STUDIES ON FISH LIPASES

THESIS

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DECLARATION

I hereby declare that this thesis is a record of bonafide research carried out by me under the supervision of Dr. K.Gopakumar, Scientist S-3, CIFT, Cochin-29, and that it has not previously been formed the basis for award of any degree, diploma, associateship, fellowship or other similar titles from this or any other University or Society.

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This is to certify that this thesis entitled "Studies on Fish Lipases" embodies the results of original work conducted by Shri M.K.Mukundan under my supervision and guidance from 25-2-1977 to 7-5-1982. I further certify that no part of this thesis has previously been formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles of this or any other University or Society. He has also passed the Ph.D. qualifying examination of the University of Cochin held in June 1980.

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ABBREVIATIONS USED IN THIS THESIS

ACTH	-	Adrenocortico tropic hormone
AMP	-	Adenosine 5' monophosphate
ATP	-	Adenosine triphosphate
BDH	-	British Drug House
BIS	-	Bis-acrylamide
cm	-	Centimetre
DEAE	-	Diethylaminoethyl
DFP	-	Diisopropyl Fluorophosphate
DNP	-	Diethyl-P-nitrophenyl phosphate
EDTA	-	Ethelene diamine tetraacetic acid
Exptl	-	Experimental
g	-	gram
GLC	-	Gas liquid chromatography
hr	-	hour
IUB	-	International Union of Biochemists
Km	-	Michaelis constant
M	-	Molar
mA	-	milli ampere
min	-	minutes
ml	-	millilitre
N	-	Normal
NEM	-	N-ethylmaleimide
nm	-	nanometre

No.	-	number
PCMB	-	p. chloromercuri benzoate
PHMB	-	p-hydroxymercuri benzoate
ppm	-	parts per million
r.p.m.	-	revelutions per minute
S	-	Swedberg constant
SDS	-	sodium dodecyl sulphate
SH	-	sulphhydril
TEMED	-	NN N'N', tetramethyl ethelene diamine
TLC	-	Thin layer chromatography
TRIS	-	Tris (hydroxymethyl) amino methane
TSH	-	Thyroid stimulating hormone
ug	-	microgram
ul	-	micro litre
UV	-	ultra violet
v	-	velocity of reaction
V	-	Maximal velocity
viz	-	namely
vs	-	versus

CONTENTS

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M.K.MUKUNDAN

1. INTRODUCTION

I. INTRODUCTION

Lipids constitute a significant portion of the biomass of earth and lipolytic enzymes play a very important role in lipid turn over. Apart from their biological significance, lipolytic enzymes are also very important in the fields of nutrition, food technology, medicine and preparative and analytical lipid biochemistry.

Recent developments in the study of proteins and enzymes have largely benefited the study of lipolytic enzymes, that some of these enzymes were isolated in pure form. Even today there is a continuous search for new and potent sources of these lipolytic enzymes. The zest for elucidating the structure and mechanism of action of the enzymes obtained in pure form for biochemist still remains unabated.

The lipolytic enzymes, lipases and esterases form an important group of enzymes as they are involved in fat metabolism. These enzymes are widely distributed in plants, animals and microorganic tissues and fluids and are physiologically important as they degrade fats and oils into free fatty acids, glycerol, and partial glycerides thereby facilitating absorption, transportation, storage, oxydation and synthesis of triglycerides.

The lipolytic enzymes are classified into two groups namely lipases and esterases. Often these terms are used interchangeably, as these enzymes catalyse the hydrolysis of carboxylic acid ester bonds and are termed hydrolases belonging to the class 3, 1, 1 of IUB classification. The term esterase is assigned to the enzymes capable of hydrolysing ester bonds between a carboxylic acid and a monohydric alcohol, and do not include lipases. According to IUB nomenclature lipases 3,1,1,3 are enzymes capable of hydrolysing triglycerides to glycerol/monoglyceride/diglyceride and free fatty acids.

$$\text{Triglyceride} + H_2O = \text{Glycerol/Monoglyceride/Diglyceride} + \text{fatty acids}$$

Essentially the above reaction is not a complete one since the lipase action may carry the reaction to monoglyceride or even to the glycerol stage. With a substrate such as triolein in the above reaction, a lipase may be considered as an enzyme hydrolysing fatty acid esters or liberating long chain fatty acids from esters (Balls and Matlack, 1938). However, for many lipase reactions both of these definitions are not applicable simultaneously. Anyway it has been well established that the lipase would act only on a heterogenous substrate or only very slowly if at all on a water soluble substrate.

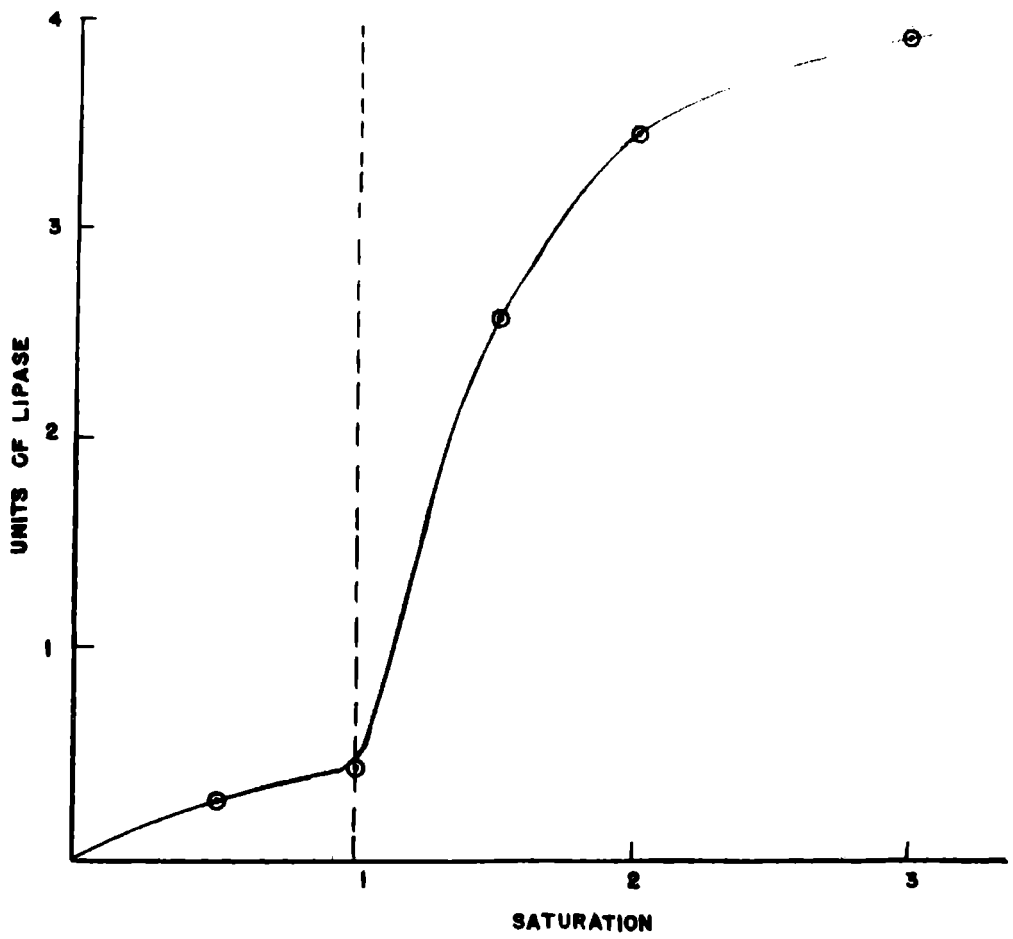


FIG.1 a TYPICAL LIPASE REACTION
HYDROLYSIS OF TRIACETIN BY PURE PANCREATIC LIPASE. THE LIMIT BEYOND WHICH THE SOLUTION IS OVER SATURATED AND CONSEQUENTLY EMULSIFIED PARTICLES BEGIN TO FORM IS INDICATED BY THE VERTICAL DOTTED LINE (FROM SARDA AND DESNUELLE, 1958)

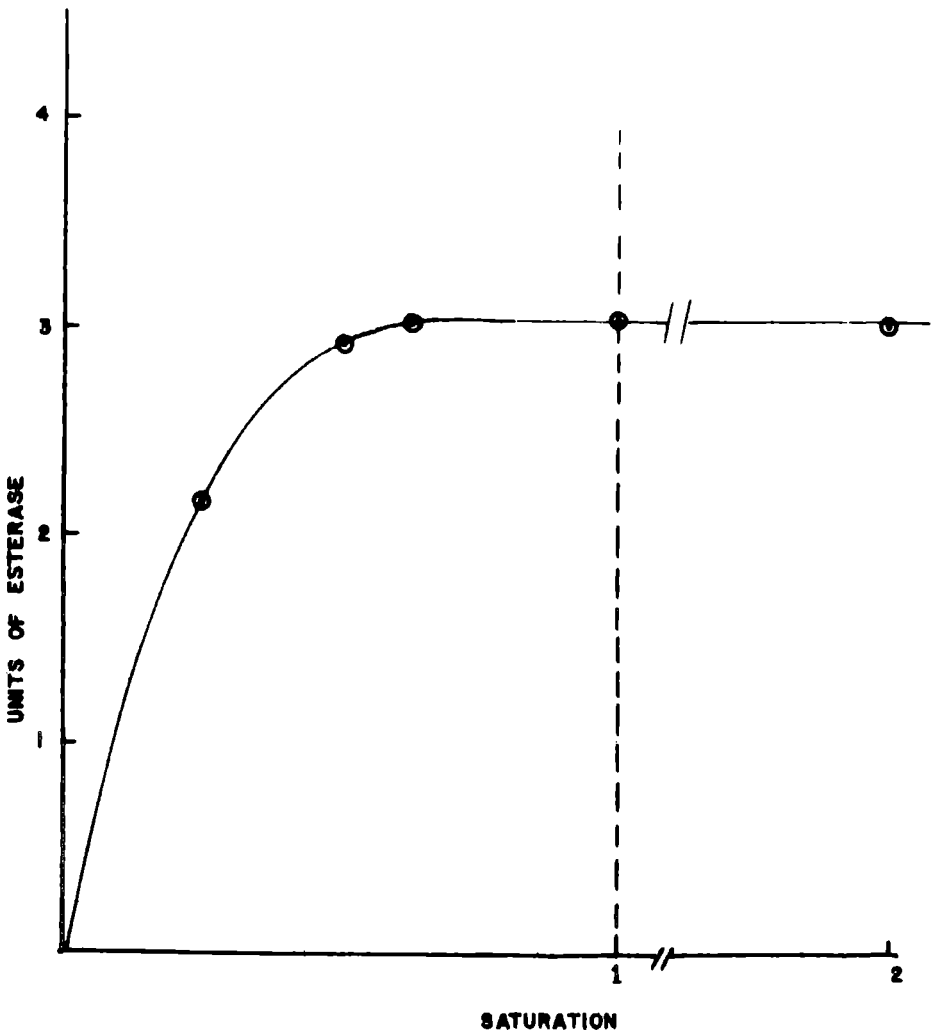


FIG.1b TIPICAL ESTERASE REACTION
HYDROLYSIS OF TRIACETIN BY PURIFIED HORSE LIVER ESTERASE. THE DOTTED LINE INDICATES THE POINT OF SATURATION (FROM SARDA AND DESNUELLE, 1958)

These peculiar properties of lipases and esterases are shown in figure 1 (a and b) which shows the reaction velocity of pancreatic lipase and horse liver esterase as a function of substrate concentration. The vertical dotted line indicates the point of saturation of the solution with tri acetin To the left of the dotted line tri acetin is soluble forming a homogenous layer and to the right of the dotted line tri acetin exists in heterogenous phase viz; emulsion. In the case of lipase the rate of reaction is negligible in the soluble area and it shoots up once the saturation point is crossed, while esterase reaction rate reaches maximum in the soluble region itself and flattens off in the over saturated area. It has been further shown that lipases hydrolyse the esters not only in the emulsified form but also in the micellar form and that pancreatic lipase hydrolyse substrates other than triglycerides (Desnuelle, 1972). Thus the lipases can be distinguished from the state of the substrate it acts upon, that they hydrolyse only emulsified or micellar (heterogenous) substrates, where as esterase can hydrolyse soluble (fully dispursed) substrates.

Recently lipases have assumed greater importance in food industry. The uncontrolled presence of these enzymes in fatty food such as fish and fish products, meat

milk etc. will cause lipolysis leading to undesirable odour, taste and rancidity. However, their presence in required amounts is essential for the development of certain characteristic flavours.

Lipases are one of the oldest of the enzymes known. Literature shows that lipase activity was first demonstrated in pancreas in 1846 and gastric juice in 1958. The presence of lipase in plant seeds was first noticed in 1871. The first organised study on the properties and characteristics of lipase was done by Willstator and his colleagues in 1920's and 1950's saw the pioneering work of Desnuelle and his associates, on purification and characterisation of hog pancreatic lipase.

During the past twenty years numerous reports came about lipases from different animal sources. Notable among them are the study of rat pancreatic lipase by Gidez (1968) bovine pancreatic lipase by Khan (1968) lipase from milk (Chandan and Shahani, 1963 a and b). This period also saw many studies on lipases from microbial sources, such as staphylococcus aureus (Vadhera and Harmon 1967), Penicillium roqueforti (Eitenmiller et al. 1970), Achromobacter lipolyticum (Khan et al. 1967), Geotrichum candidum (Alford and Smith 1965, Tsujisaka et al. 1972) etc. While the above lipases have been studied in detail

numerous reports are there on the presence of lipase in various animal tissues, organs and fluids, plant leaves and seeds and many microorganisms (Brokerhoff and Jensen, 1974).

The advent of microorganic technology and enzyme immobilisation technique are gradually making revolutionary changes in chemical processes, organic synthesis and chemical analysis. These changes along with the ever increasing use of enzymes in food manufacture and medicine are necessitating large scale production of many enzymes such as lipases, carbohydrases and proteases, will place heavy stress on the existing sources of these enzymes.

The situation being this, there is great need for an organised study of the lipases and other enzymes from newer sources. The literature shows no record of such an effort for the study of lipases from marine sources. The fact that many fishes like oil sardine, mackerel, cat fish, seer etc. contains large amounts of lipid shows the possibility of the existence of lipases in significant amounts necessitating their exhaustive study. Such a study will, not only provide alternate sources for lipase but also will provide methods to curb lipolysis and the resultant rancidity and off flavour development in fish and fishery products.

2. REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Ever since the discovery of lipase activity in pancreas in 1846, lipases have been detected in many tissues, organs and fluids of animals, plants and microorganisms. Lipases from some of the these sources have been studied in detail. For purposes of ease and lucidity the available data on lipases can be classified into that pertaining to.

2.1 Pancreatic lipases

2.2 Tissue lipases

2.3 Milk lipases

2.4 Plant lipases

2.5 Microbial lipases and

2.6 Fish lipases

2.1 PANCREATIC LIPASE

The work on pancreatic lipase perhaps form the major portion of work done on lipases. Consequently the work done on pancreatic lipase form the basis and model for studies of other lipases. Detailed reviews on pancreatic lipases have been published by Desnuelle (1961, 1971 and 1972) Coleman (1963) and Litchfeild (1972).

Mammals, other vertibrates and arthropods cannot assimilate intact dietary glycerides. Triglycerides must

be partially hydrolysed before they are absorbed from the intestinal tract and this hydrolysis is done by lipases elaborated by pancreas in mammals or other corresponding organs in lower animals. The enzyme is produced by acinar cells of pancreas and is released along with some other enzymes into the duodenum, where it plays an important role in digestion and absorption of dietary lipids. The hog pancreatic lipase has been studied most extensively, presumably because of its higher concentration (2.5% of the proteins of hog pancreatic proteins) and high turnover number.

The location of lipase activity in pancreas by Bernard in 1946 was followed by many attempts to purify and characterise pancreatic lipase. Notable attempts were made by Willstator and his colleagues in 1920's and Desnuelle and his associates in 1950's and 1960's, the latter being active even today and responsible for the isolation and characterisation of hog pancreatic lipase. Their initial procedure reported in 1957 involving preparation of an acetone powder, fractionation with ammonium sulphate, acetone and alcohol and starch gel electrophoresis was extensively modified over the years by Sarda et al. (1964), Benzonana et al. (1964) and Verger et al. (1969). One of the most widely accepted procedure consists of preparation of a defatted pancreatic powder,

removal of the acidic phosphatide from the aqueous extract of the powder by partition between butanol and ammonium sulphate solution, DEAE-cellulose chromatography, Sephadex G-100 gel filtration and finally CM cellulose chromatography giving two molecular species of lipases LA and LB. Both these lipases have molecular weight of about 48000, isotonic points 4.9 and 5 and almost similar amino acid composition. Garner and Smith (1972) adopting a small modification of the above method isolated two homogenous isoenzymes of porcine pancreatic lipase and showed them to be glycoproteins containing 2.9 moles of N-acetyl glucosamine per mole of enzyme.

Baskys et al. (1963) noted the loss of enzyme activity of lipase towards olive oil emulsion on DEAE cellulose chromatography and that a boiled pancreatic extract restored the enzyme activity. Morgan et al. (1969) identified a heat stable cofactor from rat pancreatic lipase and was named colipase. Figarella et al. (1972) showed the presence of colipase in human duodenal contents and Julien et al. (1972) in bovine pancreas. This colipase has now been purified and has been shown to be a small protein of approximate molecular weight 8000 attached to lipase (Malie et al. 1971).

The amino acid composition of pancreatic lipase has been determined (Verger et al. 1969). However, its

chemical structure has not yet been established. The existance of six disulphide bridges and two free -SH groups has been established, eventhough substitution of SH groups with phenyl mercuric radicals did not show any loss in activity, showing pancreatic lipase to be a nonsulphhdril enzyme (Verger et al. 1971)

Low concentration of diiso-propyl fluro phosphate (DFP), which is a powerful inhibitor of esterases and proteolytic enzymes do not inactivate pancreatic lipase, but relatively high concentrations do. Also, diethyl p-nitrophenyl phosphate hardly inhibits the enzyme when present in solution, but in an emulsion form it inhibits the enzyme markedly (Maylie et al. 1969). Pancreatic lipase is probably a "serine histidine enzyme", since diethyl p-nitrophenyl phosphate binds to a serine residue in the enzyme and there exists a correlation between photo oxidative inactivation of the enzyme and the oxidative loss of a histidine moiety (Blow, 1971 and Semeriva et al. 1971).

Pancreatic lipase shows an absolute requirement for sodium chloride and that calcium and bile salts do not increase the initial lipolytic velocity, but in lower concentrations they help to retain the velocity, as the reaction proceeds by counteracting the inhibitory effects of fatty acids by soap formation (Shahani, 1975). It is also shown that bile salts and calcium increase the

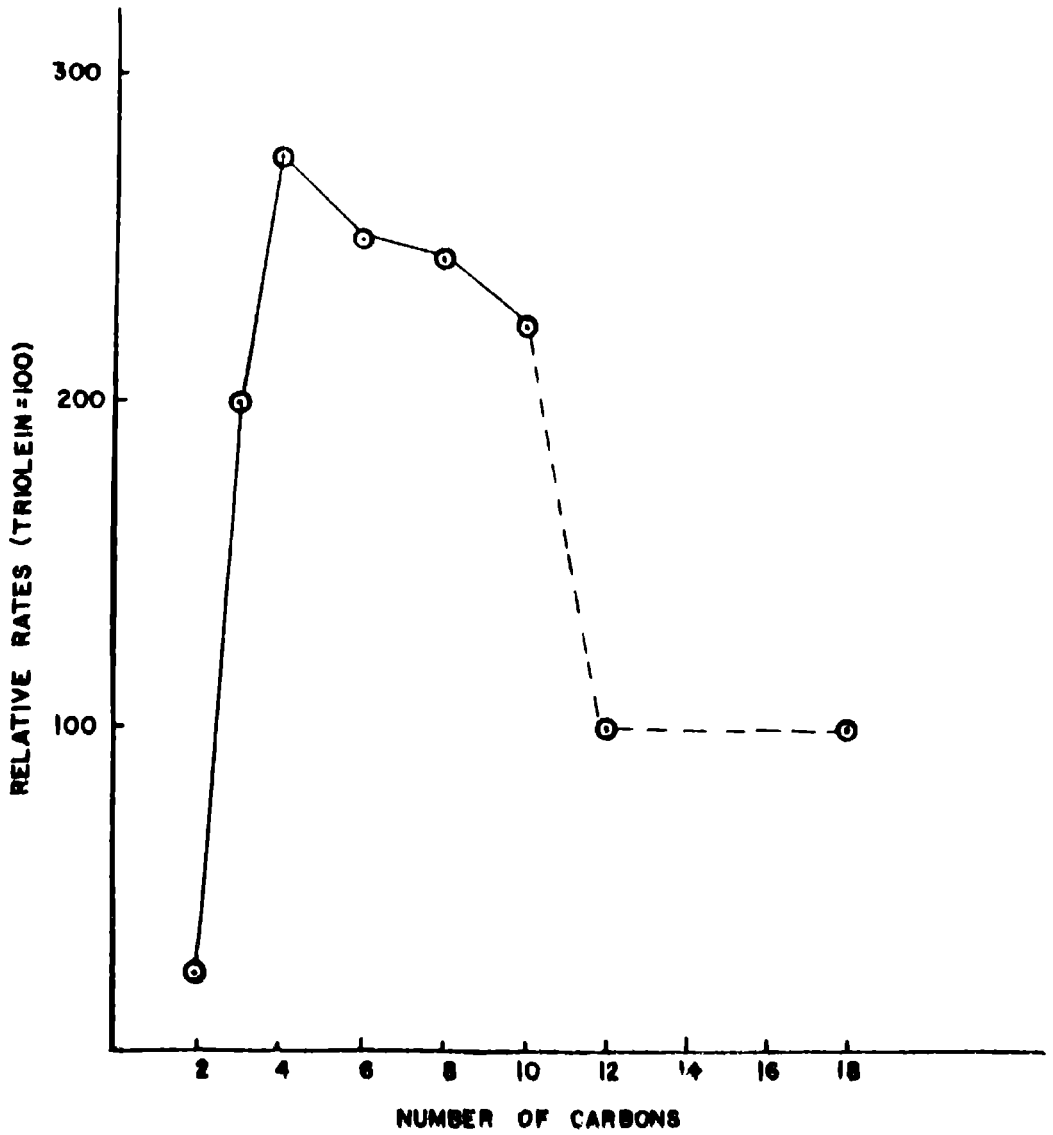


FIG.2 RELATIVE RATES OF HYDROLYSIS BY PORCINE PANCREATIC LIPASE OF TRIGLYCERIDES OF FATTY ACIDS OF DIFFERENT CHAIN LENGTHS (FROM ENTRESSANGLES *et al.* 1961b)

temperature stability of the enzyme (Desnuelle, 1961) and that bile salts also affect the pH optimum shifting it from 8.0 to 6.0 (Borgstrom, 1954). Most of the anionic detergents inhibit lipase, where as cataionic detergents increase lipolysis significantly (Wills, 1965).

It has been shown by many workers that pancreatic lipase has not absolute substrate specificity (Brokerhoff and Jensen, 1974). However, there exists a gradation in lipolysis depending upon the nature of substrate. Thus pancreatic lipase hydrolyse triglycerides more rapidly than monohydric alcohol esters. Among triglycerides the carbon chain length of fatty acids affects the rate of lipolysis, provided the substrate is in the emulsion form.

Consequently pancreatic lipase is more active towards short chain triglycerides and that tributrin and tripropionin are hydrolysed at a higher velocity (Entressangles et al. 1961 b). As is evident from figure 2, the the relative rates of lipolysis by pancreatic lipase increase from C_2 to C_4 and then with higher chain length the rate rapidly decreases. Also digestion of rac glyceryl 1-palmitate 3 butyrate by pancreatic lipase shows 72% butric acid and 28% palmitic acid in the fatty acid mixture (Entressangles et al. 1961 a and b) indicating the preference for short chain fatty acids. However as shown in table 1, studies with glycerol 1-palmitate 2,3-dibuty-

Table 1. FREE FATTY ACIDS DERIVED FROM PANCREATIC LIPOLYSIS OF EQUIMOLECULAR MIXTURES OF BUTYRATE GLYCERIDES AND TRIOLEIN

Triglyceride mixture	Free fatty acid mole %		
	P	B	O
PBB + OOO	28.2	28.8	48.0
Theoritical	26.4	26.4	47.2
PBP + OOO	55.7	-	44.3
Theoritical	51.8	-	48.2
PPB + OOO	23.6	28.2	48.2
Theoritical	24.7	24.7	50.6

From Sampugna *et al.* 1967.

P = Palmitate; B = Butyrate; O = Oleate

rate (PBB), glycerol -1-butyrate 2-3-dipalmitate (PPB) and glycerol 2-, Butyrate 1-, 3-, dipalmitate (PBP) mixed with equimolar concentrations of triolein (OOO) revealed that from the same triglyceride both short and long chain acids are liberated at the same rate (Sampugna et al. 1967) and that it is the total size of the triglyceride and not the carbon chain length that determines the rate of lipolysis. These two contradicting results were finally settled in favour of the latter theory considering the chances of acyl migration in experiments of Entressangles et al. (1961 a and b).

Similar work was also done to decide the effect of the degree of unsaturation of fatty acid, on lipolysis. The general conclusion being that the degree of unsaturation has no pronounced effect even though Brokerhoff (1965) and Bottino et al. (1967) have shown that pancreatic lipase has a preference for saturated fatty acid esters.

The strict positional specificity of pancreatic lipase for α or α' position is well established (Entressangles et al. 1966; Brokerhoff, 1968; Mattson and Volpenhein, 1968). The fact that some lipolysates contain α - monoglyceride may in part be due to isomerisation of β to α monoglyceride, in accordance with the observations of Jensen (1971) that in short time lipolysates α monoglycerides are absent. Further analysis of pancreatic lipo-

lysates of synthetic sn-glyceryl 1,2 dipalmitate 3-oleate and glycerol 1-oleate 2,3 dipalmitate have demonstrated that pancreatic lipase hydrolyses primary positions α and α' at random, indicating absence of stereo specificity (Tattrie et al. 1958).

2.2 TISSUE LIPASES

Hollenberg et al. (1961) observed the stimulation of lipolysis in homogenates of rat epididymal adipose tissue by epinephrine or ACTH. The enzyme was called hormone sensitive lipase.

The preparations of hormone sensitive lipase so far obtained have very high apparent molecular weights. The S_{105} lipase of Hutten et al. (1970 a,b and c) of molecular weight 7.2×10^6 is reminiscent of the fast pancreatic lipase (Sarda et al. 1964). The second lipase fraction described by Pittman et al. (1972) appears to have a molecular weight of several hundred thousands, an improbable size for a hydrolase.

The function of hormone sensitive lipase is the mobilisation of fatty acids of depot fat and that all three fatty acids of the triglycerides are hydrolysed with equal speed (Steinberg 1972). The enzyme would then differ from pancreatic lipase in prefering a more open lipid surface being able to hydrolyse hydrated fatty acid esters. Such a hydrophobic affinity is likely, as the lipase acts

on an oil droplet, which probably has a surface containing phospholipids (Brokerhoff and Jensen, 1974).

A number of reagents like ATP, cyclic AMP, adnylate cyclase and protein kinase were also found to be activators of hormone sensitive lipase (Steinberg, 1972). Khoo et al. (1972) also demonstrated that these activation can be stopped by the addition of protein kinase inhibitor. Further Marsh and George (1969) using isolated fat cells found that a ten second exposure to N-ethyl maleimide inhibited the lipase activation effect of TSH, theophilline and cyclic 3', 5' AMP, suggesting that sulphydril groups are essential for the lipolytic activity.

Several other reports on lipolytic activity in adipose tissue homogenates are also available. Schnatz (1966) found lipolytic activity in human adipose tissue homogenates at pH 7.0 at 37°C in the presence of albumin and, which was not activated by epinephrin. Mann and Tove (1966) purified a lipase from a rat adipose tissue to 140 fold and found it to be specific for primary esters in tri and diglycerides and without any activity on monoglycerides. Similar activity towards non physiological substrates were demonstrated by many other workers (Wallach et al. 1962; Schnatz, 1964, 1966; Schnatz and Cortner 1967; Schnatz and Cumminsky, 1969 and Crum et al. 1970). The pertinence of these activities to long chain fatty acid triglyceride

lipases in adipose tissue remains obscure.

The detection of lipolytic activity in liver homogenate was first reported by Vavrinkova and Monsinger (1965). This was followed by numerous reports on the distribution and location of lipase particles (Guder et al. 1969; Stoffel and Greten, 1967; Mahadevan and Tappel, 1968; and Waite and Van Deenen, 1967). Guder et al. (1969) have purified liver lysosomal lipase to 300 fold and recorded a pH optimum in the range 4 to 5. The enzyme was inhibited by PCMB but not by NEM, DFP or Protamine (Hayase and Tappel 1970) and that the enzyme was specific for primary ester bonds. A number of other activators and inhibitors are also reported (Claycomb, 1972).

The flight muscles, fat bodies and eggs of various insects were also analysed for lipase activity and found a general specificity of the enzyme for monoglycerides Crabtree and Newsholme, 1972) and that the enzyme have an optimum pH around pH 8.

2.3 MILK LIPASE

Study of lipolytic enzymes of milk started only by 1950, eventhough it has been known that milk from cow and other mammals contain lipase. Large scale use of pipeline milkers, an improved method for obtaining raw milk was in a way responsible for the study of milk lipases. It was noted that excessive forming of warm raw

milk in pipelines, generally caused by air leaks, activated a lipase, which upon subsequent storage at low temperature, partially hydrolysed the milk fat. Relatively large quantities of butric and capric acids are released as both these acids are located at sn 3-position of triglycerides (Pitas et al. 1967; Breckenridge and Kuksis 1968) and milk lipase is specific for 1 and 3 positions (Jensen, 1964). Milk lipases and lipolysis of milk have been reviewed by Jensen (1964, 1971), Chandan and Shahani (1964), Shahani (1966), Groves (1971) and Shahani et al. (1973).

The milk of all mammals contain lipases. However, lipase in cows milk received maximum attention from scientists. Chandan found the following activities for lipase present in milk from different sources (Table 2).

Observations by Tarassuk and Franknel (1957) have indicated the presence of two lipases in milk of which one is believed to be present in normal milk from all cows. The lipase activity is associated with the casein fraction and activation occurs as a result of homogenisation and foaming of warm raw milk. The milk from some cows contain another lipase that is absorbed to the fat globule as a result of cooling (Brokerhoff and Jensen, 1974).

The first effort to purify lipase from milk goes to the credit of Skean and Overcast (1961). The same group were also the first to locate lipase activity in a

Table 2. LIPASE ACTIVITIES OF MILK FROM VARIOUS SOURCES*

Milk source	Activity u moles of fatty acid/min/100 ml
Human	13
Cow	132
Goat	39
Sheep	9
Sow	141

* Chandan et al. 1968

specific casein fraction. They applied continuous paper electrophoresis to the casein obtained by both acidification and centrifugation of milk at room temperature in absence of added calcium. The casein was separated into three fractions viz; α, β, γ Lypolitic activity was associated with the α component and that the activity of casein obtained by centrifugation, was twice as much as that of casein obtained by acidification. Saito (1963) confirmed the association of the lipase with casein micelles. Yaguchi et al. (1964) using dialysed skim milk as the starting material noted that lipase activity accompanied several fractions obtained by DEAE cellulose chromatography and containing K casein. All fractions also contained relatively large amount of sialic acid. Only negligible activity was detected in the casein fraction. Later Fox et al. (1967) observed that lipase could be removed from K casein by treatment with dimethyl formamide, leading to the conclusion that lipase is a minor component of the casein system.

Later Downy and Andrews (1965 a and b and 1969) observed 4 to 5 overlaping lipases in skim milk four of them with molecular weights ranging from 62000 to 112000 and another one with a much smaller molecular weight. Their activities were tested against triacetin tributrin, triolein and milk fat emulsion and found that emulsified

substractes gave the maximum activity. Most of these fractions were labile and found to contain sialic acid. These results led Downey and Murphy (1970) to suggest that milk lipases are distributed in an equilibrium between micellar and soluble casein.

The credit to purify lipase from milk goes to Fox and Tarassuk (1968), who coagulated fresh skim milk with rennin and separated the curd from the whey by centrifugation. The curd containing most of the lipase was solubilised with 1 M sodium chloride and centrifugation, and the lipase was concentrated by precipitation with ammonium sulphate. Further purification was achieved by DEAE chromatography, solubilisation with diethyl formamide, precipitation with ammonium sulphate and sephadex G-200 filtration to give an electrophoretically homogenous preparation. The extend of purification and yields are presented in Table 3. Molecular weight of the homogenous preparation was 210000; Patel et al. (1968) further characterised the preparation by determining the nitrogen (14.8%), phosphorous (16%) and sialic acid (0.6%) content in the lipase. Saito and Igarashi (1971) obtained fractions with enhanced lipase activity by sephadex gel filtration of skim milk, but the enrichment was not sufficient for further purification.

Chandan and Shahani (1963 a and b) obtained a

Table 3. PURIFICATION, SPECIFIC ACTIVITY AND RECOVERY OF MILK LIPASE*

Fraction	Specific activity	Purification	Yield %
Skim milk	0.03	1	100
Rennin & Nacl extraction	0.15	5	60
First (NH_4) SO_4	0.18	6	60
DEAE cellulose	0.9	30	40
Second $(NH_4)_2$ SO_4	3.0	100	24
Sephadex	15	500	10-15

*From Fox and Tarassuk (1968)

homogenous lipase with molecular weight of about 7000 from the sediment resulting from the passage of milk through a clarifier. Eventhough this enzyme had many similarities (Shahani, 1966) to the enzyme studied by Downey and Andrews (1965 a) the latter challenged the identity. However, the isolation of a low molecular weight (8500) lipase from clarifier sediment by Ritcher and Randolf (1971) supported the contention of Chandan and Shahani.

The present conclusion is that, at least one milk lipase is a low molecular weight (7000 - 8000) glycoprotein adsorbed specifically to K casein. The other high molecular weight lipases are considered to be either pancreatic or tissue lipases which have possibly leaked into the mammary gland through blood (Jensen, 1964).

The amino acid composition of milk lipase has been studied by Patel et al. (1968), which is said to differ much from porcine and rat pancreatic lipases (Brokerhoff and Jensen 1974). The enzyme contained 0.6% sialic acid similar to several milk proteins (Graham et al. 1970).

PCMB and N-ethyl maleimide partially inhibit milk lipase (Tarassuk and Yaguchi, 1959; Chandan and Shahani, 1965; Robertson et al. 1966 and Patel et al. 1968), suggesting that free sulphhydryl groups are located in the vicinity of the reactive site but are not involved in the catalytic mechanism, a similar situation found in pancreatic

lipase (Verger et al. 1971). However, Chandan and Shahani (1965) found one milk lipase to be completely blocked by PCMB. DFP at 1 x 10^{-3} M and DNP at 5 x 10^{-4} M distroy the activity completely (Robertson et al. 1966; and Patel et al. 1968). Franknel and Tarassuk (1959) and Patel et al. (1968) observed rapid inactivation of the enzyme by photo-oxidation, suggesting milk lipase to be a 'histidine serine' type enzyme.

As is the case with most lipases, milk lipase too hydrolyse tributrin more rapidly than other long chain acyl glycerols, (Patel et al. 1968) and so milk lipase is considered specific for short chain fatty acids. Further milk lipase acting on homogenised raw milk yielded predominantly 2-monoglycerides (Jensen et al. 1960), showing the preferential hydrolysis of primary position. Jensen et al. (1961 and 1964) using lipase preparation of Forster et al. (1959 and 1961) and Chandan and Shahani (1963 a and b) confirmed the specificity of milk lipase for primary position. This data is shown in Table 4.

However, when it comes to select between position and chain length it has been shown that milk lipase has a preference for a positional rather than chain length specificity (Gander et al. 1961).

Milk lipase has a pH optimum between 8 and 9 and a temperature optimum range of 35°C to 40°C. Calcium and

Table 4. FATTY ACID COMPOSITION OF THE INTACT TRIGLYCERIDES AND THE PRODUCT OF MILK LIPASE HYDROLYSIS OF AN EQUIMOLAR MIXTURE OF *rac*-GLYCEROL 1-PALMITATE 2,3 DIBUTYRATE AND TRIOLEIN*

Substrate and products of hypolysis	Fatty acids mole %		
	4:0	16:0	18:1
Intact Triglyceride	31.6	15.8	52.6
Residual Triglyceride	25.8	12.1	62.2
Free fatty acids	37.0	33.0	30.0
Diglyceride	36.2	12.8	52.0
Monoglyceride	78.4	7.0	14.6

*From Jensen *et al.* 1964

sodium ions have been found to enhance lipolysis (Brokerhoff and Jensen, 1974). Milk lipase is also inhibited by oxygen, light, iodoacetate, hydrogen peroxide (Shahani 1966), some antibiotics (Shahani and Chandan 1962) and 2, 4 dinitrofluonobenzene (Robertson et al. 1966), while glutathion stabilises it (Shahani 1966). The phospholipids lecithin and phosphatidyl ethanolamine were also found to reduce the activities of milk lipase (Campbell et al. 1968).

2.4 PLANT LIPASES

Most fruits, vegetables, plant tissues and seeds are known to exhibit lipase activity. However, lipases from only a few of these sources have been studied so far, in detail.

Among plant lipases, the acid lipase of dormant castor bean (Ricinus Communis) is the most extensively investigated lipase (Ory, 1969). But the seed on germination shows the presence of another lipase with a pH optimum near 7 (Yamada, 1957). The spherosomes of the endosperm is the seat of the acid lipase. Castor bean lipase is obtained by maecrating the beans with pH 7.8 cysteine EDTA buffer, filtration and centrifugation of the filtrate. At this stage the lipase will be concentrated at the top fatty layer. The fatty layer is then extracted with ether, treated with saturated salt solution to give a particulate matter containing lipase.

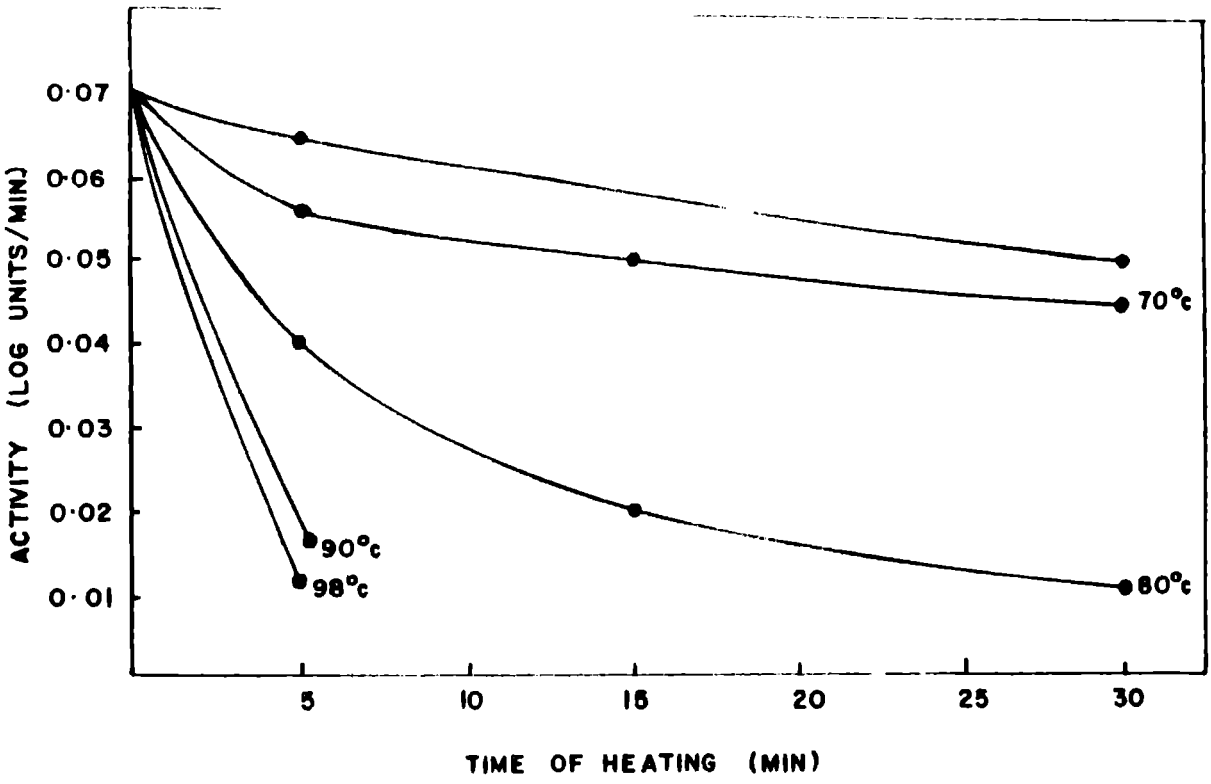


FIG.3 EFFECT OF HEAT ON THE ACTIVITY OF CASTOR BEAN LIPASE. ENZYME WAS HEATED IN A WATER BATH AT DESIGNATED TEMPERATURES FOR TIMES INDICATED, COOLED AND TESTED (FROM ORY 1969)

it is dialysed and freeze dried to give a fairly active and stable dry preparation. As this powder was insoluble in water further purification became difficult (Ory, 1969).

As is seen from figure 3, castor bean lipase is fairly heat stable. It posses a rather sharp pH optimum of 4.2 to 4.31 with activity droping off rapidly below pH 4.0 and above pH 5.0. PCMB and mercuric chloride strongly inhibit the enzyme, which can be relieved partially by cysteine and EDTA, indicating it to be a sulphydril enzyme. Further, DFP has no inhibitory effect (Ory and Altschul, 1964 and Ory, 1969).

Castor bean lipase is shown to be a three component system made of an apoenzyme, a lipid factor and a glycoprotein activator (Ory et al. 1964). The castor bean lipase do not require added emulsifier, probably because the lipid factor acts as a natural emulsifier. The three components have been seperated and purified. The lipid cofactor was found to be a cyclic tetramer of ricinoleic acid and the activator to be a castor bean allergen (Ory et al. 1967 a and b and Ory, 1969)

In general castor bean lipase hydrolyse triglycerides actively, eventhough butyl and methyl recinoleates are hydrolysed very slowly, showing it to be a true lipase. It is interesting to note that the enzyme shows maximum lipolytic activity on triglycerides of C_8 fatty acid and

that the activity with triglycerides of unsaturated fatty acids triolein, trilinolein and cotton seed oil are half that of C_8 triglyceride. The enzyme was further shown to have no action on triacetin (Ory et al. 1960). As regards positional specificity castor bean lipase has been shown to be much similar to pancreatic and milk lipase, taking into account the possible acyl migration from 2 to 1 position (Borgstrom and Ory, 1970; and Borgstrom, 1971).

Another group of lipases studied from plant origin form the cereal lipases. Generally the germ portion of wheat, rye and barley contain a much higher level of lipase activity than the endosperm. The Scutellum and aleuron layers are also rich in lipase (Engel, 1947; Sulliwan and Howe, 1933). Apart from crude and partially purified lipase preparations, literature shows no report of a homogenous lipase from cereals. Of the cereal lipases studied, the one obtained from wheat deserves some mention.

Singer and Hostee (1948) prepared a wheat germ lipase by homogenising ground wheat germ with water followed by ammonium sulphate fractionation and lyophilisation. The lipase so obtained had a pH optimum of 7.4 with simple and soluble esters as substrates. The preparation was also active on emulsified substrates. Further attempts to purify wheat germ lipase resulted in the separation of 3 enzymes viz; an esterase a tributyrinase and lipase (Stauffer

and Glass 1966 and Fink and Hay, 1969) of which the tributyrinase was shown to have a pH optima of 6.5, with high activity towards tributyrin (100) and no or negligible activity towards triacetin (5), trolein (0), ethyl acetate (0), ethyl propionate (4).

However as most of the earlier studies were done using triacetin and ethyl acetate as substrates, it is not certain whether the hydrolase activity detected is true lipase activity. Further the enzyme showed a peculiar property, that its activity and temperature optimum were affected by moisture content. The activity being 5 times higher at 15% moisture than at 8% moisture and that the lower the moisture content the higher the temperature optimum (Rothe, 1958).

Another interesting example of plant lipase is provided by Vernonia anthilmintica (Krewson and Scot 1964) Krewson et al. (1962) observed that on incubation of the ground seed, which contain trivernolin released vernolic acid and 1,3-divernolin indicating the presence of a lipase specific for β position. Further studies, with a ten fold purified enzyme and synthetic triglycerides as substrates, revealed that the lipase was capable of successively spliting both primary and secondary ester links and that the results of Krewson et al. (1962) were due to isomerisation of 1,2- and 2,3- diglycerides to 1,3- diglyceride (Olney et al. 1968).

2.5 MICROBIAL LIPASES

The remarkable stability of microbial lipases and their commercial use in medicine and industry has attracted many workers world over. A variety of microorganisms of the genera candida and Torulopsin, yeast, Mucor molds and bacteria are shown to elaborate lipase. Thus Rhizopus, Penicillium, Aspergillus, Geotrichum, Pseudomonas, Achromobacter and Staphylococcus produce exocellular lipases (Brokerhoff and Jensen, 1974). In some cases lipase production can be induced by inclusion of lipid in the culture media. Thus Achromobacter lipolyticum (Khan et al.1967) and Geotrichum candidum (Tsujisaka et al. 1972) can be induced to produce lipase in multiple amount by inclusion of olive oil in the culture medium. There are also instances like Penicillium roqueforti lipase production being inhibited by added lipid and Staphylococcus aureus and Pendcillium crustorum, whose lipase production is not at all affected by the presence of lipid (Eitenmiller et al. 1970). As most of the microorganisms produce extracellular lipase the isolation procedure start with the culture broth. The broth is subjected to fractional precipitation with ammonium sulphate followed by DEAE cellulose, sephadex G-100 column chromatography and lyophilisation to give pure lipase (Tsujisaka et al. 1973). Some of the microbial lipases were shown to contain sugar moeity attached to

them (Semeriva et al. 1969). The role of the glycopeptide is attributed to the transport mechanism of the enzyme across the cell wall (Eylar, 1966).

The properties of the microbial lipases vary considerably depending on source. The lipase of aspergillus niger has the lowest pH and temperature optima and the same are the highest for the lipase from Staphylococcus aureus. Although the rate of lipolysis is the highest at temperatures between 32°C and 45°C, several microbial lipases actively hydrolyse substrate at freezing temperatures. Table 5, shows the temperature and pH optima and the heat inactivation data of various microbial lipases studied (Shahani, 1975). In general most of the microbial lipases are more heat stable than the lipases of animal origin, a notable example being Achromobactor lipolyticum lipase whose inactivation requires heating at 99°C for 40 minutes (Khan et al. 1967).

Almost all microbial lipases hydrolyse numerous natural oils and fats and synthetic triglycerides to a greater extent. Thus crystalline Aspergillus niger lipase can hydrolyse 48 to 93% of coconut oil, castor oil, soy bean oil, cotton seed oil, linseed oil, olive oil etc. Triglycerides containing hydroxyacids and conjugated double bonds are hydrolysed at a lower rate (Shahani 1975). Penicillium lipase hydrolyse tributrin most readily than

Table 5. TEMPERATURE AND pH OPTIMA AND HEAT INACTIVATION OF VARIOUS MICROBIAL LIPASES*

Source of lipase	pH optimum	Temperature optimum °C	Heat inactivation time: temperature
Penicillium chrysogenum	6.2 - 6.8	37	15:72
Psuedomonas fragi	7.0 - 7.2	32	15:50
Rhizopus delamer	5.6	35	15:45
Aspergillus niger	5.6	25	15:45
Penicillum roqueforti	8.0	37	10:50
Staphylococcus aureus	8.5	45	30:70
Geotrichum candidum	8.2	37	15:60
Achromobacter lipolyticum	7.0	37	40:99

*From Shahani 1975

any other triglycerides. On the other hand Achromobacter lipase was shown to hydrolyse triolein more rapidly than tributrin (Khan et al. 1967) and Geotrichum candidum lipase too have a preference for triolein with a specificity for 18:1 acids, irrespective of its position in the triglyceride (Jensen et al. 1965). The capacity of Achromobactor lipase to release oleic, linoleic and linolenic acids preferentially from a triglyceride mixture has been established using emulsified milk fat as substrate (Shahani, 1975).

Low concentration of calcium, sodium, potassium and magenisum salts activate lipolysis, whereas heavy metal salts like iron, barium, etc strongly inhibit most of the microbial lipases (Khan et al. 1967; Iwai et al. 1970). PHMB and iodo acetate have little inhibitory effect on some of the microbial lipases indicating these enzymes to be nonsulphydril enzymes (Finklestein et al. 1970; Trotter and Bozeman, 1970). Antibiotics like, Penicillin, pimaricin and mycostatin exert an inhibitory effect on microbial lipase and that eventhough 50 p.p.m. of these antibiotics inhibit the cell growth of Achromobactor only mildly, it inhibits the lypolytic activity completely (Chandan et al. 1962). Antibiotics are considered competitive inhibitors of lipase. In studies with streptococcal lipase formaldehyde, mercapto ethanol, glutathion and terramycin were found to be inhibitors where as hydrogen peroxide,

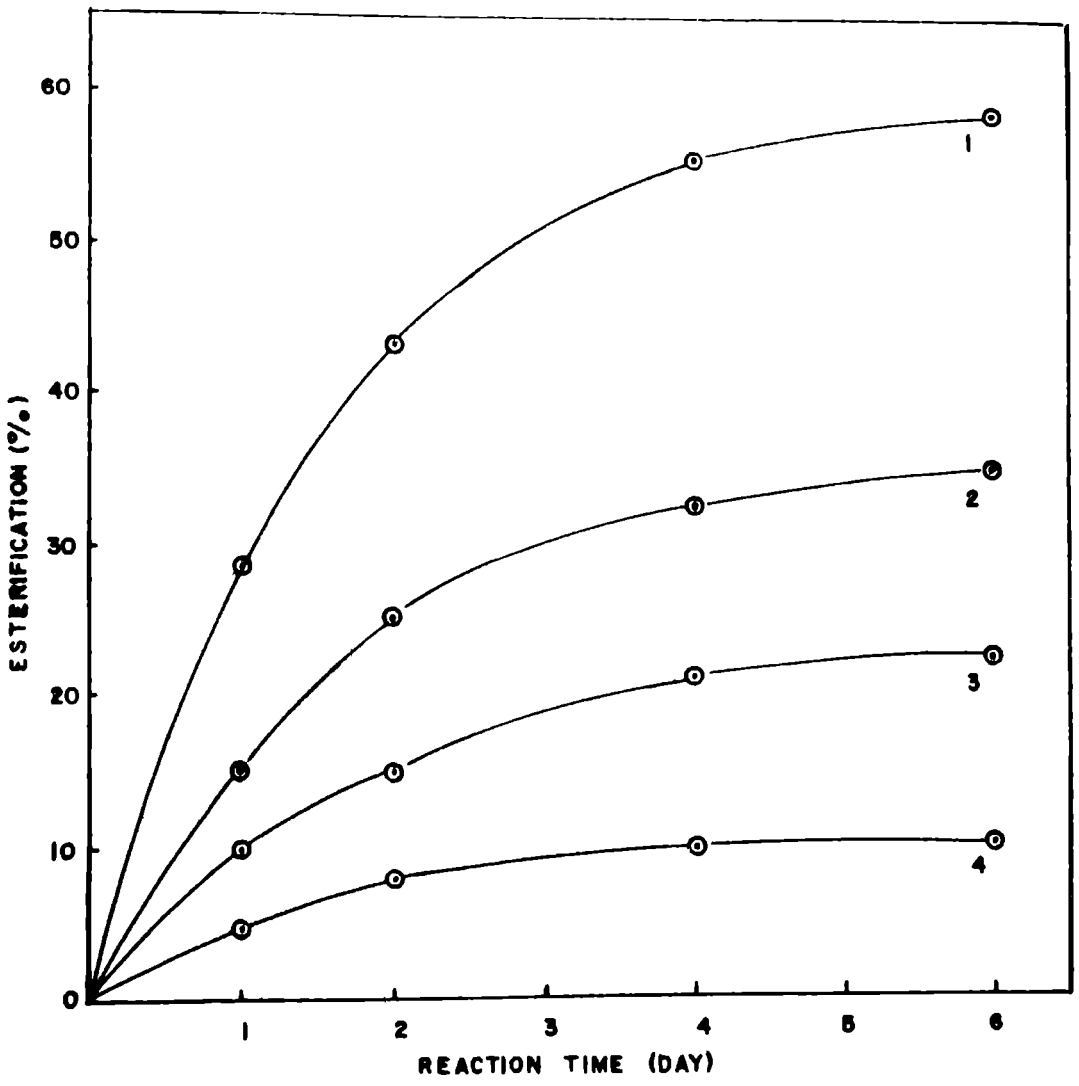


FIG.4 ESTERIFYING ACTION OF THE ASPERGILLUS NIGER LIPASE EFFECT OF VARYING GLYCEROL CONCENTRATION OF THE MIXTURE. ALL MIXTURES CONTAINED 1 ml OLEIC ACID PLUS ENZYME SOLUTION AND (1) 9 ml GLYCEROL (2) 3 ml GLYCEROL (3) 1 ml GLYCEROL AND (4) 0.3 ml GLYCEROL
(FROM IWAI et al. 1964)

streptomycin and sodium taurocholate act as activators (Vadhera, 1974).

Similar to bovine pancreatic lipase and milk lipase, Aspergillus niger lipase has been shown to synthesise triglyceride from fatty acids and glycerol (Iwai et al. 1964). It was also shown that the triglyceride synthesis is rapid when glycerol concentration is high and water concentration low (see figure 4). The lipases obtained from Rhizopus delemar, Geotrichum candidum and Penicillium cyclopium were also capable of triglyceride synthesis but with lesser velocity than Aspergillus niger lipase. (Iwai et al. 1964).

The type of lipolysis by microbial lipases vary depending on the source of the enzyme and are classified according to their positional specificy as is shown in Table 6. Most of the microbial lipases attack primary bonds of triglycerides and so is similar to pancreatic and milk lipases in positional specificity. On the other hand Staphylococcus aureus and Aspergillus flavus show no positional specificity (Alford et al. 1964). Another microbial lipase with a unique specificity is the lipase excreted by Geotrichum candidum, which is specific towards cis 9 or cis 9,12 unsaturated fatty acids regardless of its position in the triglyceride, with pH optimum of 8.2 and that it can be stored at -20°C in the lyophilised form for 8 years (Jensen et al. 1965; Jensen, 1973).

Table 6. PRINCIPAL TYPES OF LIPOLYTIC ACTIVITY EXHIBITED BY VARIOUS MICROORGANISMS*

Specificity	Microbial lipase source
Attacks 1- or 3- Position	Psuedomonas fragi, Psuedomonas fluorescence, Psuedomonas geniculata, Candida lipolytica Phycomyces nitens, Mucor sufu, Penicillium roqueforti, Rizhopus oligosporus, Chaitostylum fresnei, Thamnidium elegans
Attacks 1-, 2- or 3- position	Staphylococcus aueres Aspergillus flavus
Attacks oleic acid linkage regardless of position	Geotrichum candidum

*Alford et al. (1964)

2;6 FISH LIPASES

There are also some reports on the distribution and properties of lipases in fish. Cartilaginous fish posses a discrete pancreas and hence the study of the enzyme is convenient. The lipase from the pancreas of skate Ria radiata, was studied in some detail (Brokerhoff and Hoyle 1965). It was observed that the specific activity of an acetone powder of the pancreas approached that of the pig pancreas powder and the enzyme hydrolysed the primary ester bonds of a triglyceriede of known fatty acid distribution without any stereospecificity.

In bony fish the pancreas is diffuse. Still there were some efforts to study the lipases of bony fish. Thus Brokerhoff (1966) found the lipase of cod, Gadus morrhua to be non stereospecific. The lipase from trout, Salmo gairdneri has been partially purified (Leger et al. 1970; Leger, 1972) to give a specific activity of 15, with 8.5% yield. The enzyme was specific for primary esters but also showed a preference for oleic acid esters, regardless of position (Leger and Bauchart, 1972). Berner and Hammond (1970) observed a lipase in cray fish, Cambanus virilis hepatopancreas with a partial specificity for primary position. Brokerhoff et al. (1967, 1970) found a lipase in American lobster Homavus americanus, with a molecular weight of 43000 and pH optimum 7. The extend of activity

of the enzyme was comparable to porcine pancreatic lipase. It was found to be specific for primary ester bonds. DFP had no effect on the activity of the enzyme eventhough it was sensitive to surface denaturation, which can be prevented by addition of bile salts and albumin (Brokerhoff 1971).

Oil sardine (Sardinella longiceps) is a commercially important fish of the west coast of India. The fish is found to exhibit remarkable seasonal variation in its lipid content (Gopakumar, 1965). Triglyceride content in the body lipid is found to vary throughout the months and phospholipid content remained at a constant level. The above author also noted that spawning and variation in sea water temperature are found to have pronounced effect in the lipid as well as the fatty acid composition of the fish (Gopakumar, 1974). Hence it is thought that the fish is likely to contain a high concentration of lipolytic enzymes particularly the triglyceride lipases.

This thesis embodies the investigation on the isolation of an active triglyceride lipase from the hepatopancreas of the oil sardine. The enzyme is obtained in a high state of purity. Its specificity, inhibition molecular weight, structural homogenity and chemical kinetics are precisely worked out and discussed in the various chapters of this thesis.

3. MATERIALS

3. MATERIALS

3.1 FISH

Mature oil sardine (Sardinella longiceps) was used through out the experiment as the source of the enzyme. Fresh sardine immediately after capture and in rigor state were used. The pancreas of sardine is diffused and distributed over liver and the adjoining portion of gut, and is called hepatopancreas. The sardines were cut open and the hepatopancreas was excised out in cold condition (at 0°C to 4°C). On an average one sardine gave 2 to 3 mg hepatopancreas. At a time the hepatopancreas from at least 500 to 600 sardines were excised out and used for isolation of lipase.

3.2 REAGENTS AND CHEMICALS

All chemicals used were of the highest purity. The triglycerides tributrin, tripalmitin and tristearin were laboratory reagent grade products from BDH, Poole England. All other triglycerides, dicaprylin, monocaprilin and p-nitrophenyl acetate were of 99% purity (Sigma chemical company, Missouri, USA). Triolein was of Fluka Ag, Switzerland. The neutral triglycerides of coconut and sardine oil oil were prepared in the laboratory from commercial samples of coconut oil and sardine oil and purified by column chromatography on florisil.

The column chromatographic materials sephadex G-25, G-100, DEAE cellulose A-50 and sephacryl S-200 were from Pharmacia Fine chemicals Uppasala, Sweden. Silica gel for column chromatography and thin layer chromatography were the products of BDH India Limited. The ion exchange resins amberlite IR 120 H^+, Dowex 2x8 $HCOO^-$ and Dowex 50x12 H^+ were obtained from BDH, Poole, England.

The amino acid standards and the reagents for amino acid analysis were all from Technicon, New York, U.S.A. except methyl cellosolve, which was supplied by E. Merk India Limited. The reagents for disc gel electrophoresis viz; trishydroxymethyl amino methane (TRIS) and acryl amide were products of E. Merk, Damstardt, Germany. NNN' N', tetramethyl 1:2 Diamino ethane (TEMED), NN' methelene bis acrylamide, Bromophenol blue were of BDH, Poole, England, Riboflavin of Pfizer, India Limited and Amido black from George T.Gurr Limited, England.

The sugar standards and the fatty acid methyl ester standards excepting those of C_8, C_{12}, C_{14} and C_{18} fatty acids were from Applied Science Laboratories, USA. The methyl esters of C_8, C_{12}, C_{14} and C_{18} fatty acids were obtained from Fluka Ag, Switzerland. The column materials for GLC viz. Silar 5CP and Gas Chrom Q 80-100 were also products of Applied Science, Laboratories.

The orcinol used for carbohydrate estimation was obtained from BDH Poole England. The protein standards ribomuclease A, chymotrypsinogen A, Ovalbumin, Bovine Serum Albumin and blue Dextran were products of Pharmacia Fine Chemicals Sweden, and glyceraldehyde 3 phosphate dehydrogenase from Sigma Chemical Co. USA.

ISOLATION OF TRIGLYCERIDES FROM COCONUT OIL AND SARDINE OIL

The triglycerides of coconut oil and sardine oil were separated according to Litchfeild (1972). Silica gel for column chromatography was activated overnight by heating at 140°C. 60 g of activated silica gel was slurried with pure benzene and uniformly packed in a glass column (3 x 30 cm). The silica gel bed was covered with a filter paper disc and solvent (benzene) flow was adjusted to 3 ml/min. The solvent level was lowered to gel surface and about 1.5 to 2 g coconut oil sample was applied in 1 ml pure benzene. The column was eluted with pure redistilled benzene. The first 200 ml of the eluent containing the triglycerides was collected and vacuum dried in flash evaporator to give free triglycerides. Similarly the triglycerides of sardine oil was also obtained. These triglyceride mixtures were used as substrates for lipase reaction.

3.3 ANALYTICAL EQUIPMENTS USED

1. Spectronic-20. All colorimetric measurements reported were made with spectronic-20, Colorimeter/spec-

trophotometer, Bosch and Lomb, New York, USA.

2. Spectrophotometer. Scanning of absorption spectrum, UV absorbance measurements, TLC plate scanning acrylamide disc gel scaning were done with the aid of a double beam, double wave length spectrophotometer with TLC and gel scanning attachment, Model No.556 Hitachi Limited, Tokyo, Japan.

3. Amino acid Analyster. The Technicon NC-2P single column Ion Exchange Amino acid analyser system, Technicon Instruments Corporation, New York, USA was used for amino acid analysis. The column was packed with type C_3 sulphonic acid cation exchange resin in the size range 8 to 12 microns.

4. Gas Liquid chromatograph. All gas liquid chromatography analyses were done with the aid of a dual column gas chromatograph, with temperature programmer controller type RLO 4/04 temperature controller type RLO 4/07A, electrometer amplifier type RLO 4/03 A (All Toshniwal Brothers Private Limited, Bombay, India) and Varian techtron single pen recorder, Model 135. (Matsuhita communication industrial company, Japan).

5. Columns. The chromatographic columns 2.5x100 cm and 1.5x100 cm were products of Pharmacia Fine chemicals, Uppasala, Sweden. All other columns described were fabricated locally.

6. Fraction collector and UV detector. Fractionation of the column eluent was done with the aid of 7000 Ultrorac fraction collector, and the protein elution was monitored with the help of 2089 UV cord 111; UV absorption meter and a Chopper Bar 6 channel recorder 6520 -7/8, all products of LKB Produkter, Sweden.

7. Preparative ultracentrifuge. All preparative centrifugations were done in the preparative ultracentrifuge type VAC 601 of Veb Zentrifugenbau Engellsdorf, German Democratic Republic.

8. Water bath, air oven etc. All temperatures above ambient temperature but below 80°C were attained with the help of Serological water bath; Tempo Industrial Corporation Bombay, India. The temperature below ambient temperature were achieved by manipulating the temperature control of a refrigerator. Temperatures reported above 80°C were developed by the aid of an air oven supplied by Tempo Industrial Corporation, Bombay, India.

9. Lyophiliser. Lyophilisation of all biological substances were done in a Toshniwal lypohiliser, Toshniwal Brothers Private Limited, Bombay, India.

10. Electrophoresis Apparatus. Disc gel electrophoresis was done in an appratus manufactured by Toshniwal Brothers Private Limited, Bombay using a 100 m A power

supply unit of the same party.

11. pH meter. All pH measurements and pH manipulations were made with the aid of a digital pH meter 1400 P, ES Research, Concord Instruments Private Limited, Cochin, India.

4. METHODS

4. METHODS

4.1 ASSAY OF LIPASE ACTIVITY

Lipase was assayed by titrimetric method according to Bier (1962) after minor modification of the procedure. The method consits in estimating the fatty acid liberated by the enzyme from triglyceride emulsion by titration with standard alkali.

Reagents

a. Triglyceride emulsion

10 g polyvinyl alcohol was stirred mechanically in 100C ml distilled water to give a fine disperson. To this 5 ml of 0.1 N hydrochloric acid was added and the mixture was heated at 75°C to 85°C until solution was completed. It was cooled, filtered and pH brought to 7 with dilute sodium hydroxide solution. To 100 ml of this solution tributrin or other required triglyceride was added to 0.1 M level and emulsified by blending in a waring blender for 5 minutes.

b. N/20 Calcium chloride

2.74 g $CaCl_2$ $6H_2O$ in 250 ml distilled water was prepared.

c. N/20 Sodium hydroxide

Approximately 200 ml N/10 sodium hydroxide was

prepared and its exact normality was estimated. It was then suitably diluted to give exactly N/20 sodium hydroxide.

d. Citrate phosphate buffer pH 7 (McIlvaine phosphate buffer)

Stock solutions

Solution A. 0.1 M citric acid solution.

Solution B. 0.2 M Dibasic sodium phosphate solution.

A and B were mixed in 6.5:43.5 proportion and 1:1 diluted with distilled water to give pH 7 McIlvaine phosphate buffer (Gomori, 1962). This buffer was assumed to be 0.1 M.

e. 85% alcohol

Commercially available rectified spirit was used.

f. Phenolphthalien indicator

0.01% solution in 85% ethyl alcohol.

Procedure

In a 100 ml conical flask 5 ml emulsified substrate, (triglyceride emulsion) 5 ml McIlvaine phosphate buffer, 0.5 ml N/20 calcium chloride and a suitable aliquot of enzyme preparation to be assayed were mixed and incubated at 30°C for 30 minutes with constant shaking. At the end of incubation period 20 ml 85% ethyl alcohol was added to stop the reaction and break the emulsion.

Controls were also run in the same way but 20 ml 85% ethanol was added before the addition of the enzyme. After termination of the reaction two drops of phenolphthalien indicator was added and the liberated fatty acids were titrated against N/20 sodium hydroxide soluition. The amount of enzyme was chosen in such a way as to give a titre value of 3 to 6 ml N/20 sodium hydroxide for 30 minutes of reaction.

One unit of enzyme is defined as the amount of enzyme required to liberate 1 micro mole of fatty acid from the triglyceride emulsion under study at 30°C per minute.

4.2 ENZYME HOMOGENITY TESTS

Homogenity of purified lipase was tested by:

1. Gel filtration
2. Polyacrylamide disc gel electrophoresis and
3. Absorption spectrum

4.2.1 GEL FILTRATION

Gel filtration was performed on sephacryl S-200. The gel suspension was deaerated and poured into a glass column (1.7 x 55 cm) already half filled with distilled water. The gel was poured until the bed volume reached a hight of 45 cm. The column was drained till the water level falls to the bed level at which the column was connected to a 0.01 M, pH 7 McIlvaine phosphate buffer and

the column outlet was connected to a peristatic pump adjusted to drain the column at 15 ml per hour. About 1000 ml of the buffer was passed through the column to equiliberate the gel bed. To the equiliberated column 15 mg of lipase protein was applied and eluted with 0.01 M, pH 7 McIlvaine phosphate buffer at a flow rate of 15 ml per hour. The eluent was fractionated into 6 ml volumes in Ultrorac 7000 fraction collector after monitoring the absorbance at 280 nm with UV cord III UV absorption-meter, which was recorded to give the elution pattern.

4.2.2 ELECTROPHORESIS

Polyacrylamide disc gel electrophoresis was also done to ascertain the homogenity of purified lipase according to Davis (1964).

Reagents

Solution A.

1 N hydrochloric acid	-	48 ml
Tris	-	36.6 g
TEMED	-	0.23 ml
Water to	-	100 ml

Solution B.

1 N hydrochloric acid	-	48 ml
Tris	-	5.98 g
TEMED	-	0.46 ml
Water to	-	100 ml

Solution C.

Acrylamide	-	28 gm
BIS		0.735 g
Water to	-	100 ml

Solution D.

Acrylamide	-	10 g
BIS	-	2.5 g
Water to	-	100 ml

Solution E.

Riboflavin	-	4 mg
Water to	-	100 ml

Tris glycene buffer pH 8.3

Tris	-	6 gm
Glycene	-	28.8 g
Water to	-	1000 ml

This buffer was stored at 0°C and diluted ten times just before use.

Bromophenol blue (Marker dye)

Bromophenol blue	-	50 mg
Water	-	100 ml

Amidoschwartz stain

Amidoschwartz	-	1 g
Glacial acetic acid	-	7 ml
Water to	-	100 ml

The running gel containing 7% acrylamide was composed of solutions A,C,E and water in 1:2:2:3 proportion. The spacer gel and sample gel were composed of 2.5% acrylamide and was prepared by mixing B,D and E solutions in 1:2:1 proportion and diluting with equal volume of water.

Lipase sample was prepared by mixing 1 ml lipase solution (10 mg protein) and 0.1 ml Bromphenol blue solution with equal volume of 5% acrylamide solution, prepared by mixing B,D and E solution in 1:2:1 proportion.

The gel tubes (8.5 x 0.6 cm) were filled with 2 ml running gel solution, which was over layered with a little water. The tubes (6 numbers) were placed before a flourescent tube light for 45 minutes to polymerise. After polymerisation the water layer was drained with a piece of filter paper and 0.25 ml spacer gel solution was poured, followed by a layer of water and as before allowed to polymerise before a flourescent tube light for 45 minutes. Again water layer was removed from the tube and 0.25 ml lipase sample was added and kept before flourescent lamp for 15 minutes. The tubes were then fixed to the electrophoresis apparatus and the remaining portion of the tubes were filled with Tris-glycene buffer. The two buffer chambers were also filled with the same buffer. The terminals of the apparatus was connected to the power supply unit and electrophoresis done at 6 m A per gel.

At the end of electrophoresis the gels were taken out of the tubes, fixed and stained with amido schwartz solution. The gels were destained by repeated washing with 7% acetic acid and photographed.

4.2.3 THE ABSORPTION SPECTRUM OF PURE LIPASE

The absorption spectrum of lipase protein was taken by scanning 0.6% lipase protein in 0.01 M McIlvaine phosphate buffer from 200 nm to 600 nm in a UV - visible double beam double wave lnegth spectrophotometer.

4.3 MOLECULAR WEIGHT ESTIMATION

The molecular weight of purified lipase was estimated by:

1. Gel filtration on sephadex G-100
2. Sodium dodecyl sulphate polyacrylamide disc gel electrophoresis

4.3.1 GEL FILTRATION ON SEPHADEX G-100

Gel filtration on sephadex G-100 was performed according to Andrews (1964) 10 g sephadex G-100 was suspended in water for 48 hours for swelling. The swollen sephadex was deaerated and the fine particles were removed by decantation. The volume of the gel suspension was noted and sufficient volume of 0.1 M McIlvaine phosphate buffer pH 7 was added so as to give a final buffer concentration of 0.01 M. The gel suspension was brought to 4°C and poured

into a glass column (1.5 x 100 cm Pharmacia) which was previously half filled with 0.01 M McIlvaine phosphate buffer, and maintained at 4°C. As the gel was poured, excess buffer was allowed to percolate gradually through the bed. The gel was poured until the gel bed reached a height of 85 cm, meanwhile the elution rate was controlled to 16 ml/h by means of a peristaltic pump. The column was then washed and equiliberated with 2 litres of 0.01 M McIlvaine phosphate buffer (pH 7).

To the equiliberated column blue dextran, standard proteins ribonuclease; 13700, chymotrypsinogen A; 25000, Ovalbumin; 43000 and albumin; 67000 and pure sardine lipase were applied and eluted one after another and their elution volume noted with the aid of UV Cord III UV adsorption meter. From the results Kav for each protein was calculated as per the equation.

$$Kav = \frac{Ve-Vo}{Vt-Vo}$$

Where Ve = Elution volume of the particular protein

Vo = Void volume; the elution volume of blue dextran

Vt = Total bed volume

The Kav values obtained were ploated against logaritham of molecular weight of the standard proteins. From the graph so obtained the logaritham of molecular weight

and hence molecular weight of sardine lipase protein was read out corresponding to its Kav value.

4.3.2 SODIUM DODECYL SULPHATE POLYACRYLAMIDE DISC GEL ELECTROPHORESIS

Molecular weight of sardine lipase was also determined by SDS polyacrylamide disc gel electrophoresis according to Weber and Osbern (1969).

Reagents

a. 0.05 M phosphate buffer pH 7

This was prepared according to Gomori (1962) as follows:

Solution A.

3.12 g NaH_2PO_4 $2H_2O$ in 100 ml distilled water

Solution B.

3.56 Na_2HPO_4 $7H_2O$ in 100 ml distilled water

39 ml solution A was mixed with 61 ml solution B and diluted to 200 ml to give pH 7, 0.05 M phosphate buffer.

b. Gel buffer

Gel buffer was prepared as follows:

NaH_2PO_4 $2H_2O$	-	1.95 g
Na_2HPO_4 $7H_2O$	-	9.65 g

Sod. dodecyl sulphate - 0.50 g

Water to - 250 ml

c. Sample buffer

0.05 M phosphate buffer - 20 ml

Sodium dodecyl sulphate - 100 mg

Mercapto ethanol - 0.1 ml

Water to - 100 ml

d. 0.C5% Bromophenol blue in water

e. Preparation of sample and standards

The standard proteins, Ribonuelease A, Chymotrypsinogen A, Glyceraldehyde 3-phosphate dehydrogenase, Ovalbumin and bovine serum albumin and the purified sardine lipase were separately dissolved in 5 ml sample buffer to give a concentration of 1 mg protein/ml and the solutions were incubated at 37°C for 2 hrs. Six clean 10 ml test tubes were taken. To each 3 ul of 0.05% bromophenol blue, 1 drop of glycerol, 5 ul of mecaphto ethanol and 50 ul of sample buffer were added. To one by one of these tubes 50 ul of the incubated protein solutions of Ribomuclease A, Chymotrypsimogen A, Glyceraldehyde 3-phosphate dehydrogenase, Ovalbumin, bovine serum albumin and sardine lipase were added each in one test tube and labelled. The mixture was thoroughly mixed and kept at 4°C until further use.

f. Acrylamide solution

This was prepared in the following composition.

Acrylamide	-	22.2 g
Bis-acrylamide	-	0.6 g
Water to	-	100 ml

Filtered and kept at 4°C

g. Persulphate solution

Amm. persulphate	-	150 mg
Water	-	10 ml

Freshely prepared for each experiment.

h. -N,N,N'N' tetramethyl ethylene diamine

Procedure

The gel tubes (8.5 x 0.6 cm) were thoroughly cleaned, rinced with distilled water and oven dried.

These tubes were fixed in the stand. 10 ml gel buffer was deaerated and mixed with 9 ml acrylamide solution. The mixture was again deaerated and 1.5 ml freshly prepared ammonium persulphate and 45 ul of TEMED were added. The mixture was gently shaken and 2 ml portions were added to the gel tubes without air bubble. A few drops of water were layered on top of the gel solution immediately with the help of a syringe. The tubes were then left undisturbed for 20 minutes to harden.

After the gel hardened the water layer above the gel was removed with the help of a piece of filter paper. To each of the gel tubes 100 µl of each one of the standard proteins/sardine lipase solution prepared above was added and marked. The tubes were then taken carefully fixed on the electrophoresis apparatus. The vacant space in the tubes were filled with gel buffer diluted 1:1 with water, without mixing the protein sample applied. The two chambers of the apparatus also were filled with 1:1 diluted gel buffer, and the apparatus was connected to a power supply unit and electrophorised atacurrent of 8 m A per tube. The electrophoresis was done at 20°C in an air conditioned room.

At the end of electrophoresis the gels were removed from the tubes with a syringe and stained with 1% amidoschwartz in 7% acetic acid for 10 minutes. It was then destained by repeated washing with 7% acetic acid. From the electrophorograms obtained the mobility of each protein applied was calculated according to the equation.

$$\text{Mobility} = \frac{\text{Distance of protein migration}}{\text{gel length after destaining}} \times \frac{\text{Gel lenggh before staining}}{\text{Distance of Bromophenol blue migration}}$$

The mobility obtained was plotted against log molecular weight in a graph. From the graph the molecular

weight of sardine lipase was read out corresponding to its mobility.

4.4 CARBOHYDRATE ANALYSIS

4.4.1 ESTIMATION OF CARBOHYDRATE CONTENT

Carbohydrate content of lipase was estimated by Orcinol method (Umbriet et al. 1959) after hydrolysis and separation of the carbohydrate.

10 mg of pure lyophilised sardine lipase was hydrolysed with 0.5 ml 1 N hydrochloric acid at 100°C for 8 hours, in a corning test tube sealed under vacuum. At the end of hydrolysis the tip of the test tube was broken and the contents were transferred to a centrifuge tube along with one ml of distilled water and centrifuged at 3000 rpm. The clear centrifugate containing the free sugars was passed successively through columns of Dowex 1 x 4 H^+ (1 x 15 cm) and Dowex 50 x 4 $HCOO^-$ (1 x 15 cm) to remove amino acids and eluted with distilled water. The first 50 ml of the eluent was collected and dried under reduced pressure at 50-60°C in a flash evaporator. The residue was dissolved in 1 ml water and evaporated as before. The dissolution in water and evaporation was repeated 5 times to free the residue from acid.

The final residue obtained was dissolved in 1 ml distilled water. 0.1, 0.2 and 0.3 ml portions of this

solution were transfered into separate test tubes and the volume in each tube was made up to 3 ml with distilled water. Three ml freshly prepared 1% orcinol in 0.1% ferric chloride dissolved in concentrated hydrochloric acid was then added to each test tube, shaken gently and heated in a boiling water bath for 3 minutes. Simultaneously standards containing 10, 20, 30 and 40 ug of ribose were also prepared in a similar manner. The test tubes were cooled to room temperature and the colour developed was measured at 660 nm in spectronic-20 spectrophotometer. The optical density obtained for the standards were ploated in a graph against corresponding concentrations. From the standard curve obtained the carbohydrate content of lipase was read out against its optical density.

4.4.2 DETERMINATION OF INDIVIDUAL CARBOHYDRATES

The carbohydrate composition of the lipase was analysed as alditol acetates in a gas chromatograph according to Kim et al. (1967) as follows:

0.5 ml of the sugar solution obtained as above was brought to pH 7.5 with 0.2 N ammonia and the sugars were reduced to corresponding alditols with 15 mg of sodium borohydride in a micro test tube at room temperature for 1 day. This mixture was then treated with (1 g) amberlite IR 120 H^+ till pH is below 7 and filtered. The filtrate was dried under vacuum at 60°C. The residue obtained was

dissolved in 1 ml methanol and again vacuum dried. The dissolution in methanol and vacuum drying was repeated five times to free the residue from borate.

The residue of alditols so obtained were then mixed with 0.25 ml acetic anhydride and 0.25 ml pyridine in a 5 ml corning test tube. The tube was tightly closed and the contents refluxed at 100°C in an oil bath for 2 hours. At the end the excess acetic anhydride was removed by hydrolysis with 1 ml water followed by vacuum evaporation. The residue was again dissolved in one ml water and evaporated. The process of dissolution and evaporation was repeated three times. The residue was then dissolved in 1 ml chloroform and vacuum dried and this process was also repeated 3 times to give pure alditol acetates. The final residue was dissolved in 15 ul of chloroform.

2 ul of the chloroform solution of alditol acetate was injected to a gas chromatograph equipped with dual stainless steel columns (6' x ¼") and flame ionisation detector. The column was packed with 10% silar 5 CP coated on Gaschrom Q 100-120 mesh. The detector and injection port were maintained at 250°C. To start with the column was at a temperature of 120°C and temperature programmed to give a temperature increase of 2°C per minute up to 200°C, where the columns were maintained isothermal for 60 minutes. The out put signals were

recorded at a chart speed of 10 cm/hour. From the chromatogram obtained the component sugars were identified and composition computed with the aid of a chromatogram obtained for a standard sugar mixture under identical conditions.

4.5 AMINO ACID ANALYSIS

4.5.1 AMINO ACID ANALYSIS USING AUTOMATIC AMINO ACID ANALYSER

Amino acid analysis was performed according to Hirs (1972). About 5 mg pure lipase protein was hydrolysed with 2 ml 6 N hydrochloric acid and 2 mg phenol in a clean pyrex test tube sealed under vacuum, by heating at 110°C for 24 hours. After hydrolysis the tube was broken and the hydrolysate was evaporated under vacuum at 40 to 50°C. The residue obtained was dissolved in 1 ml distilled water and again evaporated. The process of dissolution and evaporation was repeated till the hydrolysate was free from acid (5 times). The residue obtained was dissolved in 0.5 ml 0.20 N sodium citrate pH 2.2. Two microlitre of this solution was injected to the NC2P Amino Acid Analyser system. The amino acid peaks recorded were identified and computed with the help of a pattern from a standard amino acid mixture.

4.5.2 ESTIMATION OF TRYPHTOPHAN

Tryptophan being oxidisable during acid hydrolysis

was analysed separately by the spectrophotometric method of Goodwin and Morton (1946).

0.1% solution of lipase was prepared in 0.1 N sodium hydroxide and its ultraviolet absorption spectrum was taken in a double beam double wave length spectrophotometer. The molar concentration of tyrosine (y) present in lipase protein was taken from the amino acid analysis data. The molar extinction coefficients of tyrosine (A) and tryphophane (B) in 0.1 N sodium hydroxide at 280 nm, 1576 and 5225 respectively were taken from tables (Goodwin and Morton, 1946). From the absorption spectrom of lipase the Molar Extinction (E) of lipase at 294.4 nm was calculated which will be the contribution of tryphtophan and tyrosine in accordance with their molar proportion in lipase. If x is the total number of moles of tyrosine and tryphtophan put together then.

$$E = y A + (x-y) B$$

From this equation the only unknown x was calculated, from which the number of moles of tryphtophan in lipase was calculated by substracting the number of moles of tyrosine.

4.6 EFFECT OF TEMPERATURE

4.6.1 EFFECT OF TEMPERATURE ON SARDINE LIPASE ACTIVITY

The lipase assay was conducted by incubating the

reaction mixture containing 5 ul of sardine lipase at various temperatures from 4°C to 60°C for 30 minutes. The reaction was terminated by the addition of 20 ml 85% alcohol and the liberated fatty acids were titrated, against N/20 sodium hydroxide using phenolphthalien indicator. From the titre value the velocity of reaction (number of micromoles of fatty acid liberated per minute) was calculated. A graph was drawn with velocity against temperature, from which the temperature optimum was read out.

4.6.2 FFFECT OF TEMPERATURE ON STABILITY OF SARDINE LIPASE

The temperature stability of sardine lipase was worked out by incubating 5 ul of purified sardine lipase with 5 ml 0.1 M; pH 7 McIlvaine phosphate buffer and 0.5 ml N/20 calcium chloride in a 100 ml conical flask for 1 hour at various temperatures from 30°C to 70°C. At the end of 1 hour the contents of the flask were brought to 30°C and incubated at that temperature for 30 minutes after adding 5 ml emulsified tributrin. At the end of incubation the activity retained after the temperature treatment were measured by titration with 0.05 N sodium hydroxide. The value of activity obtained for each temperature were plotted in a graph against temperature.

4.7 EFFECT OF pH

4.7.1 EFFECT OF pH ON SARDINE LIPASE ACTIVITY

The effect of pH on sardine lipase activity was determined by conducting the assay as usual with 5 ml buffer having pH ranging from 4 to 12. The buffers in this pH range were prepared as follows:

a. Universal buffer (pH 2-14)

The buffer was prepared according to Lurie (1975). A mixture of 0.04 M phosphoric, boric and acetic acidswere prepared. Buffer of required pH value was obtained by adding the given amount of 0.2 N sodium hydroxide to 100 ml of the acid mixture as shown in the following table.

TABLE 7. UNIVERSAL BUFFER

pH	Volume of 0.2 N NaOH ml
2	5.0
3	18.2
4	24.5
5	35.0
6	42.0
7	52.5
8	60.0
9	67.5
10	78.0
11	83.5
12	100.0

b. The pH optimum was also determined using a buffer combination of McIlvaine phosphate buffer and Barbital buffer which were prepared as follows:

i. McIlvaine phosphate buffer (Gomori, 1962)

stock solutions

A. 0.1 M solution of citric acid

B. 0.2 M solution of dibasic sodium phosphate

x ml of A were mixed with y of ml B and the mixture made up to 100 ml to give buffer of the required pH value as per the following table.

TABLE 8. McILVAINE PHOSPHATE BUFFER

pH	x ml	y ml
3	39.8	10.2
4	30.7	19.3
5	24.3	25.7
6	17.9	32.1
6.8	9.1	40.9
7	6.5	43.5

ii. Barbital buffer (Gomori, 1962)

Stock solutions

A. 0.2 M solution of sodium barbital

B. 0.2 M hydrochloric acid

50 ml of A and x ml of B were mixed and made up to 200 ml to give required pH value as per the following table.

TABLE 9. BARBITAL BUFFER

pH	x
6.8	45
7.0	43
8.0	17.5
9.0	2.5

In case of McIlvaine phosphate buffer (pH 3-7) and Barbital buffer (pH 6.8 to 9) combination, the activities obtained, for barbital buffer pH range were elevated to McIlvaine phosphate buffer level by adding the activity difference from common pH points 6.8 and 7 to the activity at pH 8 and 9.

The activity values obtained for various pH were then ploated on a graph paper (pH VS activity) separately for universal buffer and McIlvaine-Barbital buffer combination. From these graphs the pH optimum was read out.

4.7.2 EFFECT OF pH ON STABILITY OF SARDINE LIPASE

Stability of sardine lipase to pH was determined using universal buffer in the pH range of 4 to 12. The

buffer was prepared as in Table 7. One ml 0.1 M universal buffer (pH 4-12) 5 ul of purified sardine lipase and 0.5 ml N/20 calcium chloride were incubated at 30°C for 1 hour. To the incubated mixture 5 ml tributrin emulsion and 5 ml pH 7; 0.1 M McIlvaine phosphate buffer were added and incubated at 30°C for 30 minutes under constant shaking. As usual the activity was determined by titration against N/20 sodium hydroxide. The pH and corresponding activities were ploated on a graph paper, from which the effect of pH on the stability of sardine lipase was ascertained.

4.8 EFFECT OF SUBSTRATE CONCENTRATION ON LIPOLYSIS BY SARDINE LIPASE AND K_m VALUE

The effect of substrate concentration on velocity of sardine lipase reaction was studied by conducting the lipase assay with varying concentrations of triglyceride (0.01 M to 0.1 M) in 1% polyvinyl alcohol emulsion. The reaction was carried out as in assay methods and the velocity of reaction determined by finding out the number of micromoles of fatty acid liberated per minute by 5 ul of purified sardine lipase. The values of substrate concentration and velocity were then converted to respective reciprocals and were plotted in the x and y axes of a graph. From the straight line graph obtained, its slope and y intercept were measured and used in the Michaelis -

Menten equation $\frac{I}{v} = \frac{K_m}{VS} + \frac{1}{V}$ to calculate Michaelis constant K_m; Where slope is $\frac{K_m}{V}$ and y intercept $\frac{1}{V}$. The triglycerides tributrin and triacetin were used as substrate in this study.

4.9 INHIBITION AND ACTIVATION

4.9.1 EFFECT OF ALDEHYDES AND KETONES

Acetone, acetaldehyde, formaldehyde and ethyl methyl Ketone were incorporated separately to 0.1 M concentration in 0.1 M tributrin emulsion in 1% polyvinyl alcohol. The sardine lipase assay was conducted as described earlier but with 5 ml tributrin emulsion containing acetone/ acetaldehyde/formaldehyde/ethyl methyl ketone and 5 ul of purified lipase solution. The reaction was carried out for 30 minutes and the activity for each aldehyde/ketone was noted.

4.9.2 EFFECT OF INORGANIC ANIONS AND CATIONS

The anions used were Cl^-, Br^-, I^- and F^- in the form of their sodium salt. The cations used were Fe^{2+}, Fe^{3+}, Cu^+, Cu^{2+}, Zn^{2+}, Sn^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Ba^{2+} and Ca^{2+} in the form of chlorides. 10 mM solutions of these salts were prepared separately. To determine the effect of these ions on the sardine lipase, the assay was conducted as in assay method with 1 ml of water containing 5 ul of pure

sardine lipase and 1 ml of 10 mM solution of the ion under test. Simultaneously a control was also run with 1 ml water instead of the ion solution. The reaction was terminated after 30 minutes incubation at 30°C by addition of 20 ml 85% alcohol and the activity was determined by titration against standard N/20 sodium hydroxide. From the results percentage of activity exhibited in presence of the various ions was calculated and tabulated. The effect of Fe^{3+} was studied in some more detail by preincubating mixtures of 1 ml of 0.05% lipase with 1 ml of 10 mM solution of Fe^{3+} ion and 5 ml McIlvaine phosphate buffer for various periods at 30°C. At the end of incubation 5 ml tributrin emulsion was added and assayed as usual. The control was run by adding the tributrin emulsion to one of the above reaction mixtures immediately after preparation and assyed. From the titre values the percentage degree of inactivation calculated and tabulated.

4.10 SUBSTRATE SPECIFICITY

The specificity of sardine lipase was tested by conducting the assay using:

1. Individual triglycerides and
2. The triglyceride mixtures of the natural oils of coconut and oil sardine

4.10.1 INDIVIDUAL TRIGLYCERIDES

The triglycerides used were triacetin, tributrin, trivalerin, trihexanoin trioctanoin, tripalmitin and triolein. All these triglycerides were assayed as described in assay method by incorporating them to 0.1 M concentration in 1% polyvinyl alcohol as emulsion along with controls. The assays were carried out for 30 minutes at 30°C and the activities recorded to give the relative rate of lypolysis.

4.10.2 TRIGLYCERIDES OF NATURAL OILS

One g of the neutral triglyceride of coconut oil was emulsified with 25 ml of pH 7; McIlvaine phosphate buffer and 25 ml of 1% polyvinyl alcohol in a waring blender for 5 minutes. To the emulsion 5 ml N/20 calcium chloride solution and 0.5 ml of purified sardine lipase were added and incubated for 24 hours at 30°C under constant shaking. Towards the end, the reaction was terminated by the addition of 150 ml ethanol. Simultaneously a control was also run by adding 150 ml ethanol in the beginning itself. The reaction mixtures were made neutral with N/50 sodium hydroxide and were transfered to separating funnels under label; sample and control. The remaining triglycerides in the reaction mixtures were removed by extraction with 50 ml diethyl ether. The

aqueous solution left were washed twice with 15 ml portions of diethyl ether and acidified with dilute hydrochloric acid to liberate fatty acids from their sodium salt. The fatty acids liberated were then extracted with 50 ml diethyl ether in a separating funnel. The ether extracts obtained were washed twice with 10 ml portions of distilled water and the ether extracts were each dried by shaking with 10 g anhydrous sodium sulphate for 30 minutes. The dried extracts were transfered to 100 ml distillation flasks (quick fit) marked and evaporated one by one in a flash evaporator. Similarly under identical conditions, 1 g neutral triglycerides of sardine oil were also lipolysed along with control and the fatty acids released extracted. The fatty acids so obtained were converted to respective methyl esters according to (Litchfield, 1972) as follows:

To the residue of fatty acids obtained by evaporation in each flask 15 ml methanol-boron trifluride mixture was added along with a boiling chip and refluxed on a water bath for 2 minutes, under an atmosphere of nitrogen. The reaction mixtures were then cooled and transfered to 150 ml separating funnel, 40 ml distilled water and 50 ml diethyl ether were added, the contents were gently shaken and the ether layer was allowed to separate. The

aqueous layer was run off and the ether extract remaining was washed twice with 5 ml portions of distilled water. To the ether extract left in each separating funnel 5 g anhydrous sodium sulphate was added and allowed to stand for 30 minutes with occasional shaking. The ether extracts were then slowly decanted to 100 ml distillation flasks and the ether was removed by vacuum flash evaporation until about 5 ml remained in each flask. The remaining either was then blown off with a stream of nitrogen to give methyl esters of the fatty acids from the control and the lypolysate of neutral triglycerides of coconut and sardine oils.

In a similar manner the methyl esters of the fatty acids from the total triglycerides of coconut oil and sardine oil were prepared after saponification of the oils.

The samples of methyl esters prepared above were separately dissolved in 5 ml diethyl ether. Each of these samples were subjected to gas liquid chromatography by injecting 2 ul of the sample into a gas chromatograph equipped with dual stainless steel columns (6' x ¼") and flame ionisation detector. The columns were packed with 10% silar 5 CP coated on Gaschrom Q 100-120 mesh. The detector and injection port were maintained at 250°C. In the case of sardine oil the columns were kept inothermal at 170°C for 20 minutes and then temperature

programmed to give 1°C/minute up to 216°C where the temperature was maintained for 60 minutes.

For coconut oil the column temperature was programmed from 120°C, at 2°C/minute up to 200°C and kept isothermal at 200°C for 45 minutes. The flame ionisation detector signal was amplified and recorded in a chart run at a speed of 30 cm/hr. The individual fatty acids were identified by comparison with reference standard fatty acid peaks obtained in a separate run, percentage composition computed and tabulated as reported by Gopakumar & Nair (1967) From the results the preferential hydrolysis of certain fatty acid triglycerides were noted.

4.11 SEQUENCE OF TRIGLYCERIDE HYDROLYSIS BY SARDINE LIPASE

Using tributrin. The lipase assay was run with 25 ml tributrin emulsion (0.1 M tributrin in 1% polyvinyl alcohol) 25 ml McIlvaine phosphate buffer; pH 7, 1 ml N/20 calcium chloride and 0.25 ml purified sardine lipase solution. At time intervals 0, 15, 30, 60, 120 minutes and 24 hours (infinite time) 5 ml of the reaction mixture was withdrawn and added to 20 ml ethanol in a 100 ml separating funnel. To this 25 ml ether was added, gently shaken and allowed to settle. The aqueous layer was run out and the ether extract left was washed 3 times with 5 ml distilled water, finally the ether extract was trans-

fered to a 100 ml flask and evaporated under vacuum. Similarly the ether extractives of the reaction mixture at various times were prepared and kept at -18°C until further use to prevent spontaneous isomerisation of the partial glycerides.

The residues of lypolysis products of tributrin obtained for various times were subjected to thin layer chromatography on silica gel impregnated with boric acid according to Okumura (1976). The plates (200 x 200 x 1 mm) were activated at 140°C for 1 hour and predeveloped in diethyl ether to remove organic contaminants to the top of the plate, where they were removed by scraping off a narrow band of adsorbant. The experimental samples were then dissolved in 5 ml diethyl ether and 10 ul was spot on the activated plates. Simultaneously standards of tributrin, dibutrin and monobutrin were also spotted. The plate was then developed using chloroform acetone (96:4) solvent system. The spots of the products of lipolysis were visualised by spraying uniformly 50% sulphuric acid, followed by heating at 150°C for 20 minutes. The charred spots were measured quantitatively by reflectance scanning in a double beam double wave length spectrophotometer with TLC scanning attanchment with λ_1 set at 445 nm and λ_2 at 560 nm. The concentration of distribution of mono, di, tributrin and

butric acid with time was calculated and tabulated.

4.12 POSITIONAL SPECIFICITY OF SARDINE LIPASE USING 2-MONO, 1,2-DI AND TRICAPRYLIN

To study the positional specificity of sardine lipase the assay was carried out with 5 ml McIlvaine phosphate buffer, 0.5 ml N/20 calcium chloride, 5 ul purified sardine lipase and 5 ml 0.1 M solution of mono/di/tricaprylin emulsion in 1% polyvinyl alcohol. The reaction was carried out for 30 minutes and terminated by addition of 20 ml ethanol. The fatty acids liberated were titrated and the velocity of lipolysis of mono, di and tricaprylin were calculated and tabulated.

4.13 IMMOBILISATION OF LIPASE

Lipase was immobilised in poly acrylamide gel as follows. 1 ml pure lipase solution was mixed with 9 ml distilled water and 10 ml 0.1 M McIlvaine phosphate buffer; pH 7, containing 20% acrylamide and 2% NN' methylene bis acrylamide in a 100 ml beaker. To this mixture 1 ml 4% ammonium persulphate and 40 ul of TEMED were added and mixed well. The mixture was then allowed to gel (20 minutes). The gel was then taken out disrupted in a mincer and lyophylised to give immobilised lipase. The immobilised lipase was stored in an air tight bottle at 0°C until it was used for study of its properties, such as its activity, storage life and activity on individual triglycerides as described earlier.

5. RESULTS AND DISCUSSION

5. RESULTS AND DISCUSSION

5.1 DISTRIBUTION OF LIPASE IN OIL SARDINE

Fresh oil sardine were dressed and processed to give red meat, white meat, hepatopancreas and whole viscera. They were separately minced to a homogenous paste and one gram from each sample was separately extracted with 5 ml cold distilled water for 30 minutes. The samples were centrifuged at 0°C at 10000 r.p.m. and 1 ml of the clear extract obtained was subjected to lipase assay using 0.1 M tributrin emulsion in 1% polyvinyl alcochol. The lipase activities obtained by assaying red meat, white meat, hepatopancreas and whole viscera are shown in Table 10. It is seen that lipase activity is more concentrated in hepatopancreas followed by whole viscera, red meat and white meat.

As the lipase concentration of hepatopancreas was appreciable an effort was made to purify the enzyme and study some of its characteristics and properties.

5.2 PURIFICATION OF SARDINE LIPASE

PREPARATION OF FAT FREE HEPATOPANCREAS POWDER

Freshly caught oil sardine brought to the laboratory in iced condition were used for the enzyme purification 500 to 600 sardine were used at a time. The hepato-

TABLE 10. DISTRIBUTION OF LIPASE ACTIVITY IN DIFFERENT ORGANS OF OIL SARDINE

Organ	Activity units/gram wet weight
Hepatopancreas	126.8
Red muscle	31.7
White muscle	2.4
Whole viscera	72.3

pancreas of the sardine were excised into a 3 litre beaker maintained at 0°C. All remaining operations were done between 0°C to 4°C unless otherwise specified.

The hepatopancreas was then homogenised to a uniform paste and its volume noted (approximately 1.5 litre). To this paste in a 5 litre beaker two volume of redistilled acetone at 0°C was added and stirred for 1 hour. The acetone stirred homogenate was filtered through a buchner funnel under vacuum. The residue obtained was again suspended in a 5 litre beaker containing 1000 ml cold redistilled acetone. Stirred for 30 minutes and filtered as before. The extraction with 1000 ml cold acetone was repeated 3 more times. The filter cake obtained was then suspended in 500 ml cold 1:1 acetone-ethyl ether mixture kept stirred for 30 minutes and filtered. The extraction with acetone and ethyl ether mixture was also repeated 3 more times. The residue was then suspended in 500 ml cold ethyl ether stirred for 30 minutes and filtered. The residue was once more extracted with 500 ml cold ethyl ether when it was almost free from lipid material. The filter cake along with buchner funnel was transfered to a lyophiliser chamber at -40°C and lyophilised to remove residual solvents if any to give a friable powder of sardine hepatopancreas. It was then transfered to a clean dry bottle, sealed air tight and stored at -20°C

in a deep freezer until further use. 600 sardine gave 180 g dry hepatopancreas powder.

LIPASE EXTRACTION AND AMMONIUM SULPHATE FRACTIONATION

All operations were carried out at 4°C unless otherwise specified. 100 g dry sardine hepatopancreas powder was suspended in 1 litre cold distilled water and mechanically stired for 2 hours. The homogenate obtained was centrifuged at 10000 r.p.m. and the supernatent was made up to 1000 ml. An aliquot (0.5 ml) of the sample was assayed to find out the lipase activity of this extract. To the remaining solution solid ammonium sulphate was added little by little with constant stirring to give 0.3 saturation. The solution was then kept over night in the cold room. The proteins precipitated over night, were removed by filtration through a celite bed (washed with 0.3 saturated $(NH_4)_2\ SO_4$ solution) under suction. The filtrate was collected and solid ammonium sulphate was again added to give 0.8 saturation and the solution was left over night undistrubed. The precipitated proteins which contained lipase were collected by filtration through a celite bed previously washed with 0.8 saturated ammonium sulphate solution and kept under suction. The top layer of celite bed with the protein precipitate was scraped out and suspended in 100 ml,

cold distilled water. It was triturated for 30 minutes and filtered under suction. The residue of celite was extracted two more times with 15 ml each of cold distilled water and filtered. Finally the fitrates were combined and desalted by passing through a column of sephadex G-25. The desalted lipase solution measuring about 300 ml was then concentrated by adding 35 g dry sephadex G-25. The sephadex was allowed to swell for 4 hours and the suspension was filtered under vacuum. The residue of sephadex was washed with 10 ml portions of cold distilled water. The washings and first filtrate were combined and made up to 150 ml. Lipase activity of this solution was assayed as before using 0.05 ml of the preparation.

DEAE A-50 COLUMN CHROMATOGRAPHY

To the desalted sardine lipase solution immediately after concentration, 15 ml 0.10 M McIlvaine phosphate buffer of pH 7 was added to give a final concentration of 0.01 M. The solution was fed to a column of DEAE A-50 sephadex (2.5 x 80 cm) which was washed and equilibrated with 0.01 M McIlvaine phosphate buffer of pH 7 and maintained at a flow rate of 15 ml/hr. After feeding the desalted lipase solution the column was washed with 0.01 M McIlvaine phosphate buffer pH 7 until the eluent was almost free from UV absorbing materials. At this point

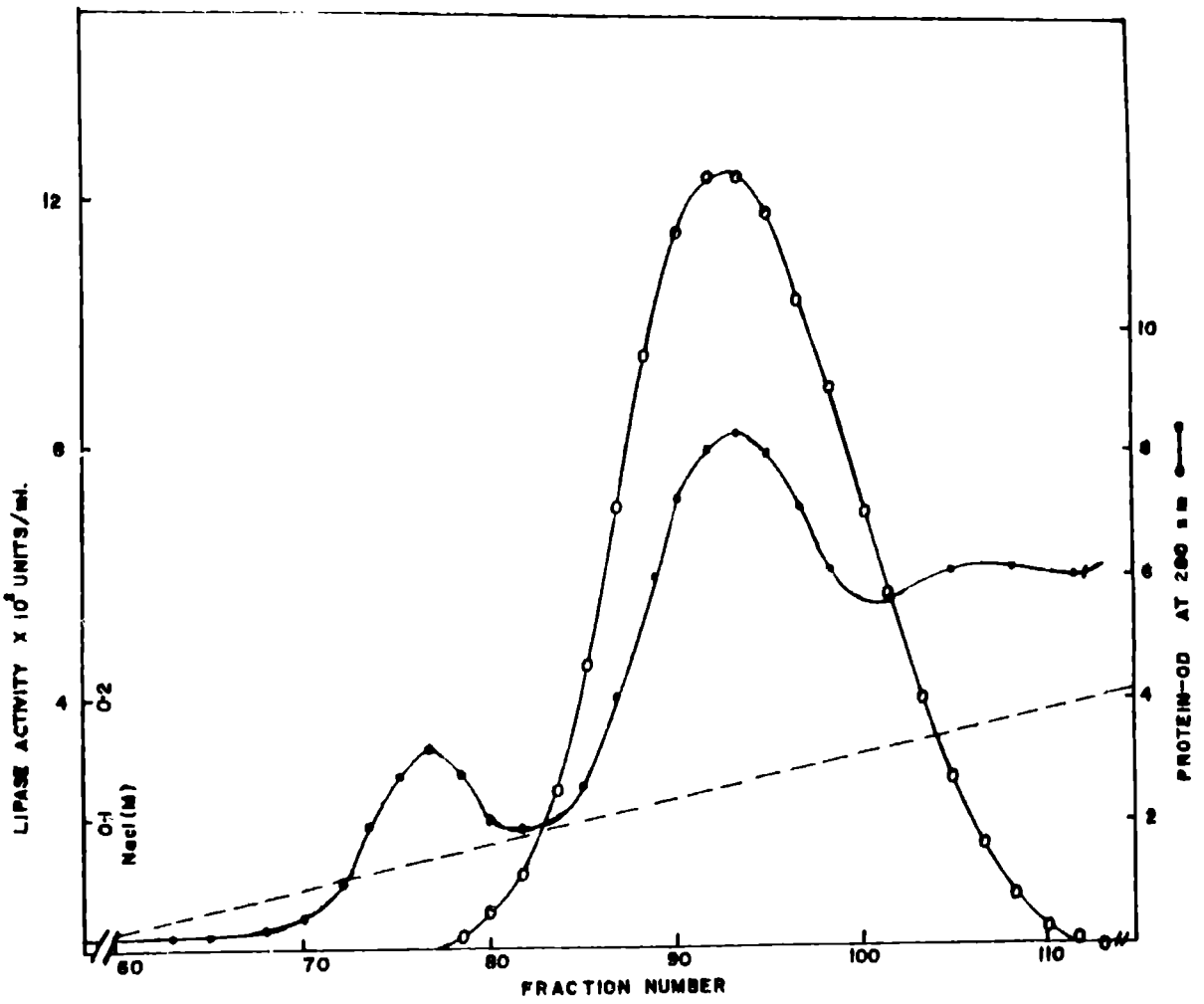


FIG.5 CHROMATOGRAPHIC PURIFICATION OF SARDINE LIPASE ON DEAE CELLULOSE. ELUTION WITH A LINEAR CONCENTRATION GRADIENT OF SODIUM CHLORIDE.

.———. PROTEIN o——o LIPASE ---- SODIUM CHLORIDE

the column was connected to a gradient developer and eluted under a linear gradient of sodium chloride from 0 to 0.4 M in 0.01 M; pH 7 McIlvaine phosphate buffer. The eluent was collected in 6 ml fractions after monitoring the optical density at 280 nm. The lipase activity of alternate fractions were assayed using a suitable aliquot of the eluent. The gradient elution profile of sardine lipase from DEAE sephadex column is shown in fig. 5. The fractions having lipase activity above 1000 units per ml and those with an activity above 100 units but below 1000 units were separately pooled and an aliquot from each of these pools were assayed for their lipase activity.

SEPHADEX G-100 CHROMATOGRAPHY

The high activity pool (30 ml) was then applied on a column of sephadex G-100 (2.5 x 100 cm) for further purification. The sephadex G-100 column was previously equiliberated with 0.01 M, pH 7 McIlvaine phosphate buffer. After application of the high activity sample, the column was connected to a buffer reservoir containing 0.01 M, pH 7 McIlvaine phosphate buffer and eluted at a flow rate of 15 ml/hr. It was fractionated into 6 ml fractions using a fraction collector after monitoring the optical density of the eluent at 280 nm with a UV

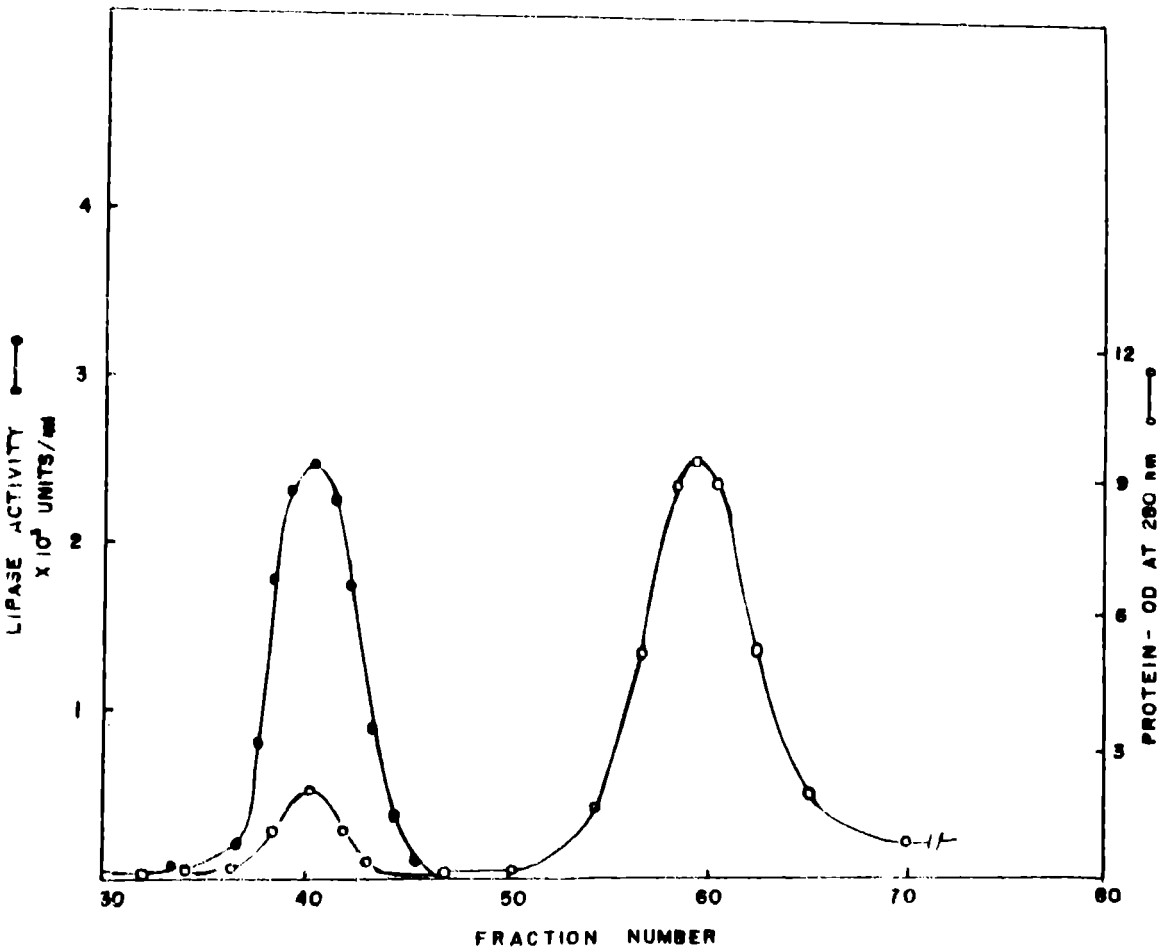


FIG.6 GEL FILTRATION OF SARDINE LIPASE OBTAINED BY DEAE CELLULOSE CHROMATOGRAPHY, ON SEPHADEX G-100

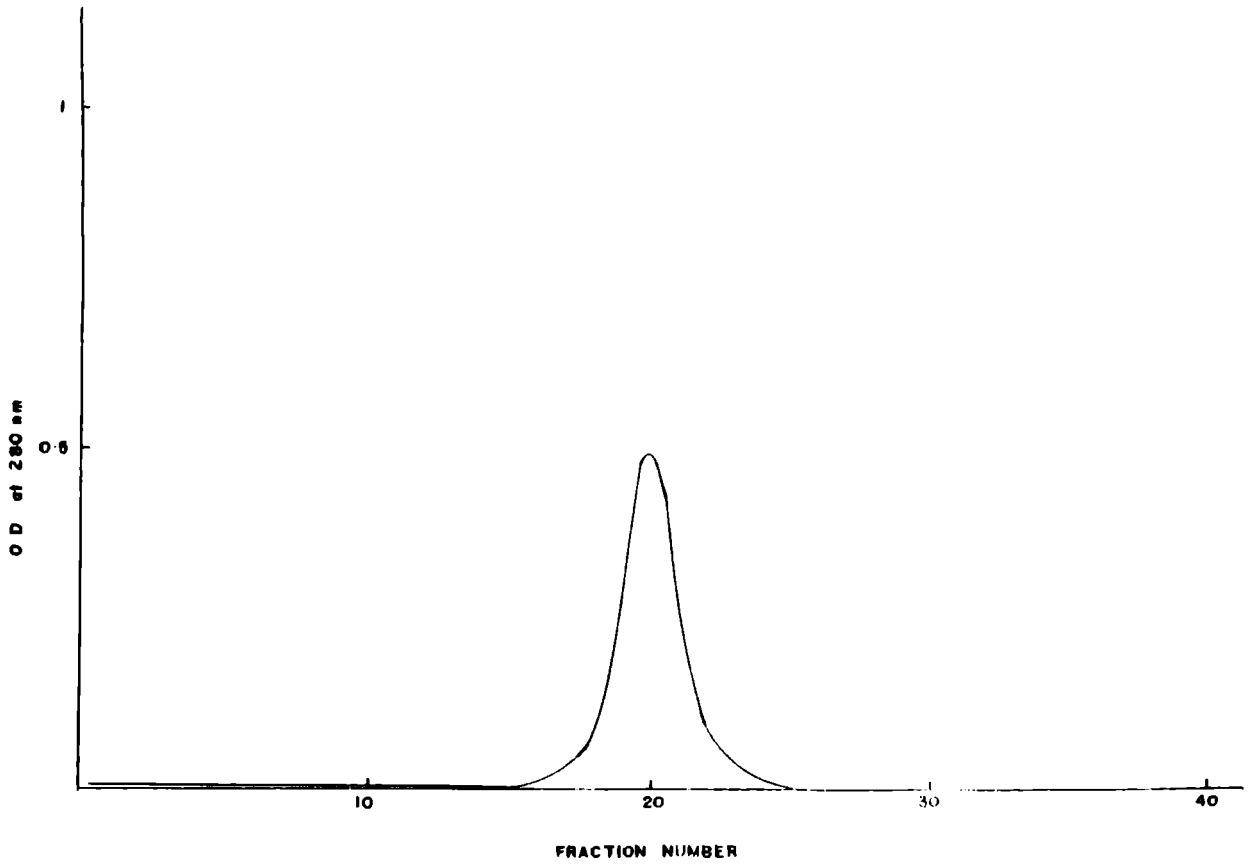


FIG.7 CHROMATOGRAPHY OF PURIFIED SARDINE LIPASE ON SEPHACRYL S-200

monitor. A suitable aliquot (5 to 50 ul) from alternate fractions were assayed for lipase activity. The elution pattern of sardine lipase, so obtained is shown in fig.6. Lipase was present in the first small peak while the other peak did not show any activity. The summary of purification up to G-100 column chromatography are shown in Table 11.

5.3 HOMOGENITY OF PURIFIED SARDINE LIPASE

5 ml of the most active fractions from sephadex G-100 chromatography was applied to a column of sephacryl S-200 (1.7 x 45 cm) equiliberated with 0.01 M, pH 7 McIlvaine phosphate buffer. The column was then eluted with the same buffer at a flow rate of 10 ml/hr. The optical density of the eluent was monitored at 280 nm and the eluent collected into 6 ml fractions. The elution pattern obtained is shown in fig.7. There was only one protein peak which was also the lipase peak.

The sardine lipase obtained after sephadex G-100 column chromatography was then subjected to poly acrylamide disc gel electrophoresis. The electrophoresis pattern obtained is shown in figure 8. There was only one protein band. The absorption spectrum of pure lipase protein obtained is shown in figure 8b.

FIG.8a DISC GEL ELECTROPHORETIC PATTERN OF PURIFIED SARDINE LIPASE

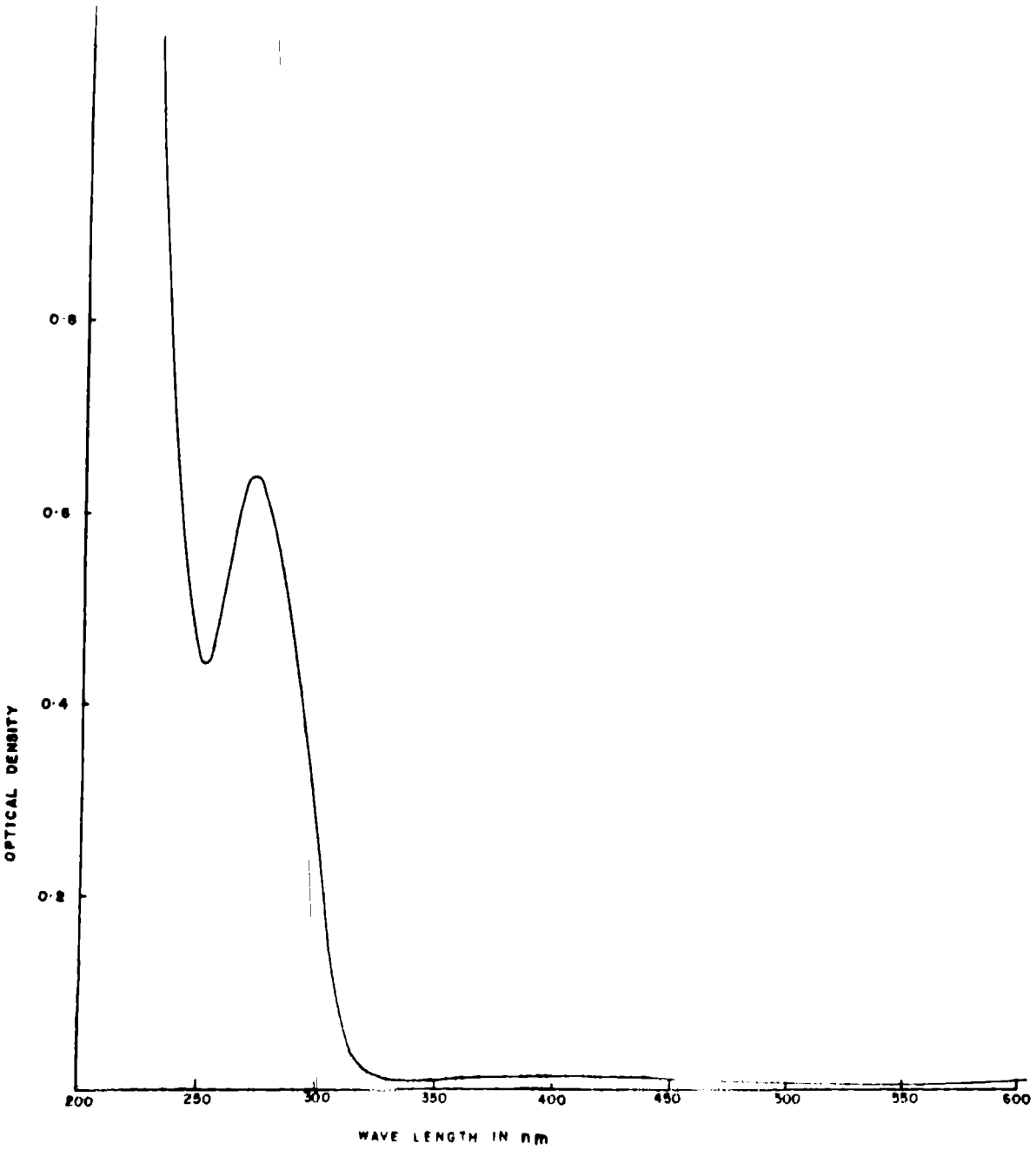


FIG.8 b ULTRA VIOLET ABSORPTION SPECTRUM OF PURIFIED SARDINE LIPASE

TABLE 11. SUMMARY OF PURIFICATION OF SARDINE LIPASE

Step	Volume ml	Protein mg/ml	Total units x 10^4	Specific activity	% recovery
1. Water extract	1000	95	9.8	10.3	100
2. Desalted solution	450	20	7.2	23.8	73
3. DEAE sephadex chromatography	90	6	4.9	92.9	50
4. *Sephadex G-100 Chromatography	18	1.3	2.2	940.1	34.7

* 30 ml DEAE eluent with optical density 7 and 1070 lipase units/ml was chromatographed and yield back calculated

Due to lack of analytical untracentrifuge facility at CIFT homogenity of the lipase preparation cannot be further tested by sedimentation experiments. However, from column chromatography on sephadex G-100, sephacryl S-200 and poly acrylamide disc gel electrophoresis the preparation of sardine lipase was found to be pure and homogenous.

From the purification data (Table 11), it is seen that oil sardine contains appreciable amounts of lipase enzyme when compared to many other sources of lipase and as regards lipase content only porcine pancreas (Verger et al. 1969), rat pancreas (Gidez, 1968) and the micro-organism Geotrichum candidum (Tsujisaka et al. 1972) are superior to oil sardine.

The purification of the enzyme is also relatively simple. Even without the use of ultrafiltration and other concentration techniques (Tsujisaka et al. 1972) and the use of lipase stabilisers like butanol (Holasek et al. 1961) and protease inhibitors like diisopropyl fluorophosphate (Verger et al. 1969) the enzyme could be purified to give a specific activity of 9.4×10^2 units. Further defatted sardine hepatopancreas can be stored hermitically sealed for more than a year at -20°C without any loss of activity. However, the solutions of the

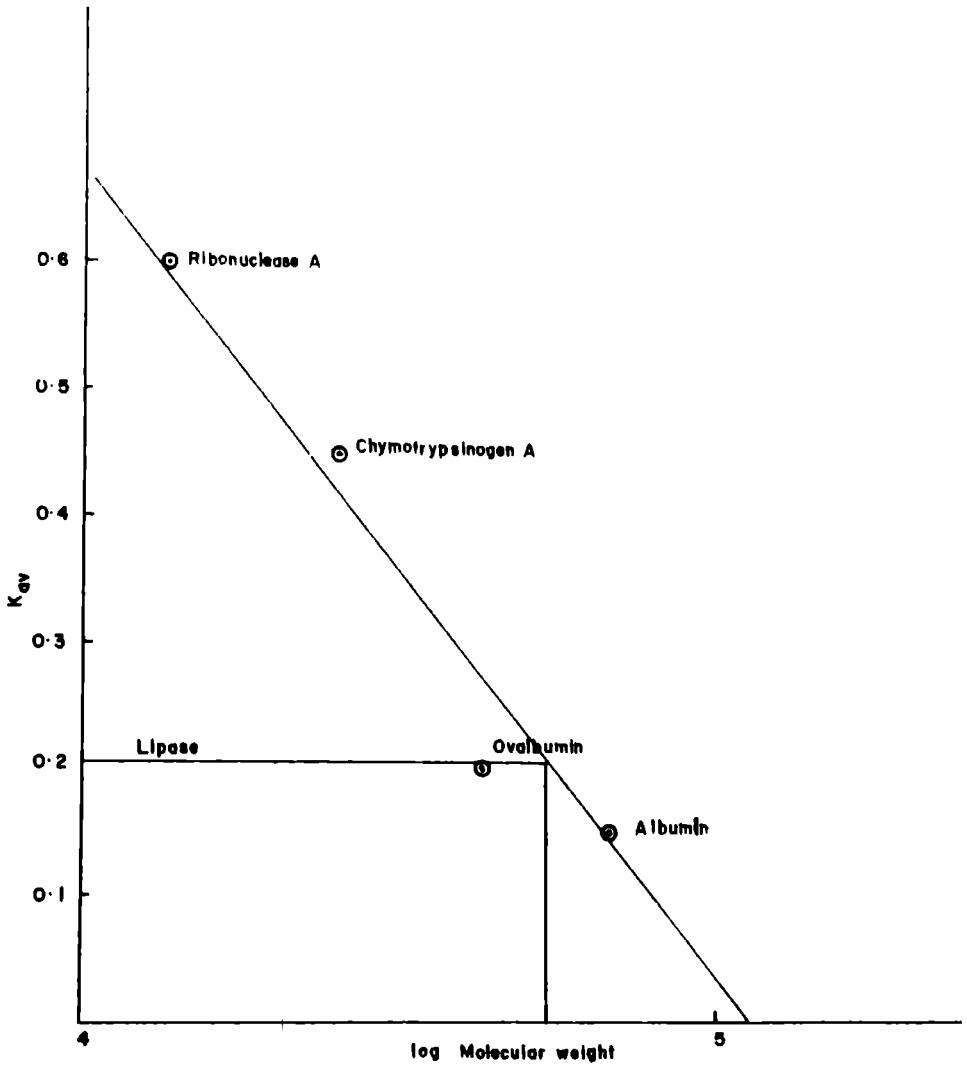


FIG. 9 ESTIMATION OF MOL. WT. OF SARDINE LIPASE BY GEL FILTRATION ON SEPHADEX - G-100

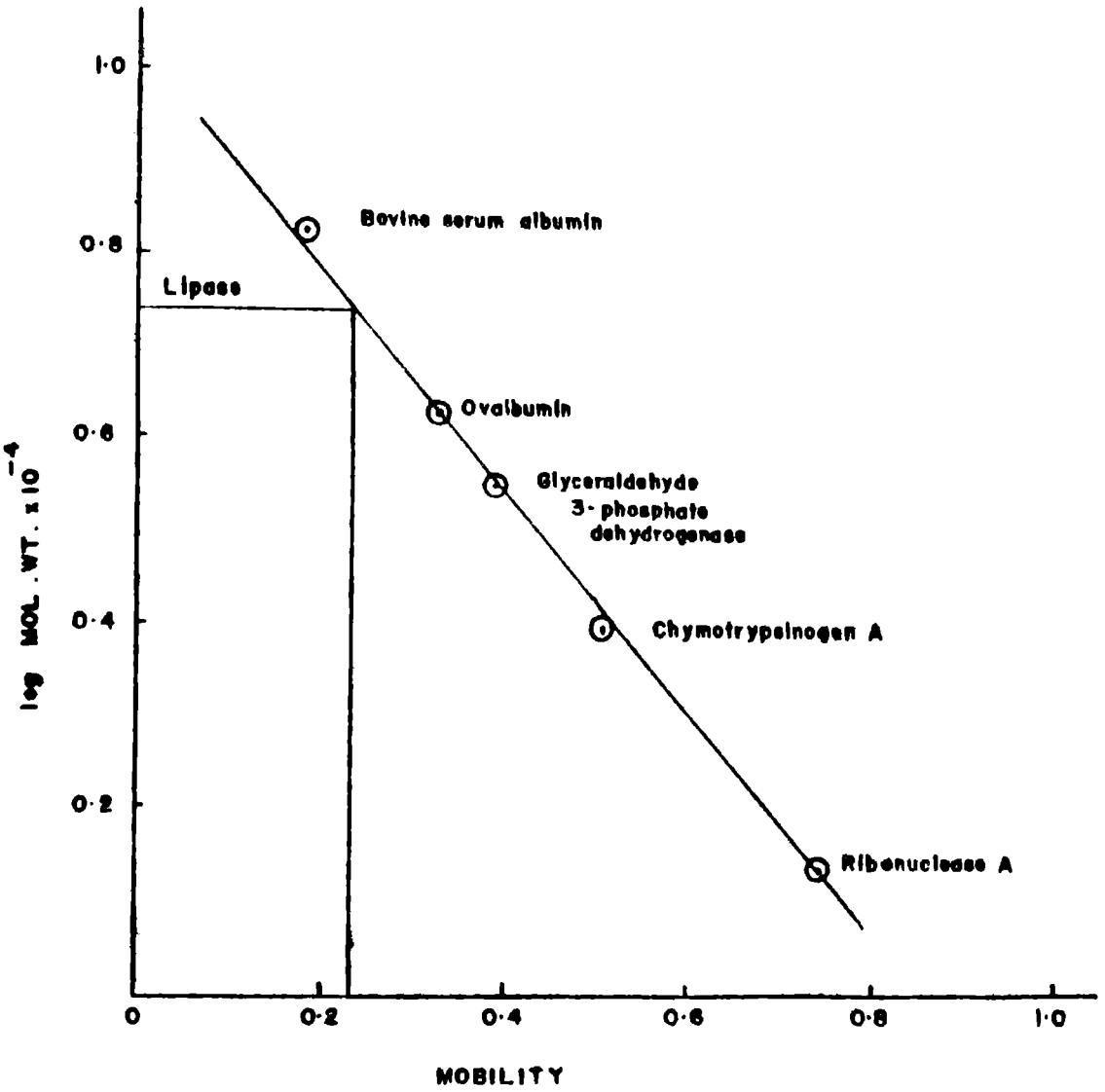


FIG.10 ESTIMATION OF MOLECULAR WEIGHT OF SARDINE LIPASE BY SDS POLY ACRYLAMIDE GEL ELECTRO-PHORESIS

lipase enzyme are not very stable at -20°C. Unlike other procedures (Bier, 1962; Verger et al. 1969 and Gidez 1968) only one extraction of the hepatopancreas powder was tried by us as subsequent extraction proved to introduce considerable dilution of the enzyme without appreciable increase in total activity. This is a major breakthrough in the methodology in purification of fish lipases.

The yield of the enzyme is also comparable to the yield of lipase preparation from other sources like porcine pancreas, rat pancreas, Milk, Geotrichum candidum etc as in seen from Table 12. Thus the method of purification of lipase from hepatopancreas of oil sardine is relatively simple and cheap. Moreover sardine hepatopancreas is a waste product during sardine processing making it probably the cheapest source.

5.4 MOLECULAR WEIGHT

Molecular weight estimation of sardine lipase by chromatography on sephadex G-100 gave a value of 53700 (Fig.9) whereas SDS electrophoresis gave 55000 (Fig.10). From these two results an average molecular weight of about 54500 is attributed to sardine lipase. Studies on lipases from other sources have shown that the molecular weight of lipases varies widely from source to source. Thus porcine pancreatic lipase has a molecular weight in the range of

TABLE 12. INITIAL AND FINAL ACTIVITIES OF LIPASE WITH YIELD FROM VARIOUS SOURCES

Source	Initial specific activity	Final specific activity	Yield of pure lipase %
Porcine pancreas[1]	150	4.5×10^3	30
Rat pancreas[2]	126	5.3×10^3	52
Milk[3]	0.03	1.5×10^1	15
Geotrichum candidum[4]	11	4.5×10^2	49
Sardine hepatopancreas	10.3	9.4×10^2	35

1. Verger *et al.* 1969
2. Gidez, 1968
3. Fox and Tarassuk, 1968
4. Tsujisaka *et al.* 1972

45000-50000, Geotrichum candidum lipase between 53000-55000, rat pancreatic lipase 40000 and Rhizopus arrhizus lipase 45000 (Semeriva et al. 1969).

Very high values for molecular weight are reported for lipase from milk, 210000 (Patel et al. 1968) hormone sensitive lipase, 7.2×10^6 from rat adipose tissue (Huttunen et al. 1970 a,b,c) and that pancreatic lipase 300000 (Ramachandran et al. 1970) vernonia anthelmintica lipa 200000 (Olney et al. 1968) and these lipases are termed fast lipases owing to the fact that they are eluted fast from sephadex columns.

The molecular weight of milk lipase is something interesting. While almost all of the lipases studied so far have molecular weights near or above 40000, milk lipase i having only a molecular weight 7000 to 8000 (Chandan et al. 1963a). It is also reported that other lipases in milk with high molecular weight are lipases from tissue or pancreases which have leaked, into the mammary gland through blood stream (Jensen, 1964).

In case of fast lipases it has been shown that they are not independant protein units but complexes with some other molecules (Brokerhoff and Jensen, 1974, Downey and Murphy, 1970). This phenomenon of lipase associating with other proteins is more pronounced in milk lipase

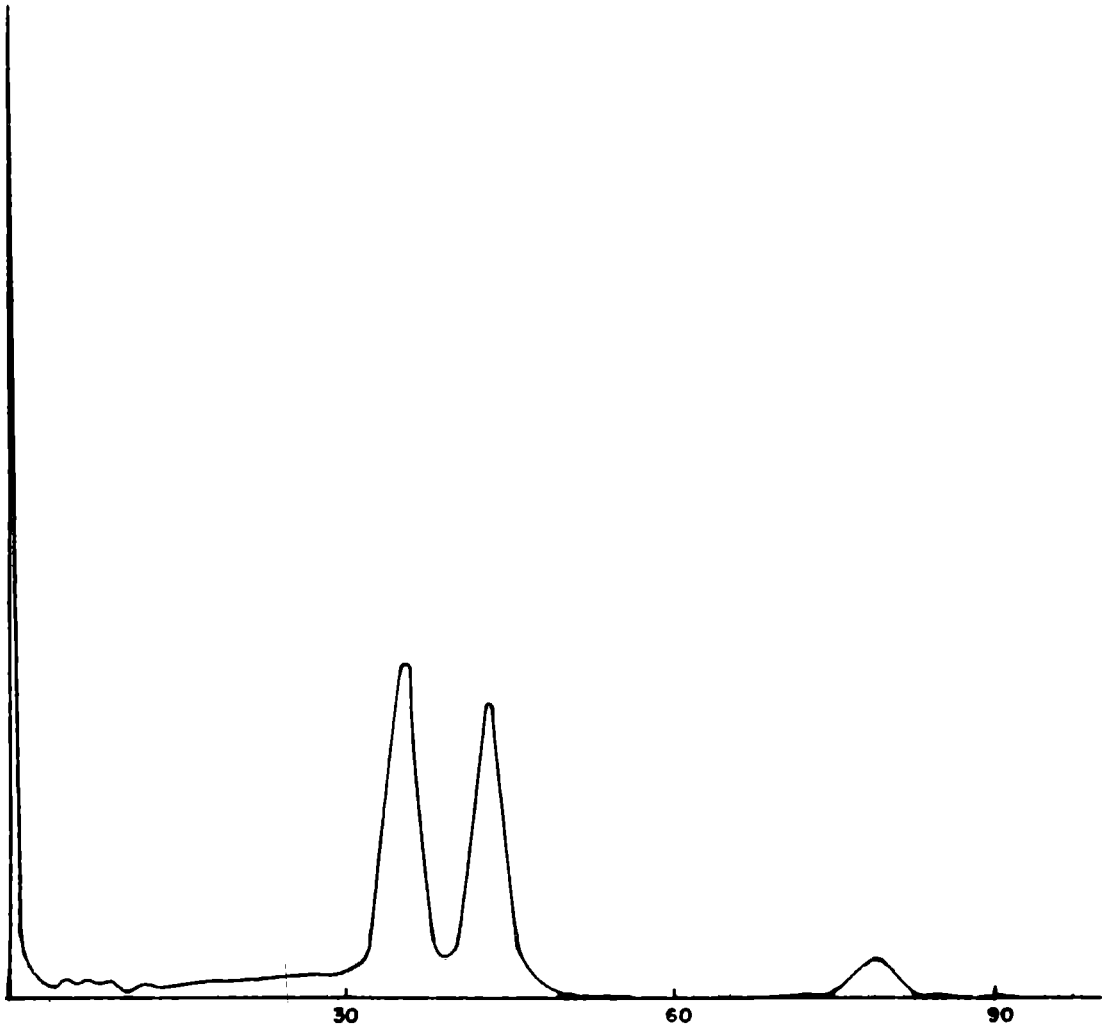


FIG.11 GAS CHROMATOGRAM OF CARBOHYDRATES OF SARDINE LIPASE. THE PEAKS WERE IDENTIFIED FROM LEFT TO RIGHT TO BE ARABINOSE, XYLOSE AND GLUCOSE

as it can be separated into a number of molar fractions (Gaffney et al. 1966). However, in sardine lipase this association behaviour was not observed.

5.5 CARBOHYDRATE CONTENT AND COMPOSITION

Analysis of purified sardine lipase showed the presence of 6.1% carbohydrate estimated as ribose and they were found to be glucose, xylose and arabinose (Fig.11) in the proportion 1:4.8:4.2 as is seen in Table 13. The presence of carbohydrate has been detected in other lipases also. Thus both porcine lipases LA and LB contain 3.1 and 2.7 residues respectively of glucosamine (Verger 1970). Plummer and Sarda (1973) in addition found the presence of 2.2 residues of mannose while Garner and Smith (1972) detected 3.8 moles of mannose and 2.9 moles of glucosamine.

Another lipase studied for carbohydrate content is that from Geotrichum candidum, which is shown to contain 7.5% carbohydrate as mannose (Tsujisaka, et al. 1972). The individual carbohydrates were found to be xylose, arabinose galactose and mannose by paper chromatography, with mannose as the main constituent.

Milk lipase was also found to contain 0.6% carbohydrate in the form sialic acid (Patal et al. 1968). The function of carbohydrate in lipase molecule is not very

TABLE 13. CARBOHYDRATE COMPOSITION OF SARDINE LIPASE

Carbohydrate	% composition
Glucose	1.0
Arabinose	4.8
Xylose	4.2

clear. However, it can be suggested that like Ribonucleases (Jakson and Hirs, 1970) the carbohydrate moieties which are hydrophilic may be remote from the active site which is hydrophobic so that lipase will get the proper orientation when brought to the vicinity of a lipid-water bilayer. Consequently the presence of carbohydrate moiety or probably some other hydrophilic group may be an essential make up for lipase activity.

5.6 AMINO ACID COMPOSITION

The amino acid composition of sardine lipase obtained is shown in Table 14. The number of each amino acid residue is calculated based on a molecular weight of 53700 for sardine lipase.

The amino acid pattern obtained for sardine lipase is very much similar to those reported for lipase from other sources (Verger et al. 1969; Vandermeer and Chistophe, 1968; Tsujisaka et al. 1972) with a very important difference that sulphur amino acids are absent in sardine lipase.

An interesting feature of this amino acid analysis is the presence of just normal amounts of apolar amino acids. According to Hatch (1965) the residues Lys, Arg, Asp, Thr, Ser, and Glu are considered to be polar amino acids and Pro, Val, Met, Ile, Leu, Phe are apolar. The

TABLE 14. AMINO ACID COMPOSITION OF SARDINE LIPASE

Amino acid	Molar ratio	No. of residues per mole of enzyme	
		Exptl.	Nearest integer
Tryptophan	0.21	5.04	5
Lysine	0.69	16.6	17
Histidine	0.38	9.1	9
Argenine	0.75	18.0	18
Aspartic acid	2.5	60.1	60
Threonine	0.75	18.0	18
Serine	1.1	26.4	26
Glutamic acid	1.41	33.9	34
Proline	1.52	36.5	37
Glycine	1.50	36.0	36
Alanine	1.37	32.9	33
Cystine	0.0	-	-
Valine	0.71	17.1	17
Methionine	0.0	-	-
Isoleucine	0.75	18	18
Leucine	1.58	38.0	38
Tyrosine	0.83	19.9	20
Pheynyl alanine	0.91	21.9	22

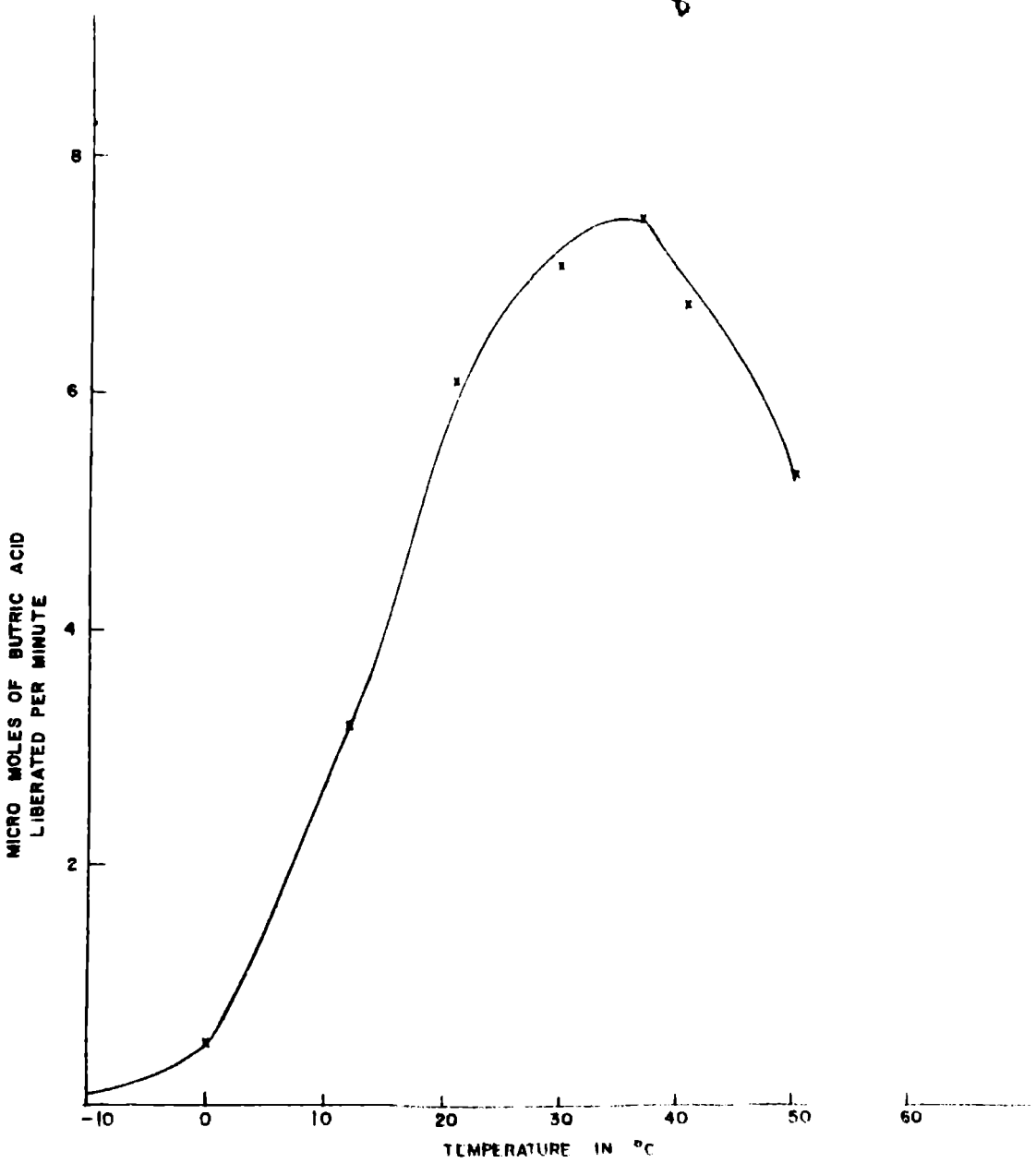


FIG.12 EFFECT OF TEMPERATURE ON SARDINE LIPASE ACTIVITY

ratio of the sum of apolar amino acid residues to polar amino acid residues is only 0.76 a value not so high to predict a hydrophobicity for sardine lipase molecule. This can be compared with the values obtained for other lipases and enzymes. They are lipase LA 0.74; LB 0.72; rat lipase 0.72 (Verger, 1970); Porcine chymotrypsinogen A 0.8, porcine ribonuclease 0.47, carboxy peptidase A 0.61; amylase I 0.67 (Hatch, 1965).

As lipases attack hydrophobic substrate by associating with lipid layer, it is logical to expect a surplus amount of apolar amino acids. Although experimental data show that this is not the case, it is still possible for the lipase molecule to form a hydrophobic end by adopting suitable secondary and tertiary structure in such a way as to expose apolar amino acids to the environment. Meanwhile the other end may be formed by exposition of polar amino acids and the carbohydrate moiety to form a hydrophilic end. These hydrophobic and hydrophilic ends in the same molecule, but sufficiently separated, may be responsible for the proper orientation of the enzyme between a bilayer to facilitate lipolysis.

5.7 EFFECT OF TEMPERATURE ON ACTIVITY AND STABILITY OF THE ENZYME

The effect of temperature on sardine lipase activity is shown in Fig. 12, which shows an optimum temperature

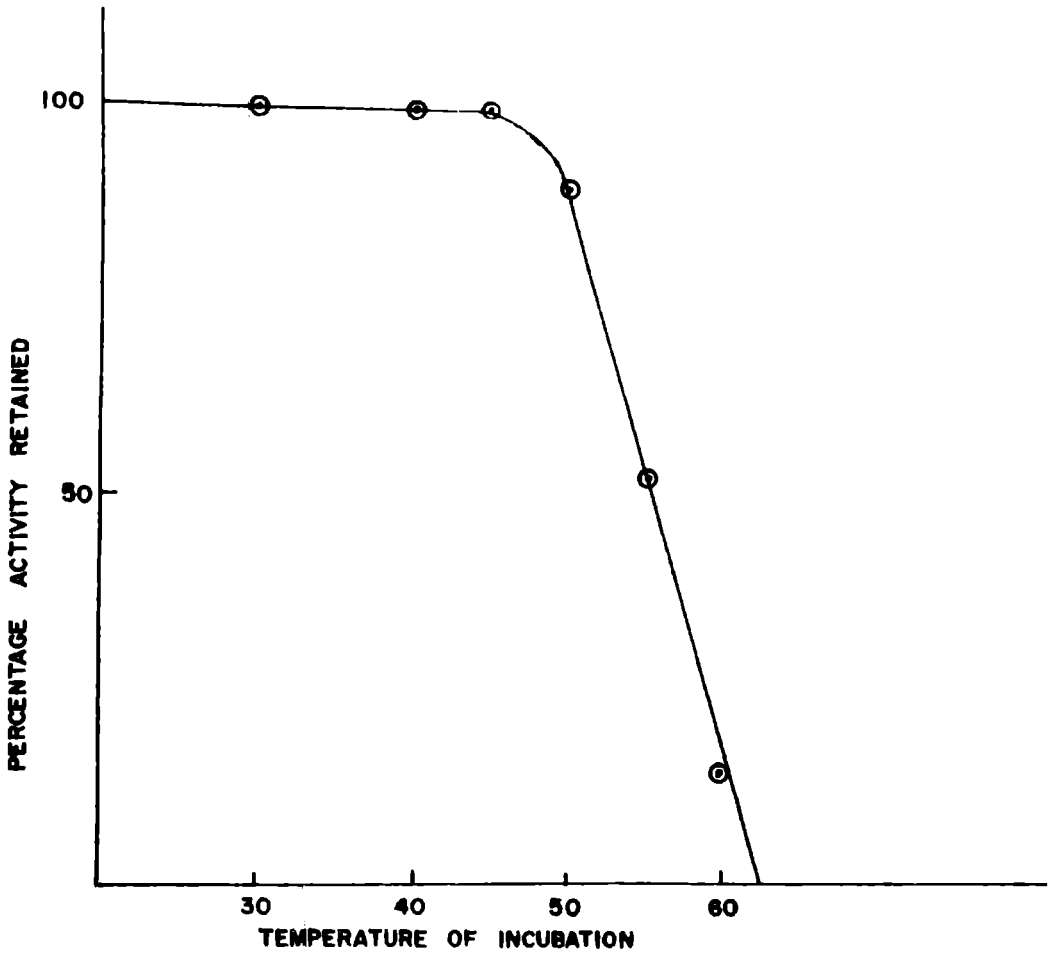


FIG.13 EFFECT OF TEMPERATURE ON STABILITY OF SARDINE LIPASE

of 37°C at pH 7. Same temperature optima is reported for pancreatic lipase with most triglycerides as substrate, eventhough with Tween as substrate in phosphate buffer a value of 25°C is reported (Archibald, 1946). The value reported for microbial lipase is still higher viz. 40°C (Tsujisaka et al. 1972).

Further, lipase is active over a wide range of temperatures. Sardine lipase was found to be active in the range -10°C to 50°C with varying rates of lipolysis. Lipases are reported to be active at still lower as well as higher temperatures. Thus lipases have been shown to be active in certain frozen foods held at -29°C (Alford and Pierce, 1961) and that lipolysis has been demonstrated in fish stored at -29°F (Lovern, 1962). On the other hand the lipase from Vernonia anthelmintica was active even at 80°C (Olney et al. 1968) an enzyme remarkably thermostable.

Fig.13 shows the graph obtained for thermostability of sardine lipase at pH 7. It is seen that sardine lipase retained 100% of its activity during a 30 minute heat treatment at various temperatures below 45°C and above 45°C the activity was partially lost. The activity was completely lost at 63°C and above. The results of Tsujisaka et al. (1972) shows that the microbial lipase from

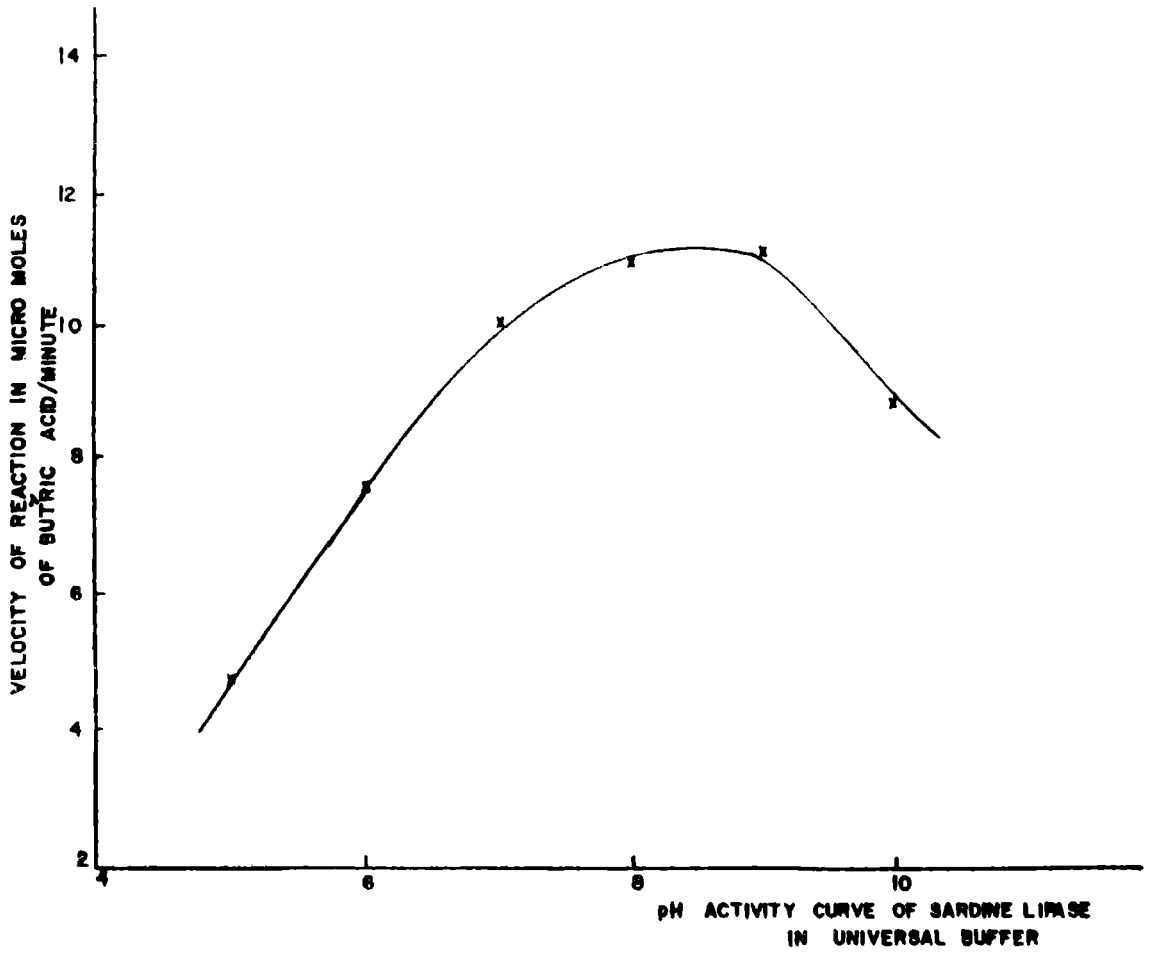


FIG.14a pH ACTIVITY CURVE

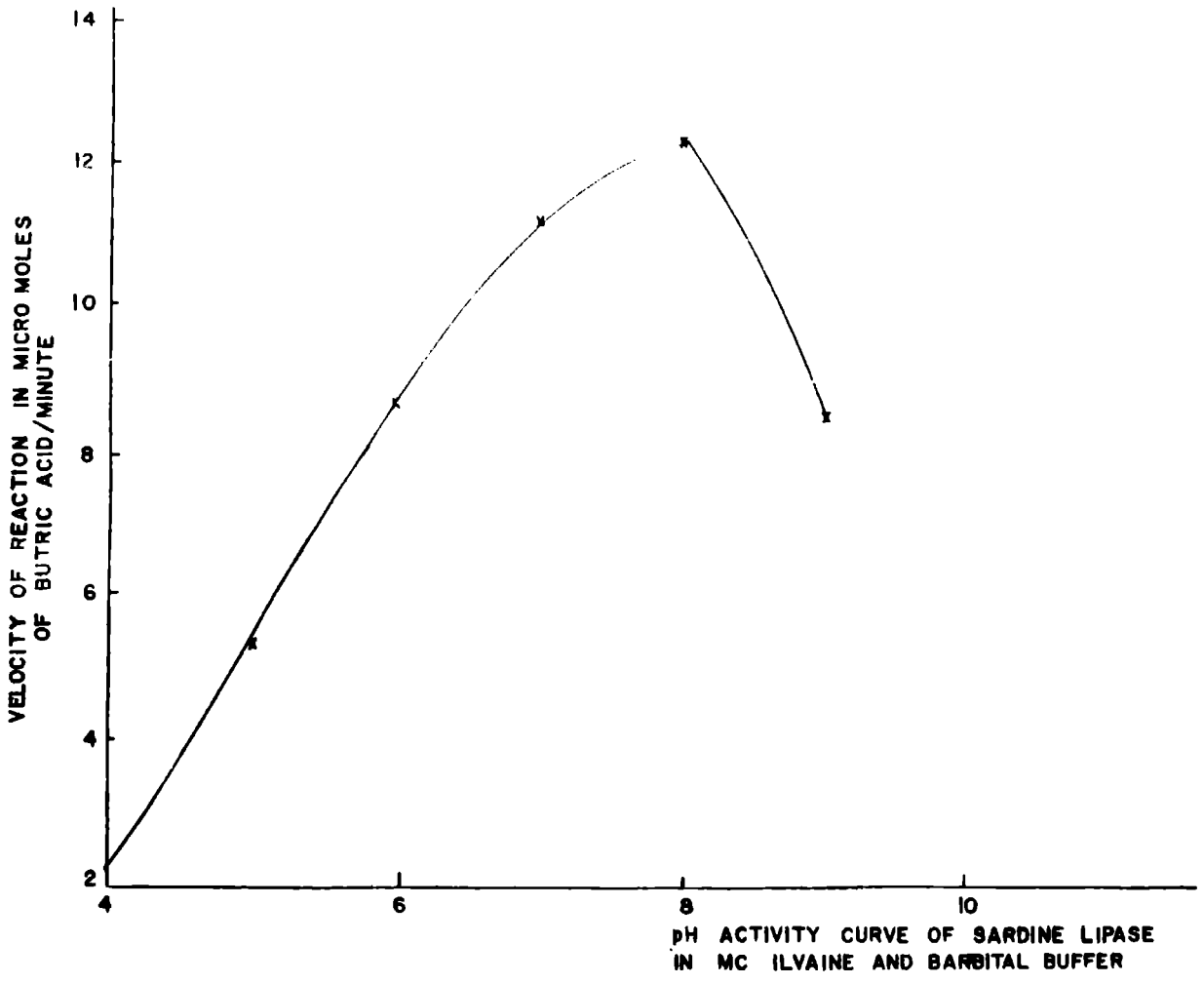


FIG.14 b pH ACTIVITY CURVE

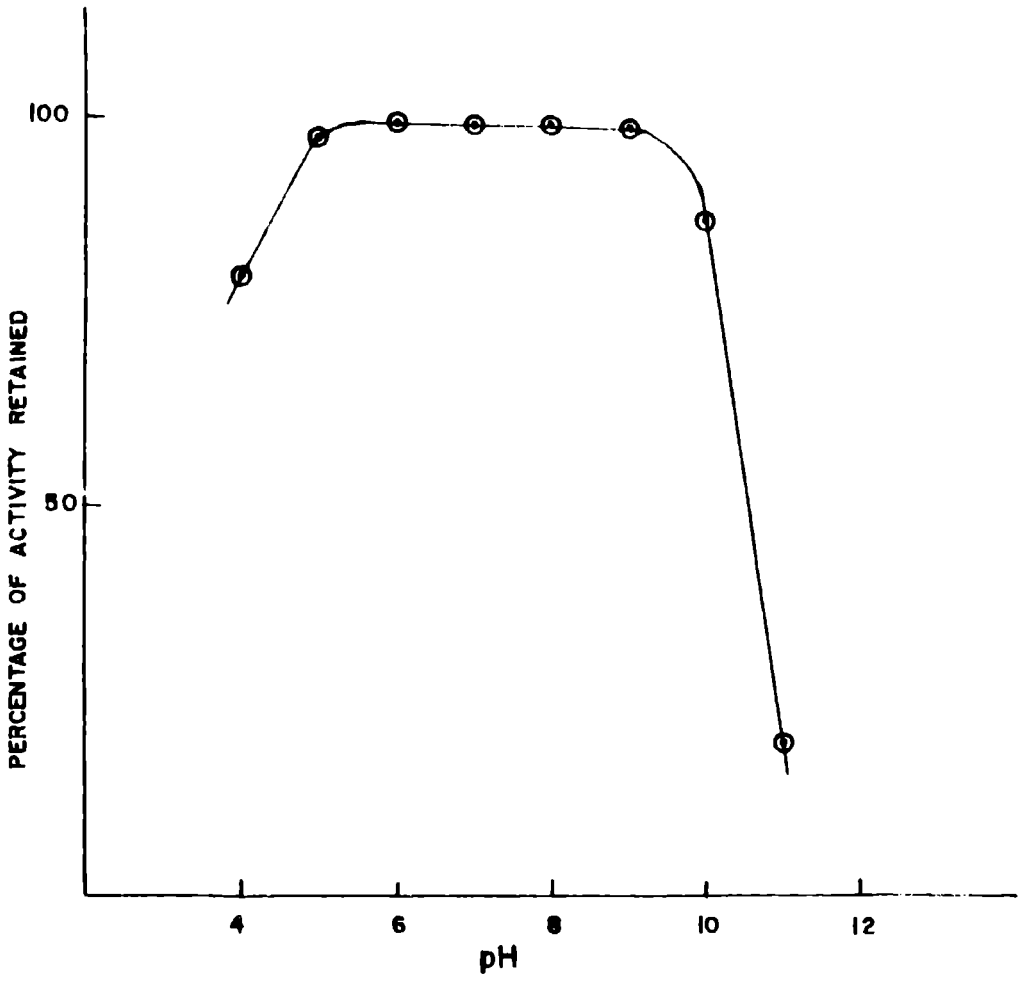


FIG.15 pH STABILITY OF SARDINE LIPASE

Geotrichum candidum can withstand temperature up to 55°C for 15 minutes and pH 5.6 without any loss in activity.

Considering these points it can be said that sardine lipase has more similarity to pancreactic lipase than Geotrichum candidum lipase in its behaviour with temperature. Sardine lipase is a thermolabile enzyme.

5.8 EFFECT OF pH ON ACTIVITY AND STABILITY OF SARDINE LIPASE

The results of pH optima study are summerised in the form of two graphs shown in Fig.14 (a & b). Fig.14a shows the pH activity plot obtained with universal buffer while Fig.14b shows the same for a combination of McIlvaine phosphate and barbital buffers. It is seen that the activity of lipase is maximum at pH 8.5 in universal buffer and pH 8 as per the merged results from McIlvaine phosphate buffer and barbital buffer.

The effect of pH on stability of sardine lipase is depicted in Fig.15, which shows that the enzyme is stable over a period of 30 minutes in the range pH 5 to pH 9.5, where the activity was intact. While at pH values below 5 and above 9.5 the sardine lipase was quickly inactivated.

Although most of the lipases depict an alkaline pH optima in the range pH 8 to 9 (Brokerhoff and Jenson, 1974; Tsujisaka et al. 1972) there exists some lipases which

are active in the acidic side. Thus castor bean lipase is most active at pH 4.2 (Ory et al. 1960), connective tissue lipase have pH optima at 4.5 and 6.5 (Lengle and Geyer, 1973) and the lipase from the microorganism Mucor pusillus have an optimum pH in range of 5 to 6 (Somkutti et al. 1969). However, almost all major sources of lipase produce an enzymes which are most active in the alkaline pH and sardine lipase is no exception. Further, most of the lipases with acid pH optima were not pure preparations. Moreover the products of lipolysis are acidic and consequently the chances of better lipolysis are more at alkaline pH values, enabling lipases to be more active in alkaline pH range.

5.9 EFFECT OF SUBSTRATE CONCENTRATION ON VELOCITY OF LIPOLYSIS BY SARDINE LIPASE

In lipolysis the substrate is dispersed in the form of an emulsion with zero concentration in aqueous phase and 100% substrate in oil phase. As such to measure the concentration of the substrate available for lipolysis in an oil emulsion for evaluation of Km and maximum velocity is a very difficult problem. Under the circumstances Schonheyder and Volqvartz (1944 a,b and 1945) suggested that lipolysis might depend on the available surface area rather than the weight or molarity of lipid material.

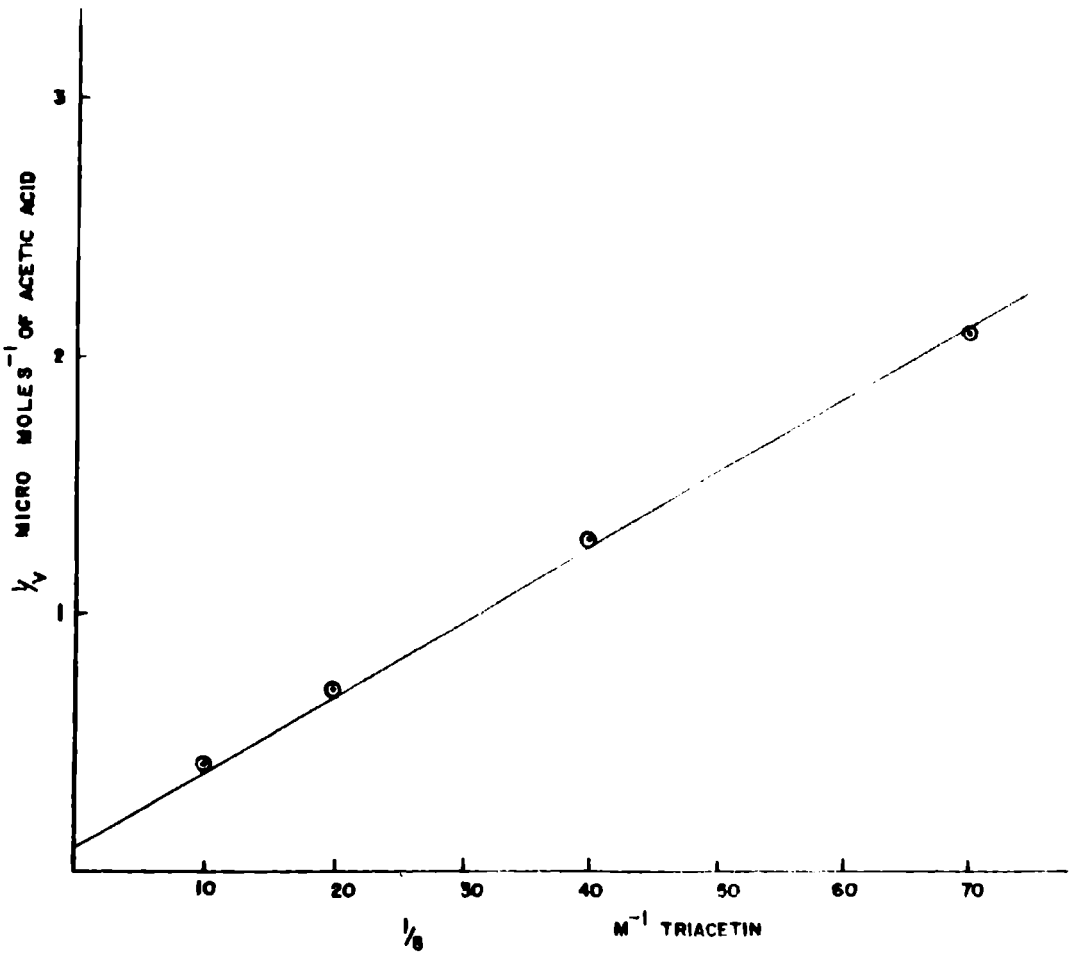


FIG.16 LINEWEAVER BURK PLOT OF LIPOLYSIS OF TRIACETIN BY SARDINE LIPASE

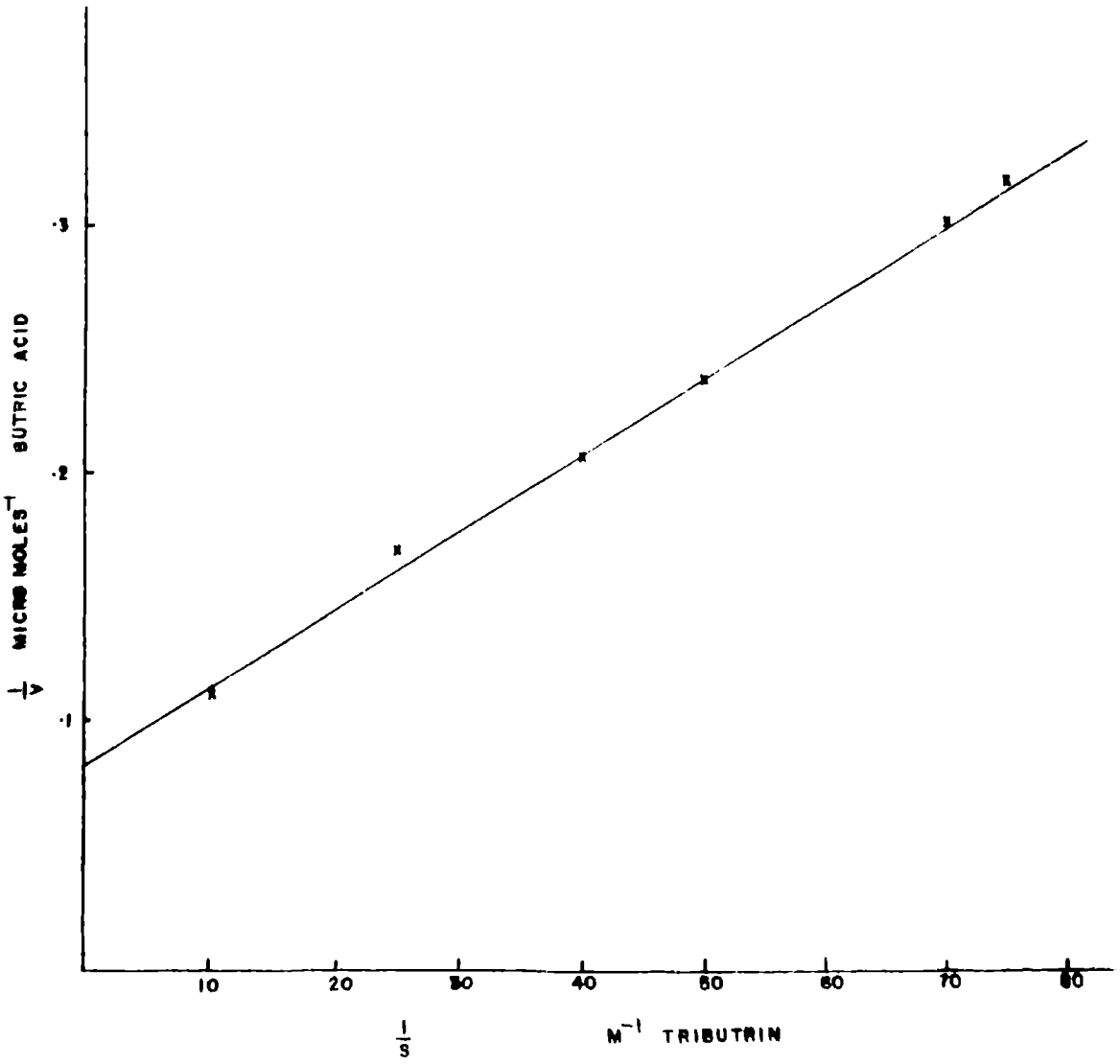


FIG.17 LINE WEAVER BURK PLOT OF LYPOLYSIS OF TRIBUTRIN BY SARDINE LIPASE

The surface area dependant interfacial reaction of porcine pancreatic lipase was further investigated by Sarda and Desnuelle (1958) and Benzonana and Desnuelle (1965) by the help of an electronic particle counter. They arrived at the conclusion that the smaller the oil particles in an emulsion the higher will be the velocity of lipolysis.

In the present study it is assumed that under identical condition of emulsification the particle size of oil droplet will be almost same in the concentration range of 0.01 M to 0.1 M triglyceride in 1% polyvinyl alcohol. Further under such conditions the total surface area of oil phase will be proportional to the concentration of oil in 1% polyvinyl alcohol, or molarity. In other words under identical conditions of emulsification, and subsequent shaking the velocity of lipolysis and molar concentration of substrate will obey, Michaelis Menten equation, eventhough the Km value as well as maximal velocity will vary with the conditions of emulsification and subsequent shaking during lipolysis.

The substrate concentrations and the corresponding velocities obtained for lipolysis of triacetin and tributrin by sardine lipase were used for the reciprocal plot. The graphs obtained are shown in Fig.16 and 17.

The Michaelis constants obtained were 6 x 10^{-6} and 12 x 10^{-6} respectively for triacetin and tributrin; which were also half maximal velocities in moles of fatty acids per minute, as per definition. Excepting Sobotka and Glick (1934), who reported a Km value of 6 x 10^{-4} for hog pancreatic lipase, there are no reports on the Km value of different lipases.

5.10 INHIBITION AND ACTIVATION

5.10.1 EFFECT OF ALDEHYDES AND KETONS ON SARDINE LIPASE ACTIVITY

The effect of the organic molecules, formaldehyde, acetone, acetaldehyde and ethylmethyl ketone were investigated. The results obtained in this regard are tabulated in Table 15. All the four aldehydes and ketones tried were found to be inhibitors of sardine lipase. The rate of inhibition of formaldehyde was > acetaldehyde > ethylmethyl ketone > acetone, against the conclusion of Bier (1962) that the extend of inhibition is proportional to the molecular volume.

The mechanism of this inhibition is not known and to work it out is beyond the scope of this thesis. As a probable course, it can be suggested that the aldehydes and ketones may undergo a condensation reaction with the free amino groups in the lipase protein and change

TABLE 15. EFFECT OF CERTAIN ALDEHYDES AND KETONES ON SARDINE LIPASE ACTIVITY

Aldehyde/ Ketone	Concentration M	Degree of inactivation %
1. Formaldehyde	0.1	31
2. Acetaldehyde	0.1	17
3. Acetone	0.1	5
4. Ethyl methyl Ketone	0.1	7

the tertiary structure of lipase and hence the molecular orientation of lipase in a bilayer in such a way as to retard the rate of lipolysis.

5.10.2 EFFECT OF METALLIC AND NONMETALLIC IONS ON LIPASE ACTIVITY

a. Metallic ions

The effect of various metallic ions studied are summarised in Table 16. Of the metallic ions studied Ferric ion exerted maximum inhibition and other metallic ions which exerted inhibition were in the order of inhibition $Fe^{3+} > Fe^{2+} > Ba^{2+} > Cu^{2+} > Cu^{+}$.

Mercuric ions did not exert any inhibitory action. This was further confirmed by incorporating PCMB in the reaction mixture up to 10 m M level. Among researchers the opinion on the effect of mercury on lipase activity is varied. While some school of thought found mercury to be of no effect on lipase activity (Tsujisaka et al. 1972, Iwai et al. 1969; Somkutty et al. 1969) some others report mercury to be inhibitory (Korn and Quigley, 1955; Hayase and Tappel, 1970; Chandan and Shahani, 1965).

From the above reports it is clear that the enzyme from sardine is much similar to the enzyme from Geotrichum candidum and that from Mucor Pusillus as far as the effect of mercury is concerned. The absence of sulphur amino

TABLE 16. EFFECT OF VARIOUS METALLIC IONS ON SARDINE LIPASE ACTIVITY

Ion	Degree of inactivation %
Fe^{2+}	14.1
Fe^{3+}	15.0
Cu^{+}	8.2
Cu^{2+}	10.2
Zn^{2+}	0.0
Sn^{2+}	0.0
Mn^{2+}	0.0
Mg^{2+}	0.0
Co^{2+}	0.0
Ba^{2+}	13.1
Ca^{2+}	-5.1
Hg^{2+}	0.0
Control	0.0

acids in sardine lipase support this behaviour, as mercury ions are mostly inhibitory owing to its capacity to combine with SH groups.

The metallic ions Sn^{2+}, Zn^{2+} Mn^{2+}, Mg^{2+} and Co^{2+} are also devoid of any effect on sardine lipase. Of the metal ions tested only Ca^{2+} gave an activation effect on sardine lipase. However, the extend of activation observed was much smaller than that observed by others (Willstator et al. 1923, Benzonana and Desnuelle, 1968; Iwai et al. 1963).

This difference is well explained from the nature of substrate used and in the light of the earlier works. At first it was noted that Ca^{2+} was neither essential for lipase reaction nor it activated lipolysis as the hydrolysis of triacetin by pancreatic lipase was independant of calcium ions (Wilestator and Memmen, 1928). Constantin et al. (1960) and Benzonana and Desnuelle (1968) demonstrated that Ca^{2+} did not increase the initial reaction rate of lypolysis. However, the same authors also observed that on prolonged incubation Ca^{++} exerted an activation effect, especially with triglycerides of long chain fatty acids.

From these observations it is concluded that the effect of calcium vary with the chain length of the fatty

acids in the triglyceride substrate. Thus, in case of lower fatty acid triglycerides lipolysis is not affected by Ca^{2+} as in triacetin (Willstator and Memmen, 1928); with medium length fatty acid triglycerides the activation effect is not as pronounced (tributrin, present study) as in the case of long chain fatty acid triglycerides like triolein (Iwai et al. 1963. Schonheyder and Volqvartz (1945) partly expalined this phenomenon by suggesting the role of calcium in lipolysis to be that of removal of inhibitory long chain fatty acids as insoluble calcium salts.

While it is generally agreed that calcium acts through the formation of calcium soaps it is not very clear how the lipolysis at the interface is affected by these soaps. It can be suggested that in the early stages of lipolysis the calcium soaps formed will stay attached to the interface with the calcium end towards the aquous phase and the fatty acid tail burried in the oil phase. Because of this association calcium will come closer to the ester group of another triglyceride molecule and activate them by polarisation of the C = O bond. It is also possible that Ca^{2+} can form a fatty acid $\cdot Ca^{+}$ cation which will attach to the oil phase as said earlier with a net positive charge. This net positive charge may exert and inductive effect upon an approaching lipase molecule, resulting in its activation.

The fact that the activation effect of calcium diminishes with lowering of chain length of fatty acids also support this theory as the calcium soaps of lower fatty acids will not be able to properly orient between oil water interphase owing to its shorter chain length, consequently calcium soaps of short chain fatty acids will not be able to activate neither the enzyme nor the ester bond as is observed in the case of triacetin.

The nature of inhibition by iron (Fe^{3+}) was studied in some more detail by preincubating the enzyme with varying amounts of Fe^{3+} ions. The results obtained are tabulated in Table 17.

It is evident from the table that prolonged incubation has no significant influence on the inhibition of sardine lipase by Fe^{3+} ions. However, on comparison of the degree of inhibitions, preincubation with ferric ions have more inhibitory effect than their presence in the reaction medium during lipolysis.

From these results it can be suggested that during preincubation of sardine lipase with ferric ions, the ions get sufficient time to interact with the enzyme to cause inhibition. Whereas in the other case the enzyme is exposed simultaneously to substrate molecules as well as inhibitor ions resulting in the formation of enzyme substrate complex as well as enzyme - inhibitor complex

TABLE 17. EFFECT OF PRETREATMENT OF SARDINE LIPASE WITH Fe^{3+} ION

Time of pretreatment (minutes)	Degree of inactivation (%)
5.	60.
15.	65.
30.	66.
60.	66.
120.	68.

leading to the formation of more reaction products.

Similar studies by Iwai et al. (1969), using Aspergillus niger lipase showed that there is no pronounced inhibition by preincubation and that the inhibition is only dependant on the final concentration of the Ferric ion in the reaction mixture. The same authors also found that the inhibitory effect of iron (ions) disappeared gradually in the course of reaction probably due to removal of Fe^{3+} ions from the inhibitory locii by fatty acids liberated. These authors were also unable to detect any inhibition by Fe^{3+} ion/added in the course of a lipase reaction.

b. Non-metallic ions (halogen ions)

The effect of halogen ions on lipase reaction are summarised in Table 18. Though halogen as a class are expected to show the same behaviour towards sardine lipase it is found not to be the case. While 1 ml of/10 m M fluride solution completely inhibited 1 ml of 0.1% lipase, bromide and iodide inhibited it, only partially. The chloride ions showed a peculiar behaviour by promoting the activity of sardine lipase by 0.5%.

Most of the inhibitors are supposed to occupy the interface of the emulsion, thereby preventing the mutual approach of the enzyme and substrate (Desnuelle, 1961;

TABLE 18. EFFECT OF HALOGEN IONS ON SARDINE LIPASE ACTIVITY

Ions	Degree of inactivation (%)
Control	0.0
Cl^-	-0.5
Br^-	27.8
I^-	10.7
F^-	100.00

Benzonama and Desnuelle, 1968; Schoor and Melius, 1969). The presence of electrical charge in these inhibitors may even result in repulsion of the enzyme. Further inhibition by substrate dilution at the interface is kinetically indistinguishable from competitive inhibition (Brokerhoff and Jensen, 1974) making inhibition of lipolysis a complex phenomenon. Consequently no attempt was made to study the kinetics of sardine lipase inhibition.

5.11 SUBSTRATE SPECIFICITY

5.11.1 RELATIVE RATES OF LIPOLYSIS OF INDIVIDUAL TRIGLYCERIDES

The action of sardine lipase on emulsified triacetin, tributrin, trivalerin, trihexanoin, trioctanoin, tripalmitin and triolein were studied as described in assay methods. The rates of lipolysis obtained for each triglyceride emulsion is shown in table 19A, assuming that the rate for tributrin (the highest value) as 100.

Maximum lipolysis rate was observed with tributrin followed by triacetin, trioctanoin, trivalerin etc. The results obtained are in agreement with those reported by Brokerhoff (1969) for pancreatic lipase. Eventhough many workers of pancreatic lipase (Sobotka and Glick, 1934; Schonhnyder and Volqvartz, 1944b; 1945) observed maximum lipolysis with tributrin, Weinstein and Wynne

TABLE 19A. RATES OF HYDROLYSIS OF TRIGLYCERIDES BY SARDINE LIPASE

Triglyceride	Rate of hydrolysis *
Triacetin	43.2
Tributrin	100.0
Trivalerin	25.6
Trihexanoin	10.9
Trioctanoin	30.7
Tripalmitin	18.1
Triolein	22.9

* Rate of tributrin = 100

(1935) found that lipolysis by pancreatic lipase was maximum with tripropionin. In the case of triolein and triacetin the difference in rate of lipolysis is more pronounced that lipases from bovine pancreas and woman milk (Schonheyder and Volqvarty, 1944 b) are considerably less active on triacetin and triolein while sardine lipase effects significant hydrolysis of these triglycerides like hog pancreatic lipase (Weinstein and Wynne, 1935 and Brokerhoff, 1969). The hydrolysis rates of various triglycerides by lipases from hog pancreas, sardine hepatopancreas and Geotrichum candidum are shown in Table 19B.

As the results obtained are the rate of lipolysis for the first 30 minutes it can be considered to be the initial rate of lipolysis. As such it can be seen that the initial lipolysis rate increases from C_3 to C_4 fatty acid triglyceride and then falls. With the exception of trihexanoin this relationship is more or less comparable to the initial rates of lipolysis obtained for various triglycerides by Entressangles et al. 1961b (see Fig.2) using pancreatic lipase.

However, some of the lipases from microorganisms like Geotrichum candidum and Aspergillus niger (Tsujisaka et al. 1972) exhibited maximum rate of lipolysis with triolein substrate which was very much higher than the

TABLE 19B. COMPARISON OF RATES OF TRIGLYCERIDE HYDROLYSIS BY LIPASES FROM SELECTED SOURCES AND SARDINE LIPASE

Triglyceride	Rate of lipolysis		
	Hog pancreas[a]	Sardine hepato-pancreas	Geotrichum candidum[b]
Triacetin	28.7	43.2	0.0
Tripropionin	137.7	-	78.7
Tributrin	100.0	100.0	100.0
Trivalerin	-	25.6	-
Trihexanoin	13.9	10.9	-
Trioctanoin	-	30.7	-
Tripalmitin	2.5	18.1	-
Tristearin	0.5	-	-
Triolein	-	22.9	2756

a. Wills, 1961

b. Tsujisaka et al. 1972

rates obtained with tributrin. In the case of the two lipases from Rhizopus delemar (Tsujisaka et al. 1972) the rates were almost equal for both the substrates tributrin and triolein.

From the foregoing results it can be concluded that lipases in general has no proportional relationship between chain length of the triglyceride fatty acid and rate of lipolysis. It varies for the lipase depending on their source. However, in the case of lipases of animal origin the maximum rate was observed with the lower fatty acid triglycerides especially tributrin and in some cases tripropionin with considerably lesser rates for higher fatty acid triglycerides like tripalmitin, triolein etc.

Further like most of other lipases sardine lipase has no absolute specificity. The rate of lipolysis also has no relationship to the chain length of the triglyceride fatty acid.

5.11.2 LYPOLYSIS OF THE MIXTURE OF TRIGLYCERIDES IN COCONUT OIL AND SARDINE OIL

Brokerhoff (1969) observed inhibition of lipolysis of a good substrate like tripropionin emulsion by the addition of a poor substrate like triacetin. This suggested that when the enzyme is exposed to a mixture of different triglycerides there is a competition between

various triglyceride molecules for the active site of the enzyme.

It was to further elaborate this point, the lipolysis of the mixed triglycerides of coconut oil and sardine oil were taken up. Table 20 gives the % composition of various fatty acids present in the coconut oil used for the study as well as the % composition of the fatty acids released by sardine lipase during lipolysis along with the ratios of the % of individual fatty acids released by the enzyme to their % in the triglyceride mixture. As the % composition of the fatty acids in natural oil are not equal, % composition of fatty acids released by lipase cannot be considered as comparative rates of lipolysis. However, the ratio of % composition of the fatty acids in oil to the % composition of the fatty acid released will be a comparative measure of the rate of lipolysis.

This result is also shown in the Table 20, from which it can be seen that the rate of lipolysis falls gradually from $C_{6:0}$ to $C_{18:1}$ with the exception of $C_{12:0}$, the deviation of $C_{14:0}$ being negligible. The result of a similar study on the total triglycerides of a sample of sardine oil is shown in Table 21. In the case of sardine oil lipolysis also the rate of lipolysis of even no number fatty acids, falls from $C_{8:0}$ to $C_{18:1}$ with the exception of

TABLE 20. SELECTIVE HYDROLYSIS OF THE TRIGLYCERIDES OF COCONUT OIL BY SARDINE LIPASE

Fatty acids	% composition in coconut oil A	% composition of fatty acids released by lipase B	B/A
$C_{6:0}$	0.9	2.5	2.78
$C_{8:0}$	3.5	9.2	2.63
$C_{10:0}$	3.6	7.3	2.03
$C_{12:0}$	31.2	55.4	2.28
$C_{14:0}$	18.9	8.1	0.43
$C_{16:0}$	13.5	7.6	0.56
$C_{18:0}$	6.0	2.6	0.43
$C_{18:1}$	15.3	5.2	0.33
$C_{18:2}$	5.9	2.3	0.39

TABLE 21. SELECTIVE HYDROLYSIS OF THE TRIGLYCERIDES OF SARDINE OIL BY SARDINE LIPASE

Fatty acids	% composition in sardine oil A	% composition of fatty acids released by lipase B	B/A
$C_{8:0}$	3.9	12.6	3.28
$C_{10:0}$	2.4	6.5	2.71
$C_{12:0}$	14.1	43.3	3.07
$C_{14:0}$	11.8	5.2	0.44
$C_{15:0}$	0.5	0.7	1.40
$C_{16:0}$	20.7	11.7	0.55
$C_{16:1}$	8.1	4.1	0.51
$C_{16:2}$	2.0	0.9	0.45
$C_{17:0}$	1.3	1.3	1.00
$C_{18:0}$	7.0	3.8	0.54
$C_{18:1}$	10.6	4.8	0.45
$C_{18:2}$	2.0	1.5	0.75
$C_{18:4}$	3.2	1.8	0.56
$C_{20:1}$	0.4	Trace	-
$C_{20:4}$	0.44	Trace	-
$C_{20:5}$	6.1	2.2	0.36
Unidentified	5.1	Trace	-

$C_{12:0}$ and minor variations of $C_{14:0}$ and $C_{18:0}$.

In both these cases it is interesting to note that $C_{12:0}$ triglyceride gives a higher rate of lipolysis by sardine lipase, than $C_{10:0}$. Further the odd number fatty acid triglycerides $C_{15:0}$ and $C_{17:0}$ exhibit considerably higher rates of lipolysis by sardine lipase than the nearby even number fatty acid triglycerides and among odd number fatty acid triglycerides the rate falls as chain length increases.

Among both these group of triglycerides the rate of lipolysis for $C_{18:2}$ triglyceride is also comparatively higher than the other even number fatty acid triglycerides excepting $C_{8:0}$, $C_{10:0}$ and $C_{12:0}$. Again in sardine oil the rate of lipolysis of $C_{20:5}$ was significantly higher (.36) than $C_{20:1}$, $C_{20:4}$ upon which sardine lipase has only very little activity.

From the above it can be suggested that when a mixture of triglycerides is presented for lipolysis by sardine lipase the rate of lipolysis falls from $C_{6:0}$ to $C_{18:1}$ in a non uniform fashion with chain length, with the exception of $C_{12:0}$ and odd number fatty acid triglycerides. This is in partial agreement with the observations of Entressangles (1961b) on the action of pancreatic lipase on various triglycerides.

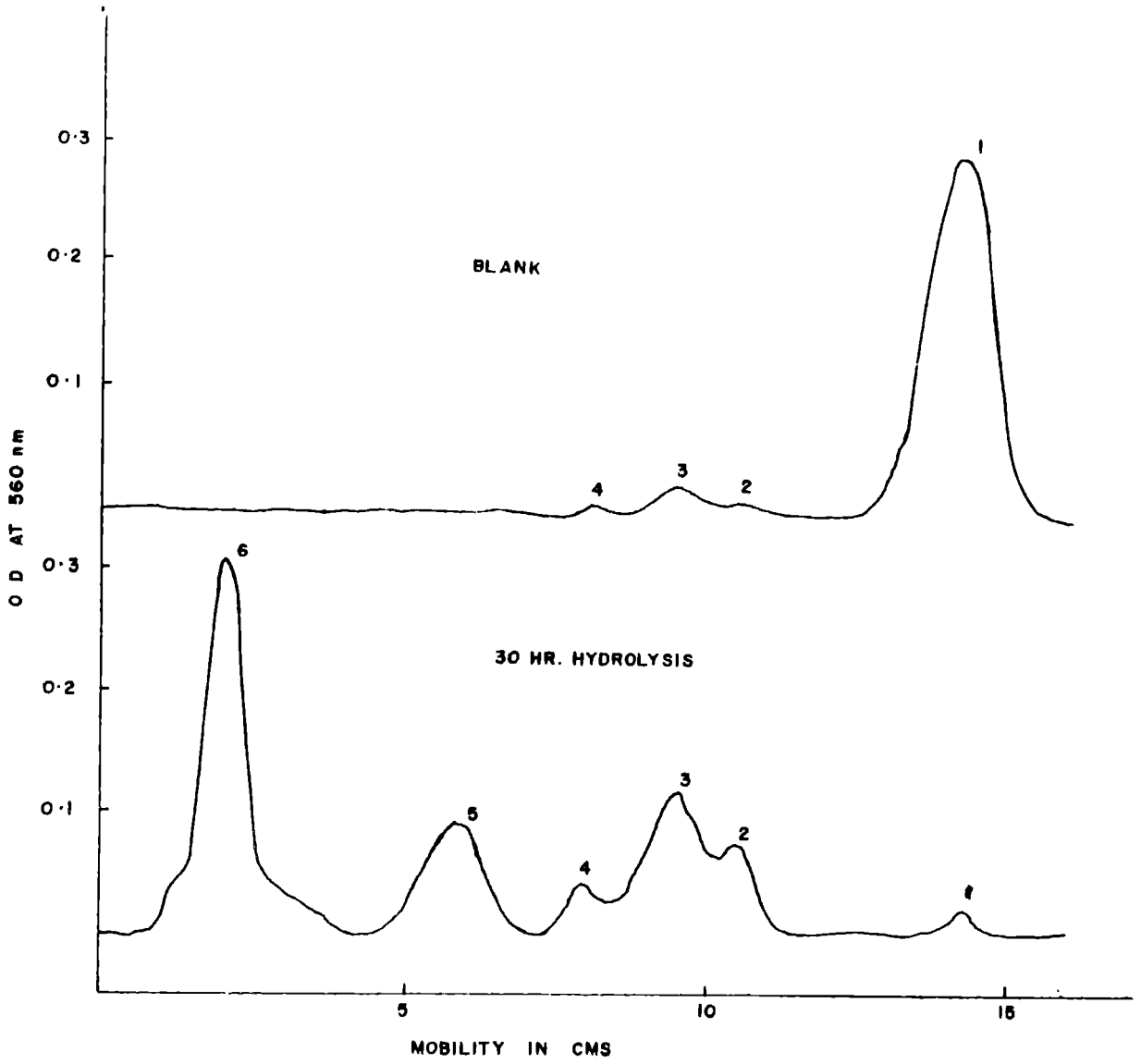


FIG.18 DISTRIBUTION OF PRODUCTS OF TRIBUTRIN HYDROLYSIS BY SARDINE LIPASE OBTAINED BY SCANNING THIN LAYER CHROMATOGRAM OF THE LIPID MATERIAL FROM THE REACTION MIXTURE AT ZERO TIME (BLANK) AND 30 HOURS. 1. TRIBUTRIN. 2 AND 3. DIBUTRINS. 5. BUTRIC ACID. 6. MONO BUTRINS. 4. SOME UNIDENTIFIED LIPID MATERIAL.

5.12 SEQUENCE OF TRIGLYCERIDE HYDROLYSIS BY SARDINE LIPASE

All lipases hydrolyse triglycerides ultimately to component fatty acids and glycerol. Stereospecifically there are 3 ester linkages in a triglyceride viz. 1,2 and 3. Of this 1 and 3 are identical (disregarding the prochiral properties of glycerol) and are termed as 1 mono glyceride and the 3rd one 2 monoglyceride. When a triglyceride is exposed to lipase which of the two positions 1 and 2 is attacked is an important point for study. Desnuelle *et al.* (1947) found that the principal products of in *Vitro* digestion of triglyceride by pancreatic lipase are the partial glycerides rather than glycerol in such a way that diglycerides are formed rapidly, monoglycerides slowly and glycerol very slowly. Constantin *et al.* (1960) took a quantitative study of the hydrolysis of triolein by pancreatic lipase and observed that glycerol is formed only after, all the triolein as well as 50% the total ester bonds were hydrolysed.

The results obtained for sardine lipase activity on tributrin emulsion with time is shown in Table 22. The distribution of the product of lipolysis at 0 time and 24 hours are shown in Fig. 18. The percentage of butric acid and monobutrin are very low compared with tributrin in the beginning. But as lipolysis proceeds

TABLE 22. DISTRIBUTION OF HYDROLYSIS PRODUCTS OF TRIBUTRIN EMULSION BY SARDINE LIPASE WITH TIME - SEQUENCE OF TRIGLYCERIDE HYDROLYSIS

Time in minutes	Tributrin %	Dibutrin %	Monobutrin %	Butric acid %
0	85.6	9.0	3.2	2.2
15	44.2	17.4	3.8	34.6
45	24.9	28.4	10.0	36.7
75	17.8	32.5	11.36	38.3
24 x 60	2.5	18.7	36.7	42.2

percentage composition of dibutrin and butric acid increases significantly at the expense of tributrin. The increase in the % composition of monobutrin is marginal (0.6%), especially in the first 15 minutes. However, during the subsequent periods of incubation both dibutrin as well as monobutrin were generated along with butric acid at the expense of tributrin. Up to 75 minutes the rate of formation of dibutrin from tributrin was more than its rate of decomposition into monobutrin and butric acid. But after 75 minutes the rate of formation of dibutrin decreases obviously due to lowering of tributrin concentration resulting in enhanced rate of break down of dibutrin thereby, reducing the dibutrin concentration.

The formation of monobutrin becomes significant only between 15 minutes and 45 minutes of incubation by which time it can be seen nearby half the ester bonds were hydrolysed. The formation of monobutrin and butric acid goes on even up to 24 hours at the expense of dibutrin. It was also observed that even after prolonging the incubation time the reaction could not be completed. This may be due to denaturation of the enzyme as a result of prolonged agitation at 30°C or due to inhibition by butric acid or due to the partial synthetic activity of the enzyme.

As the identification of such parameters was beyond the scope of this thesis no further investigation

on that aspect was under taken.

Eventhough the reaction could not be taken to completion the results obtained are very much similar to the results obtained by Constantin et al. (1960) leading to the conclusion that sardine lipase is having more affinity to triglyceride followed by diglyceride and monoglyceride, as substrate or monoglyceride is a poor substrate for sardine lipase in comparision with triglycerides and diglycerides.

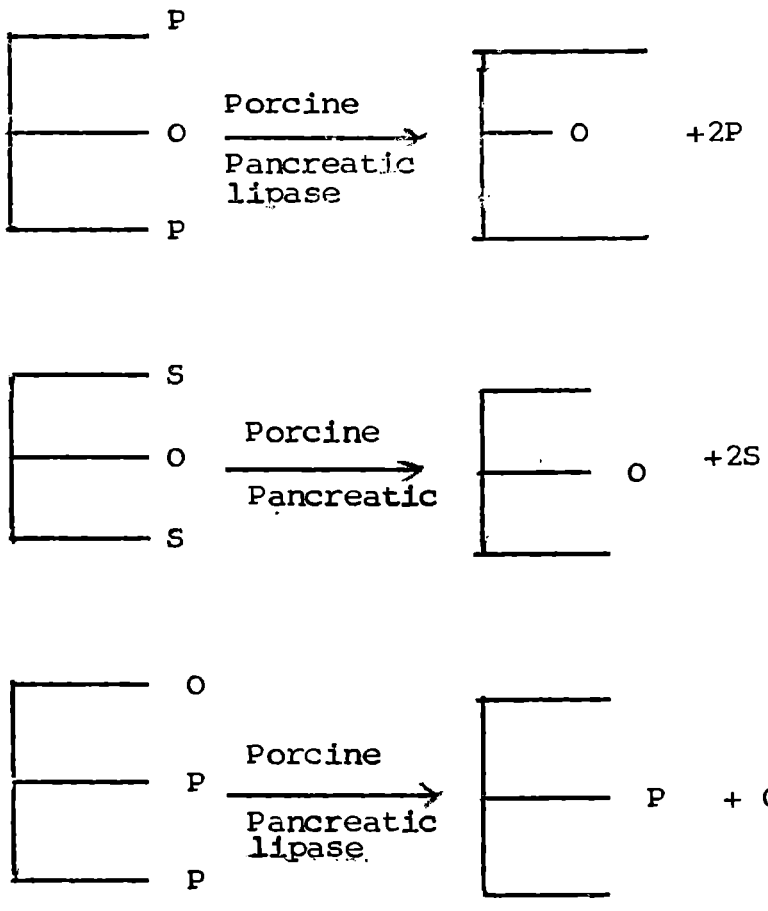
This limited role of digestive lipases on monoglycerides can be explained on the basis of the physioligical significance of 2-monoglycerides. Monoglycerides together with bile salts form the micelles that are vehicles of fatty acid absorption through the intestinal wall (Hoffman and Borgstrom, 1964). These monoglycerides then serve as starting material for the synthesis of new triglyceride molecules for formation of chylomicron of lymph or for storage in adipose tissue. It is to facilitate 2-monoglyceride to play this role, that digestive lipases leave them unhydrolysed.

5.13 POSITIONAL SPECIFICITY

As stated in previous section triglycerides have two types of ester linkages viz. 1 and 2 (1 and 1' position being identical). It is reasonable to assume some sort

of discrimination by sardine lipase for the hydrolysis of 1 and 2 ester bonds of a triglyceride as was observed with hog pancreatic lipase (Balls and Matlack, 1938).

Mattson and Beck (1955, 1956) and Savary and Desnuelle (1956) have reported specificity for porcine pancreatic lipase based on their studies on synthetic mixed triglycerides with different fatty acids at 1 and 2 positions. The $\mathcal{L}$ (1) specificity of porcine pancreatic lipase was further confirmed by Brokerhoff (1968) using synthetic mixed triglycerides as shown below.



P Palmitic
O. Oleic
S. Stearic.

The results of the studies with tricaprilyn, 1,2 dicaprilyn and 2-monocaprilyn are summarised in Table 23 along with the theoretical values for α (1) specificity. The data shows that the activity is maximum with tricaprilyn followed by 1,2 dicaprilyn and 2-mono caprilyn. While hydrolysing them, sardine lipase may have

(1) No specific preference for 1 or 2 ester bonds

(2) Preference for 2 ester bonds and

(3) Preference for 1 ester bonds.

Considering sardine lipase to be unspecific to 1 or 2 position it can be expected that the rate of lipolysis is proportional to the number of ester bonds. That is to say the rate of hydrolysis must be in the proportion 1:2:3 for mono, di and triglycerides. However, the data obtained in this regard do not conform to this conclusion. Instead the rates obtained are in the ratio 1:3.4:4.8, showing considerably higher rates of lypolysis for tricaprilyn and 1,2-dicaprylin over mono caprilyn. If 2 (β) ester bond, is the bond preferred by sardine lipase, the rates would have been more or less equal for mono, di and triglycerides as each of them contain one 2 ester bond. As the experimental data do not conform to this logic the preference for 2 ester bond can also be ruled out.

TABLE 23. COMPARITIVE RATES OF HYDROLYSIS OF MONO, DI AND TRIGLYCERIDES BY SARDINE LIPASE. POSITIONAL SPECIFICITY

Glyceride	Rate of hydrolysis	
	Experimental values	Theoretical value assuming α (1) specificity
Tricaprylin	31.15	31.15
1,2-Dicaprylin	22.10	15.60
2-Monocaprilin	6.5	0
Tributrin*	100.00	-

* Tributrin value taken as 100

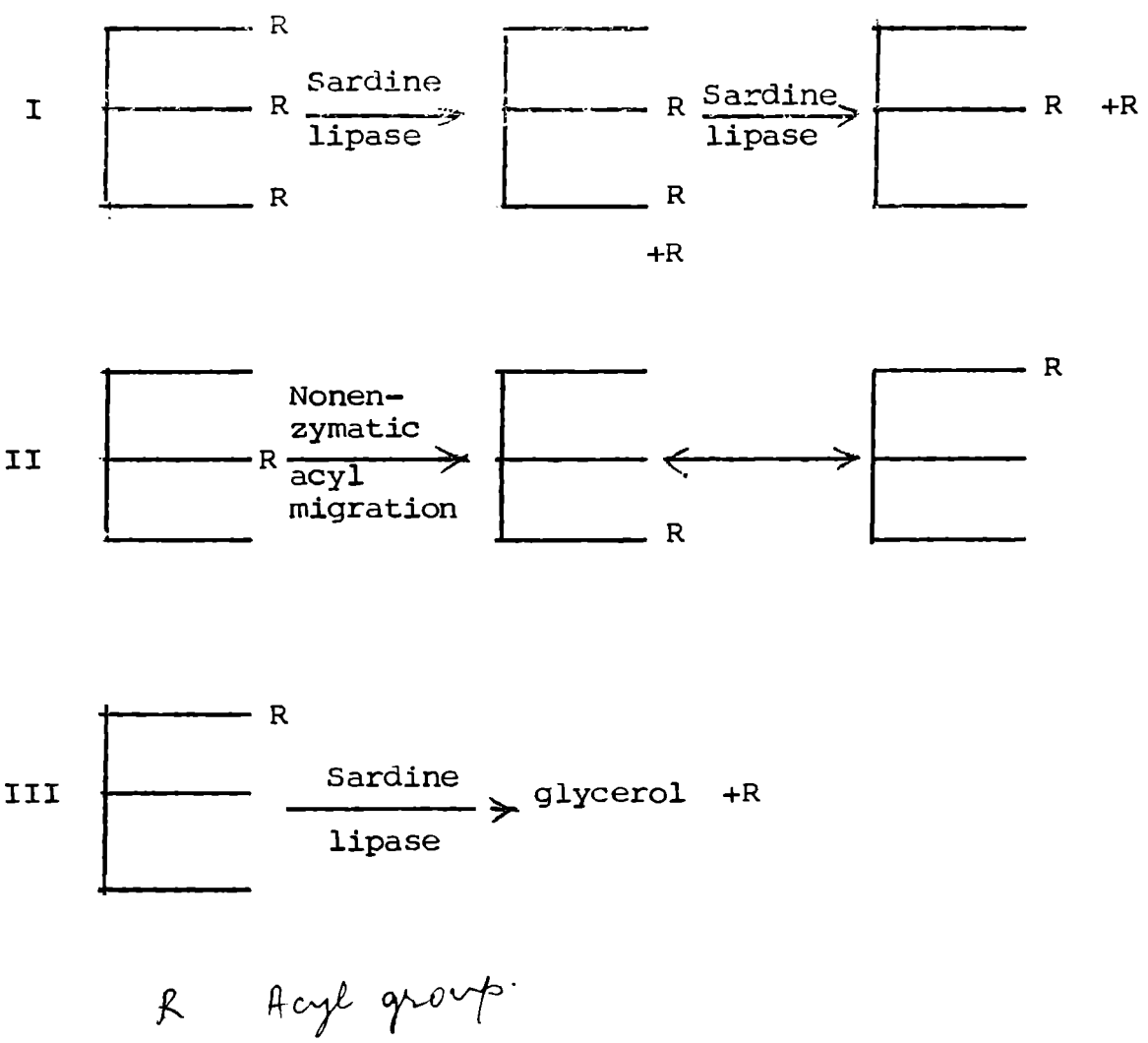
Now the only remaining alternative is sardine lipase to have an affinity for 1 ester bonds for lypolysis. Both triglyceride and the diglyceride contain, two (1 and 1') and one 1 ester bonds respectively and these glycerides should exhibit lipolysis rate in 2:1 proportion and that there should not be any lipolysis of the 2 mono glyceride. From the data obtained, it can be seen that tricaprylin and dicaprylin exhibits substantially higher rates of lipolysis than mono caprylin although these rates are not in agreement with the theoretical values.

Mattson and Beck (1956) observed acyl migration of the acid residue from 2 position to 1 or 1' positions of mono-glycerides. In the light of this acyl migration the incompatibility of theoretical values and experimental values of the above experiment (assuming 1 affinity) can be explained as follows. assuming the affinity of (1) rather b

The 2-mono caprylin, under conditions of reaction undergo acyl migration forming equilibrium amount of 1 mono caprylin which then undergo lipolysis to give caprilic acid and glycerol resulting in an apparent rate of lipolysis for 2-mono caprylin. Similarly in the 1,2 diglyceride also this acyl migration can result leading to the production of an equilibrium amount of 1, 1' diglyceride which has an equal number of 1 ester bonds as

tricaprylin. This phenomen of acyl migration in 1,2-diglyceride will subject it to a higher rate of lipolysis than is expected of theoretical calculation viz. 15.6.

In the light of the above mentioned acyl migration the experimental values of hydrolysis rates in table 23 agrees well with α (1) ester bond specificity for sardine lipase, suggesting the following mechanism for lipolysis by sardine lipase.



R Acyl group.

The reaction can be assumed to proceed in 3 stages.

I. All triglyceride molecules undergo enzymatic hydrolysis of 1 ester bonds liberating 2 mono glyceride.

II. The 2-monoglyceride undergo acyl migration, a non-enzymatic reaction cntrolled by environmental factors to form 1-monoglyceride.

III. The 1-monoglyceride formed then undergo lipolysis in presence of sardine lipase liberating glycerol and fatty acid.

5.14 IMMOBILISATION OF SARDINE LIPASE

Recently immobilised enzymes have been the subject of increased interest because they have more potential activity than free enzymes. Practically their insoluble properties make it possible to operate an enzyme engineered process continuously in place of batchwise process. They can also be applied in the field of medicine food and analytical chemistry. In research they may serve as model systems for natural enzymes bound to in vitro membrane (Katchalski et al. 1971; Zaborsky, 1972 and Gutcho, 1974)

Immobilised enzymes has been used in conjunction with auto analysers to ana_yse large number of chemical samples at lesser operational costs (Endo et al. 1979). Moreover immobilised enzyme reagents give better stability

TABLE 24. COMPARATIVE RATES OF HYDROLYSIS OF TRIGLYCERIDES BY NATIVE AND IMMOBILISED LIPASE

Triglyceride	Rate of hydrolysis*	
	Native lipase	Immobilised lipase
Triacetin	43.2	40.1
Tributrin	100.0	70.8
Trivalerin	25.6	17.5
Trihexanoin	10.9	6.6
Trioctanoin	30.7	22.4
Tripalmitin	18.1	12.0
Triolein	22.9	14.7

* Rate of tributrin hydrolysis taken as 100

and reusability than soluble enzymes. It was in this context, the sardine lipase was immobilised in poly-acryl amide gel as described in methods and its properties investigated.

The dried immobilised lipase was in the form of small flakes of rubber. By this process almost 100% of the enzyme used were immobilised. On assay the preparation exibited 65% to 70% of the lipase activity of the native enzyme. The comparative rates of lipolysis of various triglycerides by native and immobilised sardine lipase are shown in Table 24.

The immobilised sardine lipase showed excellent storage life as it can be stored at 0°C for 4 months without any significant loss in activity.

Thus the above studies clearly proves that oil sardine is an excellent source for lipase enzyme and the process delineated and described in this thesis workable for commercial production.

6. SUMMARY

6. SUMMARY

Oil sardine contains significant amounts of lipase distributed in its body organs, of which hepatopancreas has the maximum concentration viz. 126 units per gm wet weight of hepatopancreas.

A method to purify lipase from the hepatopancreas of oil sardine has been worked out. Pure sardine lipase was prepared at a yield of 35% at a specific activity of 940 units. The method consists of four major steps, which are as follows.

Step 1. Defatting hepatopancreas. Homogenised oil sardine hepatopancreas was extracted several times with (a) acetone; (b) 1:1 acetone ethyl ether mixture and (e) ethyl ether at 0°C. The fat free hepatopancreas was lyophilised to a give friable powder.

Step 2. Ammonium sulphate fractionation. The hepatopancreas powder was extracted with cold water and the proteins of the extract was fractionally precipitated first at 0.3 and then at 0.8 saturation with ammonium sulphate. The 0.8 saturation precipitate containing lipase protein was dissolved in water, desalted and concentrated with dry sephadex G-25.

Step 3. DEAE sephadex ion exchange chromatography.

The lipase solution obtained in step 2 was subjected to DEAE A50 sephadex column chromatography under sodium chloride gradient elution, with the help of a UV monitor and a fraction collector, to give partially pure lipase.

Step 4. Sephadex G-100 column chromatography. A portion of the more concentrated solution of lipase from DEAE sephadex chromatography was subjected to sephadex G-100 column chromatography, which gave two protein peaks, of which the minor peak was lipase and was a pure protein, for the following reasons.

The sardine lipase protein upon chromatography on sephacryl S-200 gave only one peak. Further on polyacrylamide gel electrophoresis it gave only one band. Molecular weight estimation by gel filtration on sephadex G-100 and SDS acrylamide electrophoresis gave a value of 54500. Carbohydrate analysis of sardine lipase showed the presence of 6.1% carbohydrate. The carbohydrates present in sardine lipase were glucose, arabinose and xylose in 1:4.8:4.2 proportion. Amino acid analysis data revealed that sardine lipase protein do not contain any sulphur amino acids. Sardine lipase is not an SH. enzyme.

Sardine lipase had pH and temperature optima at pH 8 and 37°C respectively. The enzyme was stable up to a temperature of 45°C. It was also stable in the pH range 5 to 9.5.

A variety of molecules and ions were inhibitors of sardine lipase. Thus the inhibition by aldehydes and ketones were in the order, formaldehyde > acetaldehyde > ethyl methyl ketone > acetone. The cationic inhibitors were $Fe^{3+} > Fe^{2+} > Ba^{2+} > Cu^{2+} > Cu^{+}$ in the order of their extend of inhibition. Preincubation of sardine lipase with Fe^{3+} showed a four fold increase in the degree of inhibition. The anionic inhibitors were $F^{-} > Br^{-} > I^{-}$ of which F^{-} was a powerful inhibitor. Chloride ion and calcium ion exhibited activation of sardine lipase. A number of metallic ions Hg^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Sn^{2+} and Zn^{2+} showed no inhibition.

Studies with various substrates showed that sardine lipase has no absolute specificity. Instead it hydrolyses a variety of substrates like various triglycerides, 1-monglycerides and diglycerides. The rate of lipolysis of sardine lipase has no direct relationship to the chain length of the fatty acids present in a triglyceride. Of the substrates analysed maximum rate of lipolysis was observed with tributrin followed by triacetin, trioctanoin, trivalerin etc.

Lipolysis of the neutral triglycerides of sardine oil and coconut oil by sardine lipase showed that lauric acid was released preferentially at a higher rate than the next higher acids and decanoic acid ($C_{10:0}$). Further the

acids with odd carbon number viz. $C_{15:0}$ and $C_{17:0}$ were released at substantially higher rates than the fatty acids $C_{14:0}$, $C_{16:0}$ etc with even number of carbon atoms.

A study of the effect of substrate concentration on the activity of sardine lipase revealed that the enzyme acts in accordance with Michaelis - Menten equation. Line-Weaver-Burk plot of the values of velocity and substrate concentration gave a Km value of 6×10^{-6} for triacetin and 12×10^{-6} for tributrin as substrates.

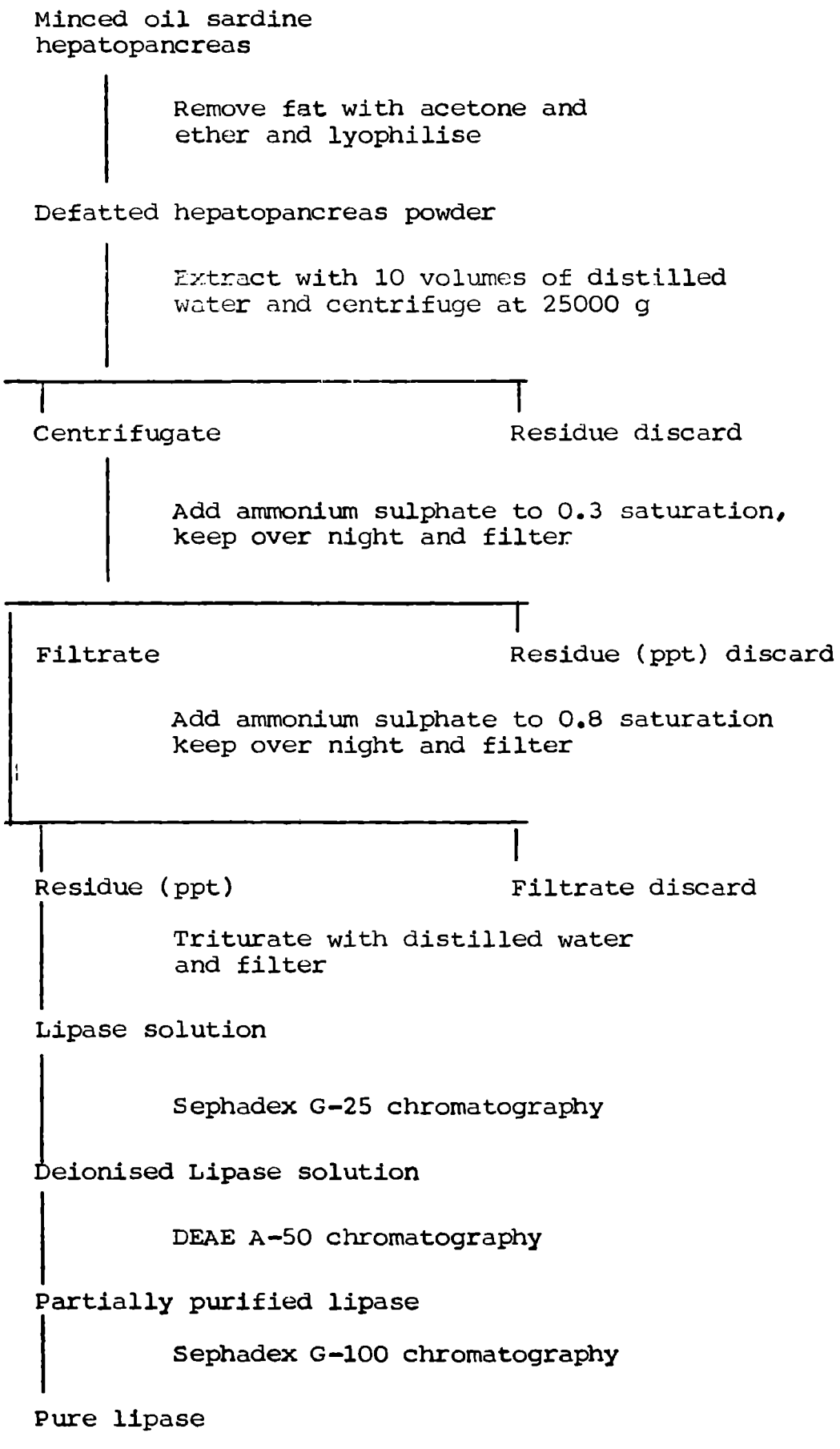
Analysis of the sequence of hydrolysis of tributrin and its partial glycerides showed that sardine lipase has maximum affinity for tributrin followed by dibutrin and monobutrin, which was confirmed from a study of lipolysis of monocaprylin, Dicaprylin and tricaprylin separately.

However, positional speciaficity with 1 monocaprylin 1,2 dicaprylin and tricaprylin revealed that sardine lipase is specific for 1 ester bond and that the lipolysis observed with 2-monocaprylin is due to the hydrolysis of 1 monocaprylin formed as a result of acyl migration of the acyl group from 2-position to 1-position.

Sardine lipase was immobilised in polyacrylamide gel. The immobilised enzyme showed 65% to 70% of the activity of the native enzyme. It was observed that immobilised sardine lipase was more stable than the native enzyme that it can be stored at 0°C for 4 months without any loss in activity.

FLOW SHEET OF PURIFICATION OF SARDINE LIPASE

(All operations at 0° to 4°C)



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