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Extraction, Purification and Characterization of the Lipase from the Viscera of Porgy (*Stenotomus chrysops*)

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A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

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ISBN: 978-0-494-66729-3
Our file Notre référence
ISBN: 978-0-494-66729-3

Suggested Short Title

Lipase From The Viscera of Porgy

ABSTRACT

Lipase, from porgy (*Stenotomus chrysops*) viscera, was purified by polyethylene glycol (PEG) 1000 precipitation, followed by dialysis and affinity chromatography on EAH-Sepharose 4B. The digestive lipase from porgy showed seasonal variation in activity with high activity found in late summer and early fall compared with a spring sample. Polyethylene glycol (PEG) precipitation fraction was used to characterize this enzyme using p-nitrophenyl palmitate (pNPP) as substrate. Porgy lipase did not behave like a bile salt activated/depended lipase because it was able to hydrolyze pNPP without bile salt (e.g., sodium cholate). Porgy lipase was stable within the pH range of pH 6.0-10.0, with an optimum activity at pH 8.5. The enzyme was quite stable at temperatures below 40°C, but lost its activity rapidly at temperatures above 40°C. The optimum activity for hydrolysis pNPP was at 40°C, but the enzyme also demonstrated relatively high activity at temperatures below 40°C (i.e., 10-40°C) as well. Detergents, Triton X-100, Tween 40 and Tween 80, at final concentrations of 0.5 mM and 1 mM were found to have inhibitory effects on porgy digestive lipase activity. However, all three tested detergents appeared to increase the activity of porgy digestive lipase at elevated temperatures (i.e., 60-80°C).

RÉSUMÉ

La lipase, de porgy (Stenotomus chrysops) viscères, a été purifiée par l'adjonction de polyéthylène glycol (PEG) 1000 précipitations, suivie par la dialyse et chromatographie d'affinité sur EAH-Sepharose 4B. La fraction purifiée partiellement suivant PEG précipitations a été utilisée pour caractériser cette enzyme en utilisant comme substrat pNPP. Le digestif de la lipase porgy montré des variations saisonnières de l'activité, avec une activité plus importante observée pour la fin de l'été et au début de l'automne échantillons par rapport au printemps échantillons. Contrairement à la bile activée la lipase, Porgy lipase a été en mesure d'hydrolyser substrat PNPP en l'absence de sels biliaires comme le sodium cholate. Porgy lipase a été stable au sein de la gamme de pH 6.0-10.0, avec une activité optimale à un pH de 8,5. L'enzyme est tout à fait stable à des températures inférieures à 40°C avec une température optimale pour pNPP hydrolyse à 40°C. L'enzyme montré relativement haute activité au sein de la gamme de températures de 10-40°C, mais au-dessus de 40°C, l'activité a été rapidement perdue. Détergents Triton X-100, Tween 40 et Tween 80 à concentrations finales de 0.5 et 1.0 mm ont été trouvés d'avoir des effets inhibiteurs sur porgy digestif activité de la lipase. Toutefois, à des températures élevées (60-80°C), l'activité de la lipase porgy semble augmenter, même en présence des trois détergents Triton X-100, de Tween 40 et de Tween 80.

ACKNOWLEDGEMENTS

I sincerely thank Dr. Benjamin Kofi Simpson, my supervisor, who gave me the opportunity to study under his supervision in the Department of Food Science at McGill University. I am eternally grateful for his guidance and immense help throughout the course of my study, and his comforting and encouragement during my academic studies was immeasurable and will never be forgotten.

Special appreciation goes to Dr. Diego Luis Garcia-Gonzalez for his help and friendship during my study.

I would like to thank to Ms. Alberta N.A. Aryee, Ms. Hanadi Al-Sarawi, and Mr. Sung-Hoon Park for giving me valuable advice and help with my work. I would also like to express my thanks Ms. Vidya Subramanian and Mr. Dean Ramanah for their friendship and sharing their knowledge with me. Nor do I forget the help and friendship of Bob Xiang, Dennis Li, Shan Bai, and Liang Yu.

I am forever grateful to my parents, who taught me the meaning of life and work, and always encouraged me during my studies.

No words could express my special gratitude and love to my wife, Lulu Wang, who has always understood my situation and provided me energy during my study. Without her love and faith, I could not have completed this thesis.

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LIST OF ABBREVIATIONS

A_{280}	Absorbance at 280 nm
A_{410}	Absorbance at 410 nm
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BSAL	Bile salt activated lipase
cAMP	Cyclic adenosine monophosphate
CEL	Carboxyl ester lipase
DHA	Docosahexaenoic acid
EC	Extinction coefficient
EDTA	Ethylenediamine tetraacetic acid
EL	Endothelial lipase
EMC	Enzyme modified cheeses
EPA	Eicosapentaenoic acid
FFA	Free fatty acid

G-6-PDH	Glucose-6-phosphate dehydrogenase
G6P	Glucose-6-phosphate
HDL	High-density lipoproteins
HIC	Hydrophobic-interaction chromatography
HK	Hexokinase
HL	Hepatic lipase
HPLC	High performance liquid chromatography
IDL	Intermediate density cholesterol
LCT	Long-chain triacylglycerol
LDL	Low density cholesterol
LPL	Lipoprotein lipase
MCT	Medium-chain triacylglycerol
NaC	Sodium cholate
NaDC	Sodium deoxycholate
NaTC	Sodium taurocholate
PEG	polyethylene glycol

PL	Pancreatic lipase
PLRP1/2	Pancreatic lipase-related protein ½
pNP	Para-nitrophenol
pNPP	Para-nitrophenyl palmitate
PUFA	Polyunsaturated fatty acid
SL	Structured lipid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBE	Equilibration buffer (pH 9, 25 mM Tris, 2mM benzamidine, and 1mM EDTA)
TC	Taurocholate
TCDC	Taurochenodeoxycholate
VLDL	Very-low-density lipoprotein

CHAPTER I

GENERAL INTRODUCTION

Lipases are a class of water-soluble enzymes that act on water-insoluble substrates to catalyze the hydrolysis of ester bonds of triglycerides. Lipases are stable in both polar and nonpolar environments (Derewenda and Sharp, 1993). Enzymes which hydrolyze triglycerides have been studied for well over 300 years and lipases which specifically catalyze the hydrolysis or synthesis of esters have been recognized for about 70 years (Hasan et al., 2006). Lipases differ greatly amongst themselves with regards to both their origins (i.e. bacteria, fungi, mammalian, etc) and their properties. Lipases can catalyze both the hydrolysis of a wide range of different carboxylic acid esters into free fatty acids and glycerols, or be involved in the reverse synthetic process (Marangoni, 2002). The most thoroughly studied lipase is pancreatic lipase. Recently, more attention is being paid to lipases produced from bacteria and fungi because they are relatively stable and are capable of catalyzing a variety of reactions (Hasan et al., 2006).

To date, enzymes from aquatic species, including lipase, have received relatively little study. Although enzymes are currently commercially extracted from cod viscera in Norway, Iceland and France, industrial scale recovery of marine enzymes is mostly at the experimental stage at present. However, about one third of the world's catch of fish is thrown back into the sea, and some estimates

suggest that 70-85% of the total weight of fishery catches is dumped in-land or hauled into the ocean (Shahidi, 1994). Approximately 30% of total landings may be considered as underutilized or unexploited (Venugopal and Shahidi, 1995). Currently, most fish by-products are turned into low value fish meal, and pig feed (Taylor and Alasalvar, 2002). The recovery of flesh by mechanical deboning and development of value-added products using mince from low-cost fishery resources has been another trend in the fishery industry. These products include surimi and surimi-based products, sausages, fermented products, protein concentrates and hydrolysates (Venugopal and Shahidi, 1995).

Fish tissue by-products are potentially rich sources of a wide range of enzymes, including lipases, with fish digestive organs and glands being the most important potential source of such enzymes in any important quantity. Therefore, extracting enzymes from fish waste is attractive from both an economic and environmental point of view. Market analyst Freedonia predicts that the world market for enzymes will grow 7.6 percent annually to $6 billion by 2011. The market growth will be driven by increases in demand for biocatalysts for pharmaceutical and other fine chemical production (Freedonia, 2007). The demand for lipases with novel properties is ever growing.

Through adaptation to their environment, fish that inhabit cold temperature environments have enzymes with relatively high catalytic efficiencies at low temperatures. Marine species could therefore be a source of unique, high-activity lipases, offering the benefits of lower thermal requirements for catalysis, and rapid deactivation by mild heat treatment. Also, the catalytic activity of marine digestive lipases towards certain substrates (e.g. fatty acid classes) has been shown to differ significantly from that of lipases from mammals, plants, or microorganisms. Gjellesvik (1991) demonstrated that the bile salt-dependent lipase from Atlantic cod (*Gadus morhua* Linnaeus, 1758) had a preference towards long-chain polyunsaturated fatty acids, whereas human bile salt-dependent lipase preferentially cleaves short and medium length fatty acids chains of a low degree of unsaturation.

Porgy (*Stenotomus chrysops* Linnaeus 1766), commonly termed Scup, is a demersal species that is generally found in warm water habitats with open sandy bottoms, but can also be found in structured habitats such as mussel beds, reefs or rough bottoms. They are primarily found from Massachusetts to South Carolina, but it has also been reported as far north as the Bay of Fundy and Sable Island Bank, and as far south as Florida (Bigelow and Schroeder, 1953; Fritz, 1965). Its body is ovate-elliptical, about half as tall as long, and laterally

compressed. Its color is dull silvery and iridescent, and somewhat darker above than below. They prefer smooth to rocky bottoms, and they appear to avoid water temperatures below 7°C, preferring to stay in waters at temperatures of 13-16°C (Fritz, 1965). The migration patterns of porgy are seasonal and have both north-south and inshore-offshore components. In winter, porgy is in offshore waters between New Jersey and North Carolina. In spring they migrate northward and inshore to New Jersey, New York, and southern New England. They stay there until fall, and a reverse migration occurs. Porgy spawn once a year, beginning in the spring, during their inshore migration (Manooch Iii, 1984). The adult fish cease feeding during spawning time, for which reason few are caught then, but they bite very greedily throughout the rest of the summer on clams, bits of crab, and sea worms.

Information on fish lipases is scarce at the moment. The overall rationale for this project is to discover possible new sources of lipase for industrial applications. Lipase from the viscera of porgy (*Stenotomus chrysops*) was selected for isolation, purification and characterization because it is an important food fish and there is a significant landing of porgy in the commercial fishery. One of the aims of this project was to evaluate the possibility of turning fishery wastes into a commercial valuable enzyme source. The specific objectives of this project were

to extract, purify and characterize lipase from the viscera of porgy. In this study, the effects of temperature, pH and inhibitors on the activity of a partially-purified lipase fraction were documented, as well as the enzyme's kinetics.

CHAPTER II
LITERATURE REVIEW

2.1 Lipase Definition

Lipases (triacylglycerol acylhydrolase EC 3.1.1.3) are carboxylic acid ester or glycerol ester hydrolase enzymes that can hydrolyze the ester bonds in tri-, di- and monoglycerides. Lipases are a class of water-soluble enzymes that act on water-insoluble substrates and are stable in both polar and non-polar environments (Brokerhoff, 1974). Lipases play an important role in lipid metabolism and are found widely in most organisms, including microorganisms (Gilbert, 1993; Jaeger et al., 1994), plants (Huang, 1984), and animals (Carriere et al., 1997; Carriere et al., 1994).

2.2 Lipases and Esterases

Lipases and esterases are both in the same group of enzymes called carboxylic acid ester hydrolases. Historically they have been classified together according to their known substrate specificity, with esterases being defined as enzymes that hydrolyze ester linkages by the addition of a water molecule (Whitaker, 1972). Later, in an effort to distinguish different esterases and lipases, either substrate specificity or site of enzymatic action has been used as a discriminatory characteristic (Carrière et al., 1998). Most esterases and lipases share the α/β hydrolase fold structure, as well as similar pI values of 5.0 to 7.8.

The global amino acid composition of both enzymes is not significantly different. However, it should be noted that there are also some distinguishing features that differentiate lipases from esterases (Fojan et al., 2000).

Lipases and esterases differ in substrate specificity, with esterases hydrolyzing soluble substrates whereas lipases display their highest activity at a water-lipid interface. Esterases preferentially break ester bonds of shorter chain fatty acids, while lipases can act on a much broader substrate range, but prefer long-chain fatty acids substrates. Long chain fatty acids are usually poorly soluble (emulsions), thus lipases have the ability to break ester bonds of insoluble or aggregated substrates (Fojan et al., 2000).

Lipases do not follow the classic Michaelis-Menten kinetics model (Aloulou et al., 2006) when they catalyze lipolytic reaction at a lipid-water interface. The lid-like structure of most lipases is important in "interfacial activation" as the "lid"-motion triggers the conformation change of lipases and facilitates the interfacial adsorption of lipase at lipid-water interfaces. Various kinetic models have been suggested to describe lipases' interfacial lipolysis reactions. Verger-De Haas's model is very similar to the classic Michaelis-Menten-Henri kinetic model and it describes the interfacial hydrolysis of synthetic short- and medium-chain lipids

generating soluble products (Verger and De haas, 1976; Verger and de Haas, 1973; Verger et al., 1973). The first step in this model is to fix a water-soluble enzyme (E) to the lipid-water interface through a reversible adsorption-desorption mechanism (Figure.2.1). The enzyme is promoted to an energy-favourable state (E*) in this step, and the enzyme then follows a two-dimensional Michaelis-Menten catalytic step. The enzyme (E*) at the interface binds to a substrate (S), and forms an enzyme-substrate (E*S) complex which subsequently decomposes. The reaction products (P*) are soluble and are instantaneously diffused away (Aloulou et al., 2006). Other kinetic models have also been proposed, such as the "surface dilution model" of catalysis with mixed micelles (Carman et al., 1995; Deems et al., 1975), and the "scooting" and "hopping" modes of enzymatic action (Jain and Berg, 1989). However, esterases (and a few lipases) do not display a lid structure or "interfacial activation" (Verger, 1997).

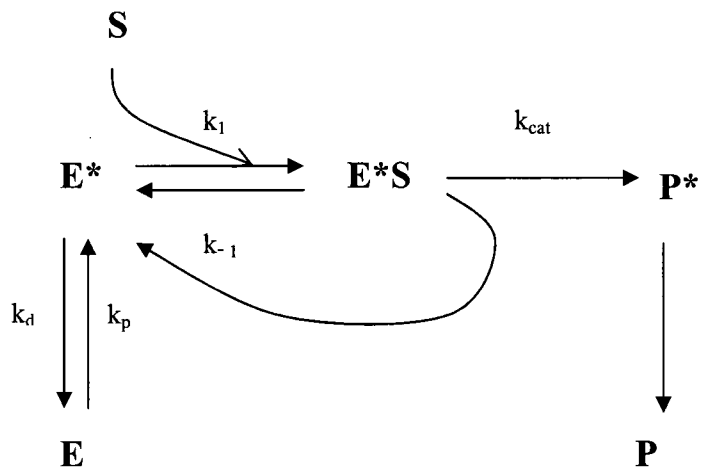


Figure 2.1 Verner-DeHaas' kinetic model for the action of soluble lipase on an insoluble substrate (Verger and Dehaas, 1976; Verger et al., 1973)

2.3 Lipase Gene Family

Based on amino acid sequence, structural similarity and gene organization, several lipases are grouped into a super family termed the lipase gene family. The members of the gene family include lipoprotein lipase (LPL), hepatic lipase (HL), and pancreatic lipase (PL) (Hide et al., 1992; Kirchgessner et al., 1989; Rader and Jaye, 2000). Later, PL-related protein 1 and 2, phosphatidylserine phospholipase A1, and endothelial lipase (EL) were also grouped into the lipase gene family (Giller et al., 1992; Grusby et al., 1990; Sato et al., 1997; van Groningen et al., 1997; Wong and Schotz, 2002).

Sequence analysis has revealed that LPL, HL, and PL are derived from a common ancestor, and that LPL and HL are more related to each other than to PL (Hide et al., 1992; Kirchgessner et al., 1989); Analyses of the gene family have indicated that EL is more related to LPL than HL, and that phosphatidylserine phospholipase A1 branched off earlier from PL than either LPL or HL (Rader and Jaye, 2000; Sato et al., 1997; Wong and Schotz, 2002) (Figure 2.2).

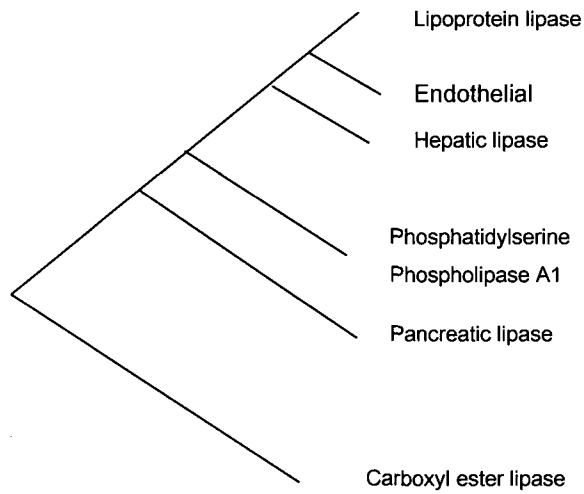


Figure 2.2 Phylogenetic relationships of the lipase gene family and Carboxyl ester lipase. Carboxyl ester lipase is used as an out-group to root the phylogeny of the lipase family (Wong and Schotz, 2002).

2.3.1 Pancreatic lipase and pancreatic lipase-related proteins 1 and 2

In mammals, pancreatic lipase (PL), the primary enzyme in dietary lipid digestion and absorption, is synthesized in pancreatic acinar cells (Lowe, 1997). Two other exocrine proteins, PL-related protein 1 (PLRP1) and 2 (PLRP2) are closely related to PL (Giller et al., 1992). Based on computer modeling of the PL backbone, PL, like LPL and HL, contains distinctive two domains (Derewenda and Cambillau, 1991).

Pancreatic lipase has a strong preference for acylglycerides over other lipids such as phospholipids and cholesterol esters (Verger, 1984). PL and its cofactor, colipase, are required for efficient dietary triglyceride digestion. It has been shown that the PL amino-terminal domain functions in the hydrolysis of ester bonds preferably at triglycerides' sn-1 position (Wong and Schotz, 2002).

The N-terminal domain of PL bears the catalytic domain and has the α/β hydrolase fold structure like other lipases and esterases (Ollis et al., 1992). Some other features in the N-terminal domain is the lid domain which is a surface loop and forms van der Waals contact with β5 loop (residues 76-85) and β9 loop (residues 204-224). The lid domain and the two loops together sterically hinder substrates from accessing the active site. Upon activation of PL, the lid domain

and the β5 loop change to a new conformation, while loop β9's position remains unchanged. These movements together expose the active site, and increase the hydrophobic area around the catalytic site (Egloff et al., 1995).

The C-terminal domain has a β-sandwich structure, and it provides the major binding surface for colipase. Colipase is a flattened molecule with three finger-shaped regions (Lowe, 2002). Colipase is able to reverse the inhibition of bile salt exhibited on PL, and PL and colipase binding creates a large hydrophobic plateau that can interact with the lipid-water interface which is believed to favour the lipase-lipid "interfacial activation" (Vantilbeurgh et al., 1993).

PLRP1 has little or no enzymatic activity against triglycerides, while PLRP2 has activities against a variety of substrates, but substrate specificity seems to depend on the species (Giller et al., 1992; Grusby et al., 1990; Roussel et al., 1998; Thirstrup et al., 1994)

2.3.2 Hepatic lipase and lipoprotein lipase

Hepatic lipase (HL) is synthesized mostly in the liver by hepatocytes and is localized at the surface of liver sinusoidal capillaries. A small amount of HL can also be found in the ovaries and adrenal gland and is suggested to be

transported via plasma transportation (Hixenbaugh et al., 1989). HL has important roles in maintaining intracellular lipid homeostasis and reversing cholesterol transportation. HL can uptake HDL cholesterol and exert both triglyceride lipase and phospholipase A1 activities, and is also involved in lipoprotein metabolism such as IDL/LDL metabolism (Karpe et al., 1993; Perret et al., 2002).

LPL can be found in various tissues, with the highest concentration in adipose tissue and muscle. LPL is bound to capillary endothelium through heparin sulphate proteoglycans. LPL functions to supply the underlying tissue with fatty acids derived from triglyceride-rich core of circulating chylomicrons and VLDL, and at the same time regulate plasma levels of triglyceride and HDL (Karpe et al., 1993; Merkel et al., 2002).

2.3.3 Endothelial lipase and phosphatidylserine phospholipase A1

Endothelial lipase (EL) is also expressed in a variety of tissues including liver and thyroid. EL is synthesized by endothelial cells and functions at the site where it is synthesized. EL has a relatively high phospholipase A1 activity, and it is also suggested that EL plays a physiological role in HDL metabolism (Choi et al., 2002; Rader and Jaye, 2000). Phosphatidylserine phospholipase A1 is less well

studied and shows a substrate preference for phosphatidylserine and lysophosphatidylserine. The function of this lipase is not very clear (Sato et al., 1997).

2.4 Carboxyl ester lipase (CEL)

In the pancreatic juice of mammals there are two major lipolytic enzymes: pancreatic lipase (PL) and carboxyl ester lipase (CEL) (Hui, 1996; Wang and Hartsuck, 1993; Zolfaghari et al., 1989). PL belongs to the lipase gene family and is the major lipolytic enzyme secreted from the pancreas. Carboxyl ester lipase, also called cholesterol esterase or bile salt-activated (or dependent) lipase (BSAL) is not in the same lipase gene family as PL, and it accounts for about 4% of total pancreatic juice protein in humans (Lombardo et al., 1978). CEL is primarily synthesized in the pancreas and lactating mammary gland (Wang and Hartsuck, 1993). CEL has relatively broad substrate specificity; it is capable of hydrolyzing cholesteryl esters, phospholipids, and tri-, di- and mono-acylglycerols, lysophospholipids, and ceramide (Hui, 1996; Wang and Hartsuck, 1993; Zolfaghari et al., 1989). In the digestive tract cholesteryl ester hydrolysis is unique to this enzyme (Hui and Howles, 2002). CEL (or BASL) has been shown to have basal activities against water-soluble substrates such as short chain fatty acid esters, but it requires bile salt to activate the hydrolytic reaction against

insoluble carboxyl esters with long chain fatty acyl groups (Wang et al., 1983). However, *in vivo*, CEL's natural substrates are long chain triglycerides, so bile salts are important in the physiological condition.

The hydrolysis of water-insoluble substrates by CEL requires bile salt containing 3α, 7α-hydroxyl groups (e.g. cholate or chenodeoxycholate and their conjugates) (Blackberg et al., 1981). It appears that trihydroxylated bile salts are more potent as activators than are the dihydroxylated bile salts, and that the 7α-hydroxyl group of bile salts is crucial in the activation of CEL (O'Connor and Wallace, 1984; Tsujita et al., 1987). The mechanism of bile salt activation of CEL is not fully understood. It has been suggested that binding bile salt causes CEL to undergo a conformational change such that the active site becomes available for the substrate molecule. Apart from this, bile salt also provides an additional fatty acid binding capacity. Bile salt can act as a fatty acid acceptor to relieve product inhibition by fatty acids during the lipolysis reaction (Wang et al., 1988). Thus, bile salts have a dual role in lipolytic enzymatic reactions; they serve as activators and as fatty acid acceptors.

2.5 Specificity of Lipases

One of the features of lipases is their enzymatic reaction specificity with various substrates. Fatty acid specificity of lipases has been studied and applied in processes such as in the making of structural lipids in foods, as well as medicinal and related applications (Aloulou et al., 2006). Lipases can be classified into four groups according to their specificity.

2.5.1 Nonspecific lipases

These groups of lipases show no positional or fatty acid specificity during their enzymatic reaction. They catalyze the complete breakdown of triglycerides to free fatty acids (FFAs) and glycerol (Figure 2.3). In the interesterification reaction catalyzed by a non-specific lipase, fatty acids are randomly distributed on the three positions of the glycerol backbone (Gupta et al., 2003). Examples of nonspecific lipases include lipases from *Candida cylindraceae*, *Corynebacterium acnes*, and *Staphylococcus aureus* (Macrae, 1983).

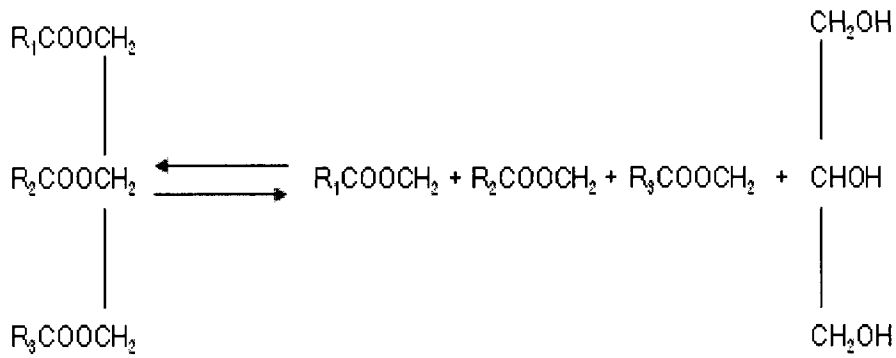


Figure 2.3 Non-specific lipase catalyzed hydrolysis of triglyceride

2.5.2 Positional specificity

The second group of lipases shows positional specificity in their catalytic reactions. Most of these lipases catalyze the release of fatty acids specifically from the outer sn-1 and sn3-positions of glycerides. The positional specificity is believed to be due to the sterically hindered sn-2 ester being unable to enter the active site of lipases. Examples of sn-1, 3 position-specific lipases are those occurring in fungi such as *Rhizomucor miehei* and *Rhizopus delemar* (Iwasaki and Yamane, 2000). Sn-1, 3 lipases are especially useful in the production of structured lipids (SLs). One strategy in producing SLs is to mix medium-chain TAG (MCT) and long-chain TAG (LCT), in the presence of a 1, 3-specific lipase

(Figure 2.4). In this reaction, all the substances (i.e., the interesterified products and unreacted substrates) are theoretically TAG, and the hydrolysis of the substrates should be suppressed. The product (i.e., a TAG fraction) is then recovered. In practice, this reaction always gives a nonhomogeneous mixture of many TAG species which are difficult to isolate. Therefore, this strategy might be used to alter the properties of a mixture of MCT and LCT rather than for preparing defined species (Fomuso and Akoh, 1998). However, sn-2 positional specific lipases do exist. One such lipase from *Candida parapsilosis* is able to hydrolyze the sn-2 position more rapidly than either the sn-1 or sn-3 position (Riaublanc et al., 1993).

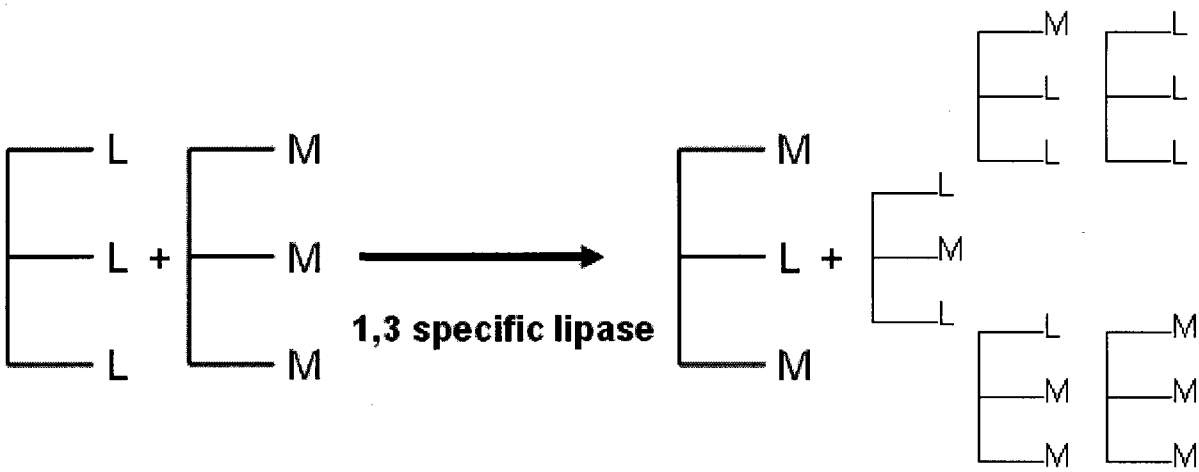


Figure 2.4 Synthesis of MLM type SLs through the interesterification reaction catalyzed by a 1, 3 specific lipase. TAGs are schematically represented. "M" and "L" indicate medium chain fatty acid and long chain fatty acid residues, respectively. Possible by-products are shown as small schemata (Iwasaki and Yamane, 2000)

2.5.3 Fatty acid specificity

Lipases are also able to catalyze the release of specific fatty acids from substrates. Fatty acid specificity likely depends on the different substrates tested. Most extracellular microbial lipases show some fatty acid specificity toward natural oils and fats (Macrae, 1983). Lipase A from yeast *Candida antarctica* shows an outstanding selectivity towards trans-fatty acids when compared with the corresponding cis-derivatives (de Maria et al., 2005). Lipases also show fatty acid chain length specificity. For example, porcine pancreatic lipase shows specificity toward medium- and short-chain fatty acids, while lipase from *Penicillium cyclopium* is specific toward long-chain fatty acids (Boyer, 1972).

2.5.4 Stereo-specificity

Stereoisomers have the same structure but a different spatial arrangement of the atoms. Stereospecificity for lipases is defined as their ability to distinguish between the sn-1 and sn-3 position of their triacylglycerol substrates. Examples for this type are human lingual (Jensen et al., 1982), dog gastric lipases, and *Candida antarctica* lipase B (Rogalska et al., 1993) for sn-3 and *Humicola lanuginose* and *Pseudomonas fluorescens* lipases for sn-1 (Rogalska et al., 1993). Enantiomers are isomers which are non-superimposable mirror images of each other; stereospecific lipases can differentiate between two enantiomers at

sn-1 and sn-3 position, and hydrolyze the racemic substrates at different rates. Chandler (1998) described *Rhizomucor miehei* lipase-catalyzed synthesis of chiral triglycerides by using the lipase's stereospecificity. Stereospecificity is determined by the source of lipase, acyl group, and also the lipid density at the interface (Jaeger et al., 1994; Muralidhar et al., 2002).

2.6 Lipase Structural Features

Lipases with known three-dimensional structures span a range of molecular weight from ~19 kDa (cutinase) to ~60 kDa (*G. candidum* lipase). Most of the lipases, with the exception of pancreatic lipase, lipoprotein lipase, and hepatic lipase, contain only one domain (Lowe, 2002; Merkel et al., 2002; Perret et al., 2002). The catalytic domains of lipases belong to the α/β protein fold and are formed by a parallel β sheet and a number of helices that flank the sheet on both sides. The minimal fragment of this α/β hydrolase fold common to all lipases is a subset of the α/β hydrolase fold described by Ollis et al. (1992). It contains a five-stranded β sheet and two α helices (B&C in Figure 2.5). The positions of the catalytic residues in this nomenclature are as follows: serine after strand β5, histidine after strand β8, and acid (aspartate or glutamate) after β7.

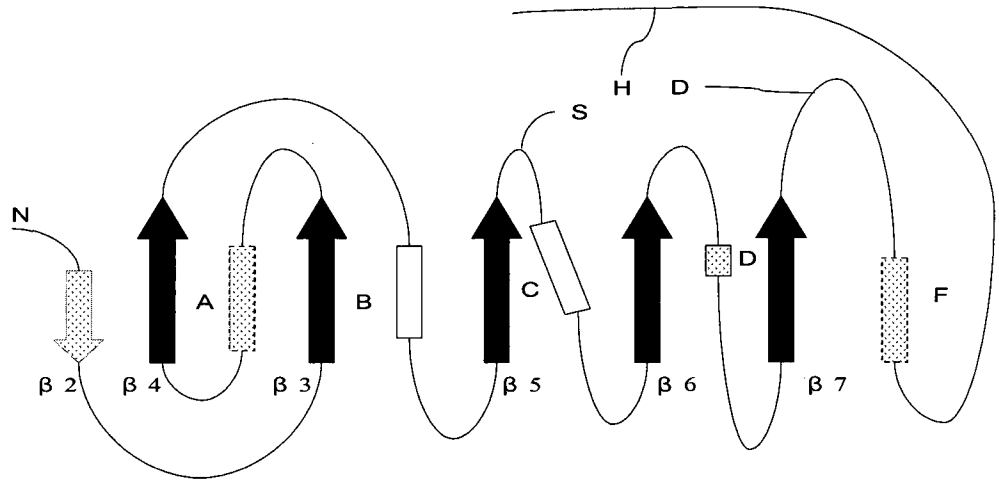


Figure 2.5 The lipase fold. Arrows indicate β strands and rectangles indicate α helices. β Strands are numbered according to the nomenclature of the α/β-hydrolase fold. Secondary structural elements shown in black or white (strands β3-β7 and helices B and C) occur in all lipases; those shown in gray (β2 and helices A, D, and F) occur in most. Helices A and F are on the concave side of the β sheet; the other helices are on the convex side. Helice D is often composed of only one turn (Ollis et al., 1992; Rubin and Dennis, 1997)

All the lipases showing the subset of the α/β hydrolase fold have some common structural features: (i) the presence of a linear sequence of nucleophile (serine)-acid (aspartate or glutamate)-His for the catalytic triad, (ii) the nucleophile is located in a strand-turn-helix nucleophile elbow, (iii) the central β sheet has at least five consecutive parallel strands, (iv) strand β6 is shorter than β7 and is followed by a sharp change in the direction of the main chain, (v) the triad handedness is opposite to that of the trypsin and subtilisin protease enzyme families, (vi) each lipase has an α helix that packs against the convex side of the

central β sheet in the crossover between strand β5 and the preceding β strand (Rubin and Dennis, 1997; Schrag and Cygler, 1997).

2.6.1 "Lid" structures of lipases: close/open conformation

A unique feature of most lipases is that the catalytic triad is covered under a "lid" of surface loop(s). In order for lipase activation to occur, the "lid" must undergo a conformational change so as to open a channel for the active site to have access to the substrates (Ferrato et al., 1997). The topological location of the lid varies among different lipases, and the length and complexity increases with the size of the molecule. While cutinase, the smallest lipolytic enzyme, does not have a lid structure it also lacks "interfacial activation" at the lipid-water interface (Martinez et al., 1992; Rubin and Dennis, 1997). A number of lipases, including pancreatic lipase from coypu (Thirstrup et al., 1994) and lipases from *Pseudomonas glumae* (Noble et al., 1993) and *Candida antarctica* (Uppenberg et al., 1994) all possess lid domains, but they do not show interfacial activation. The closed lid conformation is presumed to be predominant in the aqueous median (Cygler and Schrag, 1997). The overall surface of lipase in the closed form is relatively hydrophilic. The movement of the lid provides access to the active site and also exposes a large hydrophobic surface of the lipase molecule, which is mostly clustered around the active-site (Fojan et al., 2000).

2.6.2 Molecular mechanism of catalysis

The active site of lipases is highly conserved, and the catalytic triad consists of serine, histidine, and aspartate (or glutamate). The enzymatic catalysis in the active site of lipase consists of two steps: acylation and deacylation. In the acylation step, the Ser-OH nucleophile attacks the carbonyl carbon of the substrate to form the intermediate covalent acyl enzyme. In the second deacylation step, water attacks the acyl enzyme intermediate, and gives the product and the enzyme (Figure 2.6) (Wong, 1995).

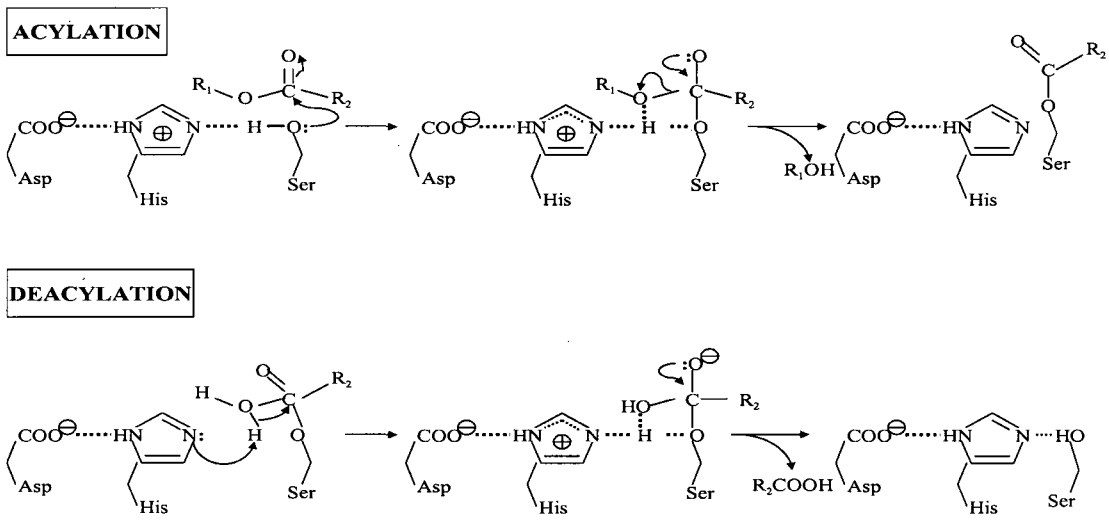


Figure 2.6 Molecular mechanism of a lipase catalyzed reaction

2.7 Fish Lipases

Although there are different types of lipases in various tissues of fish, the predominant fish lipase has been shown to be the digestive lipase which is a CEL or bile salt-activated lipase (BSAL) (Gjellesvik et al., 1992; Iijima et al., 1998; Patton et al., 1977; Taniguchi et al., 2001a; Taniguchi et al., 2001b). The major lipase sites in fish are the stomach, pyloric caeca, liver, and intestine (Borlongan, 1990; Lie and Lambertsen, 1991; Nayak et al., 2003). Unlike fish, human lipid digestion is achieved by two major lipase enzymes: pancreatic lipase with its colipase and BSAL (Gjellesvik, 1991). This is because pancreatic lipase is not efficient in cleaving certain fatty acid esters such as polyunsaturated fatty acids (PUFAs), whereas BSALs can act on a broad range of substrates (e.g., soluble, insoluble fatty acid esters, and PUFA) (Brockerhoff, 1970; Heimerma et al., 1973; Kallner, 1968). Cod feeds on relatively large amounts of polyunsaturated fats, and its only detected digestive lipase is BSAL. The cod lipase fraction showed a greater preference for hydrolysis of long-chain polyunsaturated fatty acyl esters (up to 22 carbons in length) than its human homologue (BSAL) (Gjellesvik, 1991; Lie et al., 1987). This suggests that fish could have evolved a digestive lipase that is better suited to their environment.

2.7.1 Atlantic cod (*Gadus morhua*)

Atlantic cod lipase is one of the best-studied fish digestive lipases. Lie and Lambertsen (1985, 1991), first showed that Atlantic cod lipase's activity has an absolute requirement for bile salt stimulation, and has a preference towards long-chain polyunsaturated fatty acyl esters. Gjellesvik et al. (1991) later compared this bile salt activated lipase (BSAL) with human BSAL and confirmed this finding. In further research, Gjellesvik et al. (1992, 1989) were able to purify the enzyme by cholate-sepharose affinity chromatography and gel filtration methods. Cod BSAL had an estimated molecular weight of 60 kDa by SDS-PAGE, a pH optimum of 6.5 to 7.5, and a temperature optimum in the range of 25°C to 35°C. Cod BSAL exhibited its highest hydrolysis rate on diglyceride substrates, and esters of long chain PUFAs (Gjellesvik, 1991; Gjellesvik et al., 1992). Cod BSAL was inhibited by di-isopropyl fluorophosphate and phenyl boronic acid, but not significantly by phenyl methyl sulfonyl fluoride. The enzyme was also suggested to be homologous with mammalian pancreatic BSALs (Gjellesvik et al., 1992).

2.7.2 Rainbow trout (*Oncorhynchus mykiss*)

Leger et al. (1972) partially purified and characterised rainbow trout lipase *(Oncorhynchus mykiss*, formerly *Salmo gairdneri)* using a procedure that included lyophilization, defatting, ammonium sulphate precipitation and gel

filtration. Leger et al. (1977a) later studied the lipase's characteristics, and discovered that trout lipase hydrolyzes tributyrin in the absence of Ca^{2+}, but Ca^{2+} is required for triolein hydrolysis. Thus, they believed that the cation might be an effector in the reaction. Leger et al. (1979) confirmed that a colipase played a role in the lipase reaction, and determined that the trout lipase and colipase molecular weights were 57 and 11 kDa, respectively.

Harmon et al. (1991) isolated and partially purified a triacylglycerol lipase from rainbow trout liver. The lipase showed optimal activity at pH 7.0 and 15°C. The lipase was partially purified 27,000 fold by Sepharose (Bio-gel A 0.5M, 200-400 mesh) chromatography. The molecular weight of the trout lipase was estimated as 40-43 kDa by SDS-PAGE and size-exclusion liquid chromatography. This hepatic triacylglycerol lipase was activated by cAMP/ATP-Mg^{2+}, which suggests that this lipase is regulated by phosphorylation and dephosphorylation. This was the first report of such regulation for lipid mobilization in fish.

Tocher and Sargent (1984) studied rainbow trout digestive lipolytic activity using trout pyloric ceca. Their findings suggested that CEL (or BSAL)-like lipase activity might be present in the rainbow trout pyloric ceca because primary bile salts such as cholate, taurocholate (TC) and taurochenodeoxycholate (TCDC) were

required to stimulate the lipolytic activities (Tocher and Sargent, 1984). Based on their studies, Tocher and Sargent (1984) suggested that the lipolytic activities were accomplished by 2 different enzyme proteins, one predominantly hydrolyzing triacylglycerols and the other predominantly hydrolyzing wax and sterol esters (Tocher and Sargent, 1984).

2.7.3 Oil sardine (*Sardinella longiceps*)

Mukundan et al. (1985) purified a lipase from the hepatopancreas of oil sardine *(Sardinella longiceps)* by defatting, water extraction, ammonium sulphate fractionation and chromatography on DEAE Sephadex and Sephadex G-100. The estimated molecular weight of the glycosylated enzyme (6.1% carbohydrate) was 54-57 kDa. The purified lipase showed pH and temperature optima at pH 8 and 37°C. Below 45°C, sardine lipase retained 100% activity for 1 h, whereas above 45°C activity decreased and at 63°C, activity was completely lost (Mukundan et al., 1985).

2.7.4 Spiny dogfish (*Squalus acanthias*)

Rasco and Hultin (1988) partially purified a lipase from spiny dogfish pancreas by preparative isoelectric focusing. Pre-treatments to the tissue included defatting, drying, ammonium sulphate precipitation, desalting and ultrafiltration. The

partially purified enzyme had a pH optimum of 8.5 and temperature optimum of 35°C, and it was quite stable at 45°C for up to 1 h. Above 45°C, the enzyme lost half of its activity after 1 h incubation. Dogfish lipase was active against a broad range of triacylglycerol and wax esters. The enzyme showed an increase in specific activity with an increase in sodium taurocholate (NaTC) concentration up to about 20 mM. There was some stimulation of the lipase at low concentration of sodium taurodeoxycholate (4 mM), but this effect was lost at higher concentrations of this detergent. Rasco and Hultin (1988) also suggested that the enzyme may be a PL-like lipase and that colipase may have been co-isolated with the enzyme. Therefore, further studies are needed to classify this lipase.

2.7.5 Red sea bream *(Pagrus major)*

A lipase from the hepatopancreas of red bream was purified to near homogeneity by Iijima et al. (1998). The lipase was purified by precipitation with ammonium sulphate and sequential chromatography on anion-exchange-, hydrophobic- and a second anion-exchange column followed by gel filtration and anion-exchange HPLC. The lipase was purified 339-fold, with a 2.3% yield, and it had a specific activity of 75U/mg. The lipase was able to bind tightly to a cholate-Sepharose column, and this strong interaction was confirmed through the finding that the enzyme required the presence of sodium cholate (NaC) or sodium taurocholate

for its activity. However, no activity was observed in the presence of sodium deoxycholate (NaDC). The estimated molecular weight of the purified lipase was 64 kDa by SDS-PAGE, and the pH optimum was in the range of pH 7.0-9.0. The enzyme preferentially hydrolyzed ethyl esters of polyunsaturated fatty acids, such as arachidonic acid and eicosapentaenoic acid.

2.7.6 Leopard shark (*Triakis semifasciata*)

Patton et al. (1977) used a crude pancreatic preparation to partially characterize a lipase from leopard shark. The authors classified this enzyme as a CEL type lipase because the enzyme demonstrated an absolute requirement for trihydroxy bile salts for activity. Bile salts also protected the enzyme from inactivation by *p*-chloromercuri-benzoate and trypsin treatments. The lipase extract showed a temperature optimum of 36°C and was rapidly deactivated at 50°C. Divalent metal ions were required for lipase activity with Ca^{2+} giving the greatest lipolytic stimulation.

2.7.7 Nile tilapia (*Oreochromis niloticus*)

Taniguchi et al. (2001a; 2001b) purified two lipases from tilapia (*Oreochromis niloticus,* formerly *Tilapia nilotica*) intestine and stomach. The intestinal lipase was purified using ion-exchange, chromatofocusing, and gel filtration techniques.

The lipase had a molecular weight of 46 kDa, showed its greatest activity at pH 7.5 and 35°C, and was stable at pH 6.5 to 8.5. The isoelectric point of the lipase was 4.9. The K_m of the enzyme for olive oil was calculated to be 0.7 mM.

The stomach lipase was purified using chromatofocusing and gel filtration techniques. The lipase had a molecular weight of 54 kDa, showed optimum activity at pH 6.5 and 40°C, and it was stable at pH 5.0-7.0. The K_m of the enzyme for olive oil was 0.6 mM, and the lipase activity was inhibited by Cu^{2+}, Cd^{2+}, Pb^{2+}, Ph^{2+}, Hg^{2+}, Ni^{2+}, PCMB, and EDTA.

2.7.8 Grey mullet (*Mugil cephalus*)

Aryee et al. (2007) partially purified a lipase from the viscera of grey mullet by ammonium sulfate fractionation, ultrafiltration, and affinity chromatography techniques. The partially purified enzyme was active in the pH range of 7-10, and had an optimum pH of 8. The enzyme was active in the temperature range of 20-60°C with an optimum temperature at 50°C. The enzyme showed good activity on medium and long chain fatty acids (C_{10}-C_{16}), and the hydrolytic activity was enhanced by Mg^{2+}, Mn^{2+}, NaN_3, and EDTA. Grey mullet lipase was also stable in some organic solvents, which suggests it could have valuable applications for enzyme reactions in organic solvent media.

2.7.9 Steelhead trout *(Salmo gairdneri)*

Sheridan and Allen (1984) isolated and partially purified a triglyceride lipase from the adipose tissue of steelhead trout. The adipose lipase was partially purified by heparin-sepharose affinity chromatography to approximately 71-fold over the original fraction. The lipase had an optimum pH of 7.5 and showed its highest activity at 25°C. The adipose lipase was estimated to have a molecular weight of 48 kDa by SDS-PAGE.

2.7.10 Properties of fish lipases

Some of the general properties of fish lipases are summarized in Table 2.1. For example, the optimum temperatures of lipases are from 15-50°C with most of the lipases having optimum temperatures in the range of 25-40°C. The optimum pHs of the studied lipases are mostly above neutral pH (i.e., 7.0); these trends show that most fish digestive lipases prefer an alkaline environment. However, one thing to be noted is that these studies were conducted with different standards (i.e., different lab protocols and substrates). Thus, variations in the results cannot be avoided, and general trends are not easily observed. For example, Iijima et al. (1998) observed that the optimal pH for substrate p-nitrophenyl myristate by red sea bream hepatopancreas lipase was pH 8.0 to 9.0, while using triolein as a substrate, the optimal pH was in the range of 7.0 to 9.0. The purification folds of

different studies vary considerably, and this could be due to different protocols applied in different studies. Another reason could be that individual studies used samples of different purity as a start point. Thus, purification fold may contain little information as a basis of comparison among the studies.

Table 2.1 Some properties of (partially) purified fish lipases

Fish species	Origin	Optimum Temp (°C)	Optimum pH	Molecular weight (kDa)	Activation type	Reference
Rainbow trout (*Salmo Gairdneri*)	Intercaecal tissue	ND	ND	57	Bile salt	(Leger et al., 1977b; Leger et al., 1979)
Leopard shark (*S. longiceps*)	Pancreas	36	ND	ND	Bile salt (Ca^{2+})*	(Patton et al., 1977)
Steelhead trout (*S. gairdneri*)	Adipose tissue	25	7.5	48	ND	(Sheridan and Allen, 1984)
Oil Sardine (*Sardinella longiceps*)	Hepatopancreas	37	8.0	54-57	ND	(Mukundan et al., 1985)
Dogfish (*Squalus acanthias*)	Pancreas	35	8.5	ND	Bile salt	(Rasco and Hultin, 1988)
Atlantic cod (*Gadus morhua*)	Pyloric caeca, Pancreas	25	6.5-7.5	60	Bile salt	(Gjellesvik et al., 1992)
Rainbow trout (*Oncorhynchus mykiss*)	Liver	15	7.0	40-43	Phosphorylation	(Harmon et al., 1991)
Red sea bream (*Pagrus major*)	Hepatopancreas	ND	7.0-9.0	~64	Bile salt	(Iijima et al., 1998)
Nile tilapia *(Oreochromis niloticus)*	Stomach	40	6.5	54	Bile salt**	(Taniguchi et al., 2001b)
Nile tilapia *(Oreochromis niloticus)*	Intestine	35	7.5	46	Bile salt **	(Taniguchi et al., 2001a)
Grey mullet (*Mugil cephalus*)	Viscera (pyloric ceaca, intestines, mesenteries)	50	8.0	ND	Bile salt	(Aryee et al., 2007)

ND: Not determined

*Divalent metal ions (e.g. Ca^{2+}) enhanced bile salt stimulation

**Low concentration of bile salt (e.g. 0.05-0.1%) showed greatest activation

2.8 Lipase Purification

In order to study lipases from different sources and make them useful in more industrial and environmentally friendly applications, the enzyme needs to be isolated in pure form. Lipases from various sources, such as the unicellular *Pseudomonas putida* to cod (*Gadus morhua*), have been purified and characterized (Palekar et al., 2000).

2.8.1 Pre-purification steps

Lipases from different sources are subjected to various pre-purification steps before further purification. Typically it is a one-step procedure which may involve use of ammonium sulphate [$(NH_4)_2SO_4$]. Thus lipase and some other proteins are separated from the extract solution. In some cases, it is better first to reduce the volume of extract (e.g. by ultrafiltration) before the pre-purification precipitation step (Palekar et al., 2000).

2.8.2 Chromatographic steps

Almost all purification protocols use a combination of chromatographic steps after pre-purification procedure. The order in which different types of chromatographic techniques are applied is important. However, in practice, there is always a

compromise between final product purity and process time, possibility of loss of products and economic cost for the purification.

The logical sequence of chromatographic steps would be to start with relatively robust techniques that have a concentration effect with high chemical and physical resistance and also low material cost (Palekar et al., 2000). Two methods that fit the requirements are ion-exchange chromatography and hydrophobic-interaction chromatography (HIC). Both chromatographic techniques allow a large volume sample to run through the column, which is a desirable property in starting chromatographic purification. HIC requires salt for adequate protein binding; it is preferably applied after the salt precipitation or after salt displacement from ion-exchange chromatography so that the desalting step can be excluded (Palekar et al., 2000).

The final step is to remove possible aggregates or degradation products and to condition the purified protein for use or storage. If the lipase is to be lyophilized or used for transferring to a buffer, aggregates and degradation products are preferably removed by gel filtration (Palekar et al., 2000). If the lipase is to be frozen, stored as a solution or used immediately, adsorption chromatographic techniques can be used to give relatively high lipase concentration. However, gel

filtration will dilute the sample, but can be followed by a concentration step such as ion-exchange chromatography (Palekar et al., 2000).

It has been observed that lipases purified from higher plant and animal sources need to go through many purification steps to attain the same level of purification as those obtained from microbial sources. The specific activities of the lipases from animal sources are also lower compared to microbial lipases. On the other hand, lipases purified from higher plants and animals tend to be more stable than lipases from microbial sources (Vasudevan, 2004).

2.9 Lipase Applications

Lipases occur widely in nature, and are one of the most important biocatalysts for biotechnological applications. In addition to their hydrolytic activity on triglycerides, they can catalyze other reactions such as esterification, interesterification, acidolysis, alcoholysis, and aminolysis (Figure 2.7). Lipases have been studied or applied in various fields such as in detergents, food processing, flavour enhancement, biocatalytic resolution of pharmaceuticals, production of esters and amino acid derivatives, fine chemicals and agrochemicals, for use as biosensors, in bioremediation and in cosmetics and perfumery (Hasan et al., 2006; Lee and Akoh, 1998).

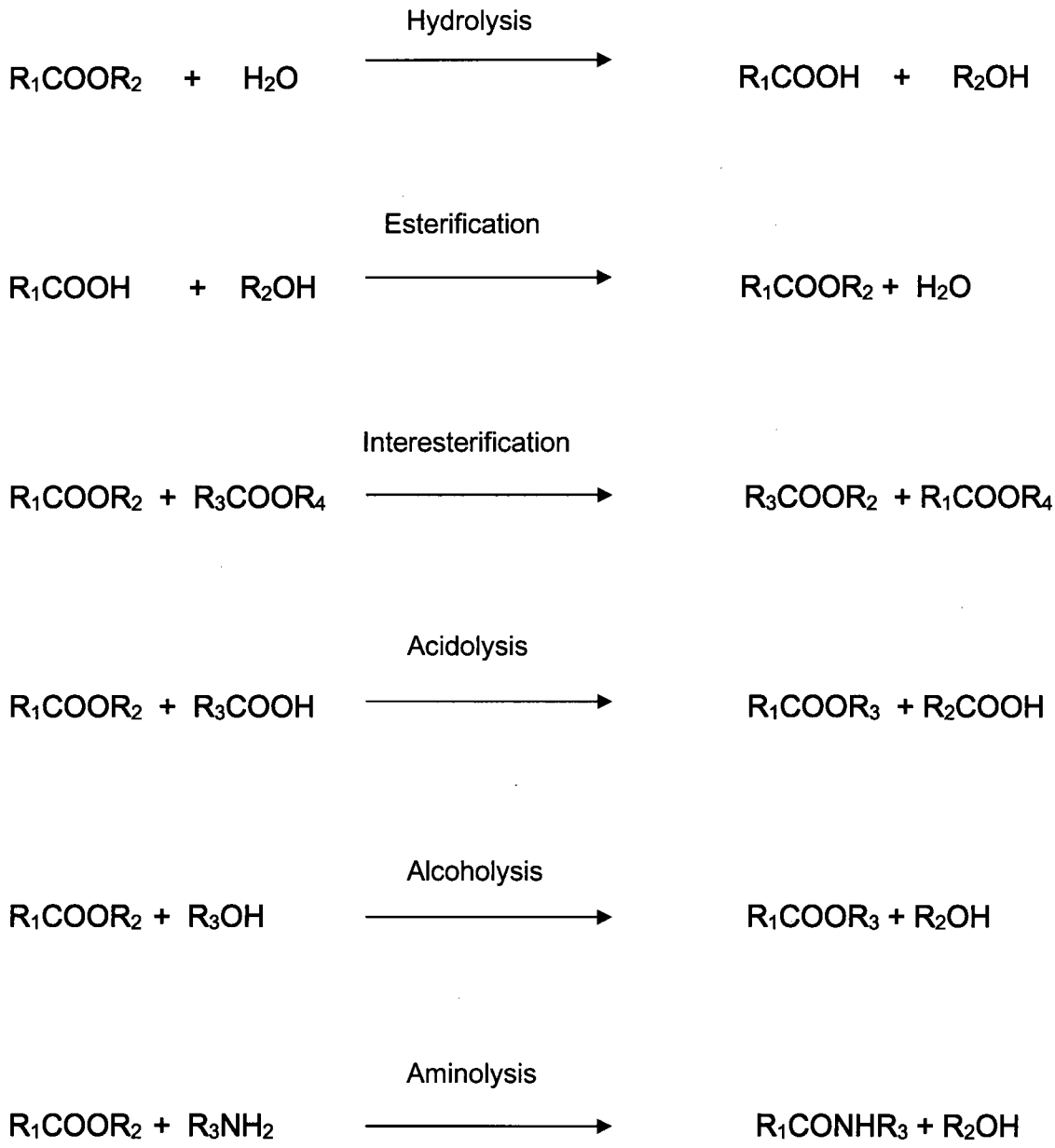


Figure 2.7 Different reactions catalyzed by lipases

2.9.1 Lipases in the fat and oleochemical industry

Fat and oil modification is one of the prime areas in food processing. One example is the production of synthetic nutritionally important structural lipids. 1, 3-regioselective lipases have been widely used to obtain new fats with nutritionally improved properties. Triglyceride absorption in the intestine is strongly dependent on the structure of triglycerides. When palmitic acid is located at the sn-2 position, such as in human milk, triglycerides are better absorbed (Innis et al., 1995). Human milkfat equivalent was synthesized by lipase catalyzed interesterification of tripalmitin with polyunsaturated fatty acids (PUFA). The result was a TAG rich in palmitic acid in position *sn*-2 and PUFAs at the 1, 3-positions. Human milkfat substitute was also produced from vegetable sources for infant formula (Andrews et al., 1988). The commercial product, Betapol from Unichema, is prepared from tripalmitin and oleic acid using lipase catalyzed interesterification aids as diet additives for premature infants (Hedeman et al., 1996).

Lipases can be used to improve PUFA content (e.g. DHA, EPA) in fish oil. A two step process was developed due to the specificity of lipase. Fish oil was first converted to partial glycerides and free fatty acids through selective hydrolysis; the partial glycerides were then esterified with free PUFA to produce PUFA-

enriched fish oil (Hou, 2002). Another example is the production of PUFA-enriched fish oil by a urea adduct and acidolysis or interesterification through a lipase catalyzed reaction (Haraldsson et al., 1989). Lipases are also used in the production of other lipids such as low calorie triglycerides and cocoa butter substitutes (Gupta et al., 2003).

2.9.2 Lipases in detergent industry

One of the commercial applications of lipases is in the detergent industry, where lipase is used in cleaning industrial and household laundry. Lipases can catalyze the hydrolysis of triglycerides present in fatty stains, such as frying fat, cosmetics and human sebum. As a result, the fatty material becomes more hydrophilic, and with the action of surfactants during the wash, the fat is removed from the textile (Aaslyng et al., 1991).

In 1987, Novozymes succeeded in launching lipolase, the first industrially produced enzyme generated through by genetic engineering. The commercially produced lipase is made by transforming the gene of a fungal lipase into an *Aspergillus* strain. Lipolase had an outstanding compatibility with detergent because it had good stability in the presence of surfactants and bleach, and a high activity at alkaline pH (Malmos, 1990). Unlike proteolytic enzymes, the

significant effects of lipolase could only be obtained after more than one wash cycle. However, lipolase ™ is an extremely effective ingredient for prespotting detergents (i.e., high enzyme concentration, low water activity) (Malmos, 1990). Novozymes later introduced three variants of lipolase: lipolase® ultra, lipoprime™, and lipex®.

Another example is a lipase (US Patent # 6265191) isolated from a *Pseudomonas* organism (such as *Pseudomonas putida*). This lipase is immobilized on the surface to facilitate oil removal from the surface and to alter wettability of the fabric surface. It can be sorbed on fabric and forms a fabric-lipase complex for oil stain removal. Lipase can be sorbed on fabric before or after an oil stain, and the lipase is active to hydrolyze an oil stain on dry fabric or fabric in laundering solutions. The sorbed lipase has enhanced stability to denaturation by surfactants and to heat deactivation (Hasan et al., 2006).

2.9.3 Lipases in tea processing

The processing of fresh tea leaves into finished black tea depends on endogenous oxidative and hydrolytic enzymes in the green leaf; a series of biochemical changes take place before the green leaf can be converted to black tea. The major enzymes involved are lipases and lipoxygenases. Lipase is able

to liberate free fatty acids (e.g. unsaturated) from the green leaf, and lipoxygenase then acts on the fatty acid and transforms them to volatile compounds (Latha and Ramarethinam, 1999). It has also been suggested that the aroma from tea is due to the volatile flavour compounds formed mainly from the conversion by lipases of unsaturated lipids (Saijyo and Takeo, 1972) to long chain aldehydes and alcohols. Lipoxygenase can act only after lipases have liberated free fatty acids from triglycerides and phospholipids, while lipase activity may occur in the absence of lipoxygenase. In one novel study, lipase from *Rhizomucor miehei* was added at the end of fermentation stage, and resulted in significant increase in the flavour volatiles compared to the control treatment. An enhancement in the aroma of lipase-treated tea was also observed by tea-tasters during organoleptic evaluation (Latha and Ramarethinam, 1999; Ramarethinam et al., 2002).

2.9.4 Use of lipases in production of biodiesel

Biodiesel (or fatty acid alkyl esters) are made from fats and oils via transesterification with an alcohol. The alkali-catalysis process has long been established and gives high conversion levels of oils to methyl esters. Compared to the alkali-catalysis process, the lipase-catalysis process is simpler as the recovery of methanol and waste-water treatment is unnecessary. Only a simple

concentration is needed to recover glycerol (Fukuda et al., 2001). Transesterification of the triglycerides in sunflower oil, fish oil, and grease with ethanol (i.e. ethanolysis) has been studied. In each case, high yields (> 80%) could be achieved using lipases from *Mucor miehei* (Selmi and Thomas, 1998), *Candida antarctica* (Breivik et al., 1997), and *Pseudomonas cepacia* (Wu et al., 1999), respectively.

However, at this stage, the cost of the lipase production is still the main issue for its industrial application. Several approaches have been studied to reduce the cost. To avoid serious degradation of lipase activity at high concentration of methanol, a new operation with stepwise addition of methanol (Kaieda et al., 2001; Kaieda et al., 1999; Shimada et al., 1999) has been developed. The use of whole cell biocatalysts immobilized within biomass support particles is advantageous because immobilization can be achieved spontaneously during batch cultivation, and complex purification is not necessary (Ban et al., 2001). Another novel approach is to use solvent-tolerant lipases. Several microorganisms has been reported to produce solvent-tolerant lipases such as 1, 3-specific lipase from *Fusarium* sp. or non-specific lipase from *Pseudomonas* and *Bacillus* sp. (Ogino et al., 1999a; Ogino et al., 1999b; Shimada et al., 1993; Sugihara et al., 1992). Further enhancement of lipase production could be

achieved by genetic engineering. High levels of expression of lipases from several microorganisms have been achieved using *Saccharomyces cerevisiae* and *F. heterosporum* as the host. (Bertolini et al., 1995; Nagao et al., 1996; Takahashi et al., 1998).

2.9.5 Lipases in cheese flavouring and bakery products

Lipases have been used extensively in the dairy industry. Most applications include flavour enhancement of cheese, acceleration of cheese ripening, manufacture of cheese-like products, and lipolysis of butter fat and cream (Saxena et al., 1999). The addition of lipases primarily releases short-chain (C_4 and C_6) fatty acids that would lead to the development of sharp, tangy flavour; while the release of medium-chain (C_{12} and C_{14}) fatty acids would tend to impart a soapy taste to the product. In addition, the free fatty acids take part in simple chemical reactions where they initiate the synthesis of other flavour ingredients such as aceto-acetate, beta-keto acids, methyl ketones, flavour esters, and lactones (Hasan et al., 2006).

Lipases play an important role in the production of so-called enzyme-modified cheeses (EMC). Any cheese that has been treated enzymatically to enhance its flavour profile is considered to be an EMC. EMCs can provide the food

manufacturer with a strong cheese note in a form that is cost effective, nutritious and natural (Sutherland, 1998). Some examples of the lipases used for EMC production are lipases from *Candida cylindracea* and *Staphylococcus aureus* (Kilcawley et al., 1998).

The use of lipase in cheese production can eliminate the need for an extremely hydrolyzed protein substrate. The generation of intense flavours by lipolysis reduces the reliance on proteinases and peptidases, and thus reduces the possibility of off-flavours (Fox and Wallace, 1997). A wide range of lipases are commercially available from a number of sources, mainly animal and microbial. Animal lipases are derived mainly from bovine and porcine pancreatic tissues.

Guys and Sahi (Guys and Sahi, 2006) showed that in cake production, the application of commercial lipase, Lipopan F, could slightly lower batter air/water surface tension and significantly lower surface viscosity, while the bulk viscosity of the batter is increased. The effects on processing were to reduce the time needed to aerate the batter. Measurements on baked cakes showed increased specific volume and some improvement in appearance such as cracks in the top crust and lack of holes in the crumb. Storage tests on the cakes over a 15 day

period showed an improvement in softness, mainly due to increased volume and in some cases to a small anti-firming effect.

2.10 Objectives and rational for the study

The demand for enzymes with specific properties is high and increasing, and various enzymes sources are being investigated (Raa, 1990). Much of the current knowledge of lipases comes from research conducted on mammals and microorganisms. The digestive lipolytic enzymes present in fish are not well understood at the moment. There are only a limited numbers of studies done on fish lipases. Some marine enzymes, like lipases, have been shown to differ from mammalian and microbial lipases in that they exhibit significant reaction rates at relatively low temperatures. From a thermodynamic point of view, these enzymes have lower free energies of activation (Haard, 1998). The lower free energy may be useful in various food-processing applications requiring low temperature treatment. Marine enzymes generally have lower thermal stabilities and respond differently to changes in pH and could have unusual kinetic and thermodynamic characteristics (Shahidi and Kamil, 2001). During commercial processing of fish, a large amount of offal and waste fish are accumulated. Recovery of enzymes such as lipases from the waste serves the purpose of waste disposal (Venugopal, 2006). Since most of the commercially-used lipases are of mammalian and

microbial origin, it is also practical to study the suitability of lipases from fish as an alternative enzyme source (Venugopal and Shahidi, 1995).

The objectives of this research were to extract, purify and characterize lipase from the viscera of porgy (*Stenotomus chrysops*). The present study presents the temperature and pH optima, inhibition effects and kinetic properties of a crude lipase fraction extracted from the porgy fish.

A lipase was extracted and purified from the viscera of porgy (*Stenotomus chrysops*). Porgy lipase showed seasonal variation in activity, with higher activity in late summer and early fall. The purified lipase from the affinity chromatography step was highly unstable, and samples with low lipase activity in the crude and PEG fraction would result in low or even no recovery from the affinity step. Further characterization was done using lipase from PEG precipitation fraction. The lipase fraction was characterized with respect to various physical and chemical properties such as kinetic properties, temperature, pH and inhibition effects.

CHAPTER III

PURIFICATION AND CHARACTERIZATION OF LIPASE FROM THE VISCERA OF PORGY (*Stenotomus chrysops)*

3.1 Abstract

Lipase was purified from the guts of porgy (*Stenotomus chrysops*) by successive steps of polyethylene glycol (PEG 1000) precipitation, dialysis, and affinity chromatography on EAH-Sepharose 4B. The affinity purified porgy lipase was unstable, and the activity of the enzyme showed seasonal variation with low activities in spring fish samples, and higher activity in late summer and early fall samples. The partially purified extract was used to characterize this lipase using p-nitrophenyl palmitate (p-NPP) as substrate. The lipase fraction was active within the pH range of 7.0-9.0, with an optimum pH of 8.5. The lipase fraction was stable from pH 6.0 to 10.00 after incubation for 30 min at 25°C, and lost its activity rapidly at pH values below 6.0 or above pH 10.0. The enzyme was active between 10°C and 60°C, and the optimum temperature for the lipase to hydrolyze p-NPP was found to be 40°C. The enzyme was relatively stable below 30°C, but unstable above 40°C. At 40°C there was about 30% residual activity after a 30 min incubation. The apparent Michaelis-Menten constant (K_m') and enzyme

catalytic efficiency (V_{max}/ K_m') for the hydrolysis of p-NPP by porgy lipase extract were measured as 0.045 mM and 0.04 s^{-1}, respectively.

3.2 Introduction

Lipases (or triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes that hydrolyze esters of long chain aliphatic acids from glycerol at the oil /water interface (Jensen, 1983). They are a class of water-soluble enzymes that act on water-insoluble substrates and are stable in both polar and non-polar environments (Brokerhoff, 1974). Most of the current knowledge of lipases comes from research done on microbial as well as mammalian lipases. The digestion of lipids in fish and the properties of lipolytic enzymes present in fish are not well understood at present. The global enzyme market increased from $1 billion in 1985 to approximately $4.1 billion in 2006, and is projected to continue to rise and reach an estimated value of $6 billion in 2011 (Chaplin and Bucke, 1990; Freedonia, 2007). The demand for enzymes with specific properties is also increasing and various enzyme sources are being investigated (Raa, 1990). Recent biotechnological developments have made production of cheaper and readily available enzymes via isolation and purification possible (An and Visessanguan, 2000). Most enzymes from fish and aquatic species are also present in terrestrial organisms (Haard, 1998). However, there are certain

variations among these two groups in terms of different molecular weights, amino acid compositions, temperature and pH optima, kinetic properties, etc. (DeVecchi and Coppes, 1996). Marine enzymes have been shown to differ from the majority of corresponding mammalian enzymes in that they have higher reaction rates at lower temperatures, respond differently to changes in pH, have lower thermal stabilities, and possess different kinetic characteristics. Therefore, marine lipases could be a possible novel source for future industrial scale enzyme applications.

Over the past few decades, various lipases had been (partially) purified from different aquatic organisms and their properties characterized. Leger et al. (1972) first partially purified rainbow trout (*Oncorhynchus mykiss*) pancreatic lipase. Subsequently, they found that lipase activity showed a linear dependence towards mixed bile salt conjugates when its substrate was triolein (Leger et al., 1977a). In 1979, these researchers detected the existence of a colipase (Leger et al., 1979). Patton and Nevenzel (1974) suggested there were two different pancreatic lipases in shark, a non-specific bile-activated lipase and one whose action was specifically on primary esters. This was further supported by Tocher and Sargent (1984) in their study with rainbow trout (*Salmo gairdnerii*) where they suggested there were two different lipolytic proteins in salmonids' ceca.

Mukundan et al. (1985) purified a lipase from the hepatopancreas of oil sardine, and showed it to exhibit optimum activity against tributyrin at pH 8.0-8.5 and 37°C. Rasco and Hultin (1988) partially purified a lipase from spiny dogfish (*Squalus acanthius*). This lipase had a pH optimum of 8.5 and temperature optimum of 35°C. Gjellesvik et al. (1992) purified a bile salt-activated lipase from Atlantic cod (*Gadus morhua*) pyloric ceca. The enzyme's molecular weight was estimated at 60 kDa, and it had a pH optimum of 6.5-7.5, and a temperature optimum of 25-35°C. Iijima et al. (1998) purified a lipase from red sea bream (*Pagrus major*), and estimated its molecular weight as 64 kDa, and its optimal pH range as 7.0-9.0. This lipase showed an absolute requirement for bile salts for activity towards esters of long chain fatty acids. Taniguchi et al. (2001a; 2001b) purified two lipases from tilapia (*Oreochromis niloticus*) intestine and stomach. The purified intestinal lipase had an estimated molecular weight of 46 kDa, and temperature and pH optima of 35°C and 7.5, respectively. Bile salts increased the intestinal lipase's activity. The stomach lipase from tilapia (*Oreochromis niloticus*) showed optimum activity at pH 6.5 and 40°C, and it was stable at pH 5.0-7.0. Its molecular weight was estimated to be 54 kDa. Aryee et al. (2007) partially purified a lipase from grey mullet (*Mugil cephalus*). Its optimum activity against pNPP occurred at pH 8.0 and its optimum temperature was 50°C.

Thus far, no information regarding the properties and characteristics of lipases from porgy (*Stenotomus chrysops*) has been documented. This study was designed to extract lipase from the viscera of porgy, and to characterize it with respect to some of its physicochemical and kinetic properties.

3.3 Materials and Methods

3.3.1 Biological specimen

Porgy (*Stenotomus chrysops*) were purchased from a local fish market (Poissonnerie O-C-N Import, Montreal, QC). The viscera were removed, stored in ice, and transported to the laboratory where they were stored at -20°C until needed.

3.3.2 Materials

The following chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA): gum arabic, benzamidine hydrochloride, bovine serum albumin (BSA), 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide, 1,4-dioxane, p-nitrophenyl palmitate (pNPP), polyethylene glycol (PEG) 1000, and sodium cholate (≥99%). Acetone, 1-butanol,calcium chloride, citric acid, chloroform, cupric sulphate pentahydrate, diethyl ether, ethylenediamine tetraacetic acid (EDTA), hydrochloric acid, mercuric chloride, sodium carbonate, sodium chloride, sodium hydroxide, sodium hydrogen carbonate, 2-propanal, polyoxyethylenesorbitan monopalmitate (Tween 40), polyoxyethylenesorbitan monooleate (Tween 80), polyethylene glycol *tert*-octylphenyl ether (Triton X-100), and tris (hydroxymethyl) aminomethane (Tris) were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). EAH-Sepharose 4B and standard low molecular weight markers

[comprised of phosphorylase b (97,000), albumin (66,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-Lactalbumin (14,400)] were purchased from GE Health care (Baie d'Urfe, Quebec, Canada).

Lipase activity was determined with pNPP as substrate using a Beckman Coulter DU 800 UV/Visible spectrophotometer (Mississauga, Ontario, Canada) at 410 nm. The activity of porgy lipase was measured using a pNPP substrate-buffer reaction mixture. The final concentration of pNPP in the reaction mixture/substrate solution was 0.167 mM. The stock substrate pNPP solution was made by dissolving 0.03152 g pNPP in 5ml of 2-propanal to obtain 16.7 mM of pNPP. Then 100 µL of the pNPP stock solution was added to 9.9 ml of 20 mM Tris-HCl buffer (pH 8.0, containing 20 mM $CaCl_2$, 5 mM sodium cholate (NaC), and 0.01% gum arabic).

3.3.3 Experimental methods

3.3.3.1 Preparation of defatted powder

The procedure for preparation of defatted powder, and the subsequent extraction and purification of porgy lipase is summarized in Figure 3.1. The frozen viscera were partially defrosted at 25°C, and then chopped into small pieces and rapidly frozen in liquid nitrogen. The frozen pieces were then comminuted to a fine

powder with a Waring blender. The powder was defatted using a modified version of the procedure of Verger et al. (1982). It involved successive changes of cold acetone, chloroform: 1-butanol (9:1 v/v), chloroform: 1-butanol (4:1 v/v), acetone and diethyl ether, all at - 20°C, with intermittent stirring. The homogenate was filtered under vacuum, and air dried at 25°C. The dry powder (about 10% of the weight of viscera) was then stored at -20°C until needed.

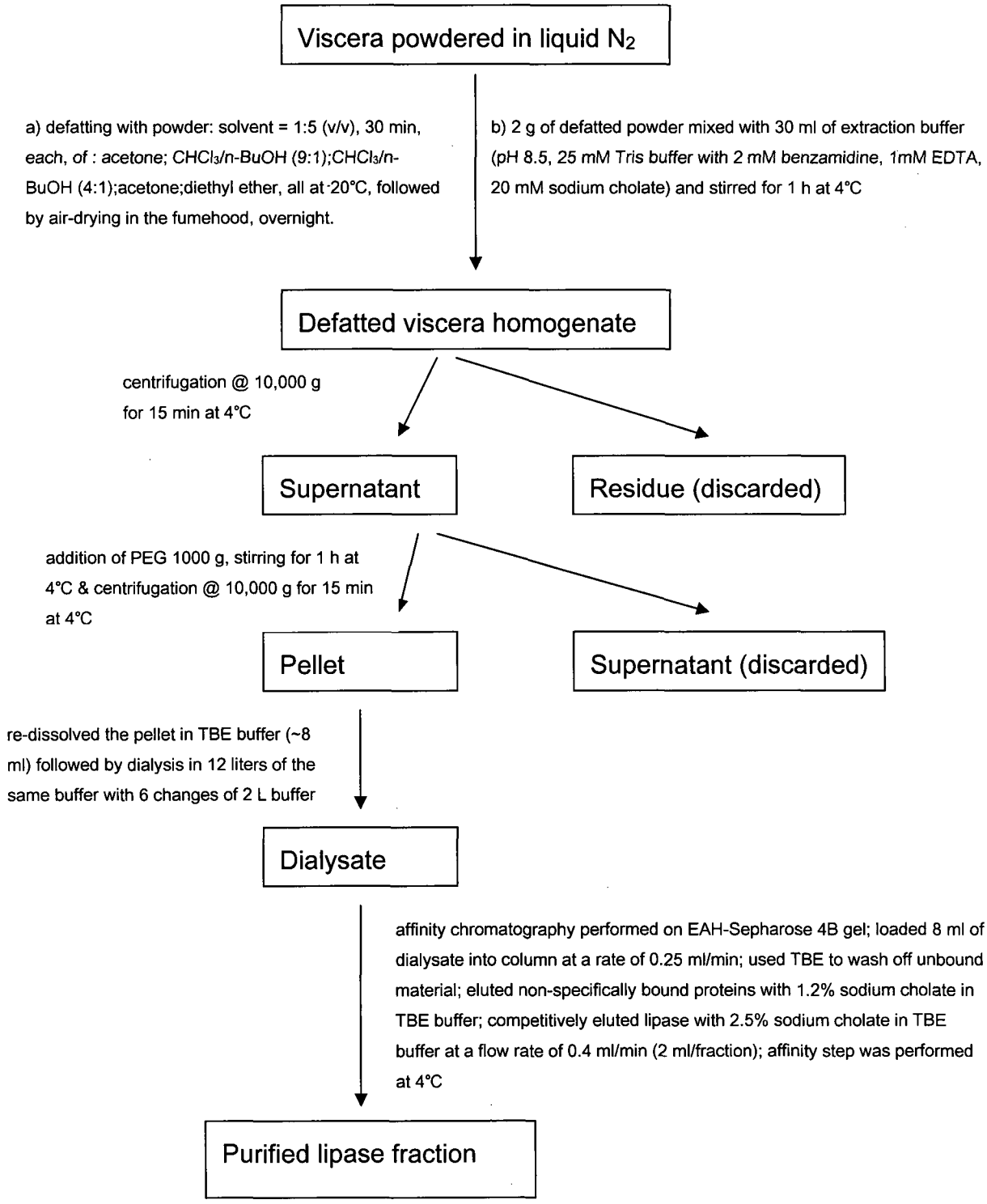


Figure 3.1 Scheme for the recovery of lipase fraction from the viscera of porgy (*Stenotomus chrysops*)

3.3.3.2 Extraction of lipase

All purification steps were performed at 4°C. Dry defatted powder was homogenized in extraction buffer (25 mM Tris containing 2 mM benzamidine, 1 mM EDTA and 20 mM sodium cholate, pH 8.5) at a ratio of 1: 15. The slurry was stirred for 1 h and centrifuged at 10,000 g for 15 min at 4°C to obtain the supernatant (i.e., crude extract).

3.3.3.3 Purification of lipase

Porgy lipase was purified by PEG 1000 precipitation at a final PEG concentration of 30%, dialysis against 6 changes of 12 L TBE buffer, followed by affinity chromatographic method performed on EAH-Sepharose 4B gel packed in a 1.0 x 10 cm column. The column was washed with TBE buffer, non-specifically bound proteins washed out with TBE buffer containing 1.2% sodium cholate (NaC), and active fractions eluted with TBE buffer containing 2.5% sodium cholate (NaC) (Figure 3.1).

3.3.3.3.1 Polyethylene glycol (PEG) precipitation

The crude extract from the lipase extraction step (as described in section 3.3.3.2) was filtered through cheesecloth, and PEG 1000 (60% stock solution) was added to the crude extract to give a final PEG concentration of 30%. The mixture was

stirred again for 1 h and finally centrifuged at 10,000 g for 15 min at 4°C to obtain the pellet.

3.3.3.3.2 Dialysis

The precipitate from the PEG 1000 step was then solubilized in a minimum amount of TBE buffer (25 mM Tris, pH 9, containing 2 mM benzamidine and 1 mM EDTA) to give a final volume of 5ml. To remove excess PEG, this fraction was dialyzed overnight using 12 kDa molecular weight cut-off dialysis tubing (cellulose membrane, Sigma-Aldrich, St. Louis, MO, USA) against 6 changes of 12 L of the same buffer. The dialysis tube was pre-activated by boiling in a solution with 10 mM sodium bicarbonate and 1 mM EDTA for 30 min.

3.3.3.3.3 Affinity chromatography

The cholate-Sepharose 4B column was prepared according to the manufacturer's instructions and the method of Gjellesvik et al. (1992) followed. Cholate was covalently coupled to the cross-linked Sepharose matrix. The pre-swollen EAH-Sepharose 4B (20 ml) was first washed according to manufacturer's instruction with pH 4.5 distilled water and 0.5 M NaCl (about 160 ml) on a sintered glass filter, and then washed with 100 ml of 50% (v/v) dioxane in water. The gel was mixed with 20 ml of 50% dioxane containing 0.8 g sodium

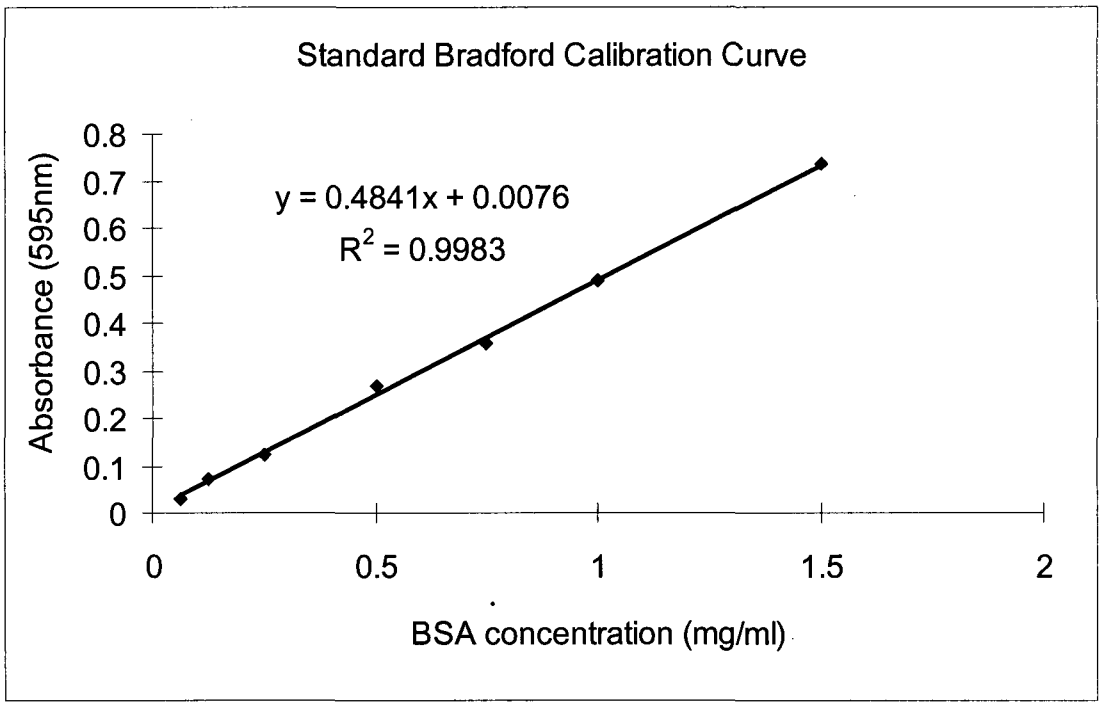
cholate (NaC), previously adjusted to pH 5 with 0.1N HCl. Next, 240 mg of N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in 5 ml 50% dioxane were added slowly to the gel slurry over 3 h. The pH of the gel slurry was maintained between pH 4-6 during the first hour of coupling using 0.1 M NaOH. The final gel slurry was rotated end-over-end overnight. Excess ligands were then washed away from the finished gel with 500 ml of 50% dioxane, followed by 0.1 M NaOH until the absorbance of the eluate at 280 nm reach a minimum stable value (e.g., Abs_{280} ≈0.0016) (Gjellesvik et al., 1992). The gel was transferred into a 1.0 x 10 cm column (Kontes Glass Company, Vineland, NJ), and equilibrated with about 5 column volumes (i.e., ~50ml) of TBE buffer.

About 8 ml of the dialysate were loaded onto the pre-equilibrated TBE column. The sample application flow rate was set to 0.25 ml/min with a microtube pump. The column was first washed with TBE buffer at a flow rate of 0.4 ml/min to elute any unbound components. This continued until the effluent had no further change in absorbance (Abs_{280} ≈ 0)(Beckman Coulter DU 800 UV/Visible spectrophotometer;Mississauga, Ontario, Canada). The column was subsequently washed at the same flow rate (0.4 ml/min) with 1.2% NaC in TBE buffer to remove non-specifically bound proteins. This continued until the absorbance of the eluate at 280 nm was minimum and stable. Finally, the tightly

bound lipase was competitively eluted with 2.5% NaC at a flow rate of 0.4 ml/min. The protein eluate from the column was collected in 2 ml fractions using a fraction collector (Model 2110 fraction collector, Bio-Rad, Mississauga, ON). Lipase activity at 30°C was measured at 410 nm using pNPP as substrate. The fractions from 2.5% NaC elution showing lipase activity were pooled and stored frozen at -20°C.

3.3.3.4 Protein determination

Protein was determined by the Bradford method (Bradford, 1976) with Bio-Rad protein assay dye reagent concentrate. One typical standard curve and one microassay protein standard curve were prepared for the Bradford assay using known amounts of bovine serum albumin (BSA) and are shown in Figure 3.2.



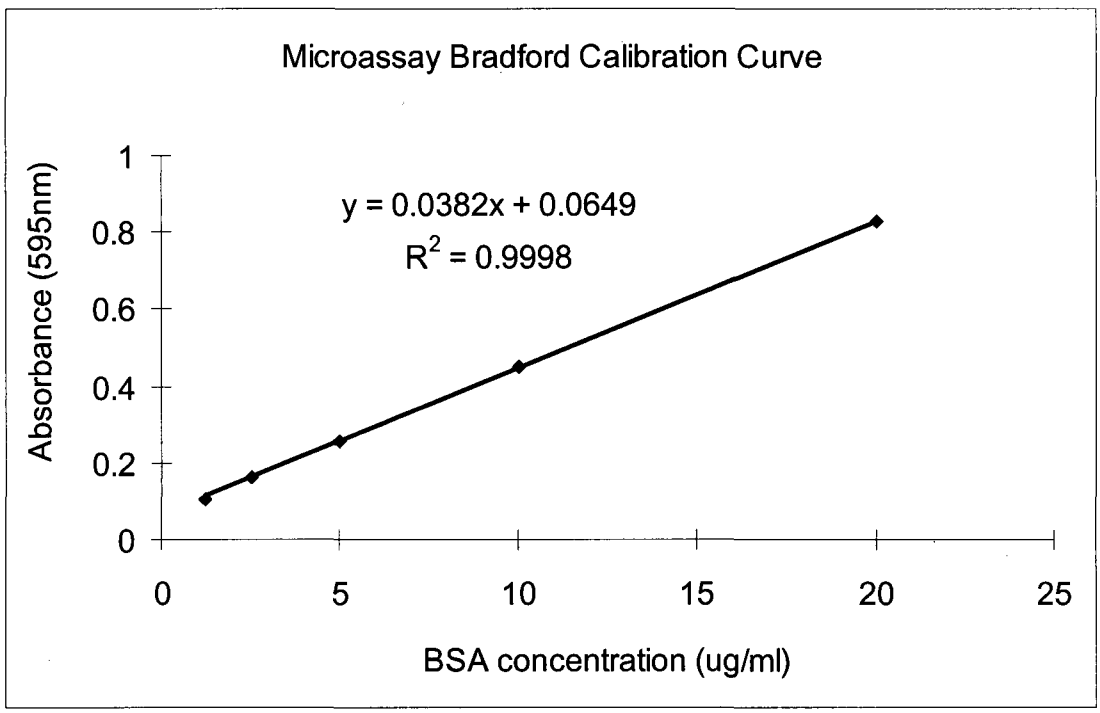


Figure 3.2 Calibration curve for protein concentration determination

3.3.3.5 Extinction coefficient determination

Extinction coefficients were determined from pH 6 to 12 using p-nitrophenol (pNP) at different concentrations (Figure 3.3).

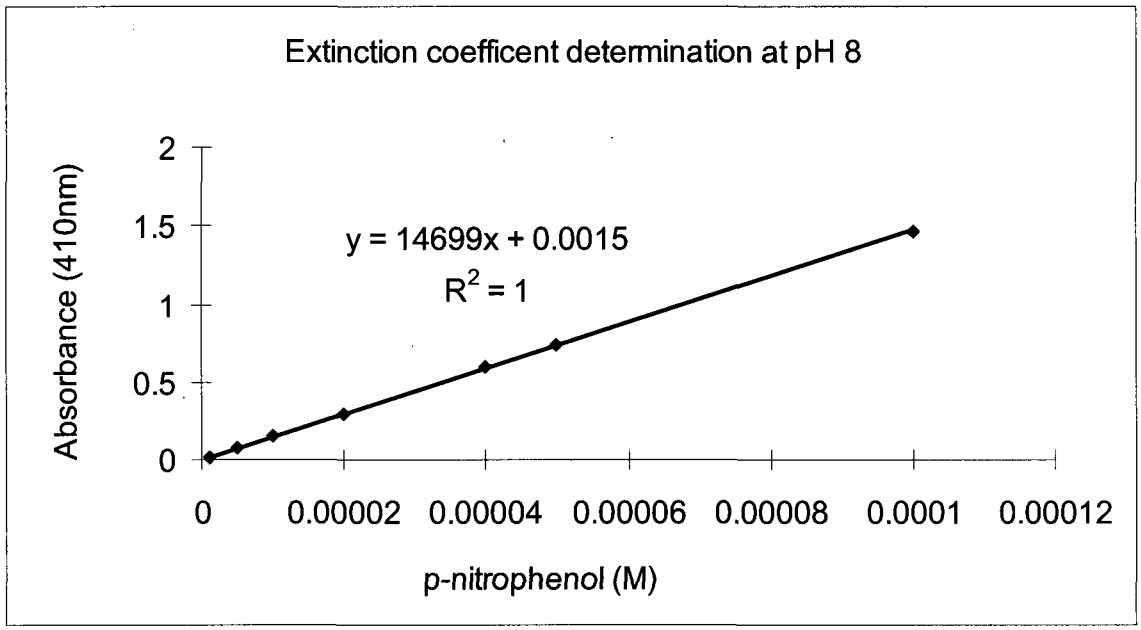


Figure 3.3 Extinction coefficient calibration curve at pH 8

3.3.3.6 Lipase assay

Lipase activity was assayed at 30°C using pNPP as substrate according to a method modified from Winkler and Stuckmann (1979). The reaction mixture was comprised of 200 µl enzyme extract plus 1.8 ml of 0.167 mM pNPP in 20 mM Tris-HCl buffer (pH 8, containing 20mM $CaCl_2$, 5mM NaC, and 1% arabic gum) to make a final reaction volume of 2 ml. The substrate solution was prepared by mixing 0.1 ml of substrate stock solution with 9.9 ml of the pH 8 Tris-HCl buffer.

The substrate stock solution was prepared by dissolving 0.03125 g of pNPP in 5 ml of 2-propanol to obtain a 16.7 mM final concentration.

The enzyme extract was replaced with the TBE buffer to establish a blank for the activity assay. Lipase activity at 30°C was assayed spectrophotometrically by measuring the rate of hydrolysis of pNPP at 410 nm (Kordel et al., 1991) using a Beckman Coulter (Model DU 800) UV/Visible spectrophotometer (Mississauga, Ontario, Canada) fitted with a high performance temperature controller. The change in absorbance at 410 nm was read at 2 seconds intervals for a total assay time of 3 min at 30°C. One pNPP unit of lipase lipolytic activity was defined as 1 µmol p-nitrophenol (pNP) released per min under the assay conditions. The extinction coefficient of pNP at pH 8.0 was calculated as 14,700 $M^{-1}cm^{-1}$ (Figure 3.3).

3.3.3.7 pH optimum of lipase activity

The effect of the pH on lipase hydrolysis rate on pNPP was determined by preparing the substrate (0.167 mM pNPP) in various buffer solutions (pH 6.0 to pH 9.5, containing 20 mM $CaCl_2$, 5 mM NaC, and 1% arabic gum). Then 0.2 ml of lipase extract was added to the different pH substrate-buffer solutions. The following pH buffer solutions were prepared for the assay: 0.1 M tris-maleate, pH

6; 0.1 M tris-maleate, pH 6.5; 0.1 M tris-HCl, pH 7.0; 0.1 M tris-HCl, pH 7.5; 0.1 M tris-HCl, pH 8.0; 0.1 M tris-HCl, pH 8.5; 0.1 M tris-HCl, pH 9.0; 0.1 M glycine-NaOH, pH 9.5; 0.1 M glycine-NaOH, pH 10.0; 0.1 M glycine-NaOH, pH 10.5; 0.1 M sodium phosphate(dibasic)-NaOH, pH 11; 0.1 M sodium phosphate(dibasic)-NaOH, pH 12. For pH 10 and pH 10.5 buffer solutions, 5 mM $CaCl_2$ was dissolved in the substrate-buffer solution because of low $CaCl_2$ solubility in high pH solutions. For pH 11 and pH 12 buffers, no $CaCl_2$ was present in the substrate-buffer solution; instead lipase extract used for the activity test contained 5mM $CaCl_2$. For assays at each pH, a blank experiment was carried out using an aliquot of TBE buffer. The activity was assayed according to the modified method of Winkler and Stuckmann (1979) as described in section 3.3.3.6.

3.3.3.8 Effect of pH on lipase stability

The pH stability of porgy lipase was investigated by incubating the lipase extract in different pH buffer solutions (pH 2.0 to pH 12.0) for 30 min at approximately 25°C, prior to assay. The compositions of the buffer solutions used were : 0.1 M hydrochloric acid-potassium chloride, pH 2; 0.1 M glycine-HCl, pH 3; 0.1 M acetate buffer, pH 4; 0.1 M acetate buffer, pH 5; 0.1 M tris-maleate, pH 6; 0.1 M tris-maleate, pH 6.5; 0.1 M tris-HCl, pH 7.0; 0.1 M tris-HCl, pH 7.5; 0.1 M tris-HCl,

pH 8.0; 0.1 M tris-HCl, pH 8.5; 0.1 M tris-HCl, pH 9.0; 0.1 M glycine-NaOH, pH 9.5; 0.1 M glycine-NaOH, pH 10.0; 0.1 M glycine-NaOH, pH 10.5; 0.1M sodium phosphate(dibasic)-NaOH, pH 11; 0.1 M sodium phosphate(dibasic)-NaOH, pH 12. The enzyme extract solution was diluted with pH buffer solutions in the ratio of 1:4 (v/v). TBE buffer was used as a blank in all pH stability assays. The enzyme activity was assayed according to the modified method of Winkler and Stuckmann (1979) as described in section 3.3.3.6., but using 200 µl of enzyme-buffer solution.

3.3.3.9 Effect of temperature on lipase activity

For optimum temperature activity, the substrate solution (0.167 mM pNPP in 20 mM Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$, 5 mM NaC, and 1% arabic gum) was equilibrated at various temperatures (10 to 65°C) prior to the assay, and the lipase activity was measured at the incubation temperatures. The substrate solution was first incubated in the previously mentioned spectrophotometer fitted with a high performance temperature controller for 20 min. For the enzyme activity assay, 0.2 ml of lipase extract was added to 1.8 ml of the temperature equilibrated substrate solution, and the rate of hydrolysis of the pNPP was measured as $\Delta A_{410\ nm}$/min in the UV/Visible spectrophotometer.

3.3.3.10 Effect of temperature on lipase stability

Thermal stability of porgy lipase was investigated by incubating the lipase extract at various temperatures from 10°C to 80°C for 10, 30 or 60 min. The lipase was then rapidly cooled in an ice bath for 2 min at the end of incubation prior to each assay. The residual lipase activity was measured at 30°C by adding 0.2 ml of residual enzyme extract into 1.8 ml of substrate solution (0.167 mM pNPP in 20 mM Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$, 5 mM NaC, and 1% arabic gum as substrate). The hydrolysis of the substrate was measured spectrophotometrically as $\Delta A_{410\,nm}$/min and used as index of residual lipase activity.

3.3.3.11 Enzyme inhibitor studies

The influence of surfactants on the activity of porgy lipase was investigated according to a method modified from Guncheva et al. (2007). The following surfactants were investigated: Tween 40, Tween 80, and Triton X-100. The reactions were done in triplicate. Relative activity was calculated with respect to the activity of the untreated enzyme.

3.3.3.11.1 Lipase stability in detergents

Detergents Tween 40, Tween 80, and Triton X-100 were solubilized in distilled water at final concentrations in 1mM and 2mM. One milliliter of lipase extract was mixed with detergent solutions in a ratio of 1:1 (v/v). The preparations were gently agitated for 1 h, and then kept at 4°C for 8 h. The lipase residual activity was determined at 30°C by adding 0.2 ml of enzyme-detergent solution to 1.8 ml of substrate solution (0.167 mM pNPP in 20 mM Tris-HCl buffer, pH 8, containing 20 mM $CaCl_2$, -5 mM NaC, and 1% arabic gum as substrate). The rate of the hydrolysis of pNPP was measured spectrophotometrically as $\Delta A_{410\ nm}$/min and used as index of residual lipase activity.

3.3.3.11.2 Effects of detergents on lipase thermostability

One milliliter of lipase extract solution was mixed with 1mM detergent solutions (i.e., Triton X-100, Tween 40, and Tween 80) at a ration of 1:1 (v/v) and then incubated for 30 min at different temperatures within the range of 30-80°C, followed by an activity assay at 30°C as described in section 3.3.3.6.. Triton X-100, Tween 40, and Tween 80 were used as controls in each assay.

3.3.3.12 Kinetics studies

The apparent Michaelis-Menten constant (K_m') and the maximum velocity (V_{max}) for the lipase hydrolysis of pNPP were determined by plotting a Lineweaver-Burk plot with pNPP concentrations from 0.05 to 0.3 mM. The catalytic efficiency (V_{max}/K_m') was also calculated.

3.4 Results and Discussions

3.4.1 Lipase extraction

The sample pre-treatment and extraction steps involved defatting using successive organic solvents at -20°C and homogenizing sample with extraction buffer for 1 h at 4°C. Only about 10-12% yield of defatted dry powder was obtained from the initial sample. Bile salts have been shown to stabilize/protect nonspecific or bile salt-dependent lipases from proteolytic degradation (Albro and Latimer, 1974; Gjellesvik et al., 1992; and Vahouny et al., 1965); therefore, 20 mM sodium cholate (NaC) was incorporated into the extraction buffer to minimize loss of lipase activity during initial extraction. Gjellesvik et al. (1992) found that lyophilization of cod pyloric caeca was necessary to maintain lipase activity throughout purification. However, lyophilization resulted in total inactivation of porgy lipase, and was not applied in the present extraction procedure.

3.4.2 Purification of porgy lipase

3.4.2.1 PEG precipitation

The stepwise purification of lipase from viscera of porgy is summarized in Table 3.1. The results were obtained from 2 g dried defatted porgy viscera powder. Polyethylene glycol (PEG) 1000 was used for the protein precipitation step based on the method of Malzert et al. (2003) and Simpson, R.J. (2004). PEGs are

nonionic, water-soluble polymers of ethylene oxide with wide ranges of molecular weights. They are well known and much used fractional precipitating agents given their benign chemical properties. PEG, unlike other organic or salt precipitating agents, shows little tendency to denature proteins even at high concentrations (Ingham, 1990). For example, PEG 400 at concentrations up to 30% (v/v) showed no detectable effect on the circular dichroic spectrum or thermal denaturation temperature of ribonuclease (Atha and Ingham, 1981). When PEG is added into a multicomponent system, it acts on the components and increases their effective concentrations. The effects of crowding lead to protein precipitation (Vergara et al., 2002). Therefore, PEG is able to regulate protein solubility without observable effects on protein structure and function.

Several studies have reported that PEG potentially increases protein stability (Castellanos et al., 2002; Uchida et al., 1998). Timasheff et al. (1985; 1992; 2002) proposed that the presence of PEG favours the stability of native proteins through preferential hydration and steric exclusion. PEG has been found to be an effective cryoprotectant. However, it is likely to destabilize protein at higher temperatures (Arakawa et al., 1993; Carpenter and Crowe, 1988).

Another point to note is that PEG may have the ability to influence the activity of some enzymes. Pancera et al. (2004) studied yeast hexokinase (HK), and reported that PEG 400 was able to increase enzyme activity by 6%, while PEG 4000 and PEG 1500 decreased enzyme activity by 7%-14%, respectively. The activity of glucose-6-phosphate dehydrogenase (G-6-PDH) in the oxidation of glucose-6-phosphate (G6P) was investigated. The presence of PEG 400 and 4000 increased enzymatic activity by 20%. However, the increase in activity was due to favourable interactions between coenzyme $NADP^+$ and PEG, not to PEG-G6P interactions (Pancera et al., 2002). In another study conducted at low temperature (-18-20°C), the activities of phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase in the presence of PEG were 1.3 and 1.55 times higher respectively, than in water (Grimonprez and Johansson, 1995). However, a recent study showed that regardless of the PEG molecular weight, PEG had no influence on lysozyme activity (Malzert et al., 2003). It is understood that enzymatic activity has complex parameters such as active site accessibility and conformation changes; therefore, it is hard to predict the effect of PEG on enzymatic activity.

There is no previous publication on fish lipase precipitation using PEG. Therefore, it is a rather novel technique to use PEG 1000 to precipitate an enzyme from

porgy. Overall the literature suggests that PEG generally has a protective effect on enzymes. Thus PEG 1000 was applied during the purification steps. Fractionation precipitation was not used in order to minimize protein loss and enzyme deactivation during purification.

In the PEG 1000 precipitation step, the pellet obtained was dissolved in a minimum amount of TBE buffer (5 ml). The protein concentration increased compared to the crude extract, however, the specific activity decreased and purification (fold) decreased as a result. The recovery from this step was calculated as 22.58% (Table 3.1).

3.4.2.2 Dialysis

The 5 ml PEG fraction was then dialyzed against TBE buffer. It is understood that a smaller volume of sequential dialysis is more efficient in removing any unwanted components than large volume dialysis with fewer changes of dialysis buffer. Therefore, the PEG fraction was dialyzed in 2 L of TBE buffer for six 2 h intervals, for a total of 12 L TBE buffer. Stirring with a magnetic stirring bar was used to facilitate the dialysis. A final volume of 16 ml of dialysate was obtained after 12 h of dialysis. The protein concentration decreased sharply compared with the PEG or crude extract fraction, and no lipase activity against pNPP was

detected in the dialysate. The reason for this lack of lipase activity could be due to the low amount of protein being retained after dialysis, i.e., the possibility of some proteins sticking to the dialysis membrane; or removal of an essential prosthetic group from the enzyme.

3.4.2.3 Affinity chromatography profile

An aliquot of 8.25 ml of dialysate was loaded onto an EAH-Sepharose 4B column previous equilibrated with about 5 column volumes of the TBE buffer. The elution profile of the affinity chromatography is shown in Figure 3.4. Three major peaks were obtained from the column elution. In order to have lipase efficiently bound to the ligand (i.e., cholate), the sample loading rate was set at a relative low speed at 0.25 ml/min. However, extending the affinity chromatographic process would possibly lead to inactivation of lipase since porgy lipase was found to be quite unstable. Therefore, a higher flow rate (i.e., 0.4 ml/min) was used in the following washing and elution steps. After loading of dialysate, TBE buffer was loaded onto the column in order to wash off any unbound materials from the affinity column. The non-specifically bound proteins were then washed off the column by 1.2% sodium cholate (NaC) in TBE buffer at a flow rate at 0.4 ml/min. As can be seen from Figure 3.4, only small amounts of proteins were eluted with 1.2% NaC. The recovery of specifically bound lipase was achieved by eluting the

affinity column with 2.5% NaC in TBE buffer at a flow rate of 0.4ml/min. There was no measurable activity in the loosely bound elution fraction and non-specifically bound protein fraction (i.e., fractions 1- 48). The increase in specific activity is showed in Figure 3.5, and fraction 52 had the highest purification fold and recovery (%).

Table 3.1 Porgy lipase purification summaries:

Purification steps	Total volume (ml)	[Protein] (mg/ml)	Total protein (mg)	Activity (IU/ml)	Total activity (IU)	Specific activity (IU/mg)	STD*	Recovery (%)	Purification (fold)
Crude extract	30	4.338	130.1	0.102	3.051	0.0234	0.00120	100	1
PEG fraction (Precipitate)	5	7.03	35.14	0.137	0.688	0.0196	0.00231	22.58	0.837
Dialysate	16	0.530	8.48	NA	NA	NA	NA	NA	NA
Fraction #52	2	0.0524	0.0974	0.0176	0.0329	0.337	0.0241	4.77	17.20

* Standard deviation for specific activity

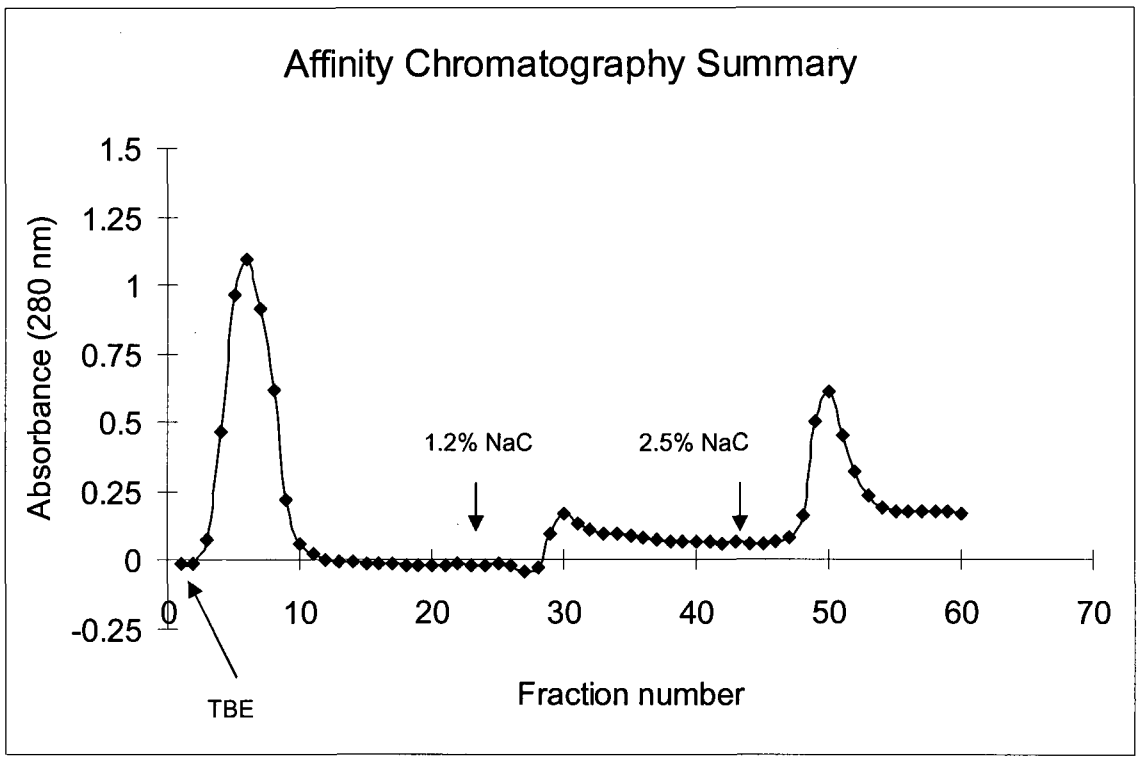


Figure 3.4 Affinity chromatography on EAH-Sepharose 4B. An aliquot of 8.25 ml of dialysate was loaded onto affinity column previously equilibrated with TBE buffer. TBE buffer was first loaded onto column (fractions 1- 22), then 1.2% NaC in TBE buffer was used to wash off non-specifically bound protein (fractions 23-42), and finally lipase was eluted with 2.5% NaC in TBE buffer (fractions 43-60)

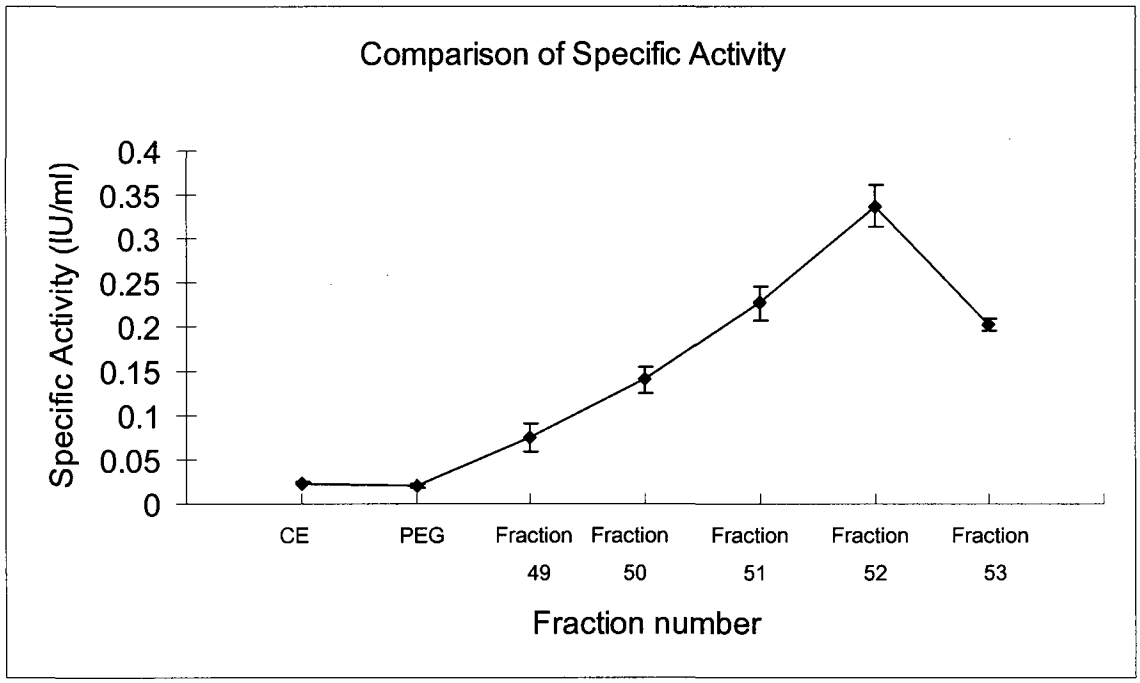


Figure 3.5 Comparison of lipase specific activity at different purification steps (CE= crude extract)

3.4.3 Characterization of porgy lipase

The activity of fish enzyme has been shown to display seasonal variation, as shown by studies of trypsin and chymotrypsin activity in cod (*Gadus morhua*). The activity of these two enzymes was found to be significantly lower in May-June compared to other seasons (Sovik and Rustad, 2004). Another study conducted on cathepsin B and collagenase from 5 cod species concluded that species, season and fishing ground significantly affected activity of cathepsin B and collagenase in cut off samples (v-cuts and belly flaps), while viscera samples were not significantly affected (Sovik and Rustad, 2006). Divakaran et al. (1999)

studied the digestive enzymes from two warm water marine finfish, pacific threadfin (*Polydactylus sexfilis*) and bluefin trevally (*Caranx melampygus*). They confirmed that different species had diverse digestive capabilities consistent with their feeding habits.

Sovik and Rustad (2005) studied the seasonal effect on lipase activity from cod (*Gadus morhua*) byproducts. They showed that lipase activity at both pH 5.0 and pH 7.0 in viscera from the Icelandic Sea were lower in May-June compared to other seasons.

Different lipases have been described in fish: triacylglycerol lipase (Gjellesvik et al., 1989), pancreatic lipase (Leger et al., 1977a; Patton, 1975), bile salt-dependent lipases (Gjellesvik et al., 1992; Patton et al., 1977), LPL (Liang et al., 2002). Many factors, besides seasons and fishing grounds, such as active swimming (Jonas and Bilinski, 1964) and fatty acid composition in the feed (Liang et al., 2002), could also have influences on porgy lipase activity. The porgy lipase activity was found to be high in late summer and early fall and low in spring (Table 3.2). Therefore, the availability of food and migration pattern of porgy might have a huge impact on their lipase activity. Affinity column purified lipase, from either summer or spring fish, was rather unstable, and the low

activity of the sample was not reliable for the initial rate analysis of enzyme activity. The PEG fraction was thus used to characterize lipase due to low lipase activity of the affinity purified lipase.

Table 3.2 Seasonal variation in porgy digestive lipase activity

Sample	Crude extract activity (IU/ml)	PEG fraction activity (IU/ml)	PEG fraction specific activity (IU/mg)
Spring	0.0925	0.113	0.0161
Summer	0.344	0.315	0.0309
Fall	0.247	0.244	0.0254

3.4.3.1 pH optimum and stability

The effect of pH on the activity of porgy lipase is presented in Figure 3.6. The optimum pH for hydrolysis of pNPP by porgy lipase was found to be 8.5. However, porgy lipase could maintain high activity in the pH range of pH 7.0-9.0. The pH activity results were similar to those obtained from previous studies (Table 2.1) which found that fish digestive lipases have optimum activities at alkaline pH values (Aryee et al., 2007; Gjellesvik et al., 1992; Iijima et al., 1998; Mukundan et al., 1985; Rasco and Hultin, 1988; Sheridan and Allen, 1984; Taniguchi et al., 2001a). Enzymatic lipase reactions cannot be measured reliably

below pH 6.5 because pNP lacks absorbance (i.e., low extinction coefficient) under acidic conditions (Gjellesvik et al., 1992).

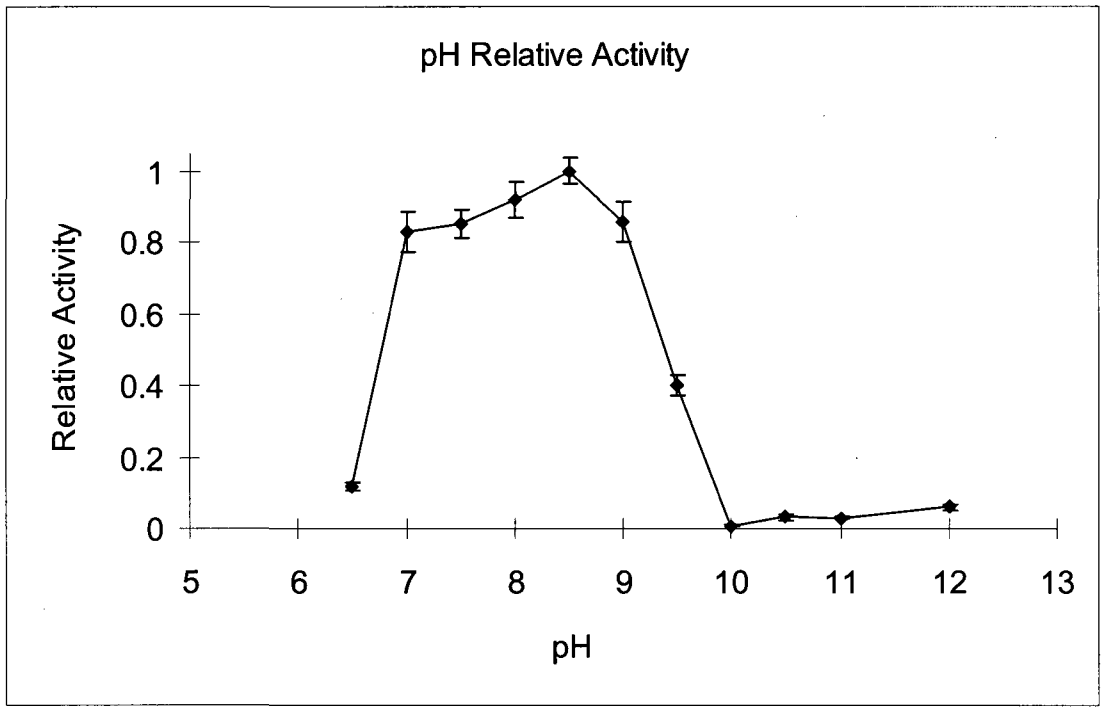


Figure 3.6 Effect of pH on the activity of porgy lipase against pNPP. 0.1 M of pH buffer-substrate solutions were prepared as described in section 3.3.3.7

The effect of pH on porgy lipase stability was evaluated by measuring enzyme residual activity after 30 min incubation in various pH buffers at 25°C (Figure 3.7). The enzyme was quite stable between pH 6.0-7.0, and retained ca. 70-75% residual activity after 30 min incubation at pH 8.0-10.0. Thus, the lipase preferred weak acidic to neutral environments. This finding concurs with that made with Nile tilapia (*Oreochromis niloticus*) stomach lipase which was stable at pH 5.0-7.0 (Taniguchi et al., 2001b). Lipase activity has been shown to be inhibited by

boronic acids (Gargouri et al., 1997), and the pH stability test on porgy lipase using a borax-NaOH buffer confirmed its inhibitory effect on porgy lipase. Therefore, glycine-NaOH buffers (pH 9.5-10) were chosen instead for pH tests. Porgy lipase lost its activity sharply at pH values over 10.0 and below pH 6.0.

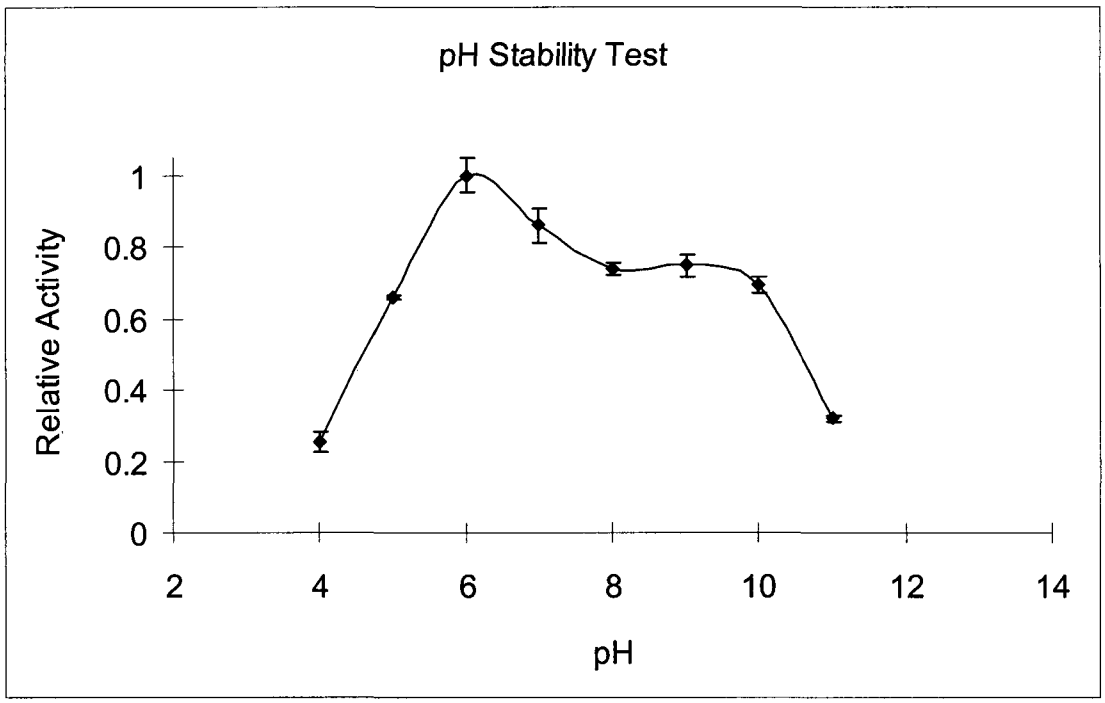


Figure 3.7 Effect of pH on the stability of porgy lipase. Lipase extract was incubated in various pH buffers for 30 min at 25°C. Relative activity was calculated based on the highest activity obtained in the assay as 100%

3.4.3.2 Temperature optimum and thermostability

The data on the effect of temperature on the activity of porgy lipase are summarized in Figure 3.8. The enzyme showed optimum activity for hydrolysis of

pNPP at 40°C. This concurs with observations made with Nile tilapia (*Oreochromis niloticus*) stomach lipase, which also showed optimum activity at 40°C (Taniguchi et al., 2001b). Reported temperature optima for other fish lipases vary, but most are lower than 40°C. For instance, Mukundan et al. (1985) reported that oil sardine (*Sardinella longiceps*) lipase has a temperature optimum at 37°C using tributyrin as substrate. Gjellesvik et al. (1992) showed that the optimum temperature for cod (*Gadus morhua)* lipase to hydrolyze 4-nitrophenyl myristate was 25°C. Harmon et al. (1991) showed the lowest optimum temperature for hepatic triacylglycerol lipase from rainbow trout (*Oncorhynchus mykiss*) to be only 15°C, when using triolein as substrate. However, it should be noted that different fish digestive lipases from various studies were of different purities and the activity tests were done using different assay methods.

The increase in activity with increasing temperature was gradual, and the activity decreased sharply beyond the optimum temperature. Based on temperature-activity data, the average temperature coefficient ($Q_{10, ave}$) for the porgy lipase against pNPP lipolytic reaction was 1.134 for the 10-40°C temperature range. In general, enzymes display Q_{10} values between 1.5 to 3.0 (Whitaker, 1994). Therefore, the low Q_{10} value suggests that porgy lipase has relatively high

lipolytic activity at lower temperatures, which could be a useful feature in low temperature enzymatic applications.

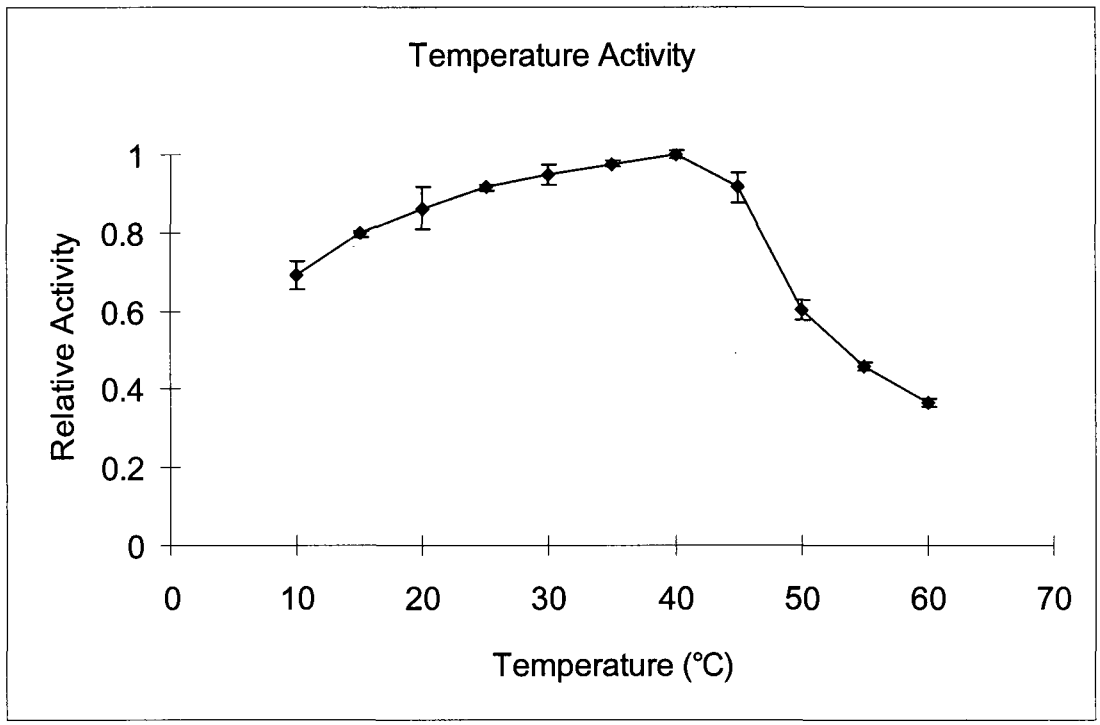


Figure 3.8 Temperature optimum of porgy lipase. Data are mean values from triplicate measurements

The thermostability of porgy lipase was studied for 10°C increment from 10°C to 80°C, with the lipase extract incubated for 10, 30 and 60 min before the activity assay (Figure 3.9). The lipase showed good stability (75-96% residual activity) from 10 to 30°C (Figure 3.10). However, the lipase lost almost half of its activity at 40°C after 10 min incubation. At 40°C with prolonged incubation, the lipase

was dramatically deactivated, with only 12% residual activity obtained after 60 min of incubation. This finding is similar to oil sardine (*Sardinella longiceps*) and spiny dogfish *(Squalus acanthias)* thermostability results. Sardine lipase retained 100% activity for a 1 h exposure to temperatures below 45°C, but above 45°C lipase activity decreased rapidly (Mukundan et al., 1985), and the enzyme entirely lost its activity at 63°C. Spiny dogfish lipase was quite stable at 45°C for up to 1 h. Above 45°C, the enzyme lost half of its activity after a 1 h incubation (Rasco and Hultin, 1988). Porgy lipase is not stable at temperatures over 40°C. For instance, at 50°C, only 54%, 16%, and 8% residual activity were measured after 10, 30, and 60 min incubations respectively. At 80°C, 10% and 7% residual activity were observed for 10 and 30 min incubation respectively, and the enzyme was deactivated by 60 min incubation at 80 °C.

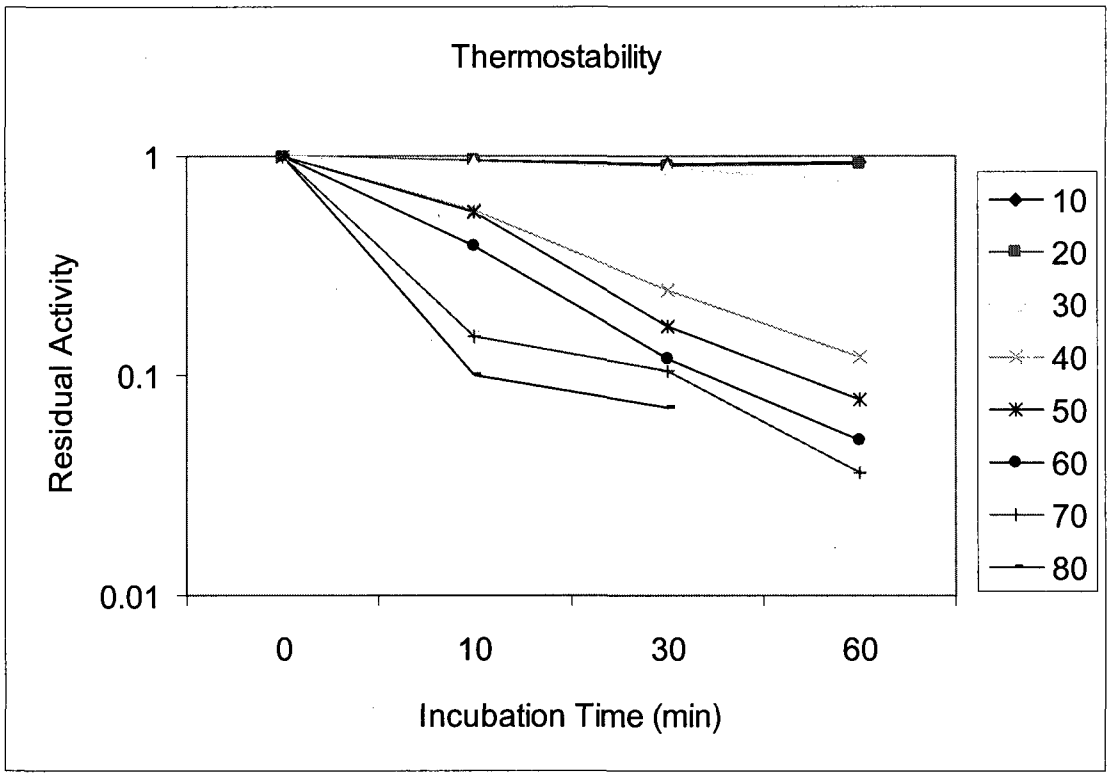


Figure 3.9 Effect of temperature on stability of porgy lipase, the residual activity (*y*-axis) is shown on a logarithmic scale. Data are mean values from triplicate measurements

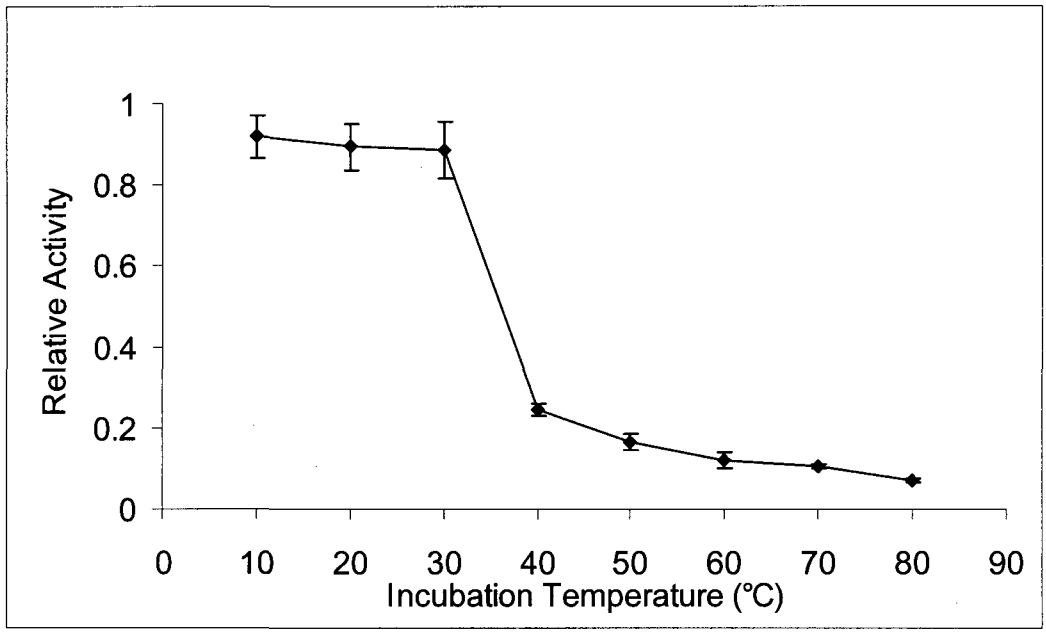


Figure 3.10 Thermostability of porgy lipase. The enzyme was incubated at temperatures from 10 to 80°C for 30 min and the remaining activity was assayed using pNPP as substrate. Data are mean values of triplicate measurements

3.4.3.3 Effects of detergents on the stability and activity of lipase

3.4.3.3.1 Lipase stability in detergents

Detergents are frequently used in the preparation of emulsions in lipolytic assays and also in the purification and characterization of lipases. However, some of them may potentially cause denatuation of enzymes by disrupting their native structure. On the other hand, they may also increase lipase activity. Lima et al. (2004) showed that lipase from *Penicillium aurantiogriseum* was stable in 0.01% Tween (109% residual activity) and 0.01% SDS (100% residual activity) after 60 min incubation at 28°C. However, incubation with Triton X-100 caused a

decrease in activity of almost half. Mogensen et al. (2005) showed that for all detergents, low concentrations enhanced *Thermomyces lanuginosus* lipase activity, while lipase activity declined at high detergent concentrations. They suggested that detergent monomers might be bound to the active site region and induce an open conformation (of the lipase lid). Inhibition occurs when additional detergent molecules bind in the active site region and block substrate access. However, other factors like carbon chain length, headgroups, and number of chains could also affect lipase activity in detergents. Guncheva et al. (2007) studied the stability of one thermostable lipase (*Bacillus stearotherophilus*) in various nonionic detergents, and reported that the enzyme had higher residual activity in lower concentrations (2.7×10^{-5} M) of sorbitan derivatives (i.e. spans and tweens). The influence of detergents on the stability of porgy lipase was investigated with the anionic detergent Triton X-100 and the nonionic detergents Tween 40 and Tween 80.

Porgy lipase showed higher residual activity when incubated in lower detergent concentration (i.e. 1 mM) for both anionic and nonionic detergents, which was similar to a previously reported study by Mogensen et al. (2005) (Figure 3.11). On the other hand, higher detergent concentrations (i.e. 2 mM) led to significant inhibition of porgy lipase activity. This could be attributed to excessive adsorption

of the surfactants on the enzyme surface, resulting in diffusional limitation on the reaction. Lipase incubated in 1 mM Triton X-100, Tween 40 and Tween 80 had higher residual activity than when they were incubated in 2 mM detergent concentrations. Lipase incubated in 2 mM tween 40 had the lowest residual activity, retaining only 30% activity.

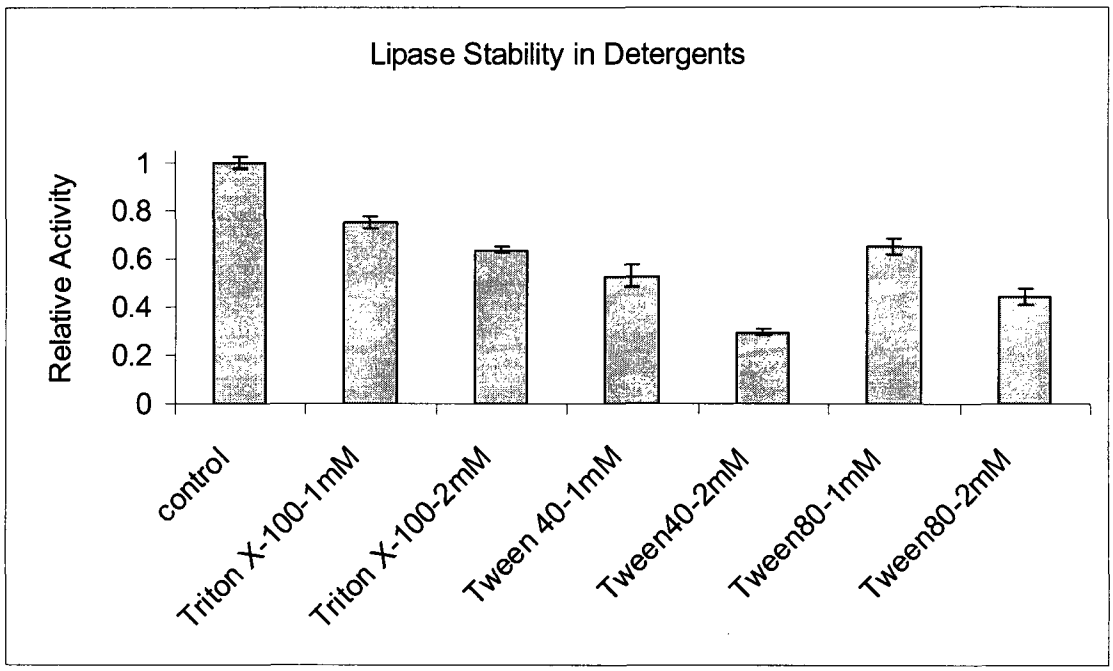


Figure 3.11 Effect of detergents on the stability of porgy lipase. Data are mean values of triplicate measurements

3.4.3.3.2 Effects of detergents on lipase thermostability

The effects of different detergents on porgy lipase thermostability were measured (Figure 3.12) at 1mM detergent concentration (i.e., 0.5 mM final detergent

concentrations). Thermal pre-treatment led to a significant reduction in enzyme catalytic activity against pNPP (Figure 3.12). All the detergents showed inhibitory effects on porgy lipase at a temperature range of 30 to 50°C. However, at higher temperatures (i.e., 60-80°C), the presence of all studied detergents had a positive impact on enzyme stability, which was similar to results previously reported by Guncheva et al. (2007). It was impressive that with detergents incorporated into the enzyme extract, the lipase residual activity was up to 28-36% compared to the native enzyme, even at 80°C. Thus, the detergents likely had a protective effect on porgy lipase at high temperatures, and the protective effect for all tested detergents was comparable.

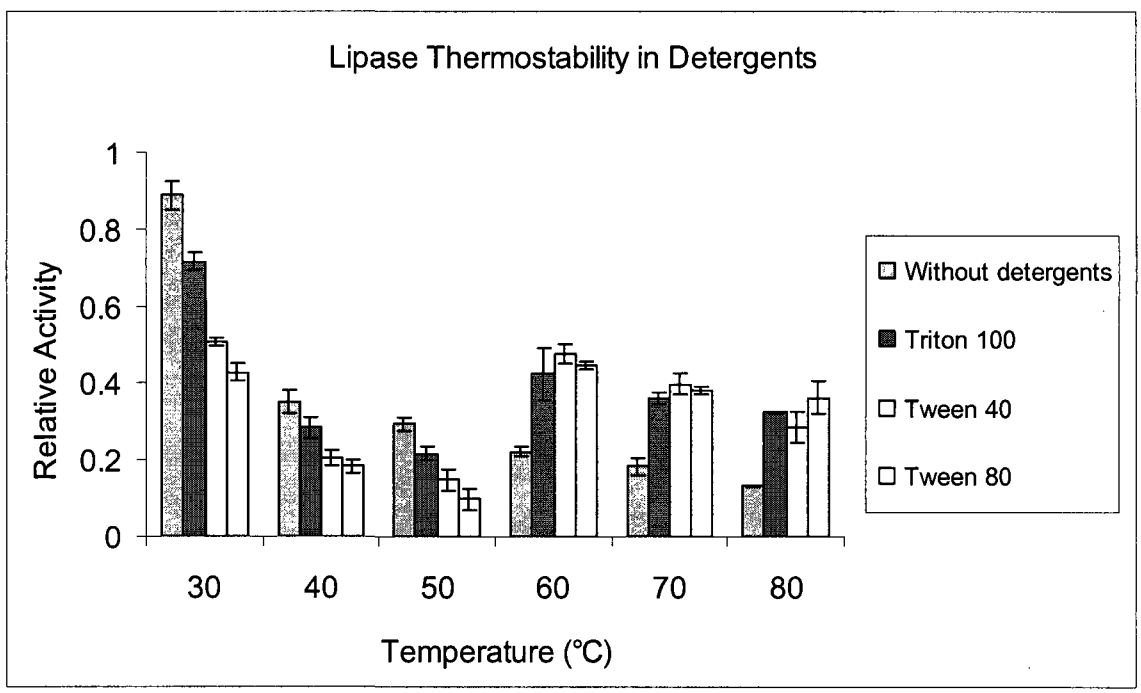


Figure 3.12 Effects of different detergents on the thermostability of porgy lipase

3.4.3.4 Kinetic studies

The apparent Michaelis-Menten constant (K_m') and maximum velocity (V_{max}) for porgy lipase lipolytic reaction was determined using a Lineweaver-Burk (double reciprocal) plot (Figure 3.13). The lipolytic rate was measured at different substrate (i.e. pNPP) concentrations, and a plot of 1/V versus 1/[S] was constructed and fitted in a linear regression as per the Lineweaver- Burk equation. The V_{max} and K_m' values were calculated from the lineweaver-Burk equation as 0.18 µmol/s and 0.045 mM respectively. Therefore, the enzyme catalytic efficiency (V_{max}/K_{cat}) is calculated as 0.04 s^{-1}.

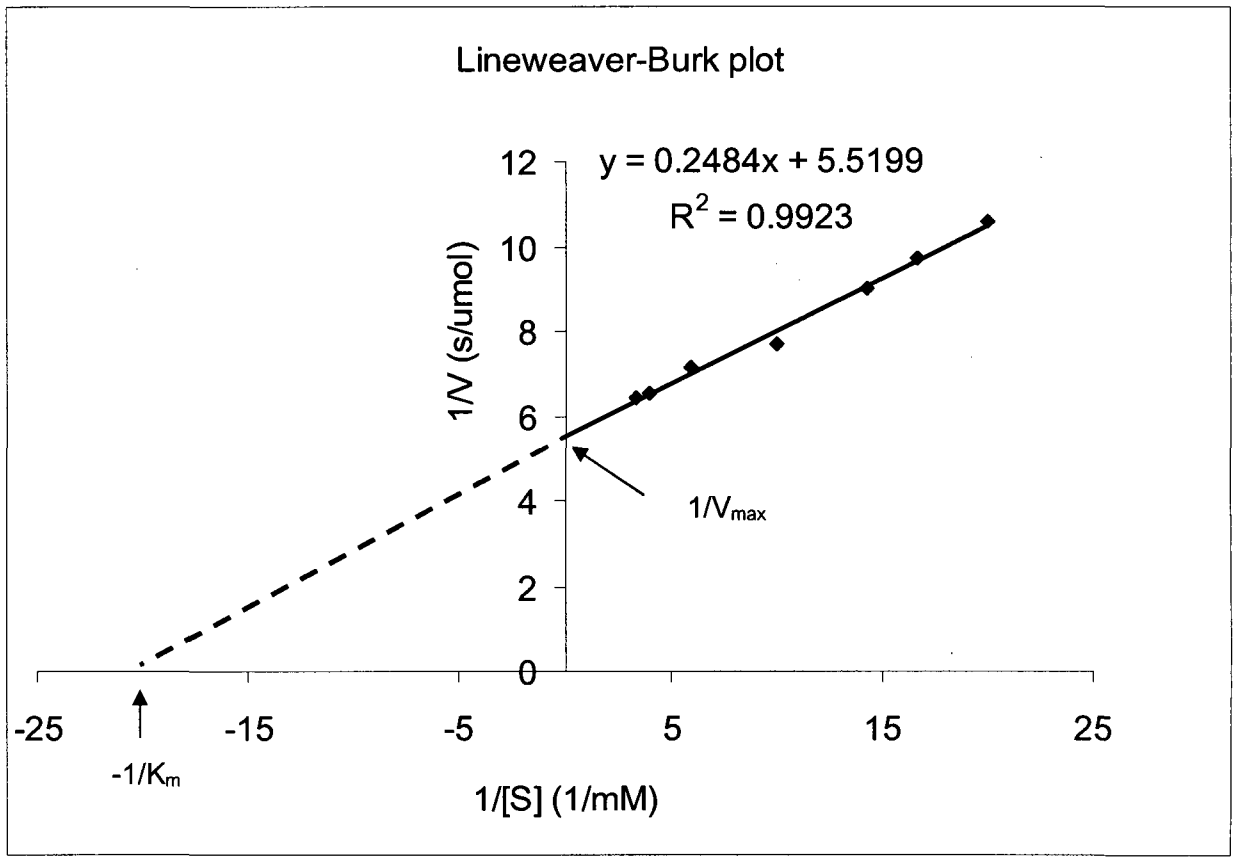


Figure 3.13 Lineweaver-Burk plot for porgy lipase kinetics

3.5 Conclusions

The use of PEG for precipitation of protein is increasing, and it is reported to increase native protein stability. Such a use of PEG has never been reported in marine fish lipase studies, and this study showed that PEG could be potentially useful in marine fish protein purification. The study showed that porgy lipase was unstable and that its activity displayed seasonal variation, possibly reflecting porgy migration patterns and feeding conditions. Consequently, the PEG precipitation fraction was used in characterization of the enzyme.

Porgy lipase was most active in the alkaline pH range (pH 7.0-9.0), which is similar to most fish lipases studied. The enzyme was stable in the range from pH 6.0 -10.0. Porgy lipase was stable below 30°C, and the optimum temperature for this enzyme to hydrolyze pNPP was 40°C. The calculated K_m' and V_{max} for this enzyme were 0.045 mM and 0.18 µmol/s. Detergents showed inhibitory effects on lipase activity, but had protective effect of the enzyme activity at high temperatures (i.e., 60-80°C).

CHAPTER IV
GENERAL CONCLUSIONS AND RECOMMENDATIONS

4.1 General conclusions

Lipase was extracted and purified from viscera of porgy and was further characterized with respect to various physical and chemical properties such as pH, temperature, the kinetic properties, and detergent effects on lipase extracts. The lipase from the warm water porgy fish showed seasonal variation in activity, with greater activity in the later summer and early fall. The lipase extract was very stable in the pH range from pH 7.0 to 9.0. The enzyme had an optimum pH and temperature of 8.5 and 40°C, respectively, for the hydrolysis of pNPP. Porgy lipase was very stable up to 30°C, but lost its activity rapidly at temperatures over 40°C. Most marine fish lipases discovered so far are bile salt-activated lipases. Porgy lipase was not absolutely bile salt-dependent for the hydrolysis of pNPP. However, the trihydroxylated bile salt NaC gave better activity than sodium taurocholate (NaTC). Detergents had a greater inhibitory effect on porgy lipase at high concentrations than at low detergent concentrations, where the detergents were able to increase the lipase activity at elevated temperatures.

Lipases from marine fish represent a special group of enzymes with different properties when compared to mammalian or microbial enzymes. Temperature optima and stability of marine fish digestive lipases vary. The optimum activity was usually obtained between 20 and 40°C, and the enzymes were deactivated at relatively high temperatures (Table 2.1). Thus, the marine fish lipases have low optimum temperature and low thermostability (Bier, 1955; Gjellesvik et al., 1992; Shahani et al., 1976). From the limited data, the pH optima for marine fish lipases are from neutral to alkaline range (pH 7.0-9.0), which is similar to mammalian lipases (Jensen, 1983).

4.2 Recommendations for future studies

Microbial and mammalian enzymes are still being intensively studied at this moment. However, the new trend is to find new enzyme sources with unique properties, and marine enzymes are the next promising tools in the biotechnology fields. Right now, the use of marine enzymes, such as lipase, in food or other industrial applications is still in its early stages. The near future of marine lipase studies possibly are still being focused on its specificity, catalytic efficiency and stability, etc. With the developing of biotechnological techniques, more marine lipases will be exploited with respect to possibilities in large scale commercial applications. The industrial scale of recovery of marine enzymes is

only at the experimental stage right now. The main obstacle in the application of marine enzymes is their sources. The cost in producing these enzymes by extraction from their natural sources is high, thus limiting their widespread use. Therefore, more extensive studies in identifying the most specific and promising enzymes and the optimal conditions for their uses should be most important at this stage. The importance of developing large-scale extraction methods should also be a priority (Shahidi and Kamil, 2001).

Finally, recombinant DNA technology offers another opportunity for future marine lipase studies. Recombinant DNA technology would allow useful marine-enzyme genes to be transferred to other organisms. Thus, the relevant genes could be cloned into a microorganism host that is more suitable for large-scale production, and make commercial production cheaper and possible on a large scale.

REFERENCES

Aaslyng, D., et al., 1991. Mechanistic studies of proteases and lipases for the detergent industry. Journal of Chemical Technology and Biotechnology. 50, 321-330.

Alasalvar, C., Seafoods: Quality, Technology and Nutraceutical Applications- an Overview. In: C. Alasalvar, T. Taylaor, Eds.), Seafoods- Quality, Technology and Nutraceutical Applications. Springer, Berlin ; New York, 2002, pp. 1-5.

Albro, P. W., Latimer, A. D., 1974. Pancreatic nonspecific lipase, an enzyme highly specific for micelles. Biochemistry. 13, 1431-1439.

Aloulou, A., et al., 2006. Exploring the specific features of interfacial enzymology based on lipase studies. Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids. 1761, 995-1013.

An, H., Visessanguan, W., Recovery of enzymes from seafood processing wastes. In: N. F. Haard, B. K. Simpson, Eds.), Seafood enzymes. Marcel Dekker, New York, NY, 2000, pp. 641-664.

Andrews, D. L., et al., 1988. Characterization of the lipid acyl hydrolase activity of the major potato (*Solanum tuberosum*) tuber protein, patatin, by cloning and abundant expression in a baculovirus vector. Biochemical Journal. 252, 199-206.

Arakawa, T., et al., 1993. Factors affecting short-term and long-term stabilities of proteins. Advanced Drug Delivery Reviews. 10, 1-28.

Arakawa, T., Timasheff, S. N., 1985. Mechanism of poly(ethylene glycol) interaction with proteins. Biochemistry. 24, 6756-6762.

Aryee, A. N. A., et al., 2007. Lipase fraction from the viscera of grey mullet (*Mugil cephalus*) - Isolation, partial purification and some biochemical characteristics. Enzyme and Microbial Technology. 40, 394-402.

Atha, D. H., Ingham, K. C., 1981. Mechanism of precipitation of proteins by polyethylene glycols - analysis in terms of excluded volume. Journal of Biological Chemistry. 256, 2108-2117.

Ban, K., et al., 2001. Whole cell biocatalyst for biodiesel fuel production utilizing *Rhizopus oryzae* cells immobilized within biomass support particles. Biochemical Engineering Journal. 8, 39-43.

Bertolini, M. C., et al., 1995. Expression and characterization of *Geotrichum-candidum* Lipase-I Gene - Comparison of Specificity Profile with Lipase-Ii. European Journal of Biochemistry. 228, 863-869.

Bhat, R., Timasheff, S. N., 1992. Steric exclusion is the principal source of the preferential hydration of proteins in the presence of polyethylene glycols. Protein Science. 1, 1133-1143.

Bier, M., 1955. Lipase. Methods in Enzymology. 1, 627-642.

Bigelow, H. B., Schroeder, W. C., 1953. Fishes of the Gulf of Maine. Fish. Bull. 53, 120-121.

Blackberg, L., et al., 1981. Bile salt-stimulated lipase in human-milk and carboxyl ester hydrolase in pancreatic-juice - are they identical enzymes. Febs Letters. 136, 284-288.

Borlongan, I. G., 1990. Studies on the digestive lipases of milkfish, chanos-chanos. Aquaculture. 89, 315-325.

Boyer, P. D. (Ed.) 1972. The Lipases, The Enzymes. Academic Press, New York.

Bradford, M. M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry. 72, 248-254.

Breivik, H., et al., 1997. Preparation of highly purified concentrates of eicosapentaenoic acid and docosahexaenoic acid. Journal of the American Oil Chemists Society. 74, 1425-1429.

Brockerh.H, 1970. Substrate specificity of pancreatic lipase influence of structure of fatty acids on reactivity of esters. Biochimica et Biophysica Acta. 212, 92-101.

Brokerhoff, H. a. J., R.G., Lipolytic Enzyme. Academic Press, New York, 1974, pp. 32-34.

Carman, G. M., et al., 1995. Lipid signaling enzymes and surface dilution kinetics. Journal of Biological Chemistry. 270, 18711-18714.

Carpenter, J. F., Crowe, J. H., 1988. The mechanism of cryoprotection of proteins by solutes. Cryobiology. 25, 244-255.

Carriere, F., et al., 1997. Molecular evolution of the pancreatic lipase and two related enzymes towards different substrate selectivities. Journal of Molecular Catalysis B-Enzymatic. 3, 55-64.

Carriere, F., et al., Gastric lipases: cellular, biochemical and kinetic aspects. In: P. Woolley, Peterson,S.B., (Ed.), Lipases--their structure, biochemistry, and application. Cambridge University Press, Cambridge, 1994, pp. 181-205.

Carriere, F., et al., 1998. Structural basis for the substrate selectivity of pancreatic lipases and some related proteins. Biochimica et Biophysica Acta-Reviews on Biomembranes. 1376, 417-432.

Castellanos, I. J., et al., 2002. Encapsulation-induced aggregation and loss in activity of gamma-chymotrypsin and their prevention. Journal of Controlled Release. 81, 307-319.

Chandler, I. C., et al., 1998. Lipase-catalyzed synthesis of chiral triglycerides. Journal of the American Oil Chemists Society. 75, 1513-1518.

Chaplin, M. F., Bucke, C., The large scale use of enzymes in solution. In: M. F. Chaplin, C. Bucke, Eds.), Enzyme technology. Cambridge University Press, London, UK, 1990, pp. 138-166.

Choi, S. Y., et al., 2002. Endothelial lipase: a new lipase on the block. Journal of Lipid Research. 43, 1763-1769.

Cygler, M., Schrag, J. D., Structure as basis for understanding interfacial properties of lipases. Lipases, Part A, 1997, pp. 3-27.

de Maria, P. D., et al., 2005. Biotechnological applications of *Candida antarctica* lipase A: State-of-the-art. Journal of Molecular Catalysis B-Enzymatic. 37, 36-46.

Deems, R. A., et al., 1975. Kinetic-analysis of phospholipase-A2 activity toward mixed micelles and its implications for study of lipolytic enzymes. Journal of Biological Chemistry. 250, 9013-9020.

Derewenda, Z. S., Cambillau, C., 1991. Effects of gene-mutations in lipoprotein and hepatic lipases as interpreted by a molecular-model of the pancreatic triglyceride lipase. Journal of Biological Chemistry. 266, 23112-23119.

Derewenda, Z. S., Sharp, A. M., 1993. News from the interface - the molecular-structures of triacylglyceride lipases. Trends in Biochemical Sciences. 18, 20-25.

DeVecchi, S., Coppes, Z., 1996. Marine fish digestive proteases - Relevance to food industry and the south-west Atlantic region - A review. Journal of Food Biochemistry. 20, 193-214.

Divakaran, S., et al., 1999. Digestive enzymes present in Pacific threadfin Polydactylus sexfilis (Bloch & Schneider 1801) and bluefin trevally *Caranx melampygus* (Cuvier 1833). Aquaculture Research. 30, 781-787.

Egloff, M. P., et al., 1995. The 2.46-angstrom resolution structure of the pancreatic lipase-colipase complex inhibited by a c-11 alkyl phosphonate. Biochemistry. 34, 2751-2762.

Ferrato, F., et al., A critical reevaluation of the phenomenon of interfacial activation. Lipases, Pt B, 1997, pp. 327-347.

Fojan, P., et al., 2000. What distinguishes an esterase from a lipase: A novel structural approach. Biochimie. 82, 1033-1041.

Fomuso, L. B., Akoh, C. C., 1998. Structured lipids: Lipase-catalyzed interesterification of tricaproin and trilinolein. Journal of the American Oil Chemists Society. 75, 405-410.

Fox, P. F., Wallace, J. M., Formation of flavor compounds in cheese. Advances in Applied Microbiology, Vol 45, 1997, pp. 17-85.

Freedonia, World Enzymes to 2011. Vol. 2008. The Freedonia Group, Cleveland, OH, 2007.

Fritz, R. L., 1965. Autumn distribution of groundfish species in the Gulf of Maine and adjacent waters, 1955-1961. Serial Atlas of the Marine Environment, Folio. 10.

Fukuda, H., et al., 2001. Biodiesel fuel production by transesterification of oils. Journal of Bioscience and Bioengineering. 92, 405-416.

Gargouri, Y., et al., 1997. Covalent inhibition of digestive lipases: An in vitro study. Biochimica et Biophysica Acta-Lipids and Lipid Metabolism. 1344, 6-37.

Gilbert, E. J., 1993. Pseudomonas lipases - biochemical-properties and molecular-cloning. Enzyme and Microbial Technology. 15, 634-645.

Giller, T., et al., 1992. 2 novel human pancreatic lipase related proteins, hplrp1 and hplrp2 - differences in colipase dependence and in lipase activity. Journal of Biological Chemistry. 267, 16509-16516.

Gjellesvik, D. R., 1991. Fatty-acid specificity of bile salt-dependent lipase - enzyme recognition and super-substrate effects. Biochimica et Biophysica Acta. 1086, 167-172.

Gjellesvik, D. R., et al., 1992. Pancreatic bile-salt dependent lipase from cod (*Gadus morhua*) - purification and properties. Biochimica Et Biophysica Acta. 1124, 123-134.

Gjellesvik, D. R., et al., 1989. Partial-purification and characterization of a triglyceride lipase from cod (*Gadus morhua*). Aquaculture. 79, 177-184.

Grimonprez, B., Johansson, G., 1995. Liquid-liquid partitioning of some enzymes, especially phosphofuctokinase, from *Saccharomyces cerevisiae* at subzero temperature. Journal of Chromatography B: Biomedical Sciences and Applications. 680, 55-63.

Grusby, M. J., et al., 1990. Cloning of an interleukin-4 inducible gene from cytotoxic lymphocytes-t and its identification as a lipase. Cell. 60, 451-459.

Guncheva, M., et al., 2007. Effect of nonionic detergents on the activity of a thermostable lipase from *Bacillus stearothermophilus* MC7. Journal of Molecular Catalysis B-Enzymatic. 49, 88-91.

Gupta, R., et al., 2003. Lipase mediated upgradation of dietary fats and oils. Critical Reviews in Food Science and Nutrition. 43, 635-644.

Guys, R. C. E., Sahi, S. S., 2006. Application of a lipase in cake manufacture. Journal of the Science of Food and Agriculture. 86, 1679-1687.

Haard, N. F., 1998. Specialty enzymes from marine organisms. Food Technology. 52, 64-67.

Haraldsson, G. G., et al., 1989. The preparation of triglycerides highly enriched with omega-3 poly-unsaturated fatty-acids via lipase catalyzed interesterification. Tetrahedron Letters. 30, 1671-1674.

Harmon, J. S., et al., 1991. Purification and characterization of hepatic triacylglycerol lipase isolated from rainbow-trout, *Oncorhynchus mykiss*. Fish Physiology and Biochemistry. 9, 361-368.

Hasan, F., et al., 2006. Industrial applications of microbial lipases. Enzyme and Microbial Technology. 39, 235-251.

Hedeman, H., et al., 1996. Fat emulsions based on structured lipids (1,3-specific triglycerides): An investigation of the in vivo fate. Pharmaceutical Research. 13, 725-728.

Heimerma, W. H., et al., 1973. Effect of double-bond position in octadecenoates upon hydrolysis by pancreatic lipase. Lipids. 8, 45-46.

Hide, W. A., et al., 1992. Structure and evolution of the lipase superfamily. Journal of Lipid Research. 33, 167-178.

Hixenbaugh, E. A., et al., 1989. Hepatic lipase in the rat ovary - ovaries cannot synthesize hepatic lipase but accumulate it from the circulation. Journal of Biological Chemistry. 264, 4222-4230.

Hou, C. T., Industrial Uses of Lipases. In: H. W. G. Tsung Min Kuo, (Ed.), Lipid biotechnology. Marcel Dekker, New York 2002, pp. 387-397.

Huang, A. H. C., Plant lipases. In: B. Borgström, H. L. Brockman., Eds.), Lipases. Elsevier, Amsterdam, 1984, pp. 419-442.

Hui, D. Y., 1996. Molecular biology of enzymes involved with cholesterol ester hydrolysis in mammalian tissues. Biochimica et Biophysica Acta-Lipids and Lipid Metabolism. 1303, 1-14.

Hui, D. Y., Howles, P. N., 2002. Carboxyl ester lipase: structure-function relationship and physiological role in lipoprotein metabolism and atherosclerosis. Journal of Lipid Research. 43, 2017-2030.

Iijima, N., et al., 1998. Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiology and Biochemistry. 18, 59-69.

Ingham, K. C., 1990. Precipitation of Proteins with Polyethylene-Glycol. Methods in Enzymology. 182, 301-306.

Innis, S. M., et al., 1995. Palmitic acid is absorbed as sn-2 monopalmitin from milk and formula with rearranged triacylglycerols and results in increased plasma triglyceride sn-2 and cholesteryl ester palmitate in piglets. Journal of Nutrition. 125, 73-81.

Iwasaki, Y., Yamane, T., 2000. Enzymatic synthesis of structured lipids. Journal of Molecular Catalysis B-Enzymatic. 10, 129-140.

Jaeger, K. E., et al., 1994. Bacterial Lipases. Fems Microbiology Reviews. 15, 29-63.

Jain, M. K., Berg, O. G., 1989. The kinetics of interfacial catalysis by phospholipase-a2 and regulation of interfacial activation - hopping versus scooting. Biochimica et Biophysica Acta. 1002, 127-156.

Jensen, R. G., 1983. Detection and determination of lipase (acylglycerol hydrolase) activity from various sources. Lipids. 18, 650-657.

Jensen, R. G., et al., 1982. Stereospecificity of premature human infant lingual lipase. Lipids. 17, 570-572.

Jonas, R. E. E., Bilinski, E., 1964. Utilization of lipids by fish .3. fatty acid oxidation by various tissues from sockeye salmon (*Oncorhynchus nerka*). Journal of the Fisheries Research Board of Canada. 21, 653-656.

Kaieda, M., et al., 2001. Effect of methanol and water contents on production of biodiesel fuel from plant oil catalyzed by various lipases in a solvent-free system. Journal of Bioscience and Bioengineering. 91, 12-15.

Kaieda, M., et al., 1999. Biodiesel fuel production from plant oil catalyzed by Rhizopus oryzae lipase in a water-containing system without an organic solvent. Journal of Bioscience and Bioengineering. 88, 627-631.

Kallner, A., 1968. Bile acids in bile of cod *gadus callarias* - hydroxylation of deoxycholic acid and chenodeoxycholic acid in homogenates of cod liver . Bile acids and steroids 200. Acta Chemica Scandinavica. 22, 2361-&.

Karpe, F., et al., 1993. Composition of human low-density-lipoprotein - effects of postprandial triglyceride-rich lipoproteins, lipoprotein-lipase, hepatic lipase and cholesteryl ester transfer protein. Atherosclerosis. 98, 33-49.

Kilcawley, K. N., et al., 1998. Enzyme-modified cheese. International Dairy Journal. 8, 1-10.

Kirchgessner, T. G., et al., 1989. Organization of the human lipoprotein-lipase gene and evolution of the lipase gene family. Proceedings of the National Academy of Sciences of the United States of America. 86, 9647-9651.

Kordel, M., et al., 1991. Extracellular lipase of *Pseudomonas* sp strain atcc-21808 - purification, characterization, crystallization, and preliminary-x-ray diffraction data. Journal of Bacteriology. 173, 4836-4841.

Latha, K., Ramarethinam, S., 1999. Studies on lipid acyl hydrolases during tea processing. Annals of Plant Physiology. 13, 73-78.

Lee, J. C., Lee, L. L. Y., 1981. Preferential solvent interactions between proteins and polyethylene glycols. Journal of Biological Chemistry. 256, 625-631.

Lee, K. T., Akoh, C. C., 1998. Structured lipids: synthesis and applications. Food Reviews International. 14, 17-34.

Leger, C., 1972. Function of specific bile acids in cholesterol esterase activity in vitro. Annales De Biologie Animale Biochimie Biophysique. 12, 431-&.

Leger, C., et al., 1977a. Some properties of pancreatic lipase in *Salmo-gairdnerii* Rich — K_m, effects of bile-salts and Ca^{2+}, gel filtrations. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology. 57, 359-363.

Leger, C., et al., 1977b. Specific distribution of fatty-acids in triglycerides of rainbow-trout adipose-tissue - influence of temperature. Lipids. 12, 538-543.

Leger, C., et al., 1979. Lipase and colipase in the rainbow-trout - some recent results. Annales De Biologie Animale Biochimie Biophysique. 19, 825-832.

Liang, X. F., et al., 2002. Effect of dietary fatty acids on lipoprotein lipase gene expression in the liver and visceral adipose tissue of fed and starved red sea bream *Pagrus major*. Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology. 132, 913-919.

Lie, O., Lambertsen, G., 1985. Digestive lipolytic enzymes in cod (*Gadus-morhua*) - fatty-acid specificity. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology. 80, 447-450.

Lie, O., Lambertsen, G., 1991. Lipid digestion and absorption in cod (*Gadus morhua*), comparing triacylglycerols, wax esters and diacylalkylglycerols. Comparative Biochemistry and Physiology a-Physiology. 98, 159-163.

Lie, O., et al., 1987. Lipid digestion in cod (*Gadus-morhua*). Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology. 88, 697-700.

Lima, V. M. G., et al., 2004. Activity and stability of a crude lipase from *Penicillium aurantiogriseum* in aqueous media and organic solvents. Biochemical Engineering Journal. 18, 65-71.

Lombardo, D., et al., 1978. Purification and characterization of a carboxyl ester hydrolase from human pancreatic-juice. Biochimica et Biophysica Acta. 527, 142-149.

Lowe, M. E., 1997. Molecular mechanisms of rat and human pancreatic triglyceride lipases. Journal of Nutrition. 127, 549-557.

Lowe, M. E., 2002. The triglyceride lipases of the pancreas. Journal of Lipid Research. 43, 2007-2016.

Macrae, A. R., 1983. Lipase-Catalyzed Interesterification of Oils and Fats. Journal of the American Oil Chemists Society. 60, 291-294.

Malmos, H., 1990. Enzymes for detergents. Chemistry & Industry. 183-186.

Malzert, A., et al., 2003. Spectroscopic studies on poly(ethylene glycol)-lysozyme interactions. International Journal of Pharmaceutics. 260, 175-186.

Manooch Iii, C. S., Fisherman Guide: Fishes of the Southeastern United States. North Carolina State Museum of Natural History Raleigh, NC (USA), 1984.

Marangoni, A. D., Lipases: Structure, Function, and Properties. In: T. M. Kou, H. W. Gardner, Eds.), Lipid Biotechnology. Marcel Dekker, New York 2002, pp. 357-386.

Martinez, C., et al., 1992. *Fusarium solani* cutinase is a lipolytic enzyme with a catalytic serine accessible to solvent. Nature. 356, 615-618.

Merkel, M., et al., 2002. Lipoprotein lipase: genetics, lipid uptake, and regulation. Journal of Lipid Research. 43, 1997-2006.

Mogensen, J. E., et al., 2005. Activation, inhibition, and destabilization of *Thermomyces lanuginosus* lipase by detergents. Biochemistry. 44, 1719-1730.

Mukundan, M. K., et al., 1985. Purification of a Lipase from the Hepatopancreas of Oil Sardine (*Sardinella longiceps* Linnaeus) and Its Characteristics and Properties. Journal of the Science of Food and Agriculture. 36, 191-203.

Muralidhar, R. V., et al., 2002. Understanding lipase stereoselectivity. World Journal of Microbiology & Biotechnology. 18, 81-97.

Nagao, T., et al., 1996. Expression of lipase cDNA from *Fusarium heterosporum* by *Saccharomyces cerevisiae*: High-level production and purification. Journal of Fermentation and Bioengineering. 81, 488-492.

Nayak, J., et al., 2003. Lipase activity in different tissues of four species of fish: rohu (*Labeo rohita* Hamilton), oil sardine (*Sardinella longiceps* Linnaeus), mullet (*Liza subviridis* Valenciennes) and Indian mackerel (*Rastrelliger kanagurta* Cuvier). Journal of the Science of Food and Agriculture. 83, 1139-1142.

Noble, M. E. M., et al., 1993. The crystal-structure of triacylglycerol lipase from pseudomonas-glumae reveals a partially redundant catalytic aspartate. Febs Letters. 331, 123-128.

O'connor, C. J., Wallace, R. G., 1984. Studies in bile-salt solutions - deoxycholate stimulation of human-milk lipase. Febs Letters. 170, 375-377.

Ogino, H., et al., 1999a. Growth of organic solvent-tolerant *Pseudomonas aeruginosa* LST-03 in the presence of various organic solvents and production of lipolytic enzyme in the presence of cyclohexane. Biochemical Engineering Journal. 4, 1-6.

Ogino, H., et al., 1999b. Purification and characterization of organic solvent-stable protease from organic solvent-tolerant *Pseudomonas aeruginosa* PST-01. Journal of Bioscience and Bioengineering. 87, 61-68.

Ollis, D. L., et al., 1992. The alpha/beta-hydrolase fold. Protein Engineering. 5, 197-211.

Palekar, A. A., et al., 2000. Purification of lipase: A review. Biocatalysis and Biotransformation. 18, 177-200.

Pancera, S. M., et al., 2002. The effect of poly(ethylene glycol) on the activity and structure of glucose-6-phosphate dehydrogenase in solution. Colloids and Surfaces B-Biointerfaces. 26, 291-300.

Pancera, S. M., et al., 2004. The effect of poly(ethylene glycol) on the activity, structural conformation and stability of yeast hexokinase. Progress in Colloid and Polymer Science. 128, 178-183.

Patton, J. S., 1975. High levels of pancreatic nonspecific lipase in rattlesnake and leopard shark. Lipids. 10, 562-564.

Patton, J. S., Nevenzel, J. C., 1974. Fat digestion in fish. Journal of the American Oil Chemists Society. 51, A521-A521.

Patton, J. S., et al., 1977. Partial characterization of bile salt-dependent triacylglycerol lipase from leopard shark pancreas. Biochimica et Biophysica Acta. 486, 322-330.

Perret, B., et al., 2002. Hepatic lipase: structure/function relationship, synthesis, and regulation. Journal of Lipid Research. 43, 1163-1169.

Raa, J., Biotechnology in aquaculture and the fish processing industry: a success story in Norway. In: M. N. Voigt, J. R. Botta, Eds.), Advances in fisheries technology for increased profitability. Technomic Publication Company, Lancaster, PA, 1990, pp. 509-524.

Rader, D. J., Jaye, M., 2000. Endothelial lipase: a new member of the triglyceride lipase gene family. Current Opinion in Lipidology. 11, 141-147.

Ramarethinam, S., et al., 2002. Use of a fungal lipase for enhancement of aroma in black tea. Food Science and Technology Research. 8, 328-332.

Rasco, B. A., Hultin, H. O., 1988. A comparison of dogfish and porcine pancreatic lipases. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology. 89, 671-677.

Riaublanc, A., et al., 1993. Peculiar properties of lipase from *Candida-parapsilosis* (Ashford) langeron and talice. Journal of the American Oil Chemists Society. 70, 497-500.

Rogalska, E., et al., 1993. Stereoselective hydrolysis of triglycerides by animal and microbial lipases. Chirality. 5, 24-30.

Roussel, A., et al., 1998. Structure and activity of rat pancreatic lipase-related protein 2. Journal of Biological Chemistry. 273, 32121-32128.

Rubin, B., Dennis, E. A. Eds.), 1997. Methods in Enzymology. Academic Press, New York.

Saijyo, R., Takeo, T., 1972. Importance of linoleic acid and linolenic acid as precursors of hexanal and trans-2-hexenal in black tea. Plant and Cell Physiology. 13, 991-998.

Sato, T., et al., 1997. Serine phospholipid-specific phospholipase A that is secreted from activated platelets - A new member of the lipase family. Journal of Biological Chemistry. 272, 2192-2198.

Saxena, R. K., et al., 1999. Microbial lipases: Potential biocatalysts for the future industry. Current Science. 77, 101-115.

Schrag, J. D., Cygler, M., Lipases and alpha/beta hydrolase fold. Lipases, Part A, 1997, pp. 85-107.

Selmi, B., Thomas, D., 1998. Immobilized lipase-catalyzed ethanolysis of sunflower oil in a solvent-free medium. Journal of the American Oil Chemists Society. 75, 691-695.

Shahani, K. M., et al., 1976. Bovine pancreatic lipase.1. Isolation, homogeneity, and characterization. Journal of Dairy Science. 59, 369-375.

Shahidi, F., Proteins from seafood processing discards. In: Z. E. Sikorski, et al., Eds.), Seafoods Proteins. Chapman and Hall, NewYork, NY, 1994, pp. 171-193.

Shahidi, F., Kamil, Y., 2001. Enzymes from fish and aquatic invertebrates and their application in the food industry. Trends in Food Science & Technology. 12, 435-464.

Sheridan, M. A., Allen, W. V., 1984. Partial-purification of a triacylglycerol lipase isolated from steelhead trout (*Salmo gairdneri*) adipose-tissue. Lipids. 19, 347-352.

Shimada, Y., et al., 1993. Purification and characterization of a novel solvent-tolerant lipase from *Fusarium heterosporum*. Journal of Fermentation and Bioengineering. 75, 349-352.

Shimada, Y., et al., 1999. Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase. Journal of the American Oil Chemists Society. 76, 789-793.

Simpson, R. J., Precipitation of Proteins by Polyethylene Glycol. In: R. J. Simpson, (Ed.), Purifying Proteins for Proteomics: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, USA, 2004, pp. Appendix 3.

Sovik, S. L., Rustad, T., 2004. Seasonal changes in trypsin and chymotrypsin activity in viscera from cod species. Journal of Aquatic Food Product Technology. 13, 13-30.

Sovik, S. L., Rustad, T., 2005. Effect of season and fishing ground on the activity of lipases in byproducts from cod (*Gadus morhua*). Lwt-Food Science and Technology. 38, 867-876.

Sovik, S. L., Rustad, T., 2006. Effect of season and fishing ground on the activity of cathepsin B and collagenase in by-products from cod species. Lwt-Food Science and Technology. 39, 43-53.

Sugihara, A., et al., 1992. Purification and characterization of a novel thermostable lipase from *Pseudomonas cepacia*. Journal of Biochemistry. 112, 598-603.

Sutherland, B. J., 1998. Developments in cheese science and technology in Australia. Australian Journal of Dairy Technology. 53, 63-66.

Takahashi, S., et al., 1998. Extracellular production of active *Rhizopus oryzae* lipase by *Saccharomyces cerevisiae*. Journal of Fermentation and Bioengineering. 86, 164-168.

Taniguchi, A., et al., 2001a. Purification and properties of lipase from Tilapia intestine - Digestive enzyme of Tilapia - VI. Nippon Suisan Gakkaishi. 67, 78-84.

Taniguchi, A., et al., 2001b. Purification and properties of lipase from Tilapia stomach - Digestive enzyme of Tilapia - VII. Nippon Suisan Gakkaishi. 67, 96-101.

Taylor, T., Alasalvar, C., Improved Utilisation of Fish and shellfish Waste. In: C. Alasalvar, T. Taylor, Eds.), Seafoods : quality, technology, and nutraceutical applications Springer, Berlin ; New York 2002, pp. 123-136.

Thirstrup, K., et al., 1994. Evidence for a pancreatic lipase subfamily with new kinetic-properties. Biochemistry. 33, 2748-2756.

Timasheff, S. N., 2002. Protein hydration, thermodynamic binding, and preferential hydration. Biochemistry. 41, 13473-13482.

Tocher, D. R., Sargent, J. R., 1984. Studies on triacylglycerol, wax ester and sterol ester hydrolases in intestinal ceca of rainbow-trout (*Salmo gairdneri*) fed diets rich in triacylglycerols and wax esters. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology. 77, 561-571.

Tsujita, T., et al., 1987. Nonspecific high-affinity binding of bile-salts to carboxylester lipases. Journal of Lipid Research. 28, 1434-1443.

Uchida, T., et al., 1998. Microencapsulation of hepatitis B core antigen for vaccine preparation. Pharmaceutical Research. 15, 1708-1713.

Uppenberg, J., et al., 1994. Sequence, crystal-structure determination and refinement of 2 crystal forms of lipase-b from *Candida antarctica.* Structure. 2, 293-308.

Vahouny, G. V., et al., 1965. Function of specific bile acids in cholesterol esterase activity in vitro. Biochimica et Biophysica Acta. 98, 607-&.

van Groningen, J. J. M., et al., 1997. nmd, a novel gene differentially expressed in human melanoma cell lines, encodes a new atypical member of the enzyme family of lipases. Febs Letters. 404, 82-86.

Vantilbeurgh, H., et al., 1993. Interfacial activation of the lipase procolipase complex by mixed micelles revealed by X-ray crystallography. Nature. 362, 814-820.

Vasudevan, P. T., 2004. Lipases and phospholipases in drug development [electronic resource] : from biochemistry to molecular pharmacology. Wiley-VCH Weinheim.

Venugopal, V., Applications of Enzymes in Fish Processing and Quality Control. In: V. Venugopal, (Ed.), Seafood processing : adding value through quick freezing, retortable packaging, and cook-chilling CRC/Taylor & Francis, Boca Raton 2006, pp. 401-424.

Venugopal, V., Shahidi, F., 1995. Value-added products from underutilized fish species. Critical Reviews in Food Science and Nutrition. 35, 431-453.

Vergara, A., et al., 2002. Mechanism of protein-poly(ethylene glycol) interaction from a diffusive point of view. Macromolecules. 35, 1389-1398.

Verger, R., Pancreatic lipase. In: B. Borgström, H. L. Brockman, Eds.), Lipases. Elsevier Amsterdam, 1984, pp. 84-150.

Verger, R., 1997. 'Interfacial activation' of lipases: Facts and artifacts. Trends in Biotechnology. 15, 32-38.

Verger, R., Dehaas, G. H., 1976. Interfacial enzyme-kinetics of lipolysis. Annual Review of Biophysics and Bioengineering. 5, 77-117.

Verger, R., et al., 1982. Novel intestinal phospholipase-a2 purification and some molecular characteristics. Biochemistry. 21, 6883-6889.

Verger, R., Haas, G. H. D., 1973. Enzyme reactions in a membrane model .1. New technique to study enzyme reactions in monolayers. Chemistry and Physics of Lipids. 10, 127-136.

Verger, R., et al., 1973. Action of phospholipase a at interfaces. Journal of Biological Chemistry. 248, 4023-4034.

Wang, C. S., Hartsuck, J. A., 1993. Bile salt-activated lipase - a multiple function lipolytic enzyme. Biochimica et Biophysica Acta. 1166, 1-19.

Wang, C. S., et al., 1988. Kinetics of acylglycerol sequential hydrolysis by human-milk bile-salt activated lipase and effect of taurocholate as fatty-acid acceptor. Biochemistry. 27, 4834-4840.

Wang, C. S., et al., 1983. Studies on the substrate-specificity of purified human-milk bile salt-activated lipase. Journal of Biological Chemistry. 258, 9197-9202.

Whitaker, J. R., 1972. Principles Of Enzymology For The Food Sciences. Marcel Dekker Inc., NY.

Whitaker, J. R., Effects of temperature on rates of enzyme catalyzed reactions. In: J. R. Whitaker, (Ed.), Principles of enzymology for the food sciences. Marcel Dekker, New York, 1994, pp. 301-328.

Winkler, U. K., Stuckmann, M., 1979. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. Journal of Bacteriology. 138, 663-670.

Wong, D. W. S., 1995. Food enzymes : structure and mechanism Chapman & Hall, New York

Wong, H., Schotz, M. C., 2002. The lipase gene family. Journal of Lipid Research. 43, 993-999.

Wu, W. H., et al., 1999. Optimizing production of ethyl esters of grease using 95% ethanol by response surface methodology. Journal of the American Oil Chemists Society. 76, 517-521.

Zolfaghari, R., et al., 1989. Expression in xenopus oocytes of rat-liver messenger-RNA coding for a bile salt-dependent cholesteryl ester hydrolase. Proceedings of the National Academy of Sciences of the United States of America. 86, 6913-6916.

APPENDICES

Raw Data

Porgy lipase pH activity test

pH 6.5

Sample 1 = 0.023002, Sample 2 = 0.021732, Sample 3 = 0.026952,

Sample 4 = 0.024807

Average Activity (IU/ml) = 0.024123583

*STD = 0.002268956

Relative Activity STD (compared to pH 8.5) = 0.011168

pH 7

Sample 1= 0.176599, Sample 2=0.174689 , Sample 3=0.151589,

Sample 4 = 0.170638

Average Activity (IU/ml) = 0.168379

STD = 0.011466

Relative Activity STD (compared to pH 8.5) = 0.056435

pH 7.5

Sample 1 =0.168739 , Sample 2= 0.181725, Sample 3= 0.163456,

Sample 4= 0.178662

Average Activity (IU/ml) = 0.173146

STD = 0.008512

Relative Activity STD (compared to pH 8.5) = 0.041896

pH 8

Sample 1= 0.192187l, Sample 2= 0.196204, Sample3= 0.173236,

Sample 4= 0.186558

Average Activity (IU/ml) = 0.187046

STD = 0.010021

Relative Activity STD (compared to pH 8.5) = 0.049323

pH 8.5

Sample 1 = 0.203787, Sample 2= 0.19273, Sample 3 = 0.209107,

Sample 4= 0.207048

Average Activity (IU/ml) = 0.203168

STD = 0.007295

Relative Activity STD (compared to pH 8.5) = 0.035907

pH 9

Sample 1 = 0.191601, Sample 2= 0.166263, Sample 3= 0.16737,

Sample 4= 0.171657

Average Activity (IU/ml) = 0.174223

STD = 0.011817

Relative Activity STD (compared to pH 8.5) = 0.058163

pH 9.5

Sample 1= 0.074694, Sample 2= 0.087857, Sample 3=0.081935,

Sample 4 = 0.081811

Average Activity (IU/ml) = 0.081574

STD = 0.005385

Relative Activity STD (compared to pH 8.5) = 0.026506

pH 10

Sample 1 = 0.001056, Sample 2= 0.00194, Sample 3= 0.00149,

Sample 4 = 0.000952

Average Activity (IU/ml)= 0.001359

STD = 0.000452

Relative Activity STD (compared to pH 8.5) = 0.002223

pH 10.5

Sample 1 =0.008588, Sample 2 = 0.004179, Sample 3 = 0.006969,

Sample 4 = 0.006947

Average Activity (IU/ml)= 0.006671

STD = 0.00183

Relative Activity STD (compared to pH 8.5) = 0.009009

pH 11

Sample 1 = 0.00599, Sample 2 = 0.006102, Sample 3= 0.004963 ,

Sample 4 = 0.006303

Average Activity (IU/ml) = 0.005839

STD = 0.000599

Relative Activity STD (compared to pH 8.5) = 0.002946

pH12

Sample 1= 0.013828, Sample 2 = 0.011215, Sample 3 = 0.010698,

Sample 4 = 0.012934

Average Activity (IU/ml) = 0.012169

STD = 0.001462

Relative Activity STD (compared to pH 8.5) = 0.007194

Porgy lipase pH stability test

pH 4

Sample 1 = 0.00943, Sample 2= 0.011607, Sample 3 = 0.009889

Average IU Activity (IU/ml) = 0.010309

STD* = 0.001147

Relative Activity STD (compared to pH 6) = 0.028156

pH5

Sample 1 = 0.027007, Sample 2 = 0.026565, Sample 3 = 0.027075

Average IU Activity (IU/ml) = 0.026882

STD = 0.000277

Relative Activity STD (compared to pH 6) = 0.006797

pH 6

Sample 1 = 0.040649, Sample 2 = 0.038869, Sample 3 = 0.042745

Average IU Activity (IU/ml) = 0.040754

STD = 0.00194

Relative Activity STD (compared to pH 6) = 0.04761

pH 7

Sample 1= 0.03744, Sample 2 = 0.034162, Sample 3 = 0.033891

Average IU Activity (IU/ml) = 0.035165

STD = 0.001976

Relative Activity STD (compared to pH 6) = 0.048475

pH 8

Sample 1 = 0.030995, Sample 2 = 0.029775, Sample 3 = 0.029843

Average IU Activity (IU/ml) = 0.030204

STD = 0.000686

Relative Activity STD (compared to pH 6) = 0.016825

pH 9

Sample 1 = 0.029154, Sample 2 = 0.030485, Sample 3 = 0.031837

Average IU Activity (IU/ml) = 0.030492

STD= 0.001341

Relative Activity STD (compared to pH 6) = 0.032915

pH 10

Sample 1 = 0.029252, Sample 2 = 0.027935, Sample 3 = 0.027635

Average IU Activity (IU/ml) = 0.028274

STD = 0.00086

Relative Activity STD (compared to pH 6)= 0.021102

pH 11

Sample 1 = 0.012798, Sample 2 = 0.013427, Sample 3 = 0.012912

Average IU Activity (IU/ml) = 0.012798

STD = 0.000335

Relative Activity STD (compared to pH 6) = 0.008227

Porgy lipase temperature activity test

10°C

Sample 1 =0.092225 , Sample 2 = 0.088517, Sample 3 = 0.083057

Average Activity (IU/ml) = 0.087933

STD = 0.004612

Relative Activity STD (Compare to 40°C) = 0.036298

15°C

Sample 1 = 0.101388, Sample 2 = 0.102466, Sample 3 = 0.100376

Average Activity (IU/ml) = 0.10141

STD = 0.001045

Relative Activity STD (Compare to 40°C) = 0.008225

20°C

Sample 1 = 0.116201, Sample 2 = 0.110447, Sample 3 = 0.10225

Average Activity (IU/ml) = 0.109633

STD = 0.007013

Relative Activity STD (Compare to 40°C) = 0.055176

25°C

Sample 1 = 0.117098, Sample 2 = 0.115166, Sample 3 = 0.11707

Average Activity (IU/ml) = 0.116444

STD = 0.001108

Relative Activity STD (Compare to 40°C) = 0.008717

30°C

Sample 1 =0.117182, Sample 2 = 0.123413, Sample 3 = 0.121779

Average Activity (IU/ml) = 0.120791

STD= 0.003231

Relative Activity STD (Compare to 40°C) = 0.025426

35°C

Sample 1 = 0.123642, Sample 2 = 0.125249, Sample 3 = 0.123275

Average Activity (IU/ml) = 0.124056

STD = 0.00105

Relative Activity STD (Compare to 40°C)= 0.008264

40°C

Sample 1 = 0.125986, Sample 2 =0.126463, Sample 3 = 0.128748299

Average Activity (IU/ml) = 0.127066

STD = 0.001476

Relative Activity STD (Compare to 40°C) = 0.01162

45°C

Sample 1 = 0.110748, Sample 2 = 0.120544, Sample 3 = 0.11776

Average Activity (IU/ml) = 0.116351

STD = 0.005048

Relative Activity STD (Compare to 40°C) = 0.039725

50°C

Sample 1 = 0.08039, Sample 2 = 0.074385, Sample 3 = 0.075456

Average Activity (IU/ml) = 0.076744

STD = 0.003203

Relative Activity STD (Compare to 40°C) = 0.025206

55°C

Sample 1 = 0.059293, Sample 2 = 0.056671, Sample 3 = 0.059011

Average Activity (IU/ml) = 0.058325

STD = 0.001439

Relative Activity STD (Compare to 40°C) = 0.011326

60°C

Sample 1 = 0.046103, Sample 2 = 0.048317, Sample 3 = 0.045821

Average Activity (IU/ml) = 0.046747

STD = 0.001367

Relative Activity STD (Compare to 40°C) = 0.010759

Porgy lipase thermostability test

Control

Sample 1 = 0.219839, Sample 2 = 0.223489, Sample 3 = 0.216259

Average Activity (IU/ml) = 0.219862

STD = 0.003616

10°C incubate for 10 minutes

Sample 1 = 0.215359, Sample 2 = 0.215646, Sample 3 = 0.201673

Average Activity (IU/ml) = 0.210893

STD = 0.007986

Relative Activity STD (Compare to control) = 0.036321

10°C incubate for 30 minutes

Sample 1 = 0.196527, Sample 2 = 0.215566, Sample 3 = 0.194534

Average Activity (IU/ml) = 0.202209

STD = 0.011611

Relative Activity STD (Compare to control) = 0.052808

10°C incubate for 60 minutes

Sample 1 = 0.196162, Sample 2 = 0.218159, Sample 3 = 0.205361

Average Activity (IU/ml) = 0.206561

STD = 0.011048

Relative Activity STD (Compare to control) = 0.050247

20°C incubate for 10 minutes

Sample 1 = 0.215363, Sample 2 = 0.206301 , Sample 3 = 0.204592

Average Activity (IU/ml) = 0.208752

STD = 0.005789

Relative Activity STD (Compare to control) = 0.026329

20°C incubate for 30 minutes

Sample 1 =0.203043, Sample 2 =0.204078, Sample 3 = 0.181962323

Average Activity (IU/ml) = 0.196361

STD = 0.012481

Relative Activity STD (Compare to control) = 0.056766

20°C incubate for 60 minutes

Sample 1 = 0.199422, Sample 2 =0.193612 , Sample 3 = 0.208439

Average Activity (IU/ml) = 0.200491

STD = 0.007471

Relative Activity STD (Compare to control) = 0.03398

30°C incubate for 10 minutes

Sample 1 = 0.208939, Sample 2 =0.19068, Sample 3 = 0.214059

Average Activity (IU/ml) = 0.204559

STD = 0.012289

Relative Activity STD (Compare to control) = 0.055895

30°C incubate for 30 minutes

Sample 1 = 0.190959, Sample 2 = 0.210994, Sample 3 = 0.181558

Average Activity (IU/ml) = 0.194504

STD = 0.015035

Relative Activity STD (Compare to control) = 0.068383

30°C incubate for 60 minutes

Sample 1 =0.166789, Sample 2 = 0.164626, Sample 3 = 0.165735

Average Activity (IU/ml) = 0.165717

STD = 0.001082

Relative Activity STD (Compare to control) = 0.00492

40°C incubate for 10 minutes

Sample 1 = 0.125531, Sample 2 = 0.120694, Sample 3 = 0.124463

Average Activity (IU/ml) = 0.123562

STD = 0.002541

Relative Activity STD (Compare to control) = 0.011557

40°C incubate for 30 minutes

Sample 1 = 0.057061, Sample 2 = 0.050388, Sample 3 = 0.052279

Average Activity (IU/ml) = 0.053243

STD = 0.00344

Relative Activity STD (Compare to control) = 0.015644

40°C incubate for 60 minutes

Sample 1 =0.029388, Sample 2 = 0.022646, Sample 3 = 0.027592

Average Activity (IU/ml) = 0.026542

STD= 0.003491

Relative Activity STD (Compare to control) = 0.015879

50°C incubate for 10 minutes

Sample 1 = 0.130473, Sample 2 = 0.121565, Sample 3 = 0.109218

Average Activity (IU/ml) = 0.120418

STD = 0.010674

Relative Activity STD (Compare to control) = 0.048548

50°C incubate for 30 minutes

Sample 1 = 0.04119, Sample 2 = 0.035551, Sample 3 = 0.032331

Average Activity (IU/ml) = 0.036357

STD = 0.004485

Relative Activity STD (Compare to control) = 0.020397

50°C incubate for 60 minutes

Sample 1 = 0.018844, Sample 2 = 0.017177, Sample 3 = 0.015568

Average Activity (IU/ml) = 0.017196

STD = 0.001638

Relative Activity STD (Compare to control) = 0.007449

60°C incubate for 10 minutes

Sample 1 =0.097331, Sample 2 = 0.081044, Sample 3 = 0.074484

Average Activity (IU/ml) = 0.084286

STD = 0.011763

Relative Activity STD (Compare to control) = 0.053504

60°C incubate for 30 minutes

Sample 1 = 0.02982, Sample 2 = 0.026486395, Sample 3 = 0.021278

Average Activity (IU/ml) = 0.025861

STD = 0.004305

Relative Activity STD (Compare to control) = 0.01958

60°C incubate for 60 minutes

Sample 1 = 0.013249, Sample 2 = 0.013439, Sample 3 = 0.007099

Average Activity (IU/ml) = 0.011262

STD = 0.003607

Relative Activity STD (Compare to control) = 0.016406

70°C incubate for 10 minutes

Sample 1 = 0.027381, Sample 2 = 0.034973, Sample 3 = 0.036312

Average Activity (IU/ml) = 0.032889

STD = 0.004817

Relative Activity STD (Compare to control) = 0.021907

70°C incubate for 30 minutes

Sample 1 = 0.023051, Sample 2 = 0.023995, Sample 3 = 0.021707

Average Activity (IU/ml) = 0.022918

STD = 0.00115

Relative Activity STD (Compare to control) = 0.00523

70°C incubate for 60 minutes

Sample 1 = 0.007231, Sample 2 = 0.009292, Sample 3 = 0.007179

Average Activity (IU/ml) = 0.007903

STD = 0.001205

Relative Activity STD (Compare to control) = 0.005481

22. 80°C incubate for 10 minutes

Sample 1 = 0.023225, Sample 2 = 0.020929, Sample 3 = 0.022333

Average Activity (IU/ml) = 0.022162

STD = 0.001158

Relative Activity STD (Compare to control) = 0.005266

23. 80°C incubate for 30 minutes

Sample 1 = 0.014459, Sample 2 = 0.016548, Sample 3 = 0.015907

Average Activity (IU/ml) = 0.015638

STD = 0.00107

Relative Activity STD (Compare to control) = 0.004869

Porgy lipase detergents stability test

Control

Sample 1 = 0.057633, Sample 2 = 0.054526, Sample 3 = 0.055424

Average Activity (IU/ml) = 0.055861

STD = 0.001599

Triton X100 1mM

Sample 1 = 0.043433, Sample 2 = 0.040902, Sample 3 = 0.04151

Average Activity (IU/ml) = 0.041949

STD = 0.001321

Relative Activity STD (Compare to control) = 0.023649

Triton X100 2mM

Sample 1 = 0.035478, Sample 2 = 0.034793, Sample 3 = 0.036363

Average Activity (IU/ml) = 0.035545

STD = 0.000787

Relative Activity STD (Compare to control) = 0.014086

Tween 80 1mM

Sample 1 = 0.03868, Sample 2 = 0.03533 , Sample 3 = 0.034935

Average Activity (IU/ml) = 0.036315

STD = 0.002058

Relative Activity STD (Compare to control) = 0.036836

Tween 80 2mM

Sample 1 = 0.025882, Sample 2 = 0.022741, Sample 3 = 0.025724

Average Activity (IU/ml) = 0.024782

STD = 0.00177

Relative Activity STD (Compare to control) = 0.031684

Tween 40 1mM

Sample 1 = 0.030685, Sample 2 = 0.026569, Sample 3 = 0.031245

Average Activity (IU/ml) = 0.0295

STD = 0.002553

Relative Activity STD (Compare to control) = 0.045707

Tween 40 2mM

Sample 1 = 0.01671, Sample 2 = 0.015938, Sample 3 = 0.016875

Average Activity (IU/ml) = 0.016508

STD = 0.0005

Relative Activity STD (Compare to control) = 0.00896

Porgy lipase detergents thermostability test

Main Control (activity of the native enzyme-no additives, no thermal pre-treatment)

Sample 1 = 0.086789, Sample 2 = 0.080381, Sample 3 = 0.083422

Average Activity (IU/ml) = 0.083531

STD = 0.003205

Temperature 30°C, incubated for 30 min

30°C Control (incubated for 30min)

Sample 1 = 0.077673, Sample 2 = 0.072134, Sample 3 = 0.072509

Average Activity (IU/ml) = 0.074105

STD = 0.003096

Relative Activity STD (compare to main Control) = 0.03706

30°C Triton X-100

Sample 1 = 0.061844, Sample 2 = 0.058871, Sample 3 = 0.058488

Average Activity (IU/ml) = 0.059734

STD = 0.001837

Relative Activity STD (compare to main Control) = 0.021988611

30°C Tween 40

Sample 1 = 0.043531, Sample 2 = 0.042033, Sample 3 = 0.042033

Average Activity (IU/ml) = 0.042532

STD = 0.000864

Relative Activity STD (compare to main Control) = 0.010349427

30°C Tween 80

Sample 1 = 0.03768, Sample 2 = 0.033803, Sample 3 = 0.035973

Average Activity (IU/ml) = 0.035819

STD = 0.001943

Relative Activity STD (compare to main Control) = 0.023265241

Temperature 40°C, incubated for 30 min

40°C Control (incubated for 30min)

Sample 1 = 0.030866, Sample 2 = 0.030823487, Sample 3 = 0.026307

Average Activity (IU/ml) = 0.029332344

STD = 0.002620032

Relative Activity STD (compare to main Control) = 0.031366

40°C Triton X-100

Sample 1 = 0.026475, Sample 2 = 0.02195, Sample 3 = 0.023031

Average Activity (IU/ml) = 0.023819

STD = 0.002363

Relative Activity STD (compare to main Control) = 0.028288

40°C Tween 40

Sample 1 = 0.016981, Sample 2 = 0.018864, Sample 3 = 0.015538

Average Activity (IU/ml) = 0.017128

STD = 0.001668

Relative Activity STD (compare to main Control) = 0.019963

40°C Tween 80

Sample 1 = 0.016194, Sample 2 = 0.013673, Sample 3 = 0.01631

Average Activity (IU/ml) = 0.015392

STD = 0.00149

Relative Activity STD (compare to main Control) = 0.017833

Temperature 50°C, incubated for 30 min

50°C Control (incubated for 30min)

Sample 1 = 0.026028, Sample 2 = 0.023319, Sample 3 = 0.024379

Average Activity (IU/ml) = 0.024575

STD = 0.001365

Relative Activity STD (compare to main Control) = 0.016343

50°C Triton X-100

Sample 1 = 0.019443, Sample 2 = 0.018806, Sample 3 = 0.016392

Average Activity (IU/ml) = 0.018214

STD = 0.00161

Relative Activity STD (compare to main Control) = 0.019272

50°C Tween 40

Sample 1 = 0.012776, Sample 2 = 0.014358, Sample 3 = 0.010078

Average Activity (IU/ml) = 0.012892

STD = 0.002164

Relative Activity STD (compare to main Control) = 0.025905

50°C Tween 80

Sample 1 = 0.011074, Sample 2 = 0.006799, Sample 3 = 0.00671

Average Activity (IU/ml) = 0.008194

STD = 0.002494

Relative Activity STD (compare to main Control) = 0.029863

Temperature 60°C, incubated for 30 min

60°C Control (incubated for 30min)

Sample 1 = 0.019611, Sample 2 = 0.0176, Sample 3 = 0.01828

Average Activity (IU/ml) = 0.018497

STD = 0.001023

Relative Activity STD (compare to main Control) = 0.012246

60°C Triton X-100

Sample 1 = 0.028921, Sample 2 = 0.038141, Sample 3 = 0.039521

Average Activity (IU/ml) = 0.035527

STD = 0.005763

Relative Activity STD (compare to main Control) = 0.068991

60°C Tween 40

Sample 1 = 0.041716, Sample 2 = 0.037643, Sample 3 = 0.040284

Average Activity (IU/ml) = 0.039881

STD = 0.002066

Relative Activity STD (compare to main Control) = 0.024739

60°C Tween 80

Sample 1 = 0.036531, Sample 2 = 0.037107, Sample 3 = 0.038145

Average Activity (IU/ml) = 0.037261

STD = 0.000818

Relative Activity STD (compare to main Control) = 0.009794

Temperature 70°C, incubated for 30 min

70°C Control (incubated for 30min)

Sample 1 = 0.013247, Sample 2 = 0.015789, Sample 3 = 0.016992

Average Activity (IU/ml) = 0.015343

STD = 0.001912

Relative Activity STD (compare to main Control) = 0.022886

70°C Triton X-100

Sample 1 = 0.031594, Sample 2 = 0.029511, Sample 3 = 0.029775

Average Activity (IU/ml) = 0.030293

STD = 0.001134

Relative Activity STD (compare to main Control) = 0.013576

70°C Tween 40

Sample 1 = 0.035958, Sample 2 = 0.031273, Sample 3 = 0.032798834

Average Activity (IU/ml) = 0.033344

STD = 0.00239

Relative Activity STD (compare to main Control) = 0.028606

70°C Tween 80

Sample 1 = 0.032758, Sample 3 = 0.03225, Sample 3 = 0.030848

Average Activity (IU/ml) = 0.031952

STD = 0.000989

Relative Activity STD (compare to main Control) = 0.01184

Temperature 80°C, incubated for 30 min

80°C Control (incubated for 30min)

Sample 1 = 0.010872, Sample 2 = 0.01158, Sample 3 = 0.01101312

Average Activity (IU/ml) = 0.011155

STD = 0.000467

Relative Activity STD (compare to main Control) = 0.004487

80°C Triton X-100

Sample 1 = 0.027342, Sample 2 = 0.026664, Sample 3 = 0.027276

Average Activity (IU/ml) = 0.027094

STD = 0.000374

Relative Activity STD (compare to main Control) = 0.004475

80°C Tween 40

Sample 1 = 0.020044, Sample 2 = 0.026175, Sample 3 = 0.025381

Average Activity (IU/ml) = 0.023867

STD = 0.003335

Relative Activity STD (compare to main Control) = 0.039919

80°C Tween 80

Sample 1 = 0.027486, Sample 2 = 0.034313, Sample 3 = 0.029031

Average Activity (IU/ml) = 0.030277

STD = 0.00358

Relative Activity STD (compare to main Control) = 0.042856

Porgy lipase kinetic test

1. 0.05mM

   Sample 1 = 0.09265 , Sample 2= 0.09105 , Sample 3 = 0.10026

   Average Vi(ΔAbs/min) = 0.094653

   STD = 0.004921

2. 0.06mM

   Sample 1 = 0.10336 , Sample 2 = 0.102286 , Sample 3 = 0.10238

   Average Vi (ΔAbs/min) = 0.102675

   STD = 0.000595

3. 0.07mM

   Sample 1 = 0.11079 , Sample 2 = 0.1204, Sample 3 = 0.10169

   Average Vi (ΔAbs/min) = 0.11096

   STD = 0.009356

4. 0.1mM

   Sample 1= 0.13263, Sample 2 = 0.1231, Sample 3 = 0.1336

   Average Vi (ΔAbs/min) = 0.129777

   STD = 0.005802

5. 0.167mM

   Sample 1 = 0.14331, Sample 2 = 0.13617, Sample 3 = 0.1422

   Average Vi (ΔAbs/min) = 0.14056

   STD = 0.003842

6. 0.2505mM

   Sample 1 = 0.15787, Sample 2 = 0.147663, Sample 3 = 0.154614

   Average Vi (ΔAbs/min) = 0.153382

   STD = 0.005214

7. 0.3mM

   Sample 1 = 0.157225, Sample 2 = 0.150343, Sample 3 = 0.158757

   Average Vi (ΔAbs/min) = 0.155442

   STD = 0.004482

*STD = Standard deviation