STUDIES ON CHOLESTEROL ESTERASE IN FISH

THESIS SUBMITTED TO
COCHIN UNIVERSITY OF
SCIENCE AND TECHNOLOGY

IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE
DOCTOR OF PHILOSOPHY

BY

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SEPTEMBER 1992

CENTRAL INSTITUTE OF FISHERIES TECHNOLOGY
(INDIAN COUNCIL OF AGRICULTURAL, RESEARCH)
COCHIN - 682 029

DECLARATION

I hereby declare that this is the record of bonafide research carried out by me under the supervision of Dr.K.Gopakumar, Director, Central Institute of Fisheries Technology, Kochi-682029 and that it has not previously been formed the basis for award of any degree, diploma, associate-ship, fellowship or other similar titles of this or any other University or Society.

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This is to certify that the thesis entitled "Studies on Cholesterol Esterase in Fish" gives the results of original work conducted by Shri.T.K.Thankappan, Scientist (SG), Central Institute of Fisheries Technology, Kochi under my supervision and guidance from 30.9.1984 to 16.9.1991. 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. q lifying examination of the Cochin University of Science and Technology held in January, 1990.

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Dr.K.GOPAKUMAR
(Director)

ABBREVIATIONS USED IN THIS THESIS

ATP	–	Adenosine triphosphate
BDH	–	British Drug House
BIS	–	Bis-acryl amide
Cm	–	Centimetre
DEAE	–	Diethyl amino ethyl
DFP	–	Diisopropyl Fluorophosphate
DNP	–	Diethyl p-nitrophenyl phosphate
EDTA		Ethylene diamine tetra acetic acid
EC	–	Enzyme commission
Exptl.	–	Experimental
g.	–	gram
GLC		Gas liquid chromatography
hr.	–	hour
Km	–	Michaelis constant
M	–	Molar
mA	–	milli ampere
min.	–	minute
ml.	–	millilitre
N	–	Normal
NEM	–	N-ethylmaleimide
nm		nanometre
No.	–	number
PCMB	–	parachloro mercuri benzoate
ppm	–	parts per million

rpm	–	revolutions per minute
s	–	Swedberg constant
SDS	–	Sodium dodecyl sulphate
SH	–	Sulphydryl
TEMED	–	NNN'N' tetramethyl ethylene diamide
TLC	–	Thin layer chromatography
TRIS	–	Tris hydroxy methyl amino methane
µg	–	microgram
µM	–	Micromole
UV	–	ultra violet
v	–	velocity of reaction
V		Maximal velocity
viz.	–	namely
vs	–	versus

CONTENTS

ACKNOWLEDGEMENT

The author expresses his deep and sincere gratitude to Dr.K.Gopakumar, Director, Central Institute of Fisheries Technology, Kochi-682029 for his able guidance and valuable suggestion during the entire course of this investigation.

He is very much thankful to his colleagues of this Institute Dr.M.K.Mukundan, Dr.Jose Stephen, Shri.A.G.Radhakrishnan, Shri.P.D.Antony, Dr.P.T.Mathew, Dr.K.G.Ramachandran Nair and Shri.P.Madhavan for their help in different stages of this study. Technical assistance rendered by Shri.A.Kassim Kunju, Shri.V.V.Johni, Shri.K.R.Kesavan, Shri.T.Mathai, Shri.P.T.Viswambharan, Shri.G.Omanakuttan Nair, Smt.V.C.Mary, Shri.P.K.Raghu, Smt. N.Leena, and Smt. S.Kamalamma is also well acknowledged.

The author is also much thankful to Shri.M.Rajendranathan Nair, former Director and Dr.K.Gopakumar, present Director of this Institute for giving permission and encouragement to conduct this investigation at this Institute.

(T.K.THANKAPPAN)

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INTRODUCTION

1. INTRODUCTION

The esterases and lipases have in common, the capacity to hydrolyse fatty acid esters and we differentiate them on the basis of their relative specificity. The esterases act on simple esters of low molecular weight fatty acids. Both enzymes are non specific in the action. A number of studies have been conducted on the assay, estimation and purification of the esterases from the rat pancreas and several other sources. But a comprehensive study on the isolation, identification and properties of the esterase enzyme from any fish has so far not been attempted.

Enzymes catalysing the synthesis or hydrolysis of cholesterol ester may be divided into three general groups (a) Enzymes that synthesise or hydrolyse sterol esters (or carry out both the activities) with no apparent requirement for a high energy source such as adenosine triphosphate (ATP) and co-enzyme A (Co A). These enzymes are collectively referred to as cholesterol esterases (sterol ester hydrolyse, EC 3.1.1.13) and have been identified in the pancrease, intestinal mucosa, liver and adrenal gland, (b) Enzyme that synthesise choleserol esters from fatty acyl Co A and free sterol (Acyl-Co A-Sterol-O-acyl transferase EC 2.3.1). Such systems have been reported in liver and adrenal gland, (c) A plasma specific enzyme which catalyse the transfer of fatty acids from the B-position of lecithin to free sterol (lecithin-cholesterol-O acyl transferase EC 2.3.1). Studies

on these three enzymes including their purification and characterisation have been reviewed (Hyun et al. 1969).

Cholesterol esterases are involved in the initial metabolic transfermentations of dietary cholesterol and its esters. In the intestinal lumen, pancreatic cholesterol esterase catalyses the hydrolysis of cholesterol esters into free sterol which is the form required for absorption (Vahouny and Treadwell, 1964). After transfering into the intestinal mucosa and prior to release into the lymphatic system, the sterol is reesterified by mucosal cholesterol esterase. There is proof that this enzyme is closely related to the enzyme of pancreas and may be identical with it (Hernandez et al. 1955; Borja et al. 1964).

Cholesterol esterase enzyme has been purified from rat pancreatic juice (Hernandez and Chaikoff ,1957), rat pancreas (Murthy and Ganguly, 1962), human pancreatic juice (Vahouny and Treadwell, 1968) and porcine pancreas (Swell, 1966). This esterase has also been identified as an intracellular enzyme in a variety of animal tissues such as rat liver (Swell and Treadwell, 1955; Hirsch et al., 1952), human iorta (Swell, L., 1966), bovine adrenal cortex (Vahouny et al., 1963; Hoffman, 1967 and Vahouny et al. 1967), human cerebro-spinal fluid (Boillei, 1960), rabbit iorta (Martin and Ames, 1963) and human leucocytes (Mattson and Volpenhein, 1966). Bacteria (Mattson and Volpenhein, 1968), moulds (Trout et al. 1960) and yeasts (Novak, M., 1965) are also the source of cholesterol esterase.

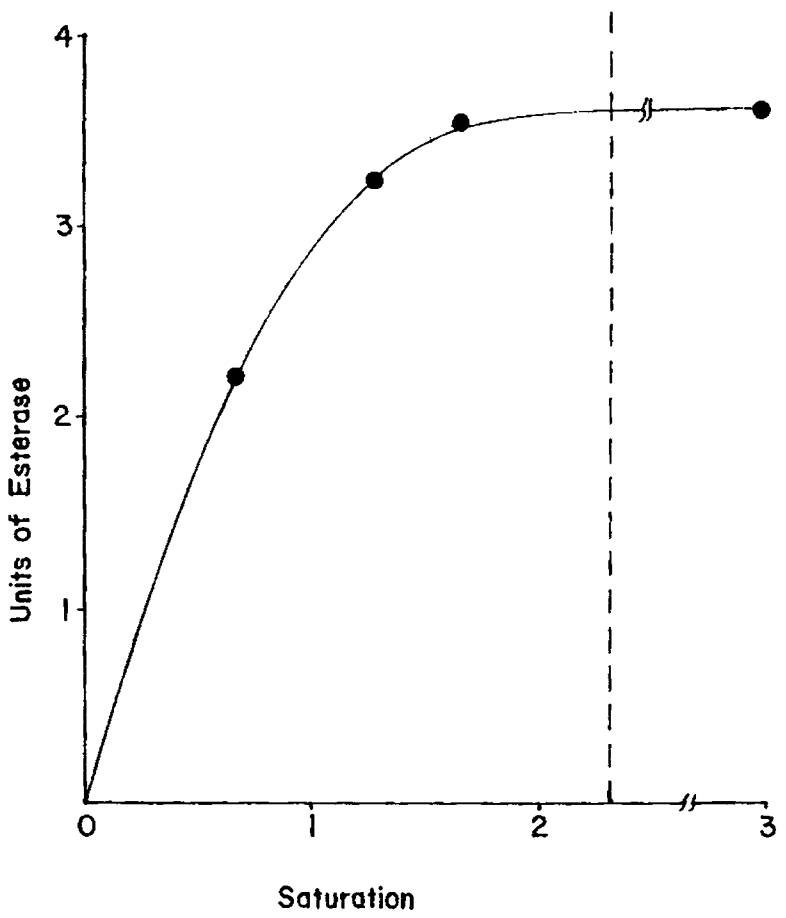


FIG. 1. TYPICAL ESTERASE REACTION

HYDROLYSIS OF TRIACETIN BY PURIFIED HORSE LIVER ESTERASE. THE BROKEN LINE INDICATES THE POINT OF SATURATION (FROM SARDA AND DESNEULLE, 1958)

Eventhough a number of studies have been carried out on the isolation, purification and properties of cholesterol esterase from animal and other sources, so far no work is reported on its isolation and purification from fish. Almost all fresh water, brackish and marine fishes may contain cholesterol esterase in their liver.

A typical esterase reaction is shown in figure 1. It shows that the reaction velocity of horse liver esterase is a function of substrate concentration. The vertical dotted line indicates the point of saturation of the solution with triacetin. To the left of dotted line triacetin is soluble forming a homogenous layer and to the right of the dotted line it exists as an emulsion. The esterase reaction rate reaches maximum in the soluble region and is steady in the over saturated area (Sarda and Desneulle 1958).

The intracellular cholesterol esterase have also been discovered in the mamalian tissue (Davis, 1964). The presence of this enzyme in pancreatic juice of dog was reported by Mueller (1915, 1916). An intestinal cholesterol esterase was detected by Klein (1938) in the cow. This enzyme is also found in the pancreas of porcine and rat (Swell, 1966).

Since a lot of work has been done on the isolation of the enzyme from animal sources, it is desirable to isolate and purify this enzyme from the aquatic sources. In a survey in our laboratory, marine cat fish liver was found to have more cholesterol esterase activity, and so this was used for the isolation and purification of the enzyme.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Enzymes which hydrolyse long chain fatty acid esters of cholesterol are found in mammalian tissues and have been named as sterol ester hydrolase (E.C. 3.1.1.13) by the Enzyme Commission. If the enzyme can hydrolyse esters of sterol that are not close relatives of cholesterol, it is preferable to name as cholesterol ester hydrolase or cholesterol esterase. The presence of this enzyme in the rat pancreatic juice and dog pancreatic juice was first demonstrated by Mueller (1915, 1916). It was also detected in the intestines of cow (Klein, 1938). Cholesterol esterase was also detected in tissues of other mammals (Hyun et al. 1969; Calama et al. 1975; Lombardo et al. 1978; Sakurda et al. 1978). The importance of esterases in the biological system of mammals was also reported by Trzeciak et al. (1974), Khoo et al. (1976), Gorbon et al. (1977) and Shino et al. (1972).

Cholesterol esterase catalyses the hydrolysis of cholesterol esters and their synthesis from cholesterol and free fatty acids. Rat cholesterol esterases has been reported to catalyse the synthesis of esters from cholesterol and free fatty acids (Hyun et al. 1969). Both hydrolysis and synthesis are important in the absorption and metabolism of cholesterol. The mammalian intestines can not as such absorb cholesterol esters. So they are split into cholesterol and fatty acids by pancreatic cholesterol esters. A number of reports are there on the isolation and purification of the

enzyme from the mammalian tissues, yeasts, moulds and microbial sources (Swell and Treadwell, 1955; Hirsch et al. 1952; Novak, M., 1965; Trout et al. 1960; Mattson and Volpenhein, 1968). The structure of cholesterol esterase and its properties has been established by many workers (Milstein, 1966; Milstein and Sayer, 1961; Vahouny et al. 1964). The salient features on the isolation of cholesterol esterase from different sources and their properties are described.

2.1 Pancreatic juice cholesterol esterase

Detailed work has been done on the isolation of cholesterol esterase from rat pancreas (Calame et al. 1975), rat pancreatic juice (Hyun et al. 1969), human pancreatic juice (Lombardo et al. 1978) and porcine pancreas (Momsen et al. 1977; Rosalind et al. 1983).

Cholesterol esterases are responsible for the initial metabolic transformation of dietary cholesterol and its esters. In the intestinal lumen pancreatic juice cholesterol esterase catalyses the hydrolysis of cholesterol esters to free sterol in which form the absorption takes place. After transfer to the intestinal mucosa and prior to the release into the lymphatic system, the sterol is re-esterified by mucosal cholesterol esterase. This enzyme system is closely related to the enzyme of pancreas and is identical in its properties (Hernandez et al. 1955; Borja et al. 1964).

A partial purification of cholesterol esterase from porcine pancreas has been reported by Hernandez and Chaikoff (1957). From ammonium sulphate (60% saturated) extract of acetone powder of porcine pancreas, they have collected a cholesterol esterase. After dialysing, fractionation and purification using Sephadex G-100 column, about 400 fold purified enzyme was obtained. On ultrafiltration and SDS gel electrophoresis it gave more than one band showing the enzyme was not pure and contained other protein frctions also. Murthy and Ganguly (1962) attempted to prepare a partially purified enzyme from the acetone powder of rat pancreas. The proteins were absorbed on clacium phosphate gel and eluted with phosphate buffer, pH 7.0. This resulted in the separation of hydrolysing and synthesizing cholesterol esterase activities. About 55 fold concentration of hydrosase was obtained. The synthetase was recovered by 0.6% ammonium sulphate extraction of the gel concentration.

Hyun et al. (1969) have isolated cholesterol esterase from rat pancreatic juice. The protein was precipitated with 35% acetone, separated on DEAE cellulose and hydroxylapatite column chromatography and elution with pH 6.8 phosphate buffer motality. Mercaptoethanol and 10% dimethyl sulphoxide were also added to the system to reduce the loss of activity in the process. Here the recovery was only 60%. The sample was stable for 2 months at 2° C and was homogenous on SDS gel electrophoresis.(Table 1, 1a).

TABLE - 1

Purification of Cholesterol esterase from
Rat pancreatic juice

Fraction	Protein mg.	Activity µ moles/ min.	Specific activity µ moles/ min./mg.	Recovery
Pancreatic juice	384	46	0.12	100
35% acetone precipitate	14.4	28	1.9	60
DEAE cellulose	2.4	20	8.0	43
Hydroxyl apatite	0.06	2.8	41	6

Hyun et al. (1969)

TABLE **1.a.**

Activity of cholesterol esterase during purification

Fraction	protein mg.	Cholesterol esterase	
		Total activity units	Specific activity units/mg/ protein
Pancreatic juice	1426.0	6863	5
35% acetone precipitate	53.6	3861	72
DEAE cellulose (0.15 M) fraction	7.9	1655	211
Hydroxyl apatite (0.20 M) fraction	0.29	805	2776

Hyun *et al.* (1969)

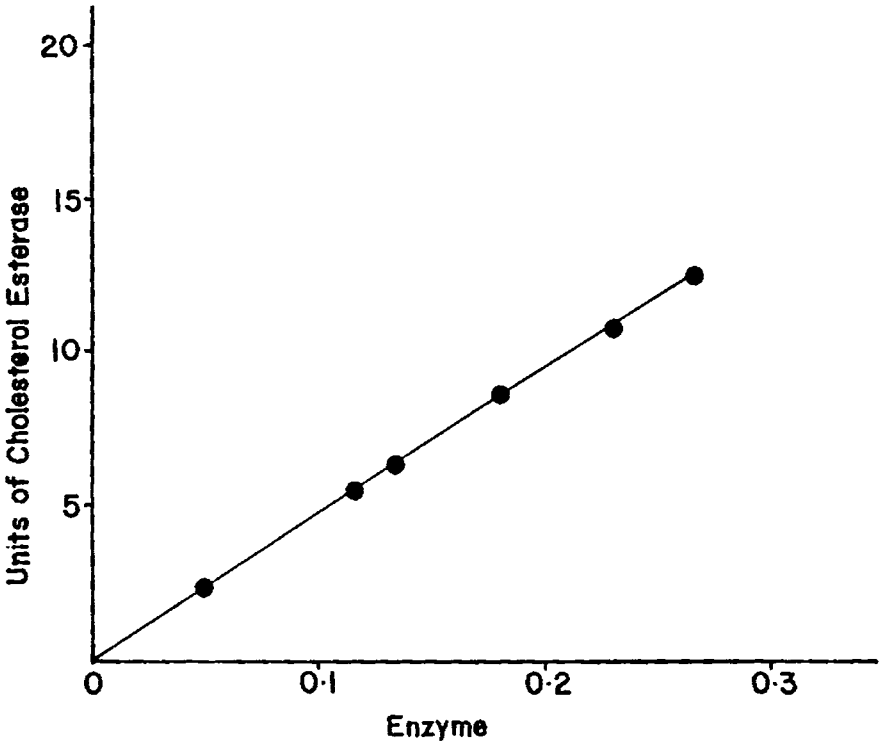


FIG:2. RELATION OF ENZYME CONCENTRATION AND ACTIVITY. (HYUN. et. al. 1969)

A cholesterol esterase from human placenta has been purified by Chen and Morin (1971). The final preparation gave about 3% yield and 350 fold purification. This gave one band on SDS disc gel electrophoresis and a specific activity of 8×10^{-6} .

The cholesterol esterase of rat pancreatic juice was found to have a molecular weight of 65000-90000 daltons (Morgan et al. 1968; Hyun et al. 1971). On precipitation with acetone it forms a dimer of molecular weight 1,35,000 daltons (Hyun et al. 1972). In the presence of cholate it gave a higher molecular weight equal to six tissues the monomer on sephadex column.

Teale et al. (1972) have reported a cholesterol esterase from porcine pancreas of molecular weight more than 8,00,000 which on lipid extraction was converted to sub units of molecular weight 15000-20000 daltons which could still hydrolyse and synthesise cholesterol esters. Human pancreatic cholesterol esterase had a molecular weight of 3,00,000 daltons by gel filtration (Erlanson and Borgstron 1970). This is a bigger size for a lipolytic enzyme and it suggests that human pancreatic cholesterol esterase may be a dissociable aggregate. Both the cholesterol esterase and lipase complexes dissociates in the bile salt solution. It is found that pancreatic cholesterol esterase is vulnerable to digestion by pancreatic proteinases much more than pancreatic lipase (Vahouny et al. 1964) but can be protected

TABLE 2

Ratio of enzymatic synthesis and hydrolysis of cholesterol oleate during purification of cholesterol esterase

Fraction	Ratio of specific activities for hydrolysis to synthesis
Pancreatic juice	1.2 ± 0.2
35% acetone precipitation	1.1 ± 0.1
DEAE cellulose fraftio (0.15 M)	1.3 ± 0.2
Before dialysis	1.1 ± 0.1
After dialysis	
Hydroxyl apatite fraction	1.2 ± 0.2

Hyun et al. (1969)

by bile salts (Hedswedski, 1936; Murthy and Ganguly, 1962). The enzyme is sensitive to surface denaturation which is evident from the loss of activity in the final preparation, which can be reduced by the addition of albumin (Vahouny and Treadwell, 1968.

The method adopted for the purification of pancreatic juice cholesterol esterase by Hyun et al. (1969) is described elsewhere. A 400 fold purification was reported by Herandez and Chaikoff (1957). The following procedure was adopted by them.

a) Acetone precipitation: Cold acetone was added to the ice cold pancreatic juice which was previously centrifuged and the final concentration was 35% by volume. The precipitate was removed by centrifugation which was redissolved in cold 0.05 M. pH 6.2 phosphate buffer. Insoluble protein was removed again by centrifugation.

b) DEAE cellulose column chromatography: DEAE cellulose previously washed several times with 0.05 M phosphate buffer was used. To the column the enzyme solution was added, eluted with phosphate buffer, collected and was dialysed to get a more purified enzyme.

c) Hydroxylaptite column chromatography: To column packed with hydroxylaptite, which was previously washed with 0.01 M phosphate buffer pH 6.5, the above enzyme solution was added and eluted with 0.01 M phosphate

buffer pH 6.2 at a flow rate of 0.25 ml/min. The eluant were collected and dialysed to get 400-600 fold purification depending on the specific activity in the original pancreatic juice. It was observed that higher concentrations of acetone increased the yield of enzyme but decreased the degree of purification. Attempts to lyophilise the fractions showed a loss in activity. Lyophilisation or freeze drying after dialysis resulted in complete loss of activity.

The enzyme prepared by the hydroxylapatite chromatography gave single band by polyacrylamide disc gel electrophoresis. With purified aldolase and γ-globulin as reference proteins in separate runs the molecular weight was found to be 1,44,000 and 1,29,000 respectively with an average of 1,36,000. The pH optimum was found to be 6.2 for the esterification. With rat and hog pancreas and rat intestine the optimum pH for the esterification was 6.1 and 6.5 (Hernandez, 1957; Murthy and Ganguly, 1962; Swell et al. 1950). The optimum pH for hydrolysis was 6.6-7.0 which is comparable to that reported for the enzyme hog and bovine pancreas and rat intestinal mucosa (Swell et al. 1955; Korenovsky et al. 1960 and Swell et al. 1950). The ratio of specific activities of the esterification and hydrolysis of cholesterol esters was 1:2 and this remained same throughout the purification process. This suggested that the two activities are due to the same enzyme protein.

The effect of ions and inhibitors on the activity of the enzyme was reported by Hernandez and Chaikoff (1957). The reaction was enhanced to about 30-35% by the addition of 10 M NaCl, LiCl, KCl or NH_4Cl at pH 6.2. It was also found that the addition of 0.1 M Na, K, NH_3 or 0.001 M Hg or Zn to the buffer solution has no effect on the enzyme activity. A requirement of free sulphydryl group was suggested by complete inhibition of both synthetic and hydrolytic activities by incubation of the enzyme with 10 M PCMB prior to the assay. This inhibition was completely prevented by 10 M cysteine or glutathione.

Like all lipolytic enzymes, the exact pH optimum for a cholesterol esterase is a function of the physical state of the substrate. The optimum pH for the synthetic reaction of the pancreatic and intestinal enzyme is 6.1 to 6.2 and for the hydrolytic reaction it is 6.7-7.0. Values as high as pH 8.6 for the hydrolytic reaction (Murthy and Ganguly, 1962) and a low pH 5.5 (Fodor, 1950) and 4.7 for synthesis (Sweel and Treadwell, 1955) are reported. In general the optimum pH for the synthetic reaction is found to be 0.5-1.0 units lower than that of the hydrolytic reaction of the same enzyme preparation. The enzyme can be assumed to catalyse a hydrolysis esterification reaction which leads to pH dependent equilibrium with esterification at low pH and hydrolysis at high pH. Such a situation has been shown for rat pancreatic cholesterol esterase (Filipek-Wender and

Borgstrom, 1971). This is analogues to that found for pancreatic lipase (Borgstrom, 1964). However both the hydrolytic and synthetic activities remains unchanged during purification processes (Hernandez and Chaikoff, 1957; Hyun et al. 1969).

Detailed studies on the amino acid composition of rat pancreatic cholesterol esterase has been conducted by Hyun et al. (1972). This was similar to that of the porcine pancreas enzyme (Verger et al. 1989) amino acid composition. Rosalind et al. (1983) have found the comparative percentage of amino acid in the procine pancreas, rat pancreas, rat pancreatic juice and human pancreatic juice (Table 3). They have observed that there was a high percentage of proline in the human enzyme and a low percentage of tyrosine in the enzyme of rat pancreas and pancreatic juice. Tryptophan was not at all detected in rat pancreas as well as pancreatic juice. There were similarities in the amino acid composition amongst the enzymes from different mammalian sources.

TABLE- 3

Amino acid composition of Cholesterol esterase

Amino acid	Porcine pancreas mol %	Rat pancreas mol %	Rat pancreatic juice mol%	Human pancreatic juice mol %
Aspartic acid	12	11	11	10
Threonine	7	6	5	3
Serine	7	10	9	6
Glutamic acid	7	10	10	6
Proline	6	7	6	13
Glycine	10	11	10	10
Alanine	10	9	11	9
Valine	4		7	7
Methionine		4	2	2
Isoleucine	5	5	5	4
Leucine	10			6
Tyrosine	5	0	3	4
Phenylaline	4	4	4	4
Histidine	2	2	2	1
Lysine	7	5	6	5
Arginine	4	5	5	3
Cysteine	1	2	1	1
Tryptophan	1	n.d	n.d	2

n.d not determined

Rosalind et al. (1983)

Studies on the specificity of fatty acids on the synthesis and hydrolysis reactions were carried out by many workers. It is observed that when both the activities are measured with the same enzyme preparation, the specificities for the individual acids are not same (Swell and Treadwell, 1955). Equimolar amounts of lauric (12:0), Palmitic (16:0), Stearic (18:0), Oleic (18:1) and linoleic (18:2) acids were added in the substrate mixture. In synthesis oleic acid is the most active acid (Hermandez and Chaikoff 1957; Murthy and Ganguly 1962; Shah et al. 1965; Hyun et al. 1969). But some times linoleic or linolenic acid dominates (Hernendez and Chaikoff 1957; Murthy and Ganguly 1962). Saturated acids are less active (Murthy and Ganguly 1962) and short chain acids C_2-C_6 are seldom reactive (Swell and Treadwell 1961; Murthy et al. 1961) since they are soluble and completely dissociated.

TABLE 4

The fatty acid specificity for enzymatic synthesis and hydrolysis of cholesterol esters

Fatty acid	Synthesis cholesterol ester formed	Hydrolysis fatty acid released
Lauric (12:0)	11	107
Palmitic (16:0)	56	104
Stearic (18:0)	43	100
Oleic (18:1)	100	55
Linoleic (18:2)	71	40

Hyun et al. (1969)

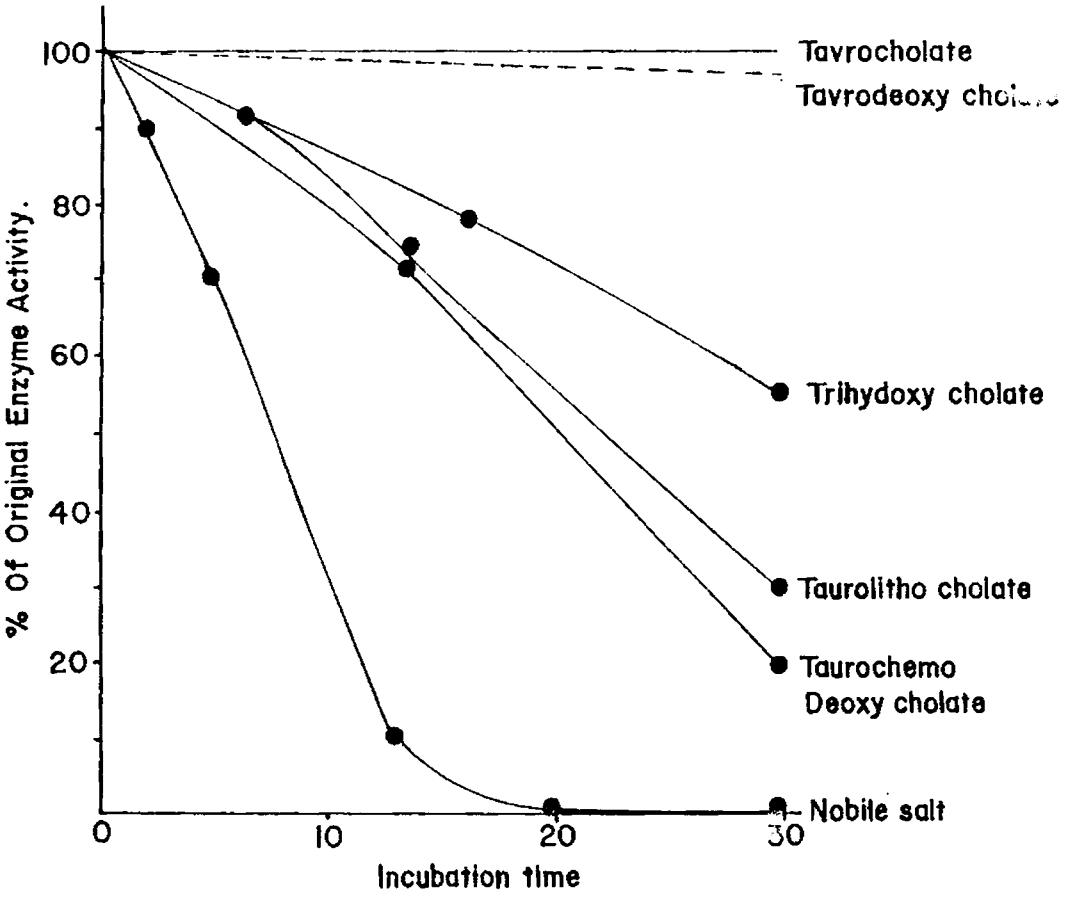


FIG. 3. COMPARISON OF VARIOUS BILE SALTS IN PROTECTION OF PANCREATIC JUICE CHOLESTEROL ESTERASE AGAINST PROTEOLYTIC INACTIVATION. (FROM HYUN et al 1969).

With hog pancreatic extract, the butyrate was the preferred substrate followed by hexanoate, acetate and oleate (Swell and Treadwell 1955). This was supported by the result with rat pancreatic juice (Vahouny et al. 1964 a). When the esters were dissolved in a mixture of lecithin and bile salts the esters of saturated (3:0 to 18:0) and unsaturated (18:1, 18:2, 18:3) fatty acids were all of the same rate. From the studies it is understood that the difference in reaction rates are not due to the substrate-enzyme action on specific binding sites but to the physiochemical properties of the fatty acid dispersion.

In the synthesis of sterol oleates, dihydrocholesterol has been found as effective as cholesterol. Substitution in the side chain reduces the activity (Swell et al. 1954; Hernandez and Chaikoff, 1957; Korzenovsky et al. 1960 a, b; Murthy and Ganguly, 1962). The hydroxyl group must be in β-position (Hernandez and Chaikoff, 1957). Dehydrogenation results in lower activity. The same pattern of specificity is observed for the hydrolysis of both oleate and butyrate esters of different sterols (Swell et al. 1954). It is observed that cholesterol esterase required a certain sterol structure in its substrate for optimal enzyme substrate interaction. Morgan et al. (1961) showed that the hydrolytic activities of rat pancreatic juice against cholesterol ester remained constant during chromatography on Sephadex and DEAE cellulose. Mattson and Volpenhein (1966 b, 1968, 1972 a) have reported an enzyme of rat pancreatic juice which

hydrolysed various primary and secondary long chain carboxyl esters on the presence of bile salts and appeared to be identical with cholesterol esterase. It is found that the relative rates of synthesis and hydrolysis depends on the electrolyte concentration (Korzenovsky et al. 1960 b).

Mueller (1966) had observed that bile salts have an effect on the activity of cholesterol esterase. Nedswedski (1936) showed that the esterification of cholesterol takes place only in the presence of bile salts and that the bile salts protected the enzyme from deactivation during storage. This is by chemically combining the bile acid with the enzyme (Nedswedski, 1937; Klein 1938) found that taurocholates and glycocholate were more potent activators. Vahouny et al. (1964 b, 1965) reported that deoxycholate and its conjugates were completely inactive. Same is the case with dehydrocholic acid (Swell et al. 1954; Murthy and Ganguly, 1962). Inactivation is due to tryptic digestion (Vahuny et al. 1964 a, 1965, 1967).

Very little is known about the esterase or lipase activity of swine milk and the specificity of the enzyme responsible for the esterase activity is now known (Augustinson and Olson, 1961). Morton (1950) has tried to isolate esterase from insects, but no reports are available on its property studies and other aspects.

2.2 Cholesterol esterase from other sources

2.2.1 Liver

A soluble cholesterol esterase was partially purified from rat liver by Deykin and Goodman (1962). The activity was precipitated by 30% saturation with ammonium sulphate. From earlier studies (Byron et al. 1953) it is shown that the activity in the liver is thousand times less than in pancreas. The final enzyme prepared was unstable and had lost its major activities in one day's storage. This enzyme had only hydrolytic ability. Its pH optimum was between 6.5 and 7.5. Zn^{++} and Cu^{++} inhibited the enzyme severely. EDTA or cations had no effect on its activity. N-Ethylmaleimide (NEM) and PCMB were strong inhibitors. Addition of DFP and reduced about 80% of its activity. It has little activity against p-nitrophenyl acetate. Detergents like Tween-20 and glycocholate inhibited the reaction.

It was found that the liver enzyme is specific towards certain fatty acids. On reacting with the cholesterol esters, the oleate and linoleate were rapidly hydrolysed, followed by acetate and palmitate. Stearate was not reacted on. Sgoutas and others (Sgoutas 1968; Guller and Sgoutas 1970; Goller et al. 1970) tested a number of cholesterol esters of cis and trans unsaturated acids and found that cis Δ^9 structure was essential for the substrate of soluble enzymes. The curve presenting the activity of esters as a function of double bond position resembles curves describing the mobility of the corresponding acids in TLC and GLC (Ackman, 1972; Gunstone et al. 1967).

The presence of lysosomal cholesterol esterase in rat liver has been reported by Stoffel and Gretten (2967) and the cholesterol esterifying activity was found in rat liver homogenates at pH 4 (Stokke, 1972 a). The human liver enzyme has a hydrolytic activity at pH 5 and synthetic activity at pH 3.8 (Stokke 1972 a, b). An acidic activity associated with plasma membranes is reported by Riddle and Glomset (2973). Detailed studies on the human liver esterase had been conducted (Burke and Schubert, 1962; Sloan and Fredrickson 1972 a, b). In the adipose tissue of rat and human, the esterase activity is well established (Arnold et al. 1974; Pitman et al. 1975). It was also found that the cholesterol esterase activity is involved either in the uptake or mobilisation of cholesterol or both.

Mohammed (1948) has described the preparation of a crystalline cholesterol esterase from horse liver but Connors et al. (1971) found that it was very low in esterase activity and the activity disappeared on recrystallisation. The latter workers described a liver esterase prepared from horse liver which is 270 fold purified over the acetone powder from the starting material. On further purification it was 277 fold containing approximately 2 mg. of solids per ml. The enzyme could be stored at 5° C for at least one month without loss of activity. This has shown fifty times activity towards methyl butyrate than towards acetyl choline. Its optimum pH for reaction was 8.0.

2.2.2 In mammalian tissues

Dailey et al. (1963) found the hydrolytic activity of esterase in the dog adrenals. The synthetic activity of this was no due to cholesterol esterase (Dailey et al. 1962). It was observed that an acetone powder extract catalysed both hydrolysis and synthesis (Brot et al. 1963). The optimum pH for esterification was between pH 3. and 5. Shyamala et al. (1965) have noticed that the optimal pH for the cell sap enzyme was pH 2.0 and the reaction was independent of ATP, coenzyme-A or Mg .

Coutts and Stansfield (1968) have shown that bovine corpus luteum hydrolysed cholesterol esters with optimum pH 6.0 and 7.5. DFP inhibited the reaction but not PCMB. About 90% of activity was noticed in the subcellular fractions. Morin (1973) observed that in the corpus luteum of pregnant rabbits the hydrolysis and synthesis activity was highest in the mitochordrial fractions whereas in the ovaries of rats the activity was in the supernatent fractions (Behrman and Armstrong, 1969). A cholesterol esterase from human placenta with a specific activity of 8×10^{-6} was purified by Chen and Morin (1971).

A detailed study on cholesterol esterase of rat brain was conducted by Eto and Suzuki (1971, 1973 a, b). It had a synthesis activity at pH 5.6 and was inhibited by bile salts. At pH 4.2 it had highest hydrolytic activity. It was activated by cholate, deoxycholate and taurocholate. Pritchard and Nichol (1964) observed hydrolytic activity in rat brain

at pH 6.6-7.6. The activity at pH 6.6 was depressed by deoxycholate but stimulated by 4 /um cholate and was activated by taurocholate.

Chooesterol esterase of arterial tissue has been studied by different workers. The hydrolytic activity has been investigated in the atherosclerotic rabbit aortas (Day and Gould-Hurst, 1965), Pigeon aortas (St. Clair et al. 1972) and in the supernatant fraction of rat and monkey aortas (Howard and Portman, 1966). In the human aorta the activity was at pH 6.6-7.4 depending on the substrate dispersion (Kothari et al. 1970). It was partially inhibited by metal ions and PCMB. Tauro cholate activated the enzyme. Similar observations were made by Kothari et al. (1973) in the acetone powder of rat and rabbit aortas.

Proudlock and Day (1972) have found an energy independent esterase with a pH optimum of 5.0 in the atherosclerotic rabbit intima. Similarly peritoneal macrophages contain an enzyme with pH 6.3 (Day and Tume, 1969). Tume and Day (1970) observed an enzyme in alveolar macrophages with a pH optimum of 4.5.

2.3 Cholesterol esterase by culturing C. versicolar

A cholesterol esterase was produced by culturing Coriolus versicolor anaerobically at 27° C for 5 days on a medium containing soyabean oil, defatted soyabean, potassium bisulphate and Magnesium sulphate (Patented by Takara Shuzo

Co. Ltd., Tokyo, Japan, 1989). The culturee filtrate containing the enzyme at a saturation of 80% ammonium sulphate, was precipitated, dialysed and lyophilised. This was dissolved in 0.1 M phosphate buffer, purified by chromatography on Sephadex and DEAE cellulose columns to get a yield of 28% with specific activity of 5.55 units/mg. protein. It has an optimum pH of 6.0 and temperature 40° C and stable at 45° C.

2.4 Cholesterol esterase from fish

Eventhough many workers have conducted detailed studies on the occurrence of cholesterol esterase in animal and human pancreas, pancreatic juice, brain, liver etc. there is so far no report available on the isolation and purification of cholesterol esterase from fish sources. And so far a fully purified and stable enzyme in sufficient quantity is not prepared by any workers. So an attempt was made to explore the possibility of isolating this enzyme from a cheaper and abundant resource like fish. On surveying the availability of cholesterol esterase in a number of species of fishes of fresh water, brackish water and marine water, it was observed that cat fish (Tachysurus thalassinus) contained the enzyme in substantial quantity. So studies were conducted on the isolation and purification of cholesterol esterase from cat fish.

This thesis gives a detailed report on the investigation and isolation of an active and highly pure and

stable cholesterol esterase from the liver of the cat fish – Tachysurus thalassinus. Its physical and chemical properties like specificity, inhibition, molecular weight, kinetics, amino acid composition and immobilisation are discussed under various heads in this thesis.

MATERIALS

3. MATERIALS

3.1 Fish

Mature cat fish (Tachysurus thalassinus) was used throughout the experiment as the source of enzyme. Fresh cat fish immediately after capture, used was in rigor state. The cat fish were cut open and the liver was removed in cold condition (0 to 4 °C). On an average, the fish contained about 200-300 g. liver. And about 500 g. liver was used in each batch of enzyme isolation.

3.2 Reagents and chemicals

All chemicals used were of extreme high purity. The substrates like cholesterol stearate, cholesterol oleate, cholesterol laurate, cholesterol palmitate, etc. were from Sigma Chemicals Co. Ltd., St. Louis, U.S.A.

The column chromatographic materials

Sephadex G-25

Sephadex G-100

DEAE Cellulose

Sephycryl S-200

were from Pharmacia Fine Chemicals, Uppsala, Sweden. Silicagel for column chromatography was the products of BDH India Ltd. The ion exchange resins

Amberlite IR $120H^+$,

Dowex 2 x 8 $HCOO^-$

Dowex 50 x 12 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 cellulose, which was the product of E. Merck India Ltd. The reagents for disc gel electrophoresis

Trishydroxymethyl amino methane (TRIS)

Acrylamide

were from E. Merck, Damstardt, Germany.

NNNN' tetramethyl 1:2 Diaminoethane (TEMED)

NN' methylene bisacrylamide

Bromophenol blue

Sodium dodecyl sulphate (SDS)

were, BDH, Poole, England.

Riboflavin - Pfizer, India Ltd.

Amido black George, T. Gurr Ltd., England.

Ethylene diamine tetraacetate (EDTA)

and other chemicals were obtained from E. Merck India Ltd.

The orcinol used for carbohydrate estimation was obtained from BDH, Poole, England. The protein standards

Ribonuclease A

Chymotrypsinogen A

Ovalbumin

Bovine serum albumin

Blue dextran

were products of Pharmacia Fine Chemicals, Uppsala, Sweden.

Glyceraldehyde-3 phosphate dehydrogenase Sigma Chemicals Co., St. Louis, U.S.A.

3.3 Analytical equipments

1. Spectronic 21 - UV Spectrophotometer - Bausch and Lomb, New York, U.S.A.

2. Spectrophotometer - Double beam double wave length spectrophotometer with TLC and gel scanning attachments - Model 1 No.556, Hitachi Ltd., Tokyo, Japan.

3. The Technicon NC - 2P single column Ion exchange amino acid analyser system. Technicon Instruments Corporation, New York, U.S.A.

4. Chromatographic columns 2.5 x 100 cm., 1.5 x 100 Pharmacia Fine Chemicals, Uppsala, Sweden.
   All other columns described were fabricated locally

5. 7000 Ultrorac fraction collector and 2089 UV Cord III; UV absorption meter and a chopper Bar B channel recorder 6520.7/8 LKB, Produkter, Sweden.

6. a) M.B.101, High speed refrigerated centrifuge is used for centrifugation upto 10000 r.p.m.
   M.B. Instruments, Ahamadabad, India.
   b) Hitachi Automatic preparative ultracentrifuge Model, Scp 85H - Hitachi Koki Co., Ltd., Tokyo, Japan.

7. a) Water bath - Haake G. West Germany
   b) Air oven - Tempo Industries Pvt. Ltd., Bombay, India.

8. Toshniwal lyophilizer - Toshniwal brothers Pvt. Ltd., Madras, India.

9. Disc gel electrophoresis apparatus and 10 mA power supply unit Toshniwal brothers Pvt. Ltd., Madras, India.

10. 2117 Multiphorelectrophoretic unit for slabgel electrophoresis - LKB, Bromma, Swden.

11. Digital pH meter, 1400 P.E. Research Concord Instruments Pvt. Ltd., Cochin, India.

METHODS

4. METHODS

4.1 Assay of cholesterol esterase activity

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

Reagents

a) Substrate 0.1 g. cholesterol acetate in 1 ml. alcohol and 0.1 g. albumin were blend with 50 ml. of 0.1M phosphate buffer (pH 7.0) for 5 min. Filtered and kept.

b) N/20 Sodium hydroxide:

Approximately 250 ml N/4 sodium hydroxide was prepared and its extract normality was estimated. It was then diluted to get exactly N/20 sodium hydroxide.

c) Phosphate buffer:

Stock solutions:

Solution A - 0.2 M solution of monobasic sodium phosphate.

Solution B 0.02 M solution of dibasic sodium phosphate.

39 ml of A and 61 ml of B diluted to 200 ml. to get a solution pH 7.0.

d) 85% alcohol:

Commercially available rectified spirit was used.

e) Phenolphthalein indicator:

0.04% solution in 85% ethyl alcohol.

Procedure:

In a 100 ml. conical flask, 5 ml. substrate and one ml. liver extract containing the enzyme were mixed well and incubated for 30 min. with occasional shaking. At the end of incubation period, 20 ml. 85% ethyl alcohol was added to arrest the reaction and break the emulsion. Control were also done in a similar way but ethanol was added before the addition of enzyme. After the reaction was over, two drops of phenolphthalein indicator was added and the liberated fatty acids were titrated against N/20 sod. hydroxide solution. The quantity of enzyme was selected in a manner to give a titre value of 3 to 6 ml N/20 sod. hydroxide for 30 minutes of reaction.

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

4.2. Enzyme homogenity tests.

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 (1.5 x 100 cm.) already half filled with distilled water. The gel was poured until the bed volume reached a height of 50 cm. The column was drained till the water level falls to

the bed level at which the column was connected to 0.01 M phosphate buffer and the column outlet was connected to a peristatic pump adjusted to drain the column at 15 ml. per hour. About one litre of the buffer was passed to equilibrate the gel bed. To this equilibrated column 10 mg. of cholesterol esterase protein was added and eluted with 0.01M, pH 7.0. Phosphate buffer at a flow rate of 15 ml. per hour. The eluant was fractionated into 5 ml. volumes in ultrorac 7000 fraction collector after monitoring the absorbance at 280 nm with UV cord III UV absorption meter, which gave the absorption pattern.

4.2.2 Electrophoresis

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

Reagents:

Solution A.

1N 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 mg.
BIS	0.735
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 glycine buffer pH	8.3
Tris	6 g.
Glycine	28.8 g.
Water to	100 ml.

This buffer was stored at 0 °C and diluted 10 times just before use.

Bromophenol blue (Marker dye):

Bromophenol blue	50 mg.
Water to	100 ml.

Amidoschwartz stain:

Amidoschwarts	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 proportion. The spacer gel and sample gel were composed of 2.5% acrylamide and was prepared by mixing B, D and E solutions in solutions in 1:2:1 ratio and diluting with equal volume of water.

Esterase sample was prepared by mixing 1 ml. cholesterol esterase solution (10 mg. protein) and 0.1 ml bromophenol blue solution with an equal volume of 5% acrylamide solution by mixing B, D and E solutions in 1:2:1 ratio.

The gel tubes (8.5 x 0.6 mm) were filled with 2 ml. running gel solution, which was overlayered with few drops of water. The tubes (6 numbers) were placed near a flourescent tubelight for 45 minutes to polymerise. After polymerisation the water layer was removed with a piece of filter paper and 0.25 ml. spacer gel solution was added, followed by a layer of water and allowed to polymerise as before for 45 minutes. Again the water layer was removed from the tube and 0.25 ml esterase 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-glycine buffer. The terminals of the apparatus was connected to the power supply unit and the electrophoresis was done at 6 mA per gel.

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

4.2.3 The absorption spectrum of pure esterase

The absorption spectrum of pure cholesterol esterase protein was taken by scanning 0.5% cholesterol esterase protein in 0.1 M phosphate buffer from 200 to 600 nm in UV visible double beam double wavelength spectrophotometer.

4.3 Molecular weight determination

The molecular weight of purified cholesterol esterase was determined 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 done according to Andrews (1964). 10 g. Sephadex G-100 was suspended in water for 48 hours for swelling. The swollen Sephadex was deacreated and the fine particles were removed by decantation. The volume of the gel suspension was noted and sufficient volume of 0.1 M phosphate buffer pH 7.0 was added for a final buffer concentration of 0.01 M. The gel suspension was brought at 4° C and poured to a glass column (1.5 x 100 cm. pharmacia) which was previously half filled with 0.01M phosphate buffer, maintained at 4° C. As the gel was poured, excess buffer was allowed to pass through the bed. Pouring of gel continued till the gel bed reached a hight of 80 cms., controlling the elution rate as 15 ml/h. by using a peristatic pump. The column was then washed and equilibrated with 2 litres of 0.01M Phosphate buffer, pH 7.0.

To this, equilibrated column blue dextran, standard proteins ribonucolease: 137000, chymotrypsinogen A: 25000, ovalbumin; 43000 and albumin; 67000 and pure cholesterol esterase were applied and eluted one after another and their elution volume was recorded with the aid of UV cord III UV absorption 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 plotted against logarithm of molecular weight and hence the molecular weight cholesterol esterase was determined corresponding to its Kav value.

4.3.2 Sodium dodecyl sulphate polyacrylamide disc gel electrophoresis

Molecular weight of cholesterol esterase was also determined by SDS-polyacrylamide disc gel electrophoresis according to Weber and Osborn (1969).

Reagents

a) 0.05 M Phosphate buffer pH 7.0

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

Solution A

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

Solution B

3.56 g. 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, 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.
Sodium dodecyl sulphate	- 0.50 g.
Water to	- 250 ml.

c) Sample buffer

0.05 M phosphate buffer	- 20 ml.
Sod. dodecyl sulphate	- 100 mg.
Mercaptoethanol	- 0.1 ml.
Water to	- 100 ml.

d) 0.05% Bromophenol blue in water

e) Preparation of sample and standards.

The standard proteins Ribonuclease A, Chymotrypsinogen A Glyceraldehyde 3-phosphate dehydrogenase, Ovalbumin and Bovine serum albumin and the purified Cholesterol esterase was 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 hours. Six clean 10 ml. test tubes

were taken. To each test tube 3 µl of 0.05% bromophenol blue, 1 drop of glycerol, 5 µl of mercaptoethanol and 50 µl of sample buffer were added. To one by one of these tubes, added 50 µl of the incubated protein solutions of Ribonuclease A, Chymotrypsinogen A, Glyceraldehyde 3-phosphate dehydrogenase, Ovalbumin, Bovine serum albumin and Cholesterol esterase and labelled. These were well mixed and kept at 4° C for further use.

f) Acrylamide solution

This was prepared as follows:

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

Filtered and kept at 4 C.

g) Persulphate solution

Ammonium persulphate	-	150 mg.
Water	-	10 ml.

Freshly prepared for each experiment.

h) NNNN' tetramethyl ethylene diamide.

Procedure:

The gel tubes (8.5 x 0.6 cm.) were thoroughly cleaned, rinsed with distilled water and dried in an air-oven at 100 ± 1° C.

Fixed the tubes in a stand. Deaerated 10 ml. gel buffer and mixed with 9 ml. acrylamide solution. The mixture

was again deaerated and 1.5 ml. freshly prepared ammonium persulphate and 45 ml. of TEMED were added. The mixture was gently shaken and 2 ml. portions were added to the gel tubes avoiding air bubbles. Few drops of water were layered on top of the gel solution immediately using a syringe. The tubes were left for 20 minutes to harden.

After the gel was hardened, the water layer above the gel was removed with filter paper. To each of the gel tubes, 100 µl of each one of the standard proteins, cholesterol esterase solution prepared was added and marked. The tubes were taken, carefully fixed on the electrophoresis apparatus. The vacant space in the tubes were filled with gel buffer diluted 1:1 with water without disturbing the protein samples applied. The two chambers of the apparatus were also filled with 1:2 diluted gel buffer, the apparatus was connected to a power supply unit and electrophorised at 6 mA per tube. The experiment 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 electrograms obtained, the mobility of each protein applied was calculated as per the equation,

$$\text{Mobility} = \frac{\text{Distance of Protein migration}}{\text{Gel length after destaining}} \times \frac{\text{Gel length before staining}}{\text{Distance of bromophenol blue migration}}$$

The mobility obtained was plotted against log molecular weight in a graph. From the graph, the molecular weight of cholesterol esterase was determined corresponding to its mobility.

4.4 Carbohydrate

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

10 mg. of pure lyophilised esterase was hydrolysed with 0.5 ml. 1 N hydrochloric acid at 100° C for 6 hrs. in a test tube sealed under vacuum. At the end of hydrolysis the tip of test tube was broken and transferred to a centrifuge along with 1 ml. distilled water and centrifuged at 5,000 rpm. The clear centrifugate containing the free sugars were passed through columns of Dowex 1 x 4 H^{+} (1 30 cm.) and Dowex 50 4$HCOO^{-}$ (1 x 30 cm) to remove the amino acids and eluted with distilled water. The first 50 ml. fractioln was collected and dried under reduced pressure 50-60° C in a flash evaporator. The residue was again dissolved in water and evaporated. This was repeated till the residue was free from acid.

The acid free residue was dissolved in 1 ml. distilled water. 0.01, 0.2 and 0.3 ml. portions of this were transferred to separate test tubes and added distilled water to make final volume to 5 ml. in each test tube. 3 ml. freshly prepared 1% orcinol in 0.1% ferric chloride dissolved in

concentrated hydrochloric acid was added to each test tube. Heated in a water bath for 5 minutes, while shaking. Similarly standards containing 10, 20, 30 and 40 µg of ribose were also prepared. The test tubes were cooled and the colour developed was measured at 550 nm in a spectronic-21 spectrophotometer. The optical density obtained for the standards were plotted in a graph against corresponding concentrations. From the graph obtained, the carbohydrate content of esterase was read out against its optical density.

4.5 Amino acid analysis

4.5.1 Amino acid analysis using automatic amino acid analyser

Amino acid analysis was carried out according to Hirs (1972). About 10 mg pure cholesterol esterase protein was hydrolysed with 10 ml 6 N HCl and a pinch of phenol in a clean test tube, filled with nitrogen gas and then heatsealed. This was kept in air-oven at 100° C for 24 hrs. After the hydrolysis was over, the tube was broken and the hydrolysate was evaporated under vacuum at 45-50° C. The residue was dissolved in few ml. distilled water and again evaporated. This was repeated till the residue was free from acid. This residue was dissolved in 10 ml. of 0.20 N sodium citrate of pH 2.2. Two microlitre of this solution was injected into the NC 2P Amino acid analyser system. The amino acid peaks recorded were identified and computed with a pattern from a standard amino acid mixture.

4.5.2 Estimation of tryptophan

Tryptophan being oxidasable during acid hydrolysis was analysed separately by the spectrophotometric method of Goodwin and Morton (1946).

0.1% solution of cholesterol esterase 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) preseerved in the cholesterol esterase protein was taken from the amino acid analysis data. The molar extinction coefficients of tyrosine (A) and tryptophan (B) in 0.1 N sodium hydroxide at 280 nM 1556 and 5225 respectively were taken from tables (Goodwin and Morton, 1946). From the absorption spectrum of cholesterol esterase the Molar Extinction (E) of cholesterol esterase at 294.4 nm was calculated which was the contribution of tyrosine and tryptophan in their molar proportion in the cholesterol esterase. If x is the total number of moles of tyrosine and tryptophan put to gether then,

$$E = yA + (x-y)\,B.$$

From this the unknown x was calculated and the number of moles of tryptophan was calculated by substracting the number of moles of tyrosine.

4.6 Effect of temperature

4.6.1 Effect of temperature on cholesterol esterase activity

The cholesterol esterase assay was conductged by incubating the reaction mixture containing 10 µl of cholesterol esterase at different temperatures from 4° C to 90° C for 30 minutes. The reaction was terminated by adding 20 ml of absolute alcohol and the liberated fatty acids were titrated against N/20 sodium hydroxide using phenolphthalein indicator. From the titre value the velocity of reaction (number of moles of fatty acid liberated per minute) was calculated. From the graph showing the velocity against temperature, the optimum temperature of reaction was found out.

4.6.2 Effect of temperature on stability of cholesterol esterase

The stability of temperature on cholesterol esterase was found out by incubating 10 µl of the purified cholesterol esterase with 10 ml of 0.1 M pH 7.0 phosphate buffer and 1 ml N/20 calcium chloride in a 100 ml. conical flask for 1 hour at temperatures of 30° C to 70° C. After one hour the temperature of the contents was brought to 30° C and incubated in the same temperature for 30 minutes and adding 10 ml of the substrate (0.1 ml. cholesterol acetate and 0.1 g. albumin in 50 ml. pH 7.0 phosphate buffer). After the incubation the activity retained after the temperature treatment were measured by titration with N/20 sodium hydroxide. The value of activity obtained for each temperature were eplotted against temperature in a graph.

4.7 Effect of pH

4.7.1 Effect of pH on cholesterol esterase activity

The effect of pH on cholesterol esterase activity was determined by conducting the assay with 5 ml. buffer of pH ranging from 4 to 12. The buffers were prepared as follows:

a) Universal buffer (pH 2-12)

This buffer was prepared according to Lurie (1975). A mixture of 0.04 M phosphoric, boric and acetic acids were prepared. Buffer of required pH value was obtained by adding 0.2 N Sodium hydroxide to 100 ml. of the acid mixture was shown in the table below:

Universal Buffer

pH	Volume of 0.2N 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 combinatin of McIlvaine phosphate buffer and Barbital buffer prepared as follows:

i) McIlvaine phosphate buffer (Gomori, 1962)

Stock solution.

A. 0.1 M citric acid solution

B. 0.2 M dibasic sod. phosphate solution.

x ml. A was mixed with y ml. B and the mixture was made upto 100 ml. to give a buffer of the required pH as shown below:

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 solution

A. 0.2 M sodium barbitol solution

B. 0.2 M hydrochloric acid

50 Ml. A and x ml. B were mixed and made upto 200 ml to give the required pH value as shown in the table.

Barbital buffer

pH	x ml.
6.8	45
7	43
8	17.5
9	2.5

In case of McIlvaine phosphate buffer (pH 3-7) and Barbital buffer (pH 6.8 to 9) combination, the activities barbital buffer pH range were elevated to McIlvaine phosphate buffer level by adding the activity difference from common pH 6.8 and 7.0 to the activities of pH 8 and 9.

The activity values obtained at various pH were then plotted (pH vs activity) for universal buffer and McIlvaine-Baribtal buffer combination. From these graphs the pH optima was found out.

4.7.2 Effect of pH on stability of cholesterol esterase

The stability of cholesterol esterase 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. of 0.1 M universal buffer (pH 4-12). 10 µl of purified cholesterol esterase and 0.5 ml of N/20 calcium chloride were incubated at 30° C for 1 hour. To this, 10 ml of the substrate emulsion and 10 ml. pH 7.0, 0.1 M Phosphate buffer were added and incubated at 30° C for 30 minutes with occasional shaking. The activity was measured by titration against N/20 sodium hydroxide. The pH

and corresponding activities were plotted on graph, from which the effect of pH on the stability of cholesterol esterase was determined.

4.8 Effect of substrate concentration on esterification by cholesterol esterase and Km value.

The effect of substrate concentration on velocity of cholesterol esterase reaction was studied by doing the esterase assay with different concentrations of the substrate (0.1 M to 0.1 M). The reaction was carried out as in assay methods and the velocity of reaction was determined by finding the number of micromoles of fatty acid liberated per minute by 10 µl of purified cholesterol esterase. The values of substrate concentration and velocity were then converted to respective reciprocate and were plotted in the and y axis of a graph. From straight line obtained its slope and y intercept were measured and applied in the Michaelis-Menten equation

$$\frac{I}{V} = \frac{Km}{Vs} \times \frac{I}{V}$$ to calculate Michaelis constant Km where slope is $\frac{Km}{V}$ and y intercept $\frac{I}{V}$

4.9 Inhibition and activation

4.9.1 Effect of aldehydes and ketones

Formaldehyde, acetaldehyde, acetone and ethyl methyl ketone were incorporated separately to 0.1 M concentrations

in 0.1 M substrate. The cholesterol esterase assay was conducted as usual but with 10 ml. substrate containing formaldehyde, acetaldehyde, acetone and ethyl methyl ketone and 10 µl of purified cholesterol esterase solution. The reaction was allowed for 30 minutes and activity with each aldehyde/ketone was determined.

4.9.2 Effect of inorganic anions and cations

The anions used were F^-, Cl^-, Br^- and I^- as their sodium salt. The cations used were Ba^{++}, Ca^{++}, Cu^{++}, Fe^{++}, Mn^{++} Mg^{++}, Sn^{++}, Zn^{++} in the form of their chlorides. 0.1 M solutions of these were prepared. To determine the effect of these ions on the esterase, assay was conducted with 1 ml. of solutions with 1 µl pure enzyme and 5 ml of the substrate. This was kept for 30 minutes at 30° C to complete the reaction. A control was also done without the enzyme. After the reaction, 20 ml. of 85% alcohol was added and titrated against N/20 sodium hydroxide. From the results the degree of activity in the presence of various ions was calculated. The control was also assayed tofind out the degree of inactivation.

4.9.3 Parachloro mercuri benzoate

0.1 M solution of PCMB was prepared. To 5 ul of the enzyme preparation was added about 5 ml of substrate and 1 ml of 0.1 M PCMB . It was kept for 30 mts. at 30° C and after the reaction, added about 20 ml. ethanol and assayed the activity as usual. The effect of PCMB was thus calculated by comparing with a control.

4.9.4 Diethyl fulrophosphate

About 0.1 M solution of DFP was prepared. To 1 ml. enzyme solution and 5 ml substrate was added about 1 ml. 0.1 M DFP and the activity was assayed as usual along with a control. From this the effect of DFP in the enzyme activity was noted.

4.10 Substrate specificity

The specificity of cholesterol esterase activity towards different fatty acid esters were verified by conducting the assay using their esters. They were cholesterol acetate, cholesterol butyrate, cholesterol heptanoate, cholesterol laurate, cholesterol stearate, cholesterol oleate, cholesterol parlmitate and cholesterol arachidate. These were individually added in equimolar amounts in the reaction mixture and enzyme, incubated for 30 minutes at 30° C and when the reaction was over, the fatty acids liberated were estimated by titrating against N/20 sodium hydroxide. The activity differences with these substrates were noted.

4.11 Immobilisation of cholesterol esterase

4.11.1 Immobilisation in polyacrylamide gel.

The enzyme was immobilised in polyacrylamide gel as follows: One ml. pure enzyme solution was mixed with 9 ml. distilled water and 10 ml. of 0.1 M phosphate buffer, pH 7.0 containing 20% acrylamide and 2% NN' methylene bisacrylamide in a 100 ml. beaker. To this, one ml. 4% ammonium

persulphate and 40 µl of TEMED were added and well mixed. This was kept for 20 minutes to gel. The gel was taken out and lyophilised to get the immobilised enzyme. This was stored in an airtight container at 0° C for the property studies such as its activity, storage life and activity in different substrates.

4.11.2 Immobilisation on chitosan

One g. chitosan was dissolved in 100 ml. 1% acetic acid. Stirred well. pH was adjusted to 5.2 by adding 0.1 N sodium hydroxide avoiding precipitation of dissolved chitosan. 100 µg of enzyme was added to the chitosan solution, kept for 2 hours with stirring. After 2 hours it was neutralised with 0.1 N Sodium hydroxide. Chitosan was precipitated. This was filtered, washed free of alkali and kept at 0-4° C. Activity of the immobilised enzyme was measured using the usual assay method. This immobilised enzyme was used for the studies like property, activity and substrate specificity etc. A comprative study on the two immobilised enzymeswere also carried out.

RESULTS AND DISCUSSION

5. RESULTS AND DISCUSSION

5.1 Results

5.1.1 Distribution of cholesterol esterase in fish

A number of different species of fresh fish were analysed for the presence of cholesterol esterase enzyme and its activity was determined. Among those, the following fishes have shown the activity in the liver, muscle and egg. They are,

Sardine	-	(*Sardinella longiceps*),
Mackerel	-	(*Rastrelliger kanagurta*),
Tuna	-	(*Thunnus albacores*),
Seer fish	-	(*Scomberomorus guttatum*),
Mullet	-	(*Mugil cephalus*),
Jew fish	-	(*Sciaena aneus*),
Cat fish	-	(*Tachysurus thalassinus*),
Catla	-	(*Catla catla*),
Rohu	-	(*Labeo rohita*),
Vala	-	(*Wallago attu*),
Pearl spot	-	(*Etroplus suratensis*),
Milk fish	-	(*Chanos chanos*),
Tilapia	-	(*Tilapia mossambica*).

Fresh fish immediately after catch was dressed and processed to give liver, muscle and egg. They were homogenised separately and one g. each was extracted with 10 ml. cold deionised water in a homogeniser and centrifuged at 0° C and 10,000 rpm for 30 minutes. The substrate was prepared by

blending a mixture of 0.1 g. cholesterol acetate in one ml. alcohol and 0.1 g. albumin with 50 ml. o.1 M phosphate buffer at pH 7.0. One ml. of the extract and 5 ml. of the substrate were incubated at room temperature for 30 minutes with occasional shaking. Towards the end of reaction 20 ml. ethanol was added and titrated the liberated acetic acid against N/20 sodium hydroxide using phenolphthalein as indicator and the activity is claculated. The activity is expressed as ml. of N/20 sodium hydroxide required to neutralise the liberated fatty acid per mg. protein of enzyme preparation. Cholesterol esterase activities of different species of fish obtained by the assay of the liver, muscle and eggs are given in Table 5. It is found that in general the enzyme activity is more in the liver of the fish and among different fish the enzyme occurs in considerable amount in cat fish. An effort has been made to purify the enzyme from cat fish liver and to study its characteristics and kinetic properties.

5.2 Purification of cholesterol esterase from cat fish liver.

Freshly caught cat fish was brought to the laboratory in iced condition and processed. The liver was separated. About 500-1000 g. liver was collected. This was brought to near 0° C washed with minimum quantity of cold water and drained. All further operations were carried out at a temperature between 0° C-4° C. The liver was homogenised in a homogeniser for 10 minutes. 0.1 M phosphate buffer pH 7.0 was added to the homogenate in 2:1 proportion, stirred well and allowed to remain for a few hours at 4° C. This was

TABLE- 5

Cholesterol esterase activity in selected tissues of different species of fish

No.	Name of fish	Liver	Muscle	Egg
		μ mol/min./g. protein		
1.	Sardine (*Sardinella longiceps*)	1.2	0.50	0.325
2.	Mackerel (*Rastrelliger kanagurta*)	0.225	0.30	0.04
3.	Seer fish (*Scomberomorus guttatus*)	0.53	0.15	0.30
4.	Mullet (*Mugil cephalus*)	0.15	0.2	0.04
5.	Tuna (*Thunnus albacores*)	0.22	0.13	0.1
6.	Jew fish (*Sciaena aneus*	0.52	0.19	0.27
7.	Cat fish (*Tachysurus thalassinus*)	2.55	1.05	1.01
8.	Catla (*Catla catla*)	0.33	0.90	0.19
9.	Rohu (*Labeo rohita*)	0.25	0.19	0.14
10.	Vala (*Wallago attu*)	0.405	0.29	0.21
11.	Pearl spot (*Etroplus suratensis*)	0.33	0.16	0.25
12.	Milk fish (*Chanos chanos*)	0.22	0.14	0.20
13.	Tilapia (*Tilapia mossambica*)	0.08	0.10	0.17

centrifuged at 10,000 rpm. The filtrate was collected and the fat layer was removed by filtering through glass wool. The residue was once more treated with phosphate buffer, centrifuged and collected the filtrate. All the filtrates were collected and centrifuged in an ultracentrifuge at 10,000 g. for 30 minutes and the residue was discarded. The centrifugate was used for further fractionation and purification.

5.2.1 Ammonium sulphate precipitation

Solid ammonium sulphate was added to the cold enzyme solution little by little to a saturation of 50% with constant stirring. This was kept overnight in cold room. The proteins precipitated was collected by centrifugation at 10,000 x g for 30 minutes. Again solid ammonium sulphate was added to the filtrate to a saturation of 75% and kept overnight. The precipitated protein which contained the enzyme was collected by centrifuging as before. The precipitate was collected in cellophane bag and dialysed. This was continued till all the ammonium sulphate was completely escaped from the enzyme protein. The remaining protein was dissolved in 1000 ml. 0.1 M phosphate buffer (ph 7.0) and subjected to desalting using Sephadex G-25. The enzyme solution was passed through a column (3.5 45 cm.) of Sephadex G-25 previously equilibrated with phosphate buffer, pH 7.0, till the whole protein was deslated out from the column. The eluate obtained was concentrated using 25 g. of dry Sephadex G-25 and filtered. This was again washed with phosphate buffer pH 7.0. The washings and first filtrate were combined and cholesterol esterase activity was assayed using 0.1 ml. of this solution.

5.2.2 DEAE column chromatography

A column of DEAE cellulose 3.5 x 36 cm. was prepared. The column was washed and equilibrated with 0.01 M McIlvaine phosphate buffer pH 7.0 and maintained at a flow rate of 30 ml/hr. The desalted enzyme solution was fed to the column.

It was washed with 0.01 M phosphate buffer pH 7.0, until the eluate was free from UV absorbing materials. Then the column was connected to a gradient developer and eluted under a lenier gradient of 0.6 M potassium chloride solution 0.1 M pH 7.0 phosphate buffer. The eluate was collected in 5 ml. fractions. The fractions were monitored for the optical density at 280 nm for determining the protein content. 0.5 ml of each fractions were assayed for the cholesterol esterase activity. The gradient elution profile of cat fish cholesterol esterase is given in fig. 4. Fractions having high esterase activity were pooled together and aliquots were assayed for enzyme activity.

5.2.3 Sephadex G-100 chromatography

Fractions having high cholesterol esterase activity were concentrated and applied on a column of Sephadex G-100 (3.5 x 80 cms.) for further purification. Sephadex G-100 column was previously equilibrated with 0.1 M, pH 7.0 phosphate buffer. Highly pure enzyme sample was applied to the column. It was then connected to a reservoir containing 0.01 M, pH 7.0 McIlvaine phosphate buffer and eluted at a flow rate of 15 ml/hr. Five ml. fractions were collected using a fraction collector attached with UV monitor for determining the protein content at 280 nm. From alternate fractions 0.5 ml. was used for the assay of cholesterol esterase activity. The elution pattern of the esterase is given in fig. 5.

TABLE 6

Purification of cholesterol esterase from different sources

Source	Fold Purification	Reference
Rat pancreatic powder	690	Murthy and Ganguly (1962)
Rat pancreatic juice	350	Chen and Morin (1971)
Rat pancreatic juice	200	Erlanson (1972)
Pork pancreas	450	Hernandez and Chaikoff (1962)
Cat fish	300	Present study

TABLE 7

Distribution of cholesterol esterase activity in different organs of cat fish

Organ	Activity units/g.Wet weight
Muscle	40.5
Liver	126.0
Egg	8.4

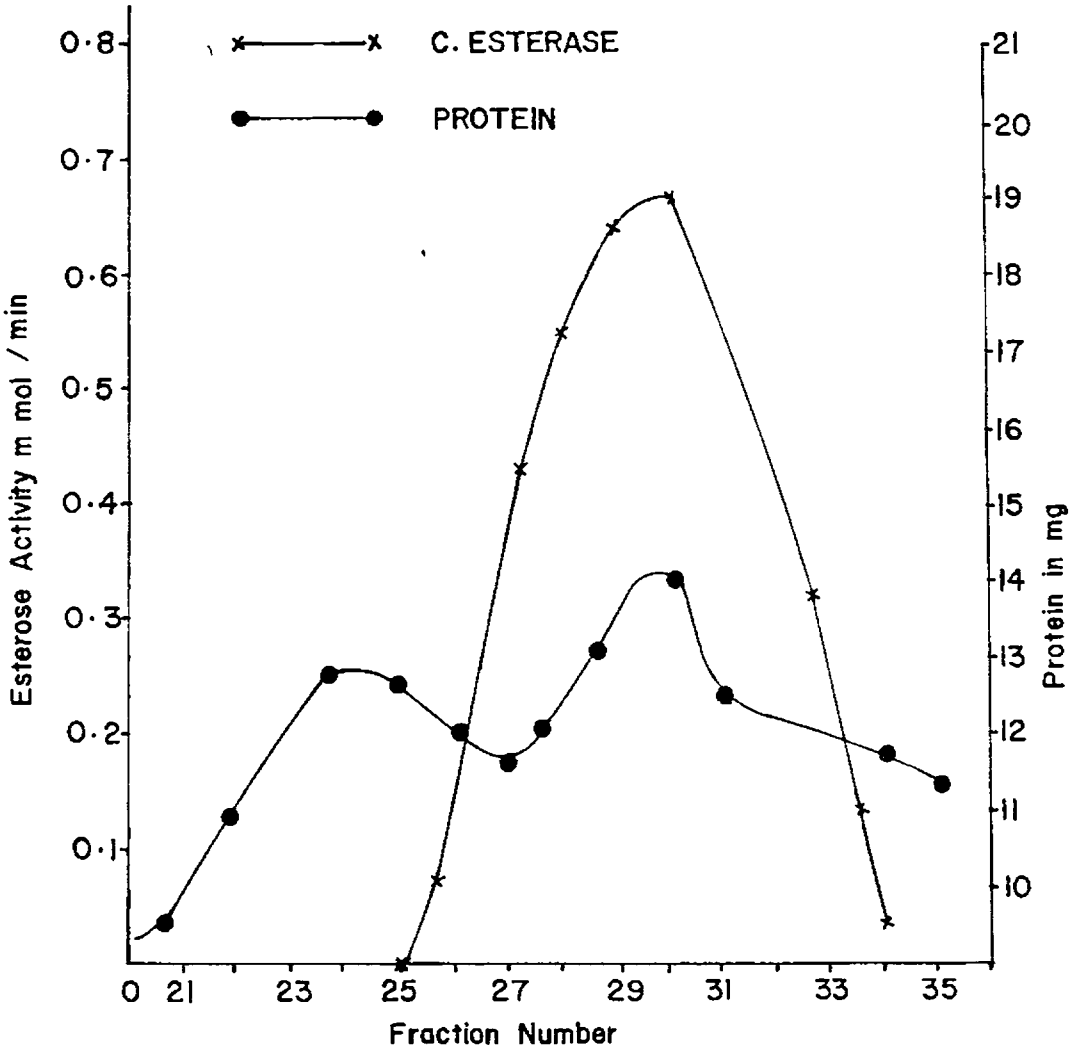


FIG: 4. CHROMATOGRAPHIC PURIFICATION OF CHOLESTEROL ESTERASE ON DEAE CELLULOSE ELUTION WITH A LINEAR CONCENTRATION GRADIENT OF POTASIUM CHLORIDE.

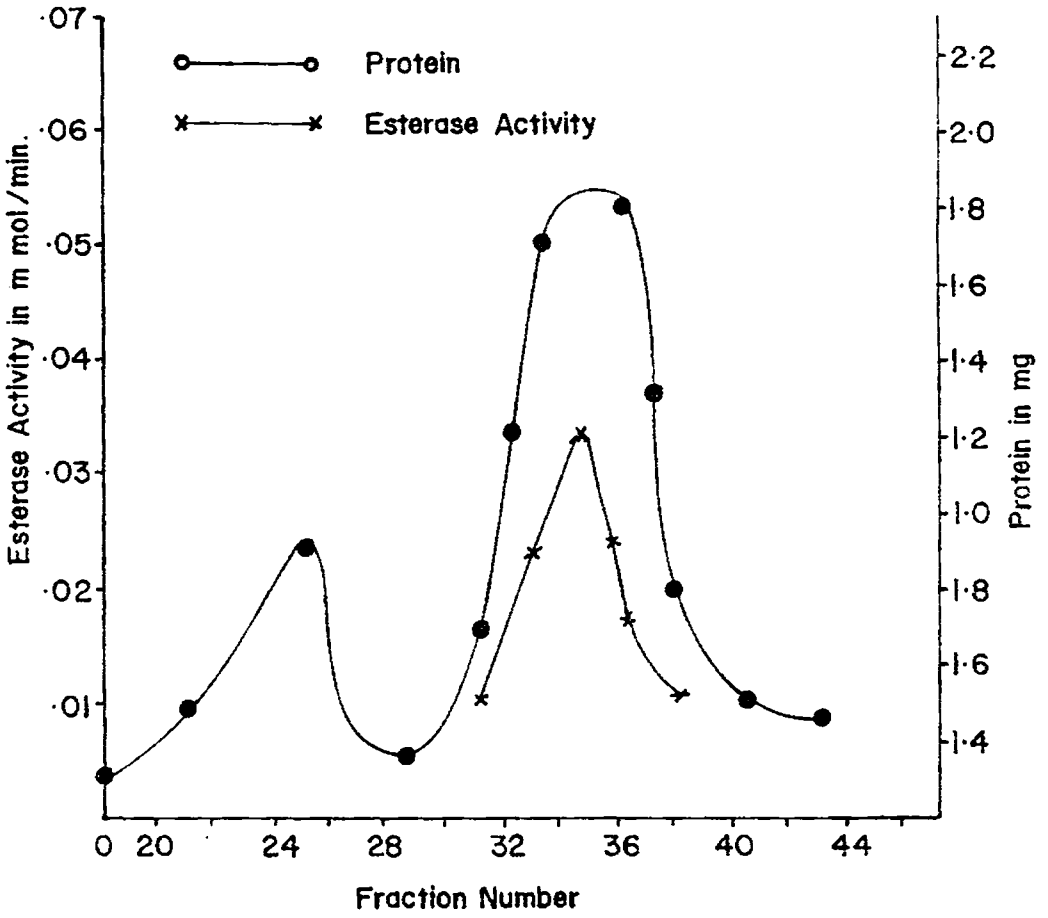


FIG : 5 . ELUTION PATTERN OF CHOLESTEROL ESTERASE FROM SEPHADEX – G – 100

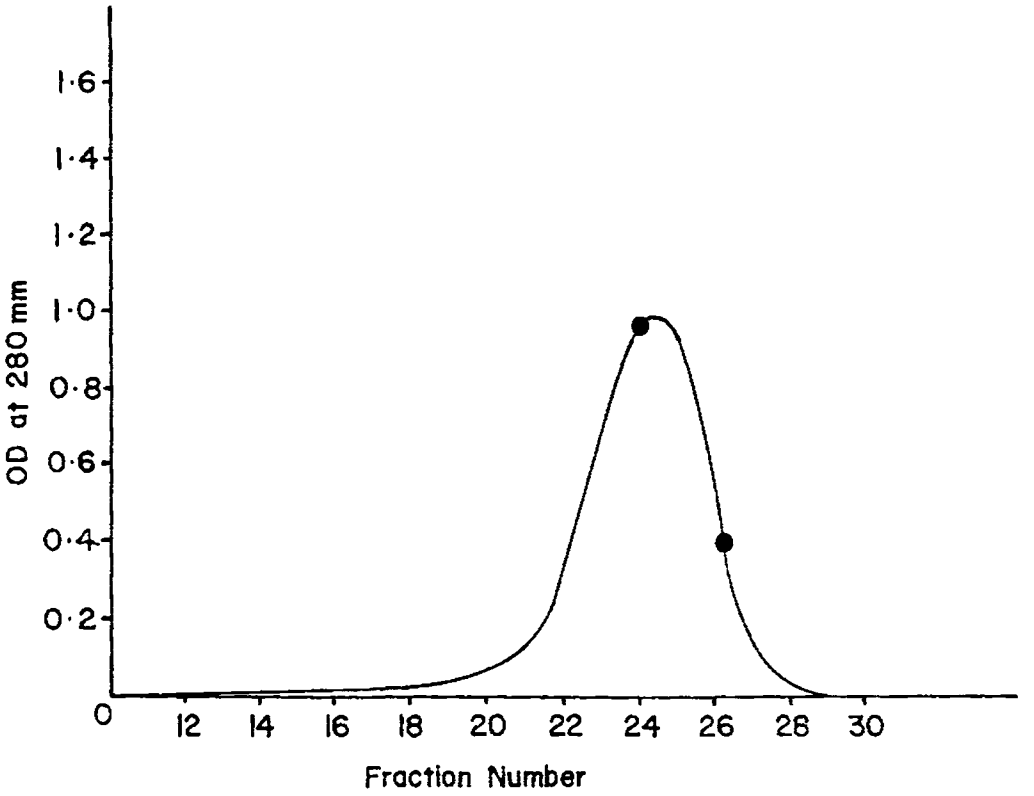


FIG : 6 . CHROMATOGRAPHY OF PURIFIED CHOLESTEROL ESTERASE ON SEPHACRYL S - 200·

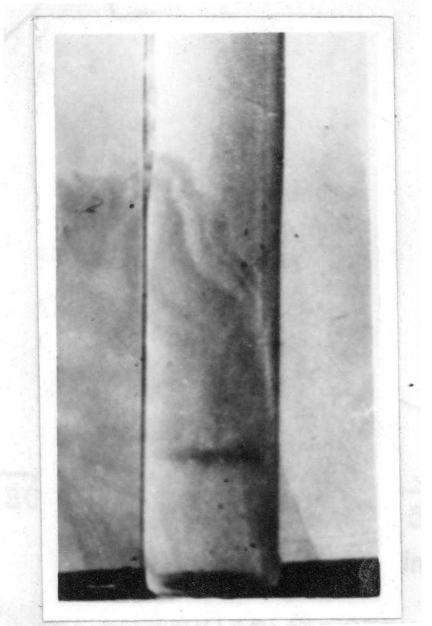


Fig. 7 GEL ELECTROPHORETIC PATTERN OF CHOLESTEROL ESTERASE.

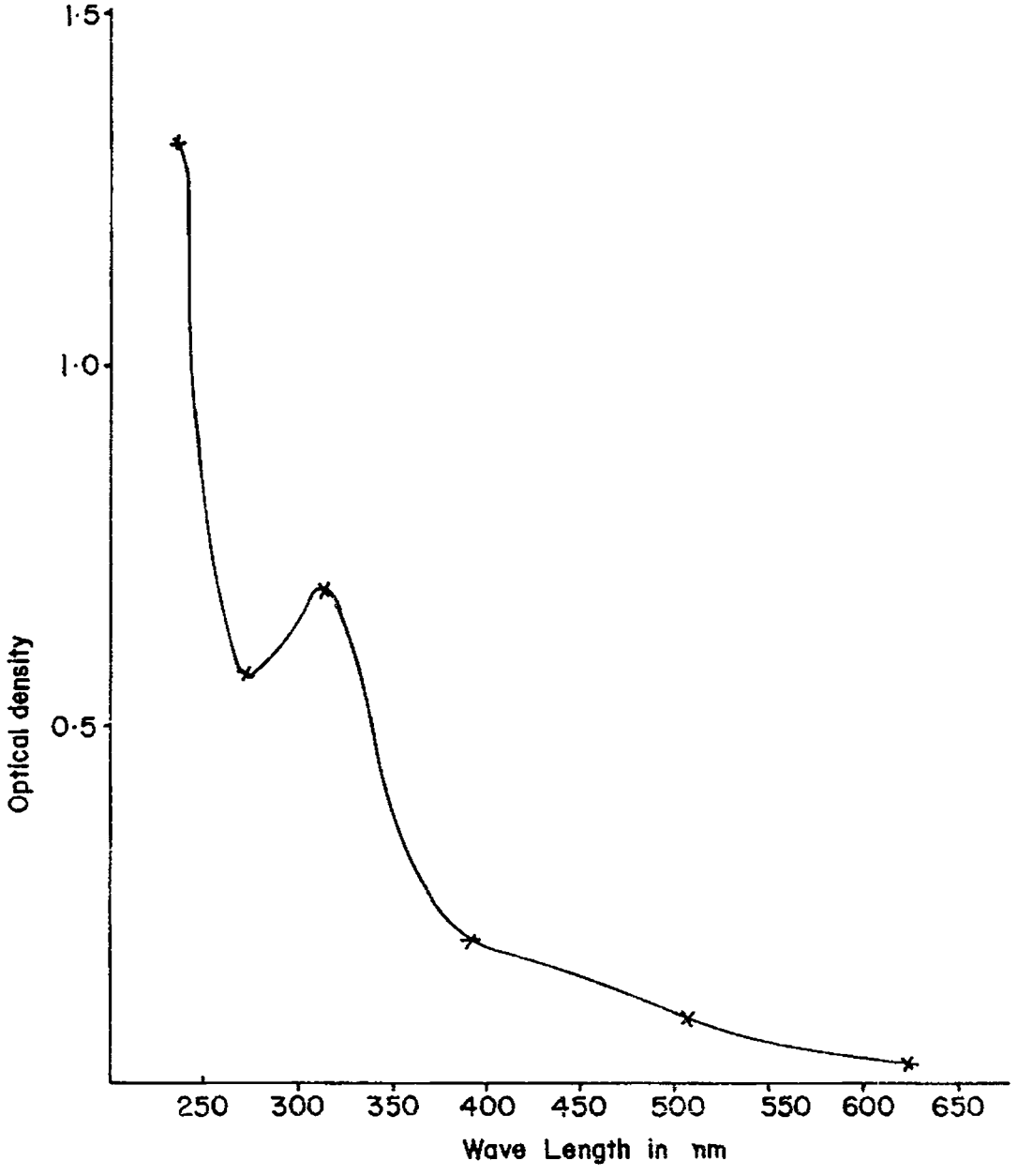


FIG. 8. ULTRAVIOLET ABSORPTION SPECTRUM OF CHOLESTEROL ESTERASE.

The fractions having high activity were collected and the total activity was again determined. This was again concentrated in a lyophyliser. The lyophylised residue was dissolved in a small quantity of pH 7.0 buffer. It is found that only one peak is obtained for the esterase on filtration on Sephadex G-100. The typical purification procedure is given in fig. 1.

5.3 Discussion

5.3.1 Homogenity of purified cholesterol esterase

About 5 ml. of the active fractions from Sephadex G-100 chromatography was applied to a column of Sephacryl S-200 (1.7 x 50 cm.) equilibrated with 0. 1 M pH 7.0 McIlvaine phosphate buffer. The column was eluted with the same buffer at a flow rate of 15 ml/hr. The optical density of the eluate was measured at 280 nm and the eluate were collected into 5 ml. fractions. There was only one protein peak obtained which was the esterase peak.

The purified cholesterol esterase obtained was then subjected to polyacrylamide gel electrophoresis (Fig. 7). The electrophoresis show that the sample contained only one band which indicates the extend of purity of the enzyme. Fig. 8 shows the UV absorption spectrum of purified cholesterol esterase. The maximum absorbance was 280 nm. The ratio of absorbance at 280 nm to 260 nm was 1.192 when the absorbance was done in 0.1% solution in 0.1 M Tris-Hcl buffer pH 7.8. By the methods of column chromatography on

TABLE 8

Purification of cholesterol esterase from cat fish liver

Fraction	Volume ml.	Units µ mol./ min.	Total protein mg.	Sp. activity units/mg./ prot.	Recovery
Water extract	500	34	2200	0.0060	100
Ammonium Sulphate fraction	325	16	1218	0.0074	60
Sephadex G-25	90	11	926	0.0093	33
DEAE cellulose	35	8.2	72	0.2104	23
Sephadex G-100	12	2	8	0.1803	16

Sephadex G-100, Sephacryl S-200 and polyacrylamide disc gel electrophoresis it is found that the cholesterol esterase prepared was pure and homogenous.

From the purification data Table 8 it was observed that cat fish liver contains appreciable amount of cholesterol esterase compared to many other sources of esterase like rat pancreatic juice (Chowand Morin, 1971; Murthy and Ganguly, 1962). Rat liver (Deykin and Goodman, 1962) Rabbit pancreas (Morin, 1962). The purity attained in this case is found to be more when compared to other works as shown in Table 6. And the yield of cholesterol esterase from cat fish liver was also comparable to other sources as shown in the Table 7. The purification steps of cholesterol esterase and the recovery is given in Table 8.

5.4 Molecular weight

The molecular weight of cholesterol esterase by chramatography on Sephadex G-100 gave a value of about 84,000 dalton whereas SDS electrophoresis gave a value of 88,000 daltons. From these results an average molecular weight of about 86,000 daltons is assigned for cat fish liver cholesterol esterase. The molecular weight of the enzyme varies from sources to source. From the rat pancreatic juice the molecular weight is 65000-69000 daltons (Morgan et al. 1968; Hyun et al. 1971). Teale et al. reported a cholesterol esterase complex from pig pancreas powder of molecular weight more than 8,00,000 daltons which can be converted by lipid

extraction to the enzyme submits of molecular weight 15000–20000 daltons which can still hydrolyse and synthesise cholesterol esters.

Human pancreatic cholesterol esterase is reported to have a molecular weight of 3,00,000 daltons according to gel filtration (Erlanson and Borgstrom, 1970). But this large size for a lipolytic enzyme and it suggests that human cholesterol esterase may be a dissociable aggregate. In the presence of oleic acid, the enzyme get aggregated to a much larger complex reminiscent of the fast pancreatic lipase. But both lipase and cholesterol esterase complexes dissociates in bile salt solution. The rat pancreatic juice purified by DEAE cellulose chromatography and hyuroxyl apatite chromatography gave an average molecular weight of 1,26,000 dalton (Hyun et al. 1969). Cholesterol esterase of molecular weight of 90,000 daltons was isolated from porcine pancreas (Rosalind et al. 1983).

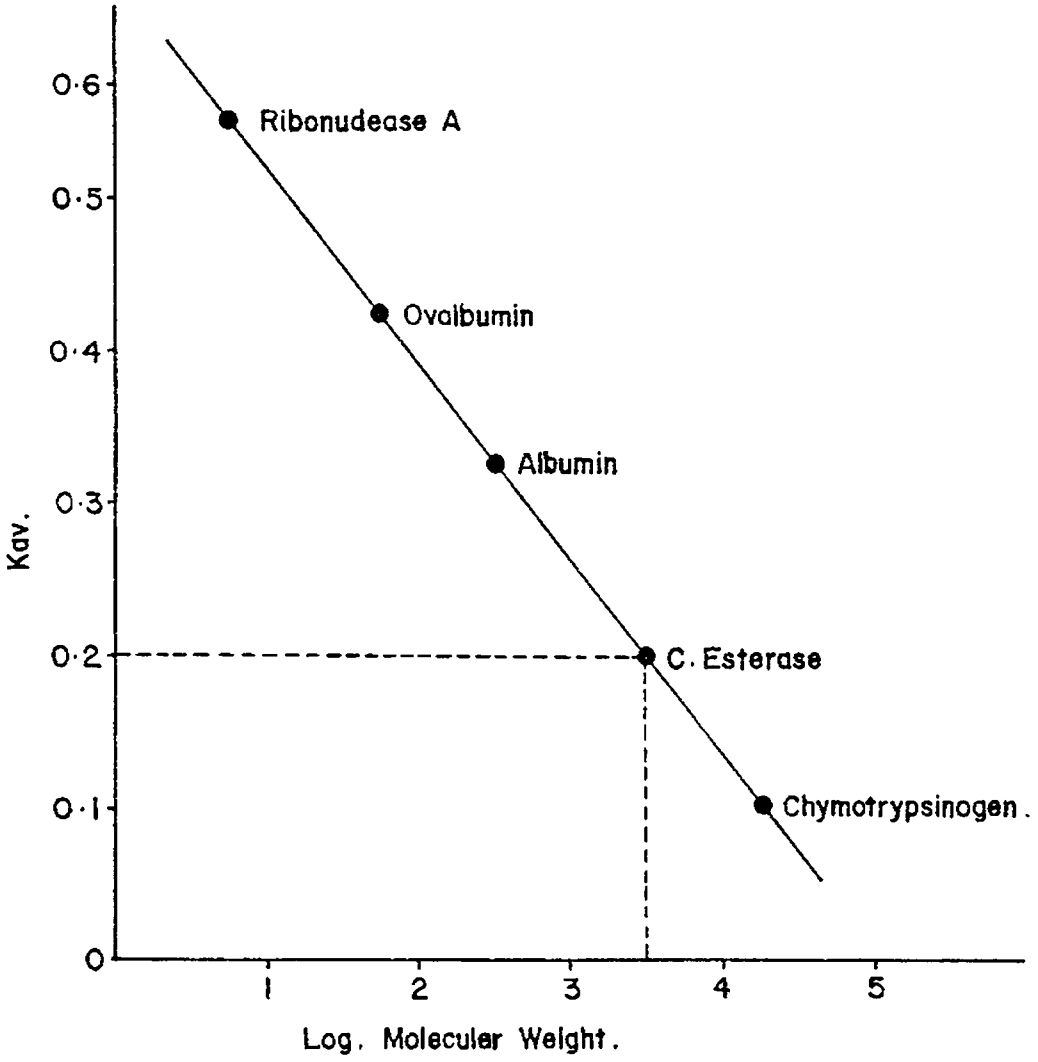


FIG. 9 . ESTIMATION OF MOLECULAR WEIGHT OF CHOLESTEROL ESTERASE BY GEL FILTRATION ON SEPHADEX - G - 100.

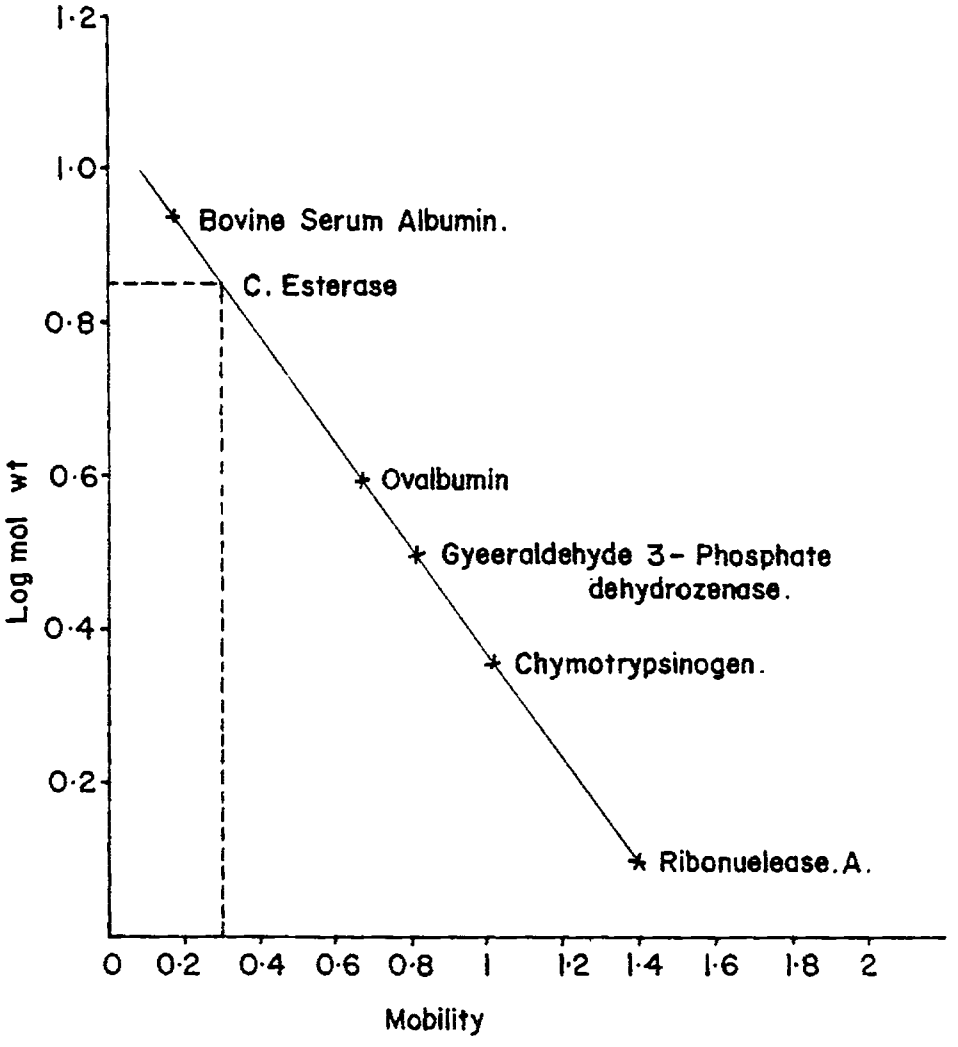


FIG: 10. ESTIMATION OF MOLEQULAR WEIGHT OF CHOLESTEROL ESTERASE BY SDS POLY ACRYLAMIDE GEL ELECTROPHORESIS.

5.5 Amino acid composition

The amino acid composition of cholesterol esterase from the liver of cat fish is given in Table 9. Number of each amino acid residue is calculated on the basis of the molecular weight of esterase as 86,000 daltons.

The amino acid pattern obtained is similar to those reported for the esterase from other sources (Hyun et al. 1972; Rosalind S. Labow et al. 1983, Verger et al. 1969) Hence tyrosine seems to be absent . However it shows that the esterases are serine enzyumes but contains sulphydryl groups there by resumbling, pancreatic lipase. It is also reported that there is a high percentage of proline in the human pancreatic juice and low percentage of tyrosine in the rat pancreas and pancreatic juice (Lombardo et al. 1978; Calome et al. 1975 and Hyun et al. 1972). From the amino acid analysis it is found that there is a considerable amount of serine and also glycine. Similarly leucine, valine, phenyl alanine etc. are also present in high quantity. It is also noted that the basic amino acid residue is about 22% and the acidic amino acids is about 30%. The hydrophobic residue is about 40%. The presence of normal amount of apolar amino acid in the esterase enzyme is also noticed. According to Hatch (1965) residues of Lysine, Arginine, Aspartic acid, Threonine and Serine are considered to be polar amino acids and Proline, Valine, Methionine, Isoleucine, Leucine and Phenyl alanine are apolar. The ratio of the sum of apolar amino acid residue to polar amino acid residue is 0.96.

TABLE 9

Amino acid composition of cholesterolesterase from liver of cat fish

Amino acid	Number of residue	Mole percentage
Aspartic acid	76	7.08
Threonine	70	6.50
Serine	104	9.50
Glutamic acid	67	6.94
Proline	40	5.08
Glycerine	92	9.10
Alanine	55	5.80
Valine	48	4.97
Cysteine	38	4.08
Methionine	60	6.36
Isoleucine	65	6.48
Leucine	52	5.04
Tyrosine	0	0
Phenyl alanine	65	6.97
Histidine	40	4.22
Lysine	56	5.43
Arginine	60	6.07
Tryptophan	20	2.10

5.6 Carbohydrate analysis

Studies were conducted on the carbohydrate content of cholesterol esterase using the orcinol method. The carbohydrate obtained was 0.0004% in total. Hammond and Papermaster (1976) tried to liberate sialic acid from the esterase. But it was not a success.

5.7 Effect of temperature on activity and stability of cholesterol esterase.

Effect of temperature on cholesterol esterase activity shown in Fig. 11 which shows an optimum temperature of 37° C at pH 7.0. Similar optimum temperature is reported for pancreatic esterase from animals. The value for a cultured cholesterol esterase is 40° C (Anon, 1981.)

The cholesterol esterase was active even in a wide range of temperatures. This was active between 0 to 45° C.

Fig. 12 shows the thermostability of the enzyme at pH 7.0. It is observed that the enzyme retained its full activity during 30 minutes heat treatment at temperature varying from 0 to 45° C and above 45° C.

5.8 Effect of pH on activity and stability of cholesterol esterase.

Effect of pH on the activity of cholesterol esterase is shown in Fig. 13 and pH stability in Fig. 14. Fig. 13 shows the pH activity plot obtained with universal buffer, McIlvaine phosphate buffer and barbital buffers. It was found that the activity of the esterase is maximum at pH 6.2 and the activity range is from pH 5.5-7.4.

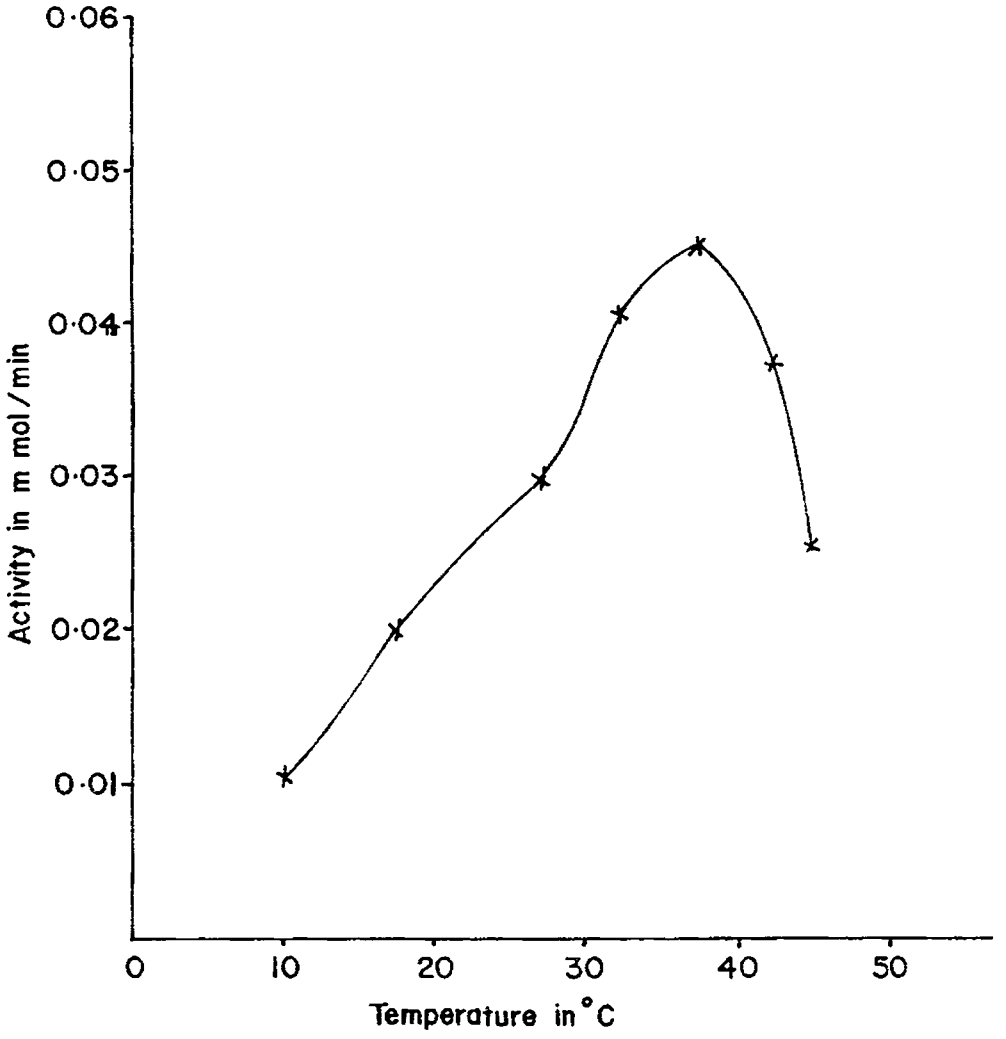


FIG: 11. EFFECT OF TEMPERATURE ON CHOLESTEROL ESTERASE ACTIVITY.

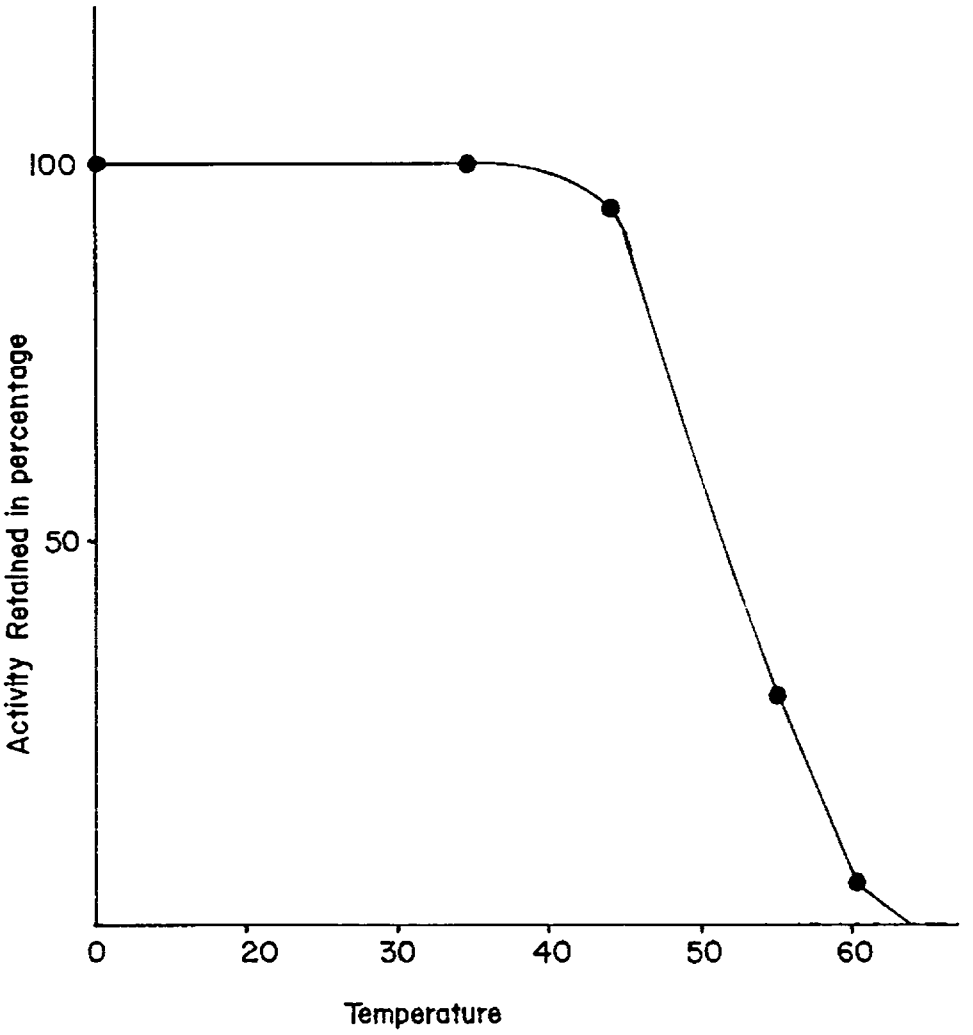


FIG: 12. EFFCT OF TEMPERATURE ON STABILITY OF CHOLESTEROL ESTERASE.

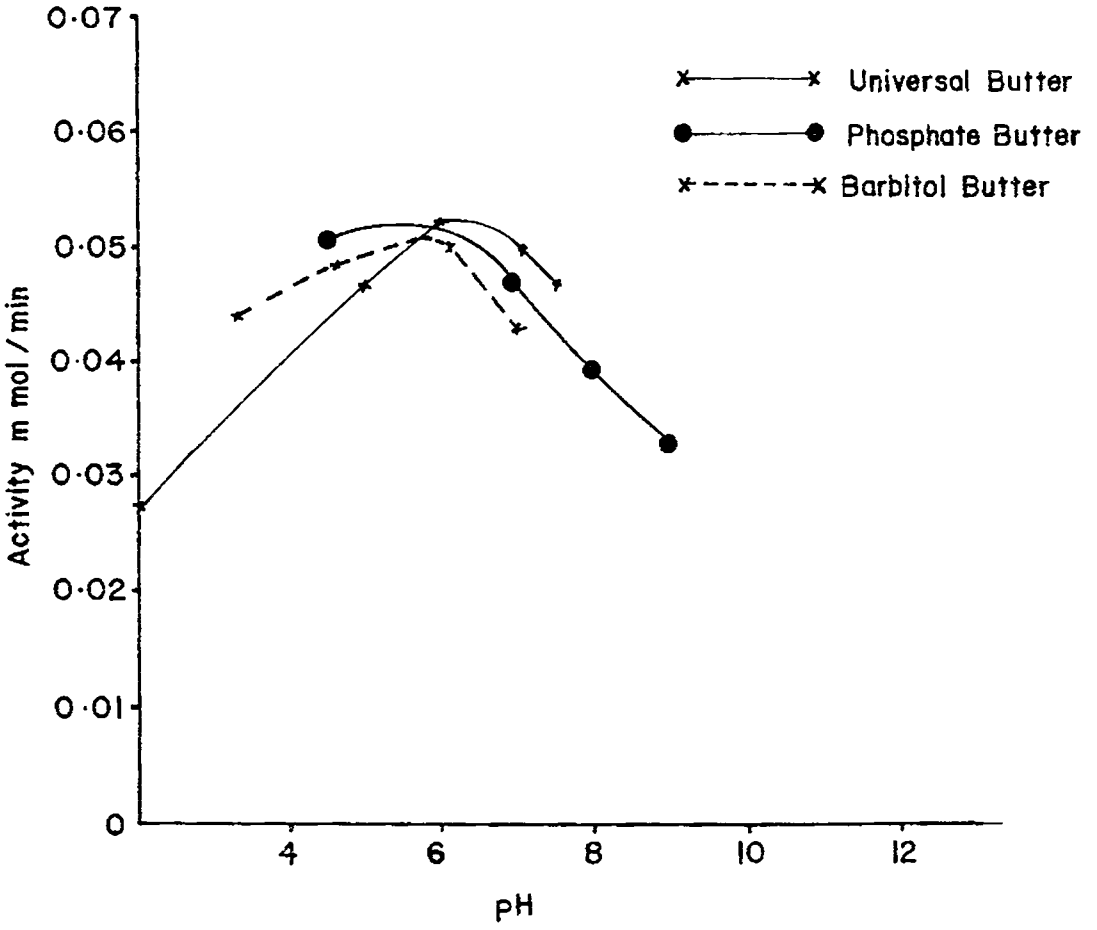


FIG. 13. EFFECT OF PH ON CHOLESTEROL ESTERASE ACTIVITY

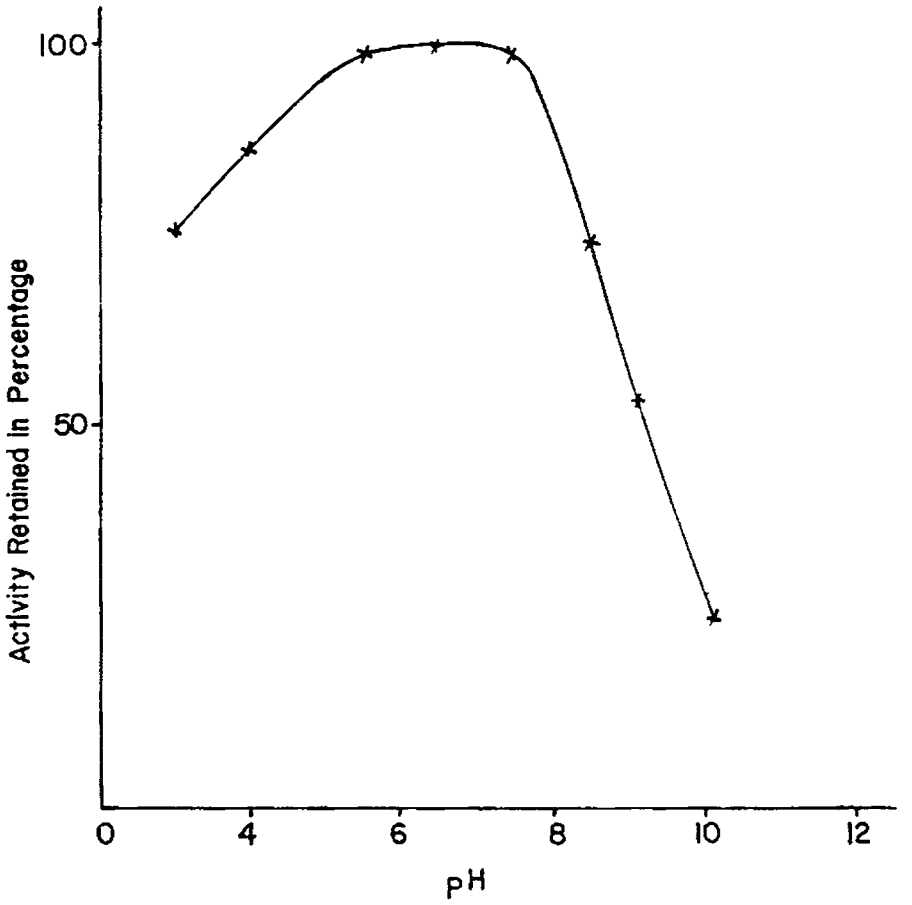


FIG : 14 ^{pH} STABILITY OF CHOLESTEROL ESTERASE

The effect of pH on stability of cholesterol esterase is given Fig. 14, which shows that the enzyme is stable for a period of minimum 30 minutes in the pH range 5.5 to 7.4. Below the pH 5.5 and above 7.4 the enzyme was found to be inactive.

Like lypolytic enzymes the pH optimum for cholesterol esterase is dependant on the physical state of substrate. The optimum pH for a synthetic reaction of pancreatic and intestinal enzymes is generally 6.0 to 6.2 and for hydrolyti reaction is 6.7-7.0. Value as high as pH 8.6 for the hydrolysis (Murthy and Gangly, 1962) and as low as pH 5.5 and pH 4.7 for a synthetic reaction (Fodor, 1950). Eto and Suzuki (1973) have found the activity of the enzyme from rat brain with a pH range of 6.1-7.2. Another cholesterol esterase with a pH optimum of 5.0 has been found in the atherosclerotic rabbit intima (Proudlock and Bay, 1992). However in all the reactions the esterase from cat fish was found to be stable and active in the pH range of 5.5 to 7.4 and optimum pH of 6.2.

5.9 Effect of substrate concentration on velocity and activity of cholesterol esterase.

In order to study the effect of substrates, detailed studies were conducted by adding different esters to the assay sample. The substrates were cholesterol sterate, cholesterol laurate cholesterol archidate, cholesterol butyrate etc., along with the albumin emulsion. It is found that the cat fish liver esterase have no absolute

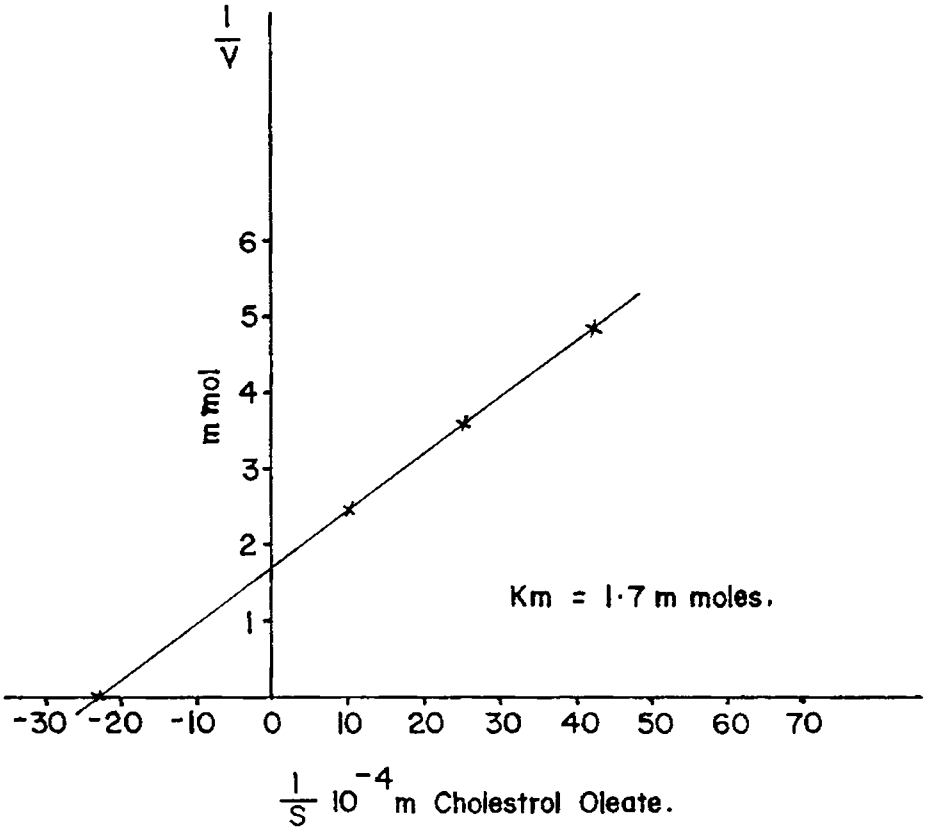


FIG. 15. LINE BEAVER - BURK PLOT OF CHOLESTEROL ESTERASE

TABLE-10

Specificity of substrates on cholesterol esterase activity

Cholesteryl ester	Activity moles/min./mg. protein
Cholesterol linolenate	14.6
Cholesterol linoleate	10.9
Chooesterol Oleate	9.2
Cholesterol palmitate	8.0
Cholesterol stearate	4.6
Cholesterol laurate	2.2
Cholesterol butyrate	1.6

specificity. However the rate of enzyme activity towards various substrates were in the order of linolenate linoleate oleate palmitate stearate laurate butyrate (Table 10).

The esterification property of the enzyme depends on the quantity of the substrate used and that too on the surface area available rather than the weight or the molarity of the lipid. It was observed (Sarada and Desneulle, 1958) that the smaller the particle size of the emulsion, higher will be the velocity of esterification. The substrate concentration and corresponding activities obtained for the esterification are given in Table 10. The Km value with respect to the enzyme found out from the Lineweaver-Burk plot obtained was 1.7 m moles (Fig. 15).

5.10 Inhibition and activation

5.10.1 Effect of aldehydes and ketones on cholesterol esterase activity.

The effect of formaldehyde, acetaldehyde, acetone and ethyl methyl ketone on the activity of cholesterol esterase was studied. The observations are given in Table 11. All these were found to inhibit the activity. The degree of inhibition was ethyl methyl ketone acetone acetaldehyde formaldehyde. The ketones were having more inhibition than the aldehydes. This is against the theory (Bier, 1962) that the inhibition is proportional to the molecular volume. The mechanism of inhibition is that the aldehydes and ketones

TABLE 11

Effect of aldehydes/ketones on cholesterol esterase activity

Aldehyde/ketone	Concentration in moles	Degree of activation %
Ethyl methyl ketone	0.1	45
Acetone	0.1	24
Acetaldehyde	0.1	11
Formaldehyde	0.1	6

TABLE 12

Effect of metal ions on cholesterol esterase activity

Ions	Concentration moles	Degree of inactivation %
Hg^{++}	0.01	100
Cu^{++}	0.01	60
Zn^{++}	0.01	54
Mn^{++}	0.01	42
Mg^{++}	0.01	15
Ca^{++}	0.01	6
Ba^{++}	0.01	2
Sn^{++}	0.01	0

undergoes condensation reaction with the free amino groups in the enzyme protein resulting in a change in the molecular structure and thereby retard the rate of activity.

5.10.2 Effect of cations and anions on cholesterol esterase activity.

a) Cations

The effect of metallic ions on the activity of cholesterol esterase was investigated and the results are given in Table 12. It was observed that ions like Zn^{++}, Cu^{++}, Hg^{++}, Mn^{++} are inhibitors with slight difference in the rate of inhibition. This is similar to the findings of Murthy and Ganguly (1962), Hernandez and Chaikoff (1957). The rate of inhibition was $Hg^{++} > Cu^{++} > Zn^{++}$ Mn^{++} Mg^{++} Ca^{++} Ba^{++} Sn. The highest being Hg^{++} and the lowest Sn^{++}. The mercury compounds also inhibit the activity. Parachloromercury benzoate (PCMB) is one such compound inhibiting the rate of reaction (Table 13). It is reported that PCMB at 1 mM level inhibited porcine pancreatic enzymes by 76% and the inhibition was prevented by adding glutathione (Hernandez and Chaikoff, 1957). An inhibition of 35-40% was found with rat cholesterol esterase with 1 µM PCMB (Murthy and Ganguly, 1962). Taurocholate (5 mM) protected the inhibition from PCMB completely (Hyun et al. 1969).

TABLE 13

Influence of PCMB and DFP on the cholesterol esterase activity

Ion	Concentration moles	Relative activity
PCBM	10 mM	8
DFP	10 mM	3

Several organophosphorous compounds that are typical serine reagents have also been found to inhibit the cholesterol esterase. Diisopropyl fluorophosphate (DFP) inhibited both the pancreatic and intestinal enzyme of the rat by 50% (Murthy and Ganguly, 1962). Diisopropyl-p-nitrophenyl phosphate, diethyl-p-nitrophenyl phosphate, tetraethyl pyrophosphate and phenyl methyl sulfonyl fluoride also caused inhibition in different rates (Brockerhoff and Jensen, 1974). It is observed that DFP is more inhibitory than PCMB, from the relative activity (Table 13).

b) Anions

The inhibition of cholesterol esterase by anions like F^-, Cl^-, Br^- and I^- were determined. Inhibitory effect of these halogen ions were in the order of F^- Cl^- Br^- I^-. It is observed from the Table 14 that the presence of iodine enabled to retain the activity almost hundred per-cent. So it can be confirmed that the enzyme isolated is not inhibited by iodine ions.

5.11 Immobilisation of cholesterol esterase.

Immobilisation of enzyme is the latest development in the enzyme technology. The immobilised enzymes are more potent that native enzyme. The advantage of using immobilised enzyme is that the enzyme reaction can be

TABLE 14

Effect of halogen ions on cholesterol esterase activity

Ions	Concentration moles	Degree of inactivation %
Control	0.01	0
Fl^-	0.01	100
Cl^-	0.01	66.5
Br^-	0.01	38.4
I^-	0.01	11.2

arrested at any desired time by simply separating the immobilised enzyme from the reaction mixture. They can be also used in the field of medicine, food, analytical chemistry and also in research as model systems for natural enzymes bound to invitro membrane (Kachalski *et al*. 1971, Zaborsky, 1972 and Gutacho, 1974). A specified amount of enzyme can be used to treat large amount of a substrate solution without removing it from the support. The enzyme does not undergo self digestion. The pH can also be optimised with some modifications. Above all immobilised enzymes give better stability, can be reused and they also accelerated the reaction rates.

Two methods were applied to immobilise cholesterol esterase. They are,

1. One ml. pure esterase was mixed with 9 ml. distilled water and 10 ml. 0.1 M McIlvaine phosphate buffer, pH 7.0 containing 20% acrylamide and 2% NN' methelene bis acrylamide in a beaker. To this 1 ml. 40% ammonium sulphate and 40 µl TEMED was added and mixed well. This was then allowed to gel for 10 minutes. This gel was taken out, minced in a mixer and lyophilised to get the immobilised enzyme. This was stored in dry and closed bottles and kept at 0° C for further studies.

2. Chitosan prepared from chitin a byproduct of the crustacean shell was used to prepare another lot of immobilised cholesterol esterase. 1% chitosan solution in 1% acetic acid was used for immobilisation. Almost

100% of the enzyme could be immobilised by both the above methods. On assaying the immobilised enzymes about 60% of the activity was retained in the first method and about 75% was retained by the second method.

It is found that the immobilised cholesterol esterase could be stored for atleast 3 months in -20 C without losing much of its activity (Table 15).

The results show that chitosan is a better immobilising agent for the enzyme.

The above studies reveal that cat fish liver is a good source of cholesterol esterase.

TABLE 15

Comparison of activities of native and immobilised cholesterol esterase

Native	Immobilised on	
	Polyacrylamide	Chitosan
100%	60%	75%
Stability at -20 C One month	1-2 months	3 months

Note: 1 g. chitosan dissolved in 100 ml. 1% acetic acid, , 0.1 mg. esterase protein was added to the solution. Neutralised with 0.1 N NaOH. The precipitated chitosan was washed free off alkali and used for the studies.

SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

Cat fish (Tachysurus thalassinus) contains considerable amount of the enzyme cholesterol esterase in the body organs of which the liver has maximum concentration, 126 units/g. wet weight of liver protein.

A method to purify the enzyme from the liver has been developed. And the purity obtained was 300 fold. The method consists of the following stages.

1. Extraction with phosphate buffer

The homogenised liver is extracted with ice cold 0.1 M McIlvaine phosphate buffer, pH 7.0 and filtered to remove fat.

2. Ammonium sulphate fractionation

The extract which contained the protein was fractionally precipitated with ammonium sulphate at 0.75 saturation. The precipitate containing the enzyme was dissolved in 0.1 M buffer.

3. Desalting

The dissolved protein was desalted using Sephadex G-25.

4. DEAE cellulose ion exchange chromatography

The cholesterol esterase solution obtained after Sephadex G-25 column chromatography was subjected to DEAE cellulose column chromatography under potassium chloride gradient elution using UV monitor and a fraction collector to get a partially purified enzyme.

5. Sephadex G-100 column chromatography.

A portion of the more concentrated solution of cholesterol esterase from the DEAE cellulose chromatography was subjectged to Sephadex G-100 column chromatography. The fraction gave only only one peak showing that it was a pure protein. This was further confirmed.

The cholesterol esterase of cat fish on Sephacryl S-200 chromatography gave only one peak. On SDS polyacryl amide gel electrophoresis it gave only one band. Molecular weight determination by gel filtration on Sephadex G-100 and SDS poly acrylamide gel electrophoresis gave an average value of 86,000 daltons.

The enzyme showed no light absorbance in the visible range. Maximum absorption was at 280 nm. The ratio of absorbance at 280 nm to 260 nm was about 1.192.

Amino acid analysis revealed that it contains more of serine along with arginine, leucine, lysine etc. The serine plays a major role in the properties and uses of the enzyme. The enzyme contains 22% basic and 30% acidic amino acid residue. The hydrophobic residues is about 40%.

Cholesterol esterase isolated from cat fish has got an optimum pH 6.2 for reaction and optimum temperature, 37° C. It was also stable in the pH range of 5.5 to 7.4 and temperature range of 0-45° C.

Studies with substrates showed that the enzyme is not so specific towards any substrates. However it showed a differential rate of activity with different substrates. The maximum rate of reaction was with cholesterol linoleate followed by cholesterol linoleate, cholesterol oleate, cholesterol palmitate, cholesterol sterate, cholesterol laurate and cholesterol butyrate.

A number of metallic and non-metallic ions were inhibitors of cholesterol esterase. The inhibition of aldehydes and ketones were in the order of methyl ethyl ketone acetone acetaldehyde formaldehyde.

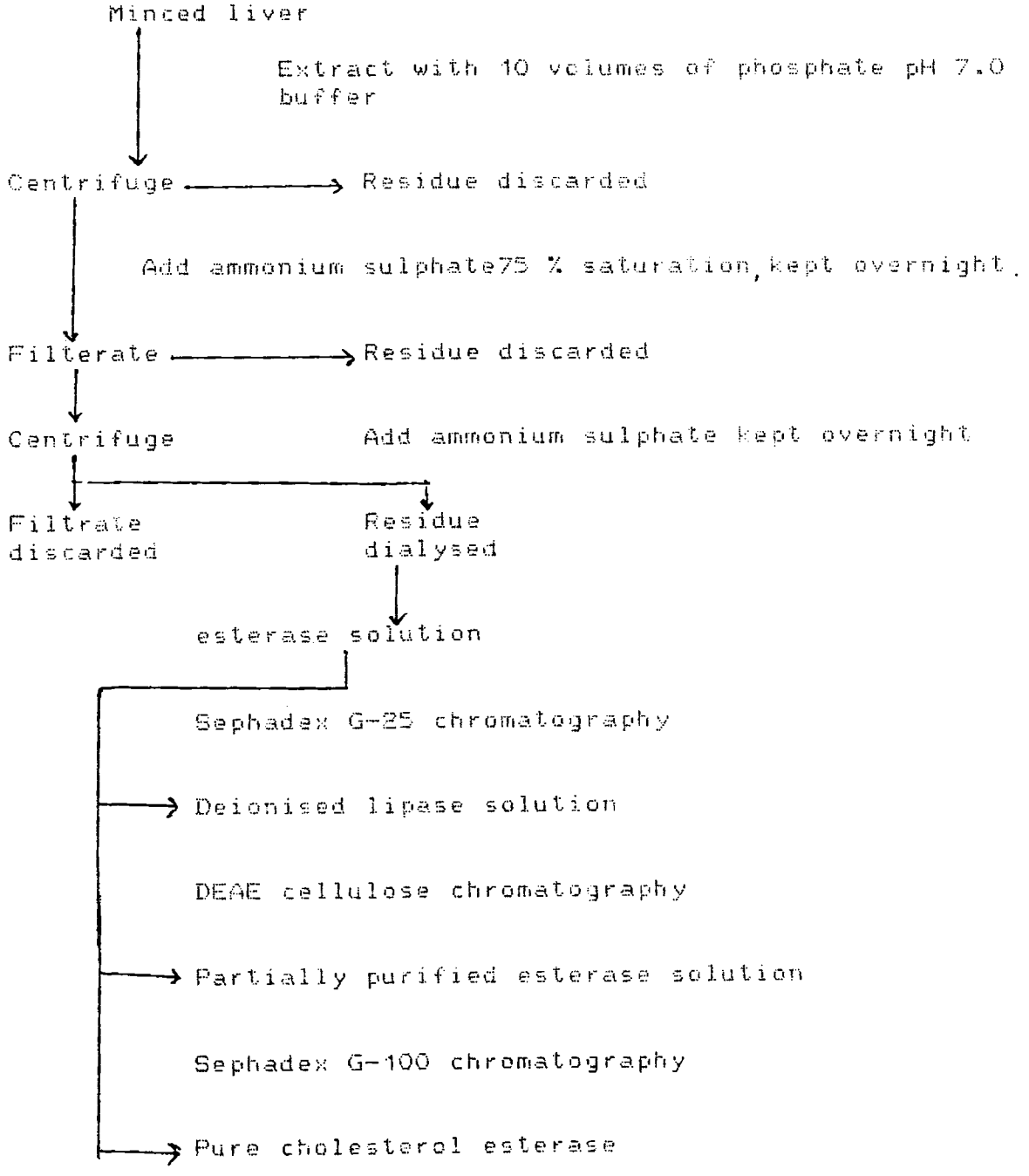
The inhibition of cation was in the order of Hg^{++} Cu^{++} > Zn^{++} Mn^{++} Mg^{++} Ca^{++} Ba^{++} Sn^{++}. And inhibition of anion was F^{-} Cl^{-} Br^{-} I^{-}

It is also found that PCMB and DFP also were inhibitors of cholesterol esterase. None of this was found to be total inhibitors.

Cholesterol esterase was immobilised using polyacryl amide gel and by chitosan. This immobilised enzyme retained 60-70% of activity of the original enzyme. It was also observed that the immobilised enzyme is more stable than the nature enzyme and also can be stored at -20° C for 3 months without significant loss in its activity.

FLOW SHEET OF PURIFICATION OF CHOLESTEROL ESTERASE FROM CAT FISH LIVER

(All operations done at 0 −4 °C)



CONCLUSION

An attempt was made to find out whether a cheap resource like fish can be utilised for the extraction of the enzyme cholesterol esterase. The method developed would be useful for large scale preparation of the enzyme which has got wide application in the pharmaceuticals and bio-technology.

The immobilisation method using chitosan is also useful for preparing a stable and active enzyme than the natural enzyme.

Isolation and purification of cholesterol esterase was a difficult task. Eventhough methods have been developed on its preparation a stable enzyme could not be produced due to various reasons. In this study a method is perfected to isolate the enzyme from a cheaper resource and the same was stabilised by immobilising with chitosan, a byproduct from the crustacean shell. The properties of pure native and immobilised cholesterol esterase were also studied.

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