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An Analysis of the Physico-Chemical Characteristics of Hilsa (Hilsa Ilisha) Fish, and Purification and Characterization of Two Lipases From its Dorsal Part

Hossain, A. K. M. Motahar

University of Rajshahi

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AN ANALYSIS OF THE PHYSICO-CHEMICAL CHARACTERISTICS OF HILSA (*Hilsa ilisha*) FISH, AND PURIFICATION AND CHARACTERIZATION OF TWO LIPASES FROM ITS DORSAL PART

Ph. D Thesis

Thesis submitted to the University of Rajshahi, Bangladesh, in fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry and Molecular Biology

Submitted by
A. K. M Motahar Hossain
B. Pharm (Honours), M. Pharm.

Session: January 2001

PROTEIN AND ENZYME RESEARCH LABORATORY
DEPARTMENT OF BIOCHEMISTRY
AND MOLECULAR BIOLOGY
UNIVERSITY OF RAJSHAHI
RAJSHAHI-6205. BANGLADESH

DEDICATED
TO

MY HEAVENLY FATHER AND BELOVED MOTHER

DECLARATION

I do hereby declare that the materials embodied in this thesis entitled "An analysis of the physico-chemical characteristics of Ilish (*Hilsa ilisha*) fish, and purification and characterization of two lipases from its dorsal part" prepared for submission to the University of Rajshahi, Bangladesh, for the degree of Doctor of Philosophy in Biochemistry and Molecular Biology, have not been previously submitted for the award of any degree or diploma anywhere.

Hossain
30-11-04.

(A. K. M Motahar Hossain)

CERTIFICATE

This is to certify that the materials included in this thesis are the original research works conducted by A. K. M Motahar Hossain. The thesis contains no material previously published or written by another person except when due reference is made in the text of the thesis

(Professor Md. Ekramul Haque)
Department of Pharmacy
University of Rajshahi
Bangladesh.
(Co-supervisor)

Nurul Absar
(Professor Nurul Absar)
Department of Biochemistry and Molecular Biology
University of Rajshahi, Bangladesh.
(Supervisor)

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---------- The author.

ABSTRACT

A comparative analysis on the nutrient contents of six different parts of hilsa fish was performed. Most of the nutrient contents of different parts were varied remarkably. The moisture and ash content of the different parts of hilsa fish were found in the ranges of 13.00-54.87% and 1.7-3.8% respectively. The ranges in the values of oil, protein, free sugar and polysaccharide composition of hilsa fish were 9.61-22.78%, 5.5-23.18%, 0.053-0.09%, and 0.03-0.065% respectively. The minerals such as iron, calcium and phosphorus were found to be varied from 199-212 mg%, 160-180 mg% and 259-278 mg% while the vitamins such as B_1 and B_2 contents were varied in the ranges of 0.35-0.48 mg% and 2.44-2.98 mg% respectively.

The oils extracted from different parts of hilsa fish were also characterized with respect to its physico-chemical properties such as specific gravity, refractive index, saponification value, iodine value, peroxide value and acid value. The specific gravity and refractive index of oil were varied from 0.920 to 0.932 and 1.4700 to 1.4722 respectively. The saponification value, iodine value, peroxide value and acid value of the oils were found to be ranged from 180.28 to 194, 80.70 to 126.40, 7 to 10 and 4.16 to 12.00, respectively.

The oils, extracted from different parts of hilsa fish were stored at temperatures $-10^{\circ}C$, $0^{\circ}C$ and $25^{\circ}C$ for 120 days. The hydrolytic deterioration of the oils were more pronounced at $25^{\circ}C$ as compared to that from $0^{\circ}C$ and $-10^{\circ}C$.

Two lipases from dorsal part of hilsa fish were purified by 85% ammonium sulphate saturation of crude fish extract followed by ion exchange chromatography on DEAE-and CM- cellulose and then subsequent gel filtration on Sephadex G-50. The homogeneity of the lipases were checked by SDS-polyacrylamide gel electrophoresis and both the lipases gave single band in the presence and absence of β-mercaptoethanol, indicating that both the enzymes are homogeneous as well as contained only one subunit. The molecular weights of hilsa fish lipases i.e. Lip-I and Lip-II, as estimated by gel filtration and SDS-polyacrylamide gel electrophoresis were about 47,500 and 41,800 respectively. Both the lipases were glycoprotein in nature and the neutral sugar content of Lip-I and Lip-II were estimated to be 8.59% and 5.18% respectively. The specific activities of Lip-I and Lip-II with respect to various substrates under investigation were found to be varied differently. Both the enzymes showed maximum activities when castor oil was used as substrate. Lip-I and Lip-II in aqueous solution gave absorption maxima around 270 and 275 nm and minima around 250 and 240 nm respectively.

The enzymes, Lip-I and Lip-II gave maximum activities in the pH values of 4.6-4.8 and 4.8-5.0 and in the temperature ranges of 34^0C and 35^0C respectively. Zn^{2+}, Hg^{+} and EDTA strongly inhibited lipolytic activities of the lipases, while the activities were slightly increased in presence of Ca^{2+} at low concentrations. The K_m values of Lip-I and Lip-II were estimated to be 0.11 mM and 0.175 mM respectively.

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CHAPTER – 1

GENERAL INTRODUCTION

HILSA FISH

Hilsa fish (bangla name- Ilish, Zoological name- *Hilsa ilisha*) is the national fish of Bangladesh. It is available in the Bay of Bengal and rivers of Bangladesh like the Padma, the Meghna and the Jamuna. In rainy season, it is extensively available in the market. Hilsa fish contributes a lot in the fish export earnings of Bangladesh. Hilsa is a great source of protein taken as food. In Bangladesh protein intake per person per day is only 57 grams (1). Hilsa is a great resource to remove the scarcity of food protein in our country.

Systemic Position of *Hilsa ilisha.*

Phylum: Chordata	Sub-order: Clupeoidei
Sub-phylum: Vertebrate	Family: Clupeidae
Super-class: Pisces	Sub- family: Alosinae
Class: Osteichthyes	Genus: Hilsa
Sub-class: Actinopterygii	Species: Ilisha.
Order: Clupeiformes	Zoological name: *Hilsa ilisha*

Habitat and Distribution of Hilsa Fish

It is statistically found on the availability of this fish that about 95% of *Hilsa ilisha* was found from the Padma and the Meghna including other rivers of the country.

Hilsa ilisha is found in the vast expanse of sea water, spreading from the Persian Gulf to the Read Sea, the Arabian Sea, the Bay of Bengal and the China Sea. Among rivers the Shatil Arab, the Tigres and the Euphrates of Iran and Iraq, the Sind of Pakistan, the rivers of Western and Eastern region

of India, the Iravati of Burma and the Padma, the Meghna, the Jamuna including the coastal rivers of Bangladesh are considered to be the real home of *Hilsa ilisha*. But it is found mainly in Bangladesh, Burma and India. Bangladesh produces 75%, India 20% and Burma 5% of the total production of *Hilsa ilisha* in the world.

Hilsa is a migratory fish. Day (2) was the first author to refer to its migratory habit and pointed out that both young as well as sexually matured fishes ascend the rivers. They can swim very fast across tide. When hilsa becomes mature they migrate to the rivers from the sea for food and reproduction.

Characteristics of *Hilsa ilisha*:

Colour: In life, silvery shot with gold and purple; a dark blotch behind gill opening, followed by a series of small spots along flanks in the immature. Fins hyaline.

Shape: Body fusiform, fairly deep and strongly laterally compressed. Dorsal and ventral profile equally convex.

Size: The average size of anadromous mature female 38-46 cm., male generally smaller. The maximum size recorded for female is 60 cm. with a weight of 2.49 Kg.; for male 43 cm. and 0.68 Kg.

Fin: Pectoral fin longer than pelvic fin. Caudal fin moderate. Caudal fin as long or slightly shorter than head.

Scale: Scales cycloid, sometimes with a pectinate border. In caudal fin partly covered with scale.

Lateral line: Lateral line mostly absent.

Teeth: Teeth wanting.

Respiration: Gillrakers fine and numerous, on lower part of first arch. Pseudo branch rather attenuated a groove present below border of pseudo branch.

Nutritional Importance of Hilsa Fish

Fish is a valuable source of food, rich in proteins. Hilsa fishes are consumed mostly as food either in the fresh condition or in the form of preserved products. They also contribute important sources of oil of medicinal and industrial importance.

Hilsa fish muscle is a rich source of protein (15-21.8%). The intracellular proteins consist of myosin (65-75%) and smaller amounts of myogen (6%), myoalbumin (7%) and globulin X (8%); collagen and elastin, constituting the stroma protein, together form 3% of the total proteins.

Hilsa fish is a good source of lipid containing 14-19% oil. Fish oils differ from much of land animal oils and vegetable seed oils in the number and composition of unsaturated fatty acids contained by them. The fatty acids range in chain length from C_{14} to C_{20} and of varying degrees of unsaturation, from mono- up to penta- and hexa- ethylenic acids.

The saturated acids, mainly palmitic acid, form only 15-20% of the total acids. Hilsa fish oils are made up of mixture of mixed triglycerides i.e., neutral lipid, glycolipids, phospholipids etc. Neutral lipid is present in highest amount in hilsa fish as compared with other lipids. Hilsa fish flesh contains negligible quantities of carbohydrates. Glycogen is present in living fish and is rapidly converted to lactic acid after death.

The mineral content in the hilsa fish muscle varies from 1-2%. The principle minerals are calcium, phosphorus, sodium, potassium, sulphur and chloride. The average values of mineral constituents calculated in the flesh are calcium 0.109, magnesium 0.133, potassium 1.671, phosphorus 1.148, sulphur 1.119 and iron 0.0055 gm%. Hilsa fish is a good source of vitamins A, D and B complex (thiamine, riboflavin, nicotinic acid, and cyano-cobalamin). Vitamin B_{12} is present in fair amounts in the flesh and in high concentrations in the liver.

Enzymes

Enzymes are efficient and specific biocatalysts for the conversion of natural substrates into specific products. They are proteins which control catalytically the vast majority of chemical reactions in all living systems like digestion, absorption, growth, differentiation, morphogenesis etc. Enzymes are soluble, colloidal organic substance, specific in action, inactive at 0°C and destroyed by moist heat at 100°C. Enzymes used in cells that make them are said intracellular enzymes. On the other hand, enzymes produced by other cells and are secreted to other parts of the body (e.g., digestive juice) are called extracellular enzymes. Enzymes can be classified into six major classes- oxidoreductases, transferases, hydrolases, isomerases, lyases and ligases.

In the past several decades an enormous number of enzymatic reactions have been discovered. Industrial enzymes have been isolated and purified from bacteria, yeast, plants and animals. In animals the pancreas, liver, heart, brain and blood are rich sources of a wide variety of enzymes. Modern

enzyme chemistry is considered to have started with the crystallization of the enzyme urease in 1926 by Summer (3), who showed this material to be a crystalline protein. A series of quantitative experiments by Northrop and co-workers (4) and by Summer and co-workers (5) conclusively proved that enzymes were indeed proteins.

The purification and crystallization of enzymes have made it possible to show that enzymes were primarily proteins, sometimes associated with a small molecule called prosthetic group. In common with all proteins, an enzyme is composed of approximately twenty amino acids in a specific and highly complex sequence. A list of the amino acid composition of approximately fifty enzymes (6) indicates that the gross composition of enzymes is not different from that of proteins.

Lipases

Lipases or lipolytic enzymes catalyze the hydrolysis of fats as well as esters of fatty acids with alcohols (7-18). According to the IUB (International Union of Biochemistry) lipases catalyze the following hydrolytic reactions:

$$\text{Fat} \xrightarrow{\text{Lipase}} \text{Mono and diglycerides} \xrightarrow{\text{Lipase}} \text{Glycerol + Fatty acids.}$$

$$\text{Fatty acid esters} \xrightarrow{\text{Lipase}} \text{Alcohol + Fatty acids.}$$

Lipases have received increased attention after they were shown to be active even in nearly anhydrous water immiscible organic solvents (19, 20) and able to be used for transesterification (21), synthesis of esters (22, 23) and peptides (24- 26) and resolution of racemic mixture into optically active alcohols and acids (27, 28).

Deficiency of Lipase

A shortage of lipase in the body may lead to high cholesterol, difficulty in losing weight, a tendency to diabetes, high urine sugar levels- which some believe could lead to arthritis, bladder problems, gall stones, hay fever, prostate problems, heart problems etc.

With too little lipase, the cell membrane permeability is not at optimum and nutrients can not enter the cell, while wastes can not leave the cell. Lipase deficiency also causes problem with electrolyte balance, muscle spasm and a spastic colon.

Conditions in which Lipase may be supportive

If inadequate fat digestion is the cause of the following health concern, then lipase may be helpful-

1. Celiac disease 2. Crohn's disease 3. Cystic fibrosis 4. Indigestion 5. Heart burn.

History of Lipases

Lipases have been studied for well over a hundred years. The literature reveals that lipase activity in the pancreas was first demonstrated in 1846

and a gastric lipase was first reported in 1858 (29). The presence of lipase in plant seeds was demonstrated by Munts in 1871 (30) and by Green in 1890 (31). Relatively little advance in the method of purification and properties of lipases was made until Willstatter and his colleagues (32) made the significant contribution to the properties and characteristics of lipase in the early 1920's. Successful estimation, purification and characterization of wheat germ lipase were made by Singer and Hopstee (33). Finally, the pioneering works of Desnuelle and his associates (34- 41) in the 1950's and, Mattson and co-workers (42- 48) witnessed the notable advances in the purification and characterization of lipases. In the 1960's numerous additional lipases have been isolated and purified from several sources such as rat pancreas (49), bovine pancreas (50), milk (51), castor bean (52), *Staphylococcus aureus* (53), *Penicillium roqueforti* (54), *Achromobacter lipolyticum* (55), *Aspergillus niger* (56) and *Rhizopus delemer* (57).

While above lipases have been studied in considerable detail, so works have also been reported on lipases found in various mammalian tissues, organs and fluids; plant tissues, leaves and seeds; and numerous microorganisms. The interest as well as the advances made in the field of lipases is further evident from the fact that during the past 25 years, there have appeared several excellent literature reviews on lipase research (58- 67).

Importance and Applications of Lipases

Lipases are physiologically important as they hydrolyze fats and oils giving rise to free fatty acids and partial glycerides which are essential metabolic processes such as fatty acid transport, oxidation and resynthesis of

glycerides and phospholipids. Additionally, these enzymes are of considerable economic significance in the food industries. If not appropriately controlled, lipases can hydrolyze lipids and produce undesirable rancid flavour in milk products, meat, fish and other food products containing fat. On the other hand, they are essential for the production of desirable and characteristic flavour in certain foods. In fact, lipases have been used in specific application for the modification of lipids. Certain fatty acids released from milk fat are crucial to the development of cheese flavour (68).

An exciting development is the new focus on lipases that can be used in the mainstream of oleochemical processing. Three key areas with potential for improvement by enzymology are fat-splitting for fatty acid production (69, 70), lipid synthesis via reversal of hydrolysis (71) and modification by ester interchange or interesterification (72). There is also currently great interest in biotechnological applications of lipases (73). One considerable advantage of lipase-mediated reactions over conventional chemical methods resides in the inherent specificity of the enzymes i.e., positional- , fatty acids- and stereospecificities (74- 76). Synthesis of esters mediated by lipases that has been under scrutiny by numerous researchers in recent years as a wide variety of such compounds are important to us. In fact, compound like triglycerides, phospholipids, galactolipids, cutin, waxes, short chain esters and steroids play many important functions: energy sources, membrane constituents, emulsifiers, viscosity builders, protective coatings, flavours. Functional properties of carboxylic esters are directly related to the length of the hydrocarbon backbone. Hence, esters of short and medium chain

carboxylic acids and alcohol moieties provide valuable oleochemical species that may function as fuel for the diesel engines, and esters of long chain carboxylic acid and alcohol moieties (typically referred to as waxes) have applications as lubricants and additives in cosmetic formulations. Esterification by lipases appears to be an attractive alternative to bulk chemical routes. In fact, ester synthesis can be performed at room temperature and normal pressure, as well as neutral pH in reaction vessels operated either batchwise or continuously. Products obtained therefore are qualitatively more pure than one obtained by alternative chemical means. Because, chemical catalysis tends to be unspecific and consequently generate several by-products. Therefore, use of lipases to carry out esterification alleviates the need for a wide variety of complex post-reaction separation processes (which are a must in chemical processes), and thus leads to lower overall operation costs.

The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry and in household dishwashers. Lipases can reduce the environmental load of detergent products, since they save energy by enabling a lower wash temperature to be used; allow the content of other chemicals in detergents to be reduced; have no negative impact on sewage treatment processes; and, do not present a risk to aquatic life.

Lipases are also used in the synthesis of ingredients for personal care products (like shampoo) e.g., isopropyl myristate, isopropyl palmitate, 2-ethyl hexyl palmitate. They are also used in the synthesis of polymers and

agrochemicals. They are used in leather industry, textile industry, baking industry and paper industry.

Lipases have important applications in pharmaceutical fields. There is a noticeable trend within the pharmaceutical industry toward the manufacture of optically pure products in preference to racemic mixtures. Lipases are very useful enzymes used in the preparation of chiral synthons to prepare optically active pure products from racemic mixtures. For example, lipases are used for the hydrolysis of epoxy alcohol esters. They are useful industrial catalysts for the resolution of racemic alcohols. Lipases were successfully applied in the regioselective modification of castanospermine, a promising drug for the treatment of AIDS. Thus lipases have numerous industrial and pharmaceutical applications.

Distribution of Lipases

Lipases are widely distributed in animals, plants and microorganisms. A comprehensive list of lipases definitely or tentatively identified in animals, plants and microorganisms were detailed in a review published in 1965 (77). Only a few main points on the distribution of lipases are presented below.

Among the animal lipases, mammalian lipases have received attention in recent years. Three groups of lipases may be distinguished in mammals: the lipases discharged into the digestive tract by specialized organs (78), tissue lipases (79) and milk lipases (80, 81). Lipases have been reported to be present in a number of tissues or organs of mammals such as heart (82), brain (83), liver (84) and adipose tissue (85). Mammalian lipases from rat liver (86- 88) and human pancreas (89, 90) were also investigated.

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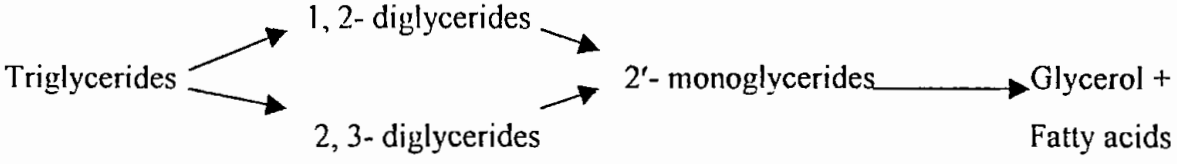
Several workers (91, 92) have investigated germinating oil seeds as sources for lipases. The best characterized enzymes of this group, castor bean lipase, is also present in dormant seeds. Lipase has been reported in wheat (93), oats (94), corn (95) and palm fruits (96).

During recent years considerable attention has been devoted to lipases produced by microorganisms presumably because of their stability and their practical medicinal and industrial applications. In the food industries, they also bear significance. A variety of microbes produce lipases. They include *Staphylococcus aureus* (97), *Pseudomonus aeruginosaa* (98), *Aspergillus niger* (99), *Rhizopus delemer* (100), *Mucor miehei* (101) etc.

Lipase Activity

The theory behind lipase action and assay is that the enzyme releases free fatty acids (FFA) from the substrate and, therefore, the lipase activity can be measured by determining, directly or indirectly, the disappearance of the substrate or the production of end products such as FFA. Lipases hydrolyze triglycerides and the products are formed in the following sequences:



Thus the rate of lipase reaction can be measured by determining either the rate of disappearance of substrate or the rate of production of fatty acids. Automatic or manual titration of the system gives a direct measure of the lipase activity per unit time (102). This assay technique was used

extensively (103- 106) for the measurement of lipase activity. As an alternative to the above method, the rate of acid production may be measured manometrically by determining the rate of liberation of carbon dioxide from a bicarbonate buffer (107). Several calorimetric methods have been described that employed a special substrate designed to give a coloured end product after hydrolysis. β-Naphthyllaurate has been recommended and used as a substrate for serum lipase. β-Naphthol liberated is coupled with tetra azotized o-dianisidine to give a coloured compound (108).

AIM OF THE PRESENT STUDY

A large number of populations in our country have been suffering from malnutrition. There are many rivers in our country having lot of fishes which are good sources of nutrients, specially proteins. Hilsa fish is one of them. The nutrients, vitamins, minerals and oils of hilsa fish are very important for functioning of different systems of human body and essential for performing various biochemical reactions in body. These nutrients are present in various parts of hilsa fish in significant amounts. Present investigation will furnish us detailed information about the distribution of nutrients in various parts of hilsa so that we can take the appropriate one.

Hilsa fish undergoes rancidification soon after their landing. Lipase present in hilsa fish causes hydrolysis of fish fat to produce fatty acids that undergo oxidation causing rancidity. Investigation on the properties of hilsa fish oil has been done to find out the way of preservation of hilsa fish.

The second part of my research work consists of purification and characterization of lipases from hilsa fish. Lipases are lipolytic enzymes that are widely distributed in plants, animals and microorganisms. Many research

works were done on lipases mainly from microbial sources and some from plant sources as well as human sources. But a very few information is available regarding fish lipases and no information has yet been reported about lipases from hilsa fish (*Hilsa ilisha*).

Lipases are very important industrial enzymes. There are various applications of lipases. They have a major role in the synthesis of pharmaceuticals and agrochemicals. Lipases were successfully applied in the regioselective modification of castanospermine, a promising drug for the treatment of AIDS (109). They are also required in dairy industry, in oleochemical industry, in the manufacture of household detergents, in the synthesis of structured triglycerides, surfactants and polymers. Lipases are also used in leather industry, textile industry, baking industry and paper industry. Thus lipases have numerous industrial applications. So, lipases should be studied vigorously to discover economic and useful one. Purification and characterization of lipase will help discovery of highly active lipases with low cost that will assist the manufacture of various chemicals and pharmaceuticals.

As hilsa fish is very abundant in Bangladesh it will be very easy to extract huge amount of lipases from this fish. It has been experienced that bad odour evolves from hilsa fish even it is stored in refrigerator. This bad odour results from degradation products of fish oil. This degradation is carried out by lipases of fish.

The present study provides a comprehensive picture of some new lipases that might be helpful to the traditional research scientists as well as industrialists for selection and production of new lipases of immense commercial value.

CHAPTER-2

CHEMICALS AND EQUIPMENTS

CHEMICALS

The chemicals used in this research work are mentioned below with their manufacturers.

1. Acetone
   BDH Chemicals Ltd. Poole. England.
2. Acrylamide
   Sigma Chemicals Co. U.S.A.
3. Ammonium per sulphate
   Bio-Rad Laboratories. Richmond. U.S.A.
4. Ammonium sulphate
   Merck, Germany.
5. α-Amylase
   Sigma Chemicals Co. U.S.A.
6. Borate (Sodium tetraborate)
   BDH Chemicals Ltd. Poole. England.
7. Bovine serum albumin (BSA)
   Sigma Chemicals Co. U.S.A.
8. Bromophenol blue
   Bio-Rad Laboratories. Richmond. U.S.A.
9. 1-Butanol
   BDH Chemicals Ltd. Poole. England.
10. Coomassie brilliant blue (R-250)
    Bio-Rad Laboratories. Richmond. U.S.A.
11. Copper sulphate
    BDH Chemicals Ltd. Poole. England.
12. DEAE-Cellulose
    Fluka Bio Chemika, Switzerland.
13. Diethyl ether
    Sigma Chemicals Co. U.S.A.
14. Disodium hydrogen orthophosphate dihydrate.
    BDH Chemicals Ltd. Poole. England.
15. Ethyl alcohol
    BDH Chemicals Ltd. Poole. England.

16. Glacial acetic acid
    BDH Chemicals Ltd. Poole. England.
17. Glycerol
    Bio-Rad Laboratories. Richmond. U.S.A.
18. Glycine
    Bio-Rad Laboratories. Richmond. U.S.A.
19. β-Galactosidase
    BDH Chemicals Ltd. Poole. England.
20. Hydrochloric acid (HCl)
    BDH Chemicals Ltd. Poole. England.
21. Lysozyme
    Sigma Chemicals Co. U.S.A.
22. N, N-Methylene bis acrylamide.
    Sigma Chemicals Co. U.S.A.
23. ortho-Phosphoric acid
    BDH Chemicals Ltd. Poole. England.
24. Petroleum ether (40°- 60° C)
    BDH Chemicals Ltd. Poole. England.
25. Phenol
    Aldrich Chemical Co. Tnc. USA.
26. Potassium sodium tartrate
    BDH Chemicals Ltd. Poole. England.
27. Potassium hydroxide
    Merck, Germany.
28. Phenolphthalein
    BDH Chemicals Ltd. Poole. England.
29. Riboflavin
    BDH Chemicals Ltd. Poole. England.
30. Sephadex G-50, 150.
    Sigma Chemicals Co. U.S.A.
31. Sodium dihydrogen orthophosphate
    BDH Chemicals Ltd. Poole. England.
32. Sodium chloride
    Merck, India.
33. Sodium carbonate.
    Hopkin & Willium, Essex, England.
34. Sulfuric acid
    BDH Chemicals Ltd. Poole. England.
35. Sodium dodecyl sulphate
    Merck, Germany.

36. TEMED (N, N, N′, N′- tetra methyl ethylene diamine)
Sigma Chemicals Co. U.S.A.
37. Tris (hydroxy methyl) amino methane
Fluka Bio-chemika, Switzerland.

EQUIPMENTS AND APPARATUS

Important equipments and apparatus used throughout the research work are listed below.

1. Centrifuge (refrigerated) Sanyo, Harrier 18/80.
2. SDS- PAGE (electrophoresis) apparatus. SJ- 1060.
3. Electronic balance (Mettler H 18)
4. Electric balance
5. Electrophoresis power supply
6. pH meter (Hanna)
7. Cold chamber (Pioneer)
8. Fraction collector SF-160 (Advantec, Japan)
9. Incubator (Gallenkamp, England)
10. Double beam spectrophotometer (Shimadzu)
11. Waterbath.
12. Freeze dryer, (Taitec corporation)
13. Freezer, Ultra low, (Sanyo, Japan)
14. Volac pipette controller.
15. Vacuum pump.
16. Micropipette (Eppendorf)
17. UV- lamp.
18. Rotary shaker.
19. Laboratory glasswares (Pyrex/Jena/Duran)

CHAPTER- 3

PHYSICO-CHEMICAL CHARACTERIZATION OF HILSA FISH AND ITS OIL

INTRODUCTION

Hilsa fish is a rich source of nutrients such as proteins, vitamins, minerals and oil that are essential for the functioning of different systems of human body. The nutrient content varies in different parts of fish. Investigation on physico-chemical properties of hilsa fish will enable us to obtain information about nutrient contents of different parts of fish. Hilsa fish rancidifies within a short time which might be due to hydrolysis of fish oil by lipase. So, physico-chemical characterization of hilsa oil will facilitate the better preservation of hilsa fish. This chapter describes the nutrient compositions as well as physico-chemical properties of oils extracted from different parts of hilsa fish.

Description of Sampling Area:

Hilsa ilisha were collected from the fish landing centers of Rajshahi Shaheb Bazar. The geographical position of Rajshahi is 24^0 North and 89^0 East. It is situated on the northern bank of the river Padma. The fishes are mainly caught from Goalund, the resources and brought to that fish landing centers. Main collections were made from these fish landing centers in different months from April, 2001 to October, 2002.

Hilsa ilisha is essentially an estuarine fish but it is abundantly caught in the Padma, the Meghna, the Jamuna and some other rivers of Bangladesh. The immature (up to 150 mm) *H. ilisha* known as "Jatka" are extensively caught in the river near Chandpur during February to May. Mature fishes are mostly caught in these rivers from middle of May to middle of October and used for experimental purposes in this study.

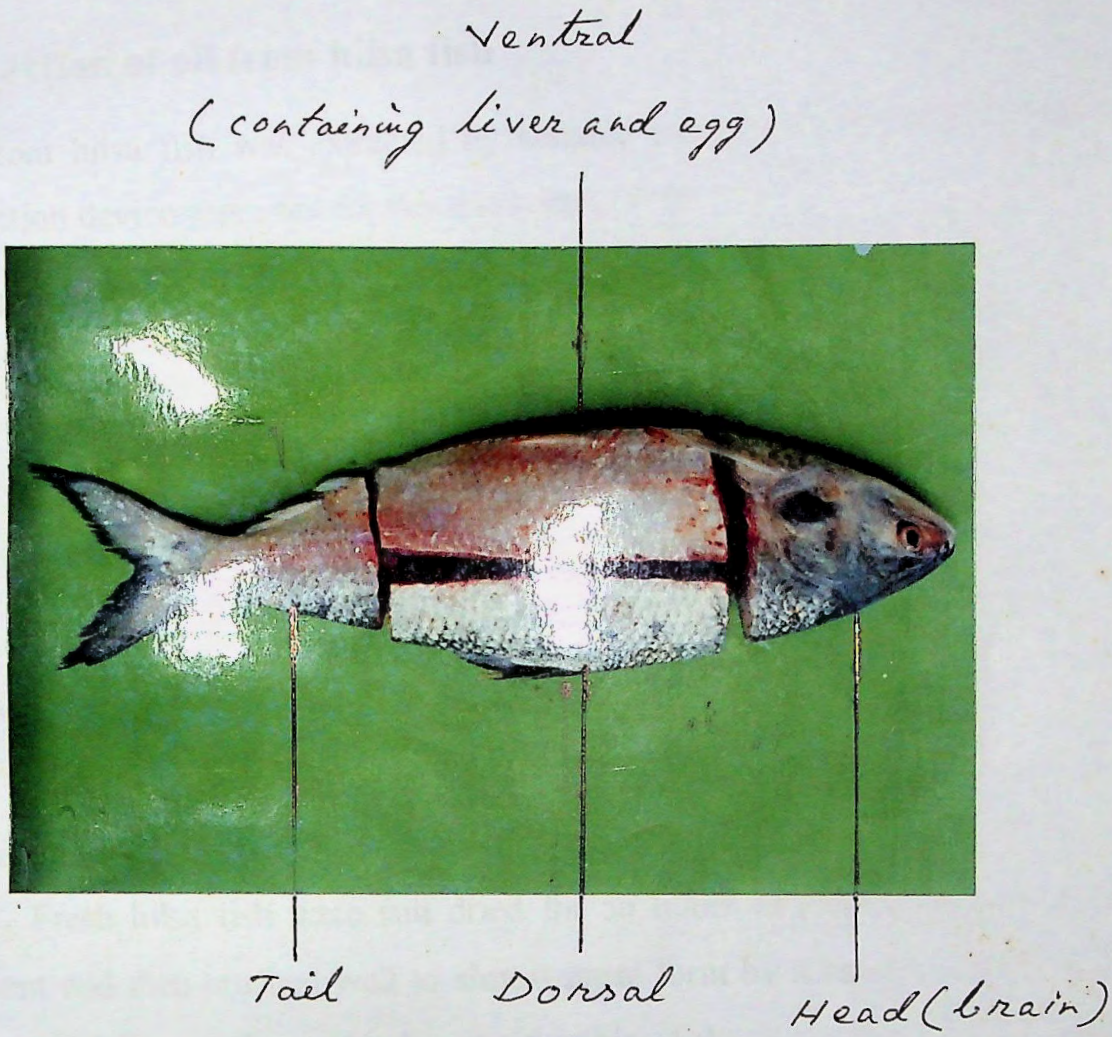


Figure- 1: Photography of hilsa fish showing different portions that have been analyzed.

MATERIALS AND METHODS

Extraction of oil from hilsa fish

Oil from hilsa fish was extracted by suitable solvent. Continuous Soxhlet extraction device was used for this extraction (110).

Apparatus:

1. Weighing balance
2. Soxhlet apparatus
3. Rotary evaporator
4. Flask

Chemicals:

n-Hexane, analytical grade

Procedure:

Fresh hilsa fish were sun dried for an hours to reduce the moisture content and then crushed well to almost paste form by a hand crusher. This (about 100 g) was then placed in the thimble of the apparatus. The oil was then extracted with n-Hexane in the continuous Soxhlet extraction apparatus. The extract was evaporated under reduced pressure (rotary evaporator) to obtain the oil.

Study of Physico-chemical Characteristics of Fish

Determination of moisture content of hilsa fish

Moisture content was determined by the conventional procedure.

Materials:

a) Porcelain crucible

b) Electrical balance

c) Oven

d) Desiccator

Procedure:

1-2 g of hilsa fish were weighed in a porcelain crucible (which was previously cleaned, heated to about 100^0C, cooled and weighed). The crucible with the sample was heated in an electrical oven for about six hours at 100^0C. It was then cooled in a desiccator and weighed again.

Calculation:

Percent of moisture content (g per 100 g of hilsa fish)

$$= \frac{\text{Weight of the moisture}}{\text{Weight of the hilsa fish}} \times 100$$

Determination of ash content of hilsa fish

Ash content was determined by the following method of A.O.A.C (111). **Materials:**

a) Porcelain crucible

b) Muffle furnace

c) Electrical balance (Mettler H-18)

d) Desiccator

Procedure:

1-2 grams of hilsa fish were weighed in a porcelain crucible (which was previously cleaned, heated to about 100^0C, cooled and weighed). The crucible with its content was placed in a muffle furnace for about four hours at about 600^0C. It was then cooled in a desiccator and weighed again. To ensure the completion of ashing, the crucible was again heated in the muffle furnace for half an hour, cooled and weighed again. This was repeated till two consecutive weights became same and the ash was almost white in colour.

Calculation:

Percent of ash content (g per 100 g of hilsa fish)

$$= \frac{\text{Weight of the ash obtained}}{\text{Weight of the hilsa fish}} \times 100$$

Determination of total protein content of hilsa fish

Protein content of the different parts of hilsa fish was determined by the method of Micro-kjeldahl (112).

Reagents and equipment's:

a) Solid potassium sulphate

b) Concentrated sulphuric acid

c) 5% $CuSO_4$ 5 H_2O in distilled water

d) 0.01N H_2SO_4

e) Concentrated sodium hydroxide (5N)

f) Few quartz chips

g) Boric acid solution containing bromocresol (receiving fluid): 10 g of boric acid was dissolved in hot water (about 250 ml) and cooled. Then 1 ml of 0.1% bromocresol green in alcohol was added and diluted to 500 ml with distilled water.

h) Nitrogen determination apparatus according to Paranas-Wagner, made of JENA Glass-all connections with interchangeable ground joints.

Procedure:

A) Digestion: Concentrated H_2SO_4 (6-8 ml), 1 g K_2SO_4, 1-2 drops of 5% $CuSO_4$ solution and some quartz chips were added (to avoid bumping) to 1-2 g of hilsa fish in a kjeldahl flask. The mixture was heated till it became light green (2-3 hours).

B) Collection of ammonia: The digestion was carried out in the steam distillation chamber of the nitrogen determination apparatus. The chamber is designed to act as a micro-kjeldahl flask and can be easily detached when needed. After completion of digestion, the steam distillation chamber containing the digested mixture was fitted back to the nitrogen determination apparatus. Boric acid solution (15 ml) in a small flask was placed, so that the tip of the condenser outlet dipped below the surface of the boric acid solution. Sufficient amount of concentrated sodium hydroxide solution (approximately 30-40 ml) was added to the digest in the chamber to neutralize the amount of acid present. Steam was generated from the steam-generating flask and the sample in the chamber was steam distilled until 20

ml of distillate collected in the boric acid solution. The condenser outlet was then rinsed with little distilled water and the receiving flask was removed.

C) Titrimetric estimation of ammonia: The ammonia in the boric acid solution was titrated with 0.01N H_2SO_4 until the solution had been brought back to its original yellow colour. The titration was repeated with a control containing only 15 ml of boric acid solution diluted to approximately the final of the titrated sample. The volume of acid required was noted.

The nitrogen was calculated using the following formula

(i) 100 ml of 1N sulphuric acid = 14 g of nitrogen

(ii) X g of N_2 = 6.25 X g of protein

Percent of protein content (g per 100 g of hilsa fish)

$$= \frac{\text{Weight of the protein obtained}}{\text{Weight of the hilsa fish}} \times 100$$

Protein contents of different parts of hilsa fish were also estimated by Lowry method.

Determination of polysaccharide content of hilsa fish

The polysaccharide content of hilsa fish was determined by the Anthrone method (113).

Reagents:

(i) Anthrone reagent (0.2% in concentrated H_2SO_4)

(ii) Standard glucose solution (10 mg /100 ml distilled water)

(iii) 1M HCl

Procedure:

1-2 g of hilsa fish was homogenized well with 10 ml of water. It was then filtered through double layer of muslin cloth. To the filtrate, twice the volume of ethanol was added to precipitate the polysaccharide, mainly glycogen. After kept it overnight in cold the precipitate was collected by centrifugation in a clinical centrifuge at 3000 g for 15 minutes. The precipitate was then dried over a steam bath. Then 40 ml of 1M hydrochloric acid was added to the dried precipitate and heated to about 70^0C. It was then transferred to a volumetric flask and diluted to 100 ml with 1M HCl. Then 2 ml of diluted solution was taken in another 100 ml volumetric flask and diluted to 100 ml with 1M HCl.

Aliquot of 1 ml of the extract of each part was pipetted into different test tubes and 4 ml of anthrone reagent was added to the solution of each tube and mixed well. Glass marbles were placed on the top of each tube to prevent loss of water by evaporation. The tubes were placed in a boiling water bath for 10 minutes, then removed and cooled. A reagent blank was prepared by taking 1 ml of water and 4 ml of anthrone reagent in test tube and treated similarly. The absorbance of blue-green solution was measured at 680 nm in a colorimeter. The amount of polysaccharide present in the hilsa fish was calculated from standard curve of glucose.

Calculation:

The percentage of polysaccharide content (g per 100 g of hilsa fish)

$$= \frac{\text{Amount of polysaccharide obtained}}{\text{Weight of hilsa fish}} \times 100$$

Determination of free sugar content of hilsa fish

Free sugar content of hilsa fish was determined colorimetrically by the anthrone method (114).

Reagents:

a. **Anthrone reagent:** The anthrone reagent was prepared by dissolving 2 g of anthrone in 1 liter of concentrated H_2SO_4.

b. **Standard glucose solution:** A standard glucose solution was prepared by dissolving 10 mg of glucose in 100 ml of distilled water.

Extraction of sugar from hilsa fish:

Extraction of sugar from hilsa fish was done by the following method as described by Loomis (115).

4 to 6 g of hilsa fish was plunged into boiling ethyl alcohol and allowed to boil for 5-10 minutes (5 to 10 ml of alcohol was used for every gram of hilsa fish). The extract was cooled and crushed thoroughly in a mortar with a pestle. Then the extract was filtered through two layers of muslin cloth and re-extracted the ground fish for three minutes in hot 80 percent alcohol, using 2 to 3 ml of alcohol for every gram of fish sample. This second extraction ensured complete removal of alcohol soluble substances. The extract was cooled and passed through muslin cloth. Both the extracts were filtered through Whatmann no-41 filter paper.

The volume of the extract was evaporated to about ¼ th the volume over a steam bath and cooled. This reduced volume of the extract was then transferred to a 100 ml volumetric flask and made upto the mark with

distilled water. Then 1 ml of the diluted solution was taken into another 100 ml volumetric flask and made upto the mark with distilled water (working standard).

Procedure:

Aliquot of 1 ml of the fish extract from each part was pipetted into different test tubes and 4 ml of the anthrone reagent was added to each of this solution and mixed well. Glass marbles were placed on the top of each to prevent loss of water by evaporation. The test tubes were heated for 10 minutes in a boiling water bath and then cooled. A reagent blank was prepared by taking 1 ml of water and 4 ml of anthrone reagent in a tube and treated similarly. The absorbance of the blue-green solution was measured at 680 nm in a colorimeter.

A standard curve of glucose was prepared by taking 0.0, 0.2,0.4, 0.6, 0.8 and 1 ml of standard glucose solution in different test tubes containing 0.0, 0.02 mg, 0.04 mg, 0.06 mg, 0.08 mg and 0.1 mg of glucose respectively and made the volume upto 1 ml with distilled water. Then 4 ml of anthrone reagent was added to each test tube and mixed well. All these solutions were treated similarly as described above. The absorbance was measured at 680 nm using the blank containing 1 ml of water and 4 ml of anthrone reagent.

The amount of free sugar was calculated from the standard curve of glucose (Fig-2). Finally, the percentage of free sugar present in the hilsa fish was determined using the formula given below:

Percentage of free sugar (g per 100 g of hilsa fish)

$$= \frac{\text{Amount of sugar}}{\text{Weight of hilsa fish}} \times 100$$

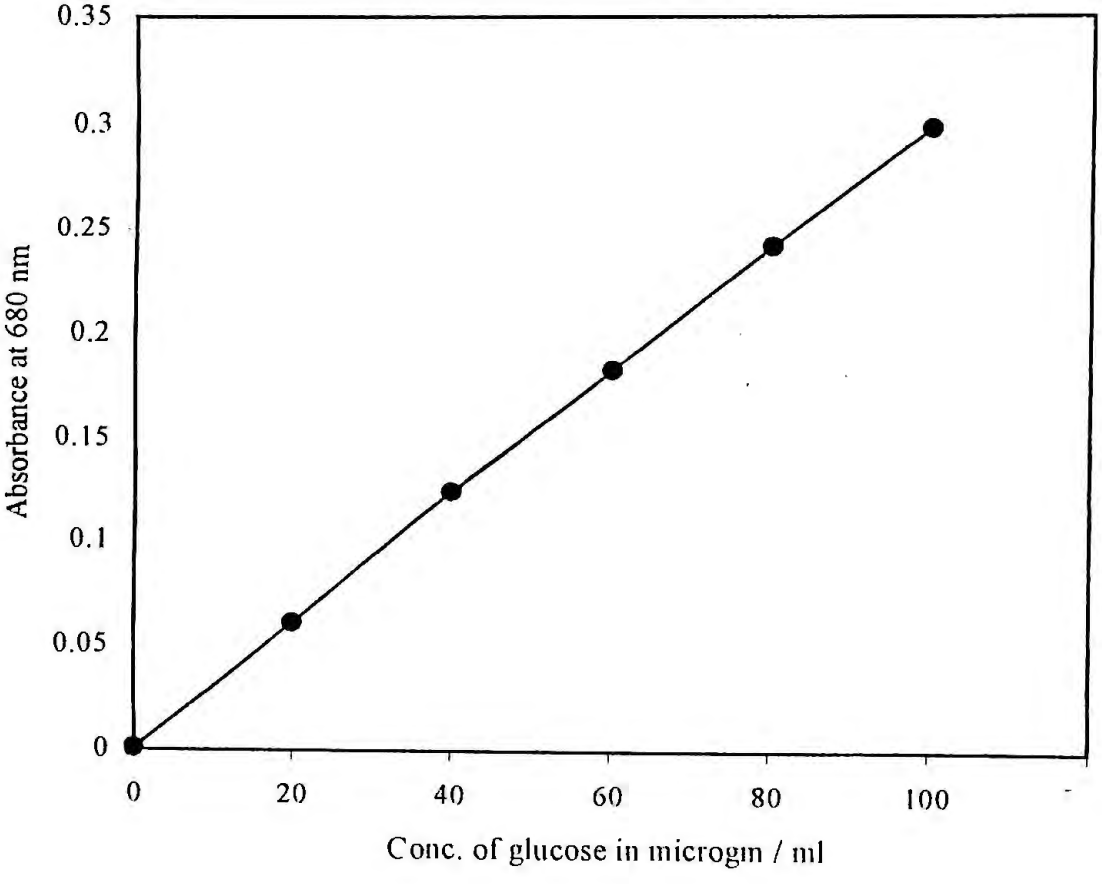


Figure-2: Standard curve of glucose for estimation of free sugar and polysaccharide.

Determination of calcium, iron and phosphorus content of hilsa fish

A) Calcium: Calcium content was determined by the titrimetric method (116).

Reagents:

a. Concentrated hydrochloric acid
b. Ammonium oxalate (6%)
c. Methyl red indicator
d. Dilute sulphuric acid (2N)
e. Strong ammonia
f. Potassium permanganate solution

Procedure:

The ash obtained as described earlier was moistened with a small amount of distilled water (0.5-1.0 ml) and then 5 ml of concentrated HCl was added to it. The mixture was evaporated to dryness on a boiling water bath. Another 5 ml of concentrated HCl was added again to the precipate and the solution was evaporated to dryness as before. Then 4 ml of concentrated HCl and a few ml of distilled water were added to the dry ash and the solution was warmed on a boiling water bath. The warmed solution was then filtered into a 100 ml volumetric flask using Whatmann no-41 filter paper. After cooling, the volume was made upto 100 ml with distilled water and suitable aliquot was used for the estimation of calcium.

25 ml of the prepared solution was taken in a conical flask and 125 ml of distilled water was added to it. A few drops of methyl red indicator was added and the mixture was neutralized with ammonia till the pink colour

changes to yellow. The solution was heated to boiling and 10 ml of ammonium oxalate was added. The mixture was then allowed to boil for a few minutes and glacial acetic acid was added to it till the colour was distinctly pink. The mixture was kept in dark at room temperature for an hour. When the precipitate settled down the supernatant was tested with a drop of ammonium oxalate solution to ensure the completion of the precipitation. The precipitate was then filtered through Whatmann no-41 filter paper and washed with warm water till the precipitate became free of oxalate (tested with $CaCl_2$). The precipitate was transferred to a beaker by piercing a whole in the filter paper and about 5 to 10 ml of dilute H_2SO_4 (2N) was poured over it. The solution was then heated to about $70^{o}C$ and titrated against N/100 $KMnO_4$ solution.

Calculation:

1 ml of N/100 $KMnO_4$ solution = 0.2004 mg of calcium

Percent of calcium content (mg per 100 g of hilsa fish)

$$= \frac{\text{mg of calcium obtained}}{\text{Weight of hilsa fish}} \times 100$$

B) Phosphorus

Phosphorus content of hilsa fish was determined by the method of Boltz (117).

Reagents:

a) **Standard phosphorus solution**: 0.434 g of potassium dihydrogen phosphate was dissolved in distilled water and diluted it to 1 litre. Now, each ml of the solution contained 0.1 mg of phosphorus.

b) **Molybdate reagent:** 100 g of molybdate was dissolved in distilled water and diluted to 1 litre.

c) **Nitric acid:** (2.5N)

Procedure:

The sample stock solution (25 ml), 5 ml of 2.5N HNO_3 and about 15 ml of distilled water was taken in a 50 ml volumetric flask. Then 5 ml of molybdate reagent was added to the above mixture and diluted it upto the mark with water. The mixture was mixed thoroughly and a yellow colour was obtained. The absorbance of the solution was then measured at 380 nm using a reagent blank, which was prepared in the usual process, taking 25 ml of distilled water instead of stock solution.

A calibration curve was constructed in the usual manner by taking 0.5, 1, 1.5, 2.5, 3.0, and 3.5 ml of the standard solution of phosphorus into six different volumetric flasks (50 ml). To each flask, 5 ml of HNO_3, 15 ml of distilled water and 5 ml of molybdate reagent were added and then diluted it to the mark. The mixture was mixed thoroughly by shaking whereby a yellow colour was obtained. The absorbances of these solutions were taken at 380 nm using reagent blank that contained distilled water instead of phosphorus solution. The calibration curve was constructed by plotting absorbance against the respective concentration of the solution (Fig-3).

Calculation:

Percent of phosphorus content (mg per 100 g of of hilsa fish)

$$= \frac{\text{mg of phosphorus obtained}}{\text{Weight of hilsa fish}} \times 100$$

C) Iron: Iron content of hilsa fish was determined spectrophotometrically by thiocyanate method (118).

Reagents:

a) 4 N HCl

b) Potassium thiocyanate solution (20%)

c) Standard iron, Fe (III) solution

Exactly 0.864 g of ammonium iron (III) sulphate was dissolved in distilled water and 10 ml of concentrated HCl was added to it. Finally the volume was made upto 1000 ml with distilled water.

Then 1 ml of the standard solution = 0.1 mg of Fe (III).

Preparation of stock solution:

The amount of ash as described was taken in a porcelain crucible and 15 ml of concentrated HCl was added to it. The resulting solution was evaporated nearly to dryness to expel excess of acid and diluted slightly with water. The iron was oxidized to the ferric state with a little bromine water and made up to 100 ml with distilled water to give the stock solution.

Procedure:

10 ml of stock solution sample, prepared from different parts of hilsa fish, was taken in 50 ml volumetric flask and 5 ml of 20% potassium thiocyanate was added in each of the flasks and mixed well. Distilled water was then added to made upto the mark. The absorbance for each of the solution was measured at 480 nm against a reagent blank. Iron contents of these solutions were determined from the standard curve (Fig-4) constructed by using standard iron solution of different concentrations in the same manner as

before. From the result obtained, amount of iron present in hilsa fish was calculated.

Calculation:

Percent of iron content (mg per 100 gms of hilsa fish)

$$= \frac{\text{mg of Fe (III) obtained}}{\text{Weight of hilsa fish}} \times 100$$

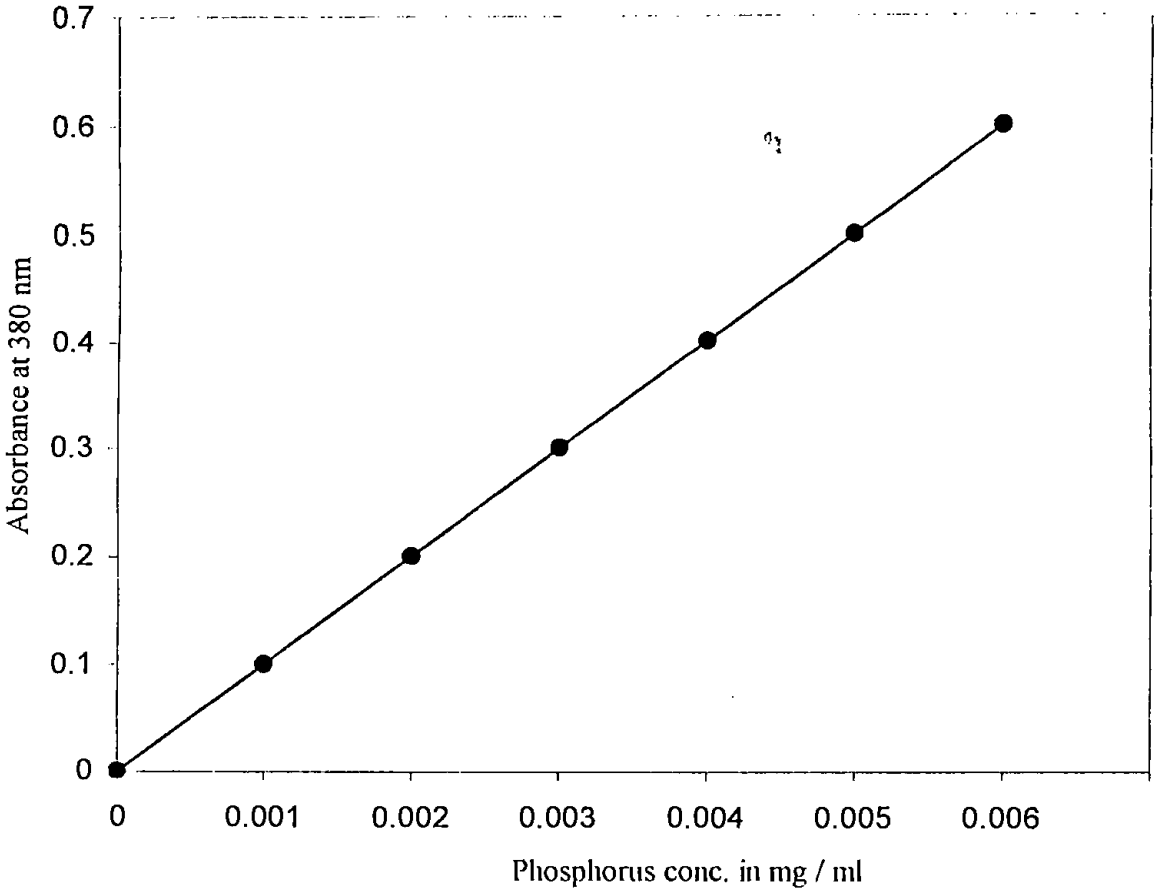


Figure-3: Standard curve for phosphorus (P) estimation

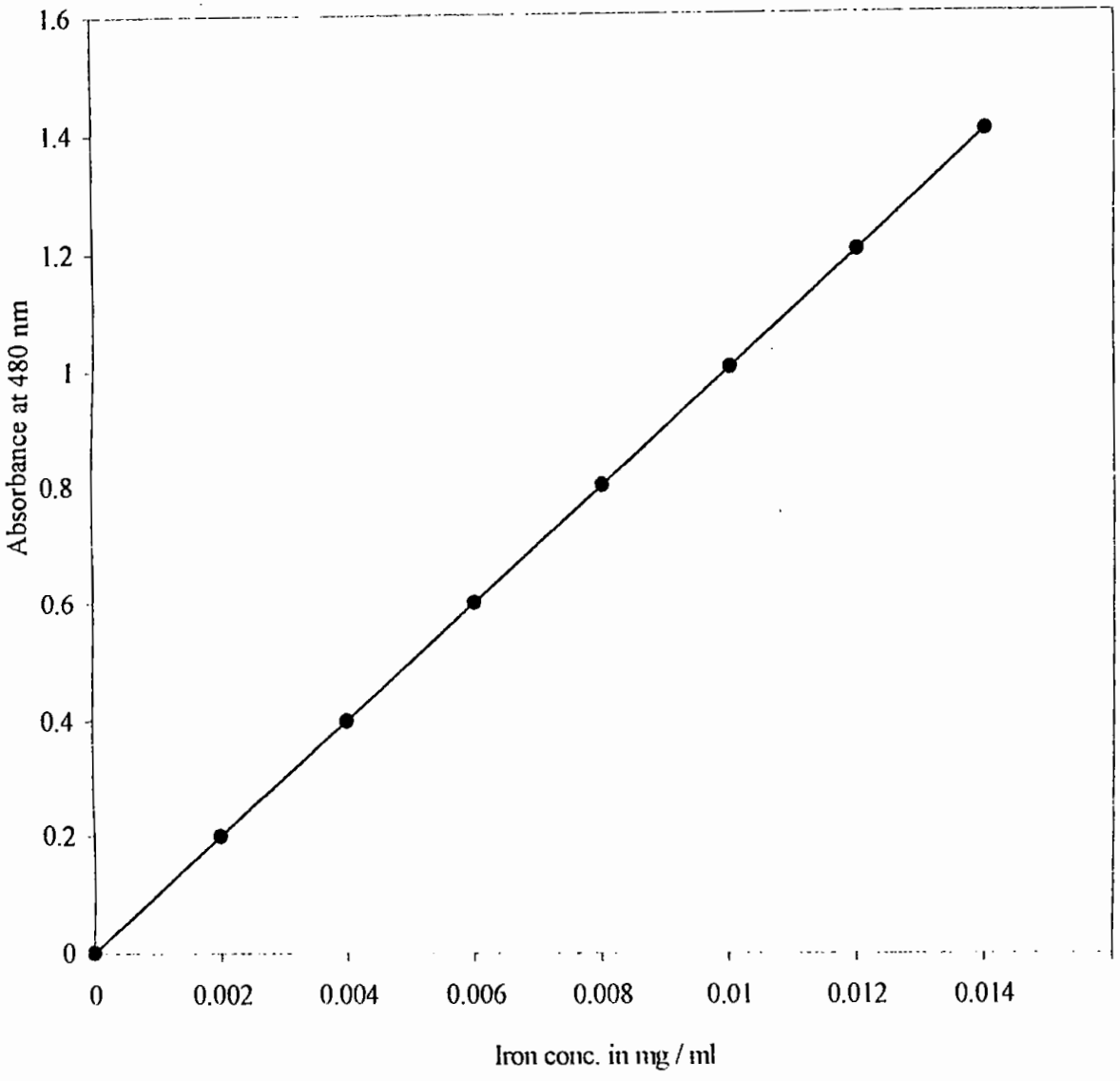


Figure-4: Standard curve for Iron (Fe) estimation.

Determination of B-vitamins content of hilsa fish

Vitamin B_1:

Vitamin B_1 content of hilsa fish was determined following the method of Anon, 1965 (119).

Reagents:

a) Potassium ferricyanate (2%)

b) Oxidizing reagents: 10 ml of 2% potassium ferricyanate was mixed with 3.5 N NaOH solution (90 ml). This solution might be used within 4 hours.

c) 0.02 N HCl

d) Alcohol

Preparation of standard thiamine hydrochloride solution:

About 25 mg of thiamine hydrochloride was transferred in 1000 ml volumetric flask and it was dissolved in 300 ml of dilute alcohol solution. The pH was adjusted to 4.0 with dilute HCl and stored in a light resistant container.

Procedure:

Fresh hilsa fish (5 g) was homogenized with 0.02 N HCl. The mixture was heated on a steam bath, then cooled and diluted. Standard thiamine-HCl solution (5 ml) was mixed rapidly with 3 ml of oxidizing reagent and 20 ml of isobutyl alcohol was added within 30 seconds, then mixed the mixture vigorously for 90 seconds by shaking the tubes manually. A blank was prepared only by substituting the oxidizing reagent with an equal volume of

3.5 N sodium hydroxide and proceeded in the same manner. 2 ml of dehydrated alcohol drawn off and transferred into cuvettes, then measured the fluorescence. Pipetted 5 ml of hilsa fish extract in different test tubes and treated in the same manner as described above.

Calculation:

The amount of mg of thiamine hydrochloride in each 5 ml of the fish extract was calculated from the formula (A-b)/ (S-d), in which A and S were the average fluorometer reading of the portions of hilsa fish extract and standard preparation treated with oxidizing reagent respectively, and b and d were the reading for the blanks of fish extract and standard preparation respectively.

Percent of vitamin B_1 (mg per 100 g of fresh hilsa fish)

$$= \frac{\text{mg of vitamin } B_1}{\text{Weight of hilsa fish}} \times 100$$

Vitamin B_2 or Riboflavin:

Vitamin B_2 content of hilsa fish was determined by the method of Anon 1965 (119).

Reagents:

a) 0.02 N acetic acid
b) 0.1N H_2SO_4
c) 0.1N NaOH
d) 0.1N HCl
e) 4% Potassium permanganate
f) Hydrogen peroxide

Procedure:

Standard preparation of 50 mg of riboflavin was mixed with 300 ml of 0.02 N acetic acid and the mixture was heated on a steam bath with frequent agitation until the riboflavin was dissolved. Then the solution was cooled

and made up to 500 ml with 0.02 N acetic acid. This solution was diluted appropriately with 0.02 N acetic acid to made final riboflavin concentration of 10 µg/ml.

Extraction of riboflavin from hilsa fish:

Fresh hilsa fish (5 g) was homogenized well with 0.1 N H_2SO_4 (about 50 ml). The mixture was heated in an autoclave at 121-123^{0}C for 30 minutes, then cooled it and filtered through double layer of muslin cloth. The filtrate was made upto 100 ml with distilled water. Then 25 ml of this solution was taken in a beaker and 25 ml of water was added to it. The mixture was agitated vigorously and adjusted the pH to 6.0 ~ 6.5 with 0.1 N NaOH. Immediately, 0.1 N HCl was added until no precipitation occurs. The extract was again filtered and pH of the extract was adjusted to 6.6 ~ 6.8 with 0.1 N NaOH.

Hilsa fish extract (10 ml) was taken in the test tube, 1 ml of water and 1.0 ml of glacial acetic acid were added to it. The mixture was then mixed with 0.05 ml of potassium permanganate solution, allowed to stand for two minutes and 0.5 ml of hydrogen peroxide solution were added, where upon the permanganate colour was destroyed within 10 seconds. The tube was shaken vigorously until excess oxygen expelled. Then 1 ml of standard riboflavin solution was pipetted in a test tube and treated in the same manner as that described for fish extract. In a suitable fluorometer, the fluorescence of solutions were measured. Then 20 mg of sodium hydrosulfite was added to each tube, mixed well and measured the fluorescence within 5 seconds.

Calculation:

The quantity in mg in each ml of the hilsa fish extract was calculated by the formula

$$0.0001\ (I_u - I_B)\ (I_S - I_u).$$

Where I_u = Average reading for hilsa fish extract

I_S = Average reading for standard preparation

I_B = Average reading after mixing with sodium hydrosulfite

Percentage of vitamin B_2 content in hilsa fish (mg per 100 g of hilsa fish).

$$= \frac{\text{mg of vitamin } B_2 \text{ obtained}}{\text{Weight of hilsa fish}} \times 100$$

STUDY OF THE PHYSICAL CHARACTERISTICS OF OIL

Determination of specific gravity of the oil

The specific gravity (Sp. Gr.) of the oil was determined by means of a specific gravity bottle using the formula (120).

$$\text{Specific gravity} = \frac{\text{Weight of oil in bottle}}{\text{Weight of the distilled water in bottle}}$$

Apparatus:

a. Specific gravity bottle

b. Water bath

c. Electrical weighing balance

Procedure:

The specific gravity of the oil was determined by means of a specific gravity bottle. The specific gravity bottle was cleaned, dried and weighed. The bottle was filled with distilled water which was previously boiled to room temperature to avoid the formation of bubbles. It was then immersed in a constant water bath at 25^0C with its stoppered end just above the level of the bath for half an hour. The bottle was removed from the bath, wiped dry with tissue paper and allowed to stand for 15 minutes and weighed. The procedure was repeated by replacing water with oil. The specific gravity was then calculated using the formula as given above.

Determination of refractive index of the oil

Refractive index of a medium is the ratio of the speed of light at a definite wavelength in vacuum to its speed in the media.

Apparatus:

(i) Abbe refractometer (standard model 60/70)

(ii) Thermostat.

Procedure:

Refractive index of the oil was determined following the Hilditchs (121) procedure. The hinged prisms of refractometer were opened and cleaned with little ether and tissue paper. Two to three drops of oil was placed on the face of the prism. The prism was then closed and the apparatus left for 2 to 3 minutes to attain equilibrium temperature. The reading was directly measured by rotating the focus telescope until the line of total refraction passed though the intersection of the two hairlines fixed in the field of view.

STUDY OF THE CHEMICAL CHARACTERISTICS OF OIL:

Determination of iodine value of the oil

Iodine value (I.V.) of the oil was determined by Hanus method (122) using the formula

$$\text{I.V.} = \frac{S \times (X - Y) \times 0.127}{W} \times 100$$

Where,

S = Strength of the sodium thiosulphate

X = ml of thiosulphate required in the blank experiment

Y = ml of thiosulphate required in the test experiment

W = wt. of oil

Apparatus:

(i) Wide necked grams bottle with ground stopper

(i) Wide necked grams bottle with ground stopper

(ii) Burette, pipette

Reagents:

a. Chloroform analytical reagent grade

b. Glacial acetic acid, free from ethanol

c. Potassium iodide solution, 15%

d. Bromine

e. Sodium thiosulphate solution, standardized before use

f. Starch indicator: Starch (1 g) was dissolved in glacial (12 ml) acetic acid. It was warmed to dissolve the iodine completely. The mixture was cooled at room temperature. Then enough bromine was added to double the halogen content, usually about 3 ml was sufficient.

Procedure:

The oil (2 g) was dissolved in 10 ml of chloroform in a dry glass stoppered bottle (500 ml). To the content, 25 ml of Hanus solution was added and the mixture was allowed to stand in the dark for exactly 30 minutes with occasional shaking. Potassium iodide solution (10 ml) was mixed to it and the mixture was shaken well. Degassed water (100 ml) was added to the mixture and the content of the bottle was titrated with sodium thiosulphate solution using starch solution as an indicator.

A blank experiment was performed exactly in the same manner without the oil.

Determination of saponification value and saponification equivalent

Saponification value (S.V) is the number of mg of KOH required to saponify 1 g of oil. The saponification value was determined (123) using the following formula

$$S.V. = \frac{56.1 \times (A - B) \times \text{Strength of acid used}}{W}$$

Where,

A= number of ml of acid required for blank experiment

B= number of ml of acid required for test experiment

W= wt. of the oil

Apparatus:

(i) Round bottle flask

(ii) Reflux condenser

(iii) Pipette and burette

(iv) Water bath

Reagents:

(i) Alcoholic solution of potassium hydroxide (0.5N approximately)

(ii) 0.5 N hydrochloric acid

(iii) Phenolphthalein solution (1% in alcohol)

Procedure:

The oil (1-2 g) was taken in a conical flask and 25 ml alcoholic hydroxide solution was added to it. The flask was then connected to a reflex condenser and heated on a boiling water bath so that the alcoholic solution boiled gently for 30 minutes. During this time the flask with its content was shaken occasionally to prevent agitation. A blank experiment (without oil) was performed simultaneously in the same manner as described. After 30

minutes, both the flasks were removed from the water bath and their contents, while still hot were titrated by hydrochloric acid (0.5 N) using phenolphthalein as indicator.

Saponification equivalent (S.E.) (124) was calculated from the saponification value using formula

$$S.E. = \frac{56100}{S.V.}$$

Determination of peroxide value of the oil

Peroxide value of (P. V.) is expressed in terms of milli equivalent of active oxygen per kg of oil. Peroxide value of the oil was determined (125) using the following formula.

$$\text{Peroxide value} = \frac{(S - B) \times N}{W} \times 1000$$

Where,

S = Titration with sample

B = Blank titration

N = Exact normality of thiosulphate

W = mass of test portion

Apparatus:

(i) Flask, glass stoppered

(ii) Burette

Reagents:

(i) Acetic acid- chloroform solution.

(ii) Potassium iodide, saturated aqueous solution, recently prepared, free from iodine and iodides.

(iii) Sodium thiosulphate, accurately standardized aqueous solution, standardized just before use.

(iv) Starch solution- 1 g starch was dissolved in 50 ml hot water and diluted to 100 ml with cold water.

Procedure:

The oil (0.5–1.0 g) was taken in a 250 ml stoppered flask and 30 ml of acetic acid-chloroform solution was added to it. The oil was mixed well and 1 ml of saturated potassium iodide solution was added. The stopper was inserted quickly and the mixture was shaken for 1 minute. It was allowed to stand for 5 minutes away from light at a temperature from 15-25^{0}C. About 75 ml of distilled water was added to the mixture. The liberated iodine was titrated with standardized sodium thiosulphate solution by shaking vigorously, using starch solution as indicator. A blank test was carried out simultaneously in the same manner as described above.

Determination of acid value and % FFA (as oleic) of the oil

The percentage of free fatty acids (%FFA) was measured (126) using the following formula (as oleic).

$$\%\ \text{FFA (as oleic)} = \frac{V \times S \times 28.2}{W}$$

Where,

V = ml of alkali required to neutralize

S = Strength of alkali

W = Weight of the oil

Apparatus:

(i) Conical flask

(ii) Burette and pipette

Reagents:

(i) Solvent mixture (1:1) of 95% (V/V) ethanol and diethyl ether. Neutralized exactly by means of KOH solution in presence of phenolphthalein indicator.

(ii) Potassium hydroxide solution (0.1N) accurately standardized

(iii) Phenolphthalein indicator in 1% ethanol

Procedure:

The oil (0.5-1.0 g) was taken in a conical flask. The oil was dissolved in the neutralized solvent mixture of ethanol and diethyl ether (1:1 V/V), 2-3 drops of phenolphthalein solution was added and shaken well. The mixture was titrated by shaking with the standardized potassium hydroxide solution until the pink colour just appear which indicate the end point of the titration.

Determination of acid value:

It is the number of mg of KOH required to neutralized the free fatty acids present in 1 g of oil. This is used for determining the rancidity due to free fatty acids.

Reagents:

Same as used in the determination of % FFA .

Procedure:

Acid value (A.V.) was determined using the formula (127).

$$A.V. = \frac{V \times S \times 56.1}{W}$$

Where,

V = ml of alkali required to neutralize

S = Strength of alkali

W = Wt. of the oil

The procedure was the same as described above for % FFA.

Determination of the quantity of unsaponifiable matter of the oil

The unsaponifiable matter is a fraction of fat or oil that remain insoluble after saponification of the fat sample by alkali. The unsaponifiable matter includes the sterols, higher alcohol's, pigment and hydrocarbons.

Apparatus:

(i) Round bottom flask (ground joint)

(ii) Reflux condenser

(iii) Desiccator

(iv) Separating funnel

(v) Water bath

(vi) Oven, regulated at $105^0 \pm 2^0C$

Reagents:

(i) Diethyl ether, analytical reagent grade

(ii) Acetone, analytical reagent grade

(iii) Alcoholic solution of potassium hydroxide (1 N approximately)

(iv) Aqueous solution of potassium hydroxide (0.5 N)

(v) Phenolphthalein indicator: 1% solution in ethanol

The amount of unsaponifiable matter present in the oil was determined using the method as described (128).

Procedure:

The oil (5 g) was taken in a round bottom flask and 1N alcoholic potassium hydroxide (50 ml) was added to it. The mixture was then refluxed for 45 minutes on a water bath at about 70^0C to 80^0C with occasional swirling.

The solution was transferred to a separating funnel and rinsed with 100 ml water. The solution while still perceptibly warm, extracted four times with ether using 50 ml each time. Each extraction was done by vigorous shaking in the separating funnel and allowing the two layer to be separated. The aqueous alcoholic layer at the bottom of the separating funnel was run off and the ethereal solution from the top was poured into another separating funnel containing 20 ml water. The total ether extracts in the separating funnel was washed thrice using 20 ml water each time.

The ethereal layer was then washed three times with 20 ml aqueous potassium hydroxide solution. Each alkali wash was followed by a wash with 20 ml water. After the aqueous alkali wash, the solution was washed with sufficient amount of water, till the washing did not show alkaline reaction to phenolphthalein solution.

The ether extract was then transferred into a weighed flask and the ether was evaporated. The residue was dried to constant weight at 80^0C. Adding 2-3 ml of acetone to the extract when nearly all the ether was evaporated assisted drying. The flask with its contents was then weighed. The quantity of

unsaponifiable matter present in the 100 gms of oil was calculated from the following formula

$$\text{Unsaponifiable matter (U.S.M.)} = \frac{\text{Weight of unsaponifiable matter}}{\text{Weight of oil taken}} \times 100$$

Storage of hilsa fish oil at different temperatures

The oil from different parts of fresh hilsa fish were placed in cellophane bags and stored in a temperature-controlled refrigerator (Lec Refrigerator Ltd. U.K.). After attaining the desired temperature i.e. -10^0C, 0^0C, the %FFAs of the oils contained in the fish samples were measured after each five days interval upto 120 days. For the storage at room temperature (25^0C- 28^0C), the fish samples were kept in the laboratory room (humidity, 60-65%) and the %FFAs were measured after each five days intervals upto 120 days.

RESULTS AND DISCUSSION:

Moisture: The moisture contents of different parts of hilsa fish were tabulated in Table-1. It was found that the moisture contents in different parts of hilsa fish were varied from 13.00% to 54.87%. Dorsal part of the hilsa fish contained the higher percentage of moisture (54.87%) whereas brain contained the lower percentage of moisture (13.00%).

Ash: As given in the Table-1, the ash content of the different parts of hilsa fish were found to be ranged between 1.7% to 3.8%.

Ash content was also found to be maximum in the dorsal part (3.8%) and minimum in the brain (1.7%).

Table-1: Moisture and Ash content of the different parts of hilsa fish

Name of the parts	Moisture (%)	Ash (%)
Dorsal	54.87 ± 0.13	3.80 ± 0.03
Ventral	51.82 ± 0.12	2.92 ± 0.02
Tail	50.82 ± 0.11	3.70 ± 0.03
Egg	19.06 ± 0.09	2.20 ± 0.02
Liver	38.50 ± 0.10	2.50 ± 0.02
Brain	13.00 ± 0.09	1.70 ± 0.01

Oil content of the different parts of hilsa fish:

The oil content of the different parts of hilsa fish were extracted with soxhlet apparatus using n-hexane as an extracting solvent and it was found to be ranged between 9.61% to 22.78% (Table-2). The ventral portion contained the highest amount of oil (22.78%) while tail contained the lowest amount (9.61%).

Table-2: Oil content of the different parts of hilsa fish

Name of the parts	Amount of oil (%)
Dorsal	14.67 ± 0.09
Ventral	22.78 ± 0.10
Tail	9.61 ± 0.07
Egg	14.40 ± 0.06
Liver	21.92 ± 0.05
Brain	12.70 ± 0.05

Protein content of the different parts of hilsa fish:

Fish muscle is a rich source of protein. Fish proteins occupy an important place in human nutrition. They have high digestibility, biological value and growth promoting value. They are well balanced with respect to essential amino acids. The amounts of protein present in the different parts of hilsa fish are shown in Table-3.

Table-3: Protein content of the different parts of hilsa fish

Name of the parts	Amount of protein (%)	
	Micro- Kjeldahl method	Lowry method
Dorsal	23.18 ± 0.11	20.58 ± 0.10
Ventral	9.83 ± 0.03	8.72 ± 0.03
Tail	19.40 ± 0.09	16.36 ± 0.06
Egg	7.10 ± 0.05	4.87 ± 0.03
Liver	6.04 ± 0.04	3.95 ± 0.02
Brain	5.50 ± 0.03	N. D

As found, in Micro- Kjeldahl method the protein content of the different parts of hilsa fish were ranged from 5.5% to 23.18%. Among the parts examined, dorsal part contained the highest amounts of protein (23.18%) whereas brain contained the lowest amount (5.5%). In Lowry method the range was 20.58% (dorsal) to 3.95% (liver).

Finding of the present results suggested that hilsa fish might be used as a potential source of protein.

Determination of polysaccharide and free sugar content of the different parts of hilsa fish:

The analytical data of the polysaccharide and free sugar contents of the different parts of hilsa fish are given in Table-4. Fish flesh contains

negligible quantities of carbohydrates. Glycogen is present in living fish and is rapidly converted to lactic acid after death. The amount of polysaccharide present in hilsa fish samples were varied from 0.03 % to 0.065 %. Liver contained the highest amount of polysaccharide (0.065 %) whereas tail contained the lowest amount (0.03 %).

The amount of free sugar present in different parts of hilsa fish were 0.053 % to 0.09 %. From the results it might be concluded that each parts of hilsa fish contained slightly higher amount of free sugar than the polysaccharide.

Table-4: Polysaccharide and free sugar content of the different parts of hilsa fish

Name of the parts	Polysaccharide (%)	Free sugar (%)
Dorsal	0.04 ± 0.003	0.065 ± 0.003
Ventral	0.06 ± 0.002	0.08 ± 0.005
Tail	0.03 ± 0.002	0.053 ± 0.003
Egg	0.045 ± 0.003	0.074 ± 0.005
Liver	0.065 ± 0.003	0.09 ± 0.004
Brain	0.035 ± 0.001	0.055 ± 0.002

Mineral content of the different parts of hilsa fish:

Calcium: Calcium is essential for formation of bone and teeth. The blood clotting processes require calcium and it is directly related to muscle contraction. A number of enzymes are also activated by calcium.

The amount of calcium present in the different parts of hilsa fish was presented in the Table-5, and its contents was found to be varied from 160 mg% to 180 mg%.

As described in the Table-5, the tail contained the highest amount of calcium (180 mg%) whereas the brain contained the lowest amount (160 mg%).

Phosphorus: Phosphorus is essential for acid base regulation, bone and teeth formation. Phosphorus is also used in the form of phosphate, in the synthesis of phospholipids, constituents of cell membrane etc.

The amount of phosphorus present in the different parts of hilsa fish was presented in Table-5. The phosphorus content of hilsa fish was found to be ranged from 259 mg% to 278 mg%. Tail contained the maximum amount of phosphorus (278 mg%) while liver contained the lowest amount (259 mg%).

Table-5: Mineral content of the different parts of hilsa fish:

Name of the parts	Calcium (mg %)	Phosphorus (mg %)	Iron (mg%)
Dorsal	178 ± 0.56	275 ± 0.74	199 ± 0.39
Ventral	174 ± 0.52	272 ± 0.68	201 ± 0.45
Tail	180 ± 0.61	278 ± 0.59	205 ± 0.42
Egg	170 ± 0.49	269 ± 0.54	210 ± 0.58
Liver	172 ± 0.51	259 ± 0.48	212 ± 0.62
Brain	160 ± 0.42	260 ± 0.34	202 ± 0.56

Iron: The primary function of iron is to form hemoglobin and for the formation and maturation of red cells. It carries oxygen in blood in the form of hemoglobin. Myoglobin is an iron containing chromoprotein. Like hemoglobin which combines with oxygen and acts as an oxygen store for muscle.

As cited in the Table-5, it was found that iron content of different parts of hilsa fish were almost same and ranged from 199 mg% to 212 mg%.

Vitamin contents of the different parts of hilsa fish :

Vitamin B_1 (Thiamine):

Thiamine is practically present in all plants and animal tissues commonly used as food. Thiamine in the form of pyrophosphate (TPP), the active form of the vitamin, which is involved in oxidative decarboxylation of certain intermediates in carbohydrate metamolism. Besides the metabolic role, thiamine has a specific role in neurophysiology, dependent of its co-enzyme function. Thiamine is essential for growth, normal appetite, digestion and healthy nerves.

Table-6 shows the analytical values of vitamin B_1 content of the different parts of hilsa fish. As shown in the Table, vitamin B_1 content was ranged from 0.35-0.48 mg%. The egg contained the maximum percentage of vitamin B_1 (0.48 mg%) followed by the liver and so on.

Riboflavin (Vitamin B_2):

Vitamin B_2, a crystalline pigment, is the principal growth-promoting factor of the vitamin B complex. It functions as a flavoprotein in tissue respiration. Riboflavin may be used as medicine, animal feed supplement, enriched flours, dietary supplement etc. It functions as a coenzyme for many flavin enzymes.

Table-6 shows the analytical values of vitamin B_2 content of the different parts of hilsa fish. Vitamin B_2 content was found to be ranged between 2.44-2.98 mg%. Of the different parts, egg contained the highest amount (2.98 mg%) while liver contained the lowest amount (2.44 mg %).

Table-6: Vitamin contents of the different parts of hilsa fish

Name of the parts	Vitamin B_1(mg %)	Vitamin B_2(mg %)
Dorsal	0.40 ± 0.02	2.50 ± 0.06
Ventral	0.44 ± 0.02	2.91 ± 0.05
Tail	0.43 ± 0.01	2.65 ± 0.03
Egg	0.48 ± 0.006	2.98 ± 0.02
Liver	0.35 ± 0.01	2.44 ± 0.03
Brain	0.40 ± 0.03	2.56 ± 0.01

CHARACTERIZATION OF THE HILSA FISH OIL:

The physical and chemical characteristics of hilsa fish oils from different parts are given in the Table-7 and 8.

Physical characteristics of hilsa fish oil:

Specific gravity (Sp. Gr.):

The specific gravity of fats or oils does not vary as general rule to an extent, which makes this property useful in discriminating between one fat and another. The specific gravity of particularly all fats or oils lies between 0.9 to 0.9544. As shown in the Table-7, the specific gravity of the hilsa fish oil presently examined from the different parts were varied from 0.920 to 0.932 at 25^0C. The specific gravity of the oil from brain gave the maximum value (0.932) while that from the dorsal and ventral gave the minimum value (0.920). The values obtained in the present studies are quite similar to that reported for rice bran oil (0.916-0.92) by Mattil (129).

Refractive index (R. I.):

The refractive power of oils or fats varies somewhat widely and is chiefly governed by the proportion and degree of unsaturated matter present. It was found that the R.I. of the oils from the different parts of hilsa fish varied from 1.4700 to 1.4722 at 25^0C (Table-7). The hilsa fish oil from dorsal and tail gave the maximum value (1.4722) while that from egg and liver gave the minimum value (1.4700). For comparison, it may be mentioned that the refractive index of Brassica, Linseed oil, Sesame oil, Sunflower oil and Olive oil are 1.470, 1.478, 1.475, 1.466 and 1.466 respectively (130, 131).

The present value of this investigation is quite similar to that of the reported values.

Table-7: Physical characteristics of hilsa fish oil extracted from different parts

Name of the parts	Specific gravity (25° C)	Refractive index (25°C)
Dorsal	0.920	1.4722
Ventral	0.920	1.4720
Tail	0.922	1.4722
Egg	0.926	1.4700
Liver	0.924	1.4700
Brain	0.932	1.4710

CHEMICAL CHARACTERISTICS OF HILSA FISH OIL:

Saponification value (S.V.) and Saponification equivalent (S.E.):

As shown in the Table-8, S.V. of the hilsa fish oils from different parts were found to be ranged from 180.28 to 194.00. It was found that the oil from dorsal and ventral gave the maximum value (194.00) while that from egg gave the minimum value (180.28). This result is found to be very similar to that reported for rice bran oil by Murti *et. al* (132).

Of the oils examined, the oil from egg gave the maximum S.E. (311.18) followed by that from dorsal, ventral (289.17) and so on in decreasing order.

Iodine value: Iodine value (I.V.) is defined as grams of iodine absorbed by 100 g of fat. As shown in the Table-8, the iodine values of hilsa fish oils

from different parts were ranged between 80.70 to 126.40. As found the oil from liver gave the maximum value (126.40) while that from brain gave the minimum value (80.70). Some vegetables oil namely Olive oil, Linseed oil, Sunflower oil and Cotton seed oil have the iodine values of 80-85, 175-200, 125-141 and 102-114 respectively. The present values of this investigation are quite similar to that of the reported values.

Peroxide value (P.V.):

The peroxide value is the milli equivalent of peroxide oxygen combined in a kilogram of oil. As described in the Table-8, the peroxide values of hilsa fish oils from different parts were ranged between 7 to 10.

Acid value and % of FFA:

Acid value (A.V.) is a measure of the hydrolysis that has occurred in a fat and is defined as the number of milligrams of potassium hydroxide required to neutralized the free fatty acids in 1 g of oil or fat.

In the present investigation, the acid value and the % FFA of hilsa fish oils from different parts were estimated in normal laboratory conditions and presented in Table-8. The acid values of hilsa fish oils from different parts were found to be varied from 4.16 to 12.00 and % FFA from 2.08 to 6.00. The oils from the liver have higher acid value and % FFA than those of oils from any other parts.

Unsaponifiable matter:

The unsaponifiable matter of oil represents the presence of a mixture of several alcohols. All fish oils contained cholesterol; other alcohols in the unsaponifiable matter include pigments, vitamin A, and D, glycerol ethers

(Salachyl, chimyl and batyl alcohols), and fatty alcohols. Vitamin E is also present in small amounts.

The unsaponifiable matters present in the presently examined hilsa fish oils from different parts were varied from 1.58% to 7.00%. Highest amount of unsaponifiable matter is present in the oil from brain and lowest amount is present in that from ventral.

Table-8: Chemical characteristics of hilsa fish oils extracted from different parts

Constants	Name of the parts					
	Dorsal	Ventral	Tail	Egg	Liver	Brain
Saponification value	194.00	194.00	192.00	180.28	180.76	182.24
Saponification equivalent	289.17	289.17	292.19	311.18	310.36	307.84
Iodine value	101.30	102.0	101.40	100.22	126.40	80.70
Peroxide value m. eq. O_2/Kg oil	8.00	8.60	8.00	9.28	10.00	7.00
Acid value	4.28	4.16	4.30	7.16	12.00	8.72
%FFA(as oleic)	2.14	2.08	2.15	3.58	6.00	4.36
Unsaponifiable matter (%)	1.66	1.58	1.82	4.60	3.72	7.00

Effect of storage on the quality of hilsa fish oil at different temperature:

The conversion of fatty oils (hilsa fish oil) isolated from different parts of hilsa fish into fatty acids by the action of lipase have been studied in the present investigation after keeping the samples at low temperature (-10^{0}C, 0^{0}C) and at room temperature (25^{0}C-28^{0}C).

Figure-5 shows the hydrolytic deterioration of different parts of hilsa fish oils at low temperature (-10^{0}C). It was found that the contents of %FFA in the oils obtained from different parts of the fish samples were initially low but increased rapidly on storage. After storage of 120 days at -10^{0}C, the oils from liver, brain, egg, ventral, dorsal and tail contained 45.82, 44.16, 42.61, 39.87, 35.88 and 30.84 %FFA respectively. From this finding, it was concluded that the lipase enzyme in hilsa fish oil is active even at temperature -10^{0}C.

Figure-6 shows the hydrolytic deterioration of hilsa fish oils from different parts at low temperature (0^{0}C). It was found that the hydrolytic deterioration of hilsa fish oils were more effective at 0^{0}C than that from -10^{0}C, which might be due to higher activity of lipase at 0^{0}C than -10^{0}C. After storage of 120 days at 0^{0}C, the hilsa fish oils from liver, brain, egg, ventral, dorsal and tail were found to be contained 63.46, 59.51, 54.00, 46.93, 44.91 and 41.00 %FFA respectively.

As shown in Figure-7, the quality of hilsa fish oils were deteriorated slightly further at 25^{0}C and after 120 days of storage at 25^{0}C, the oils from liver,

brain, egg, ventral, dorsal and tail were found to be contained 66.60, 62.00, 56.72, 51.40, 47.20 and 43.15 %FFA respectively.

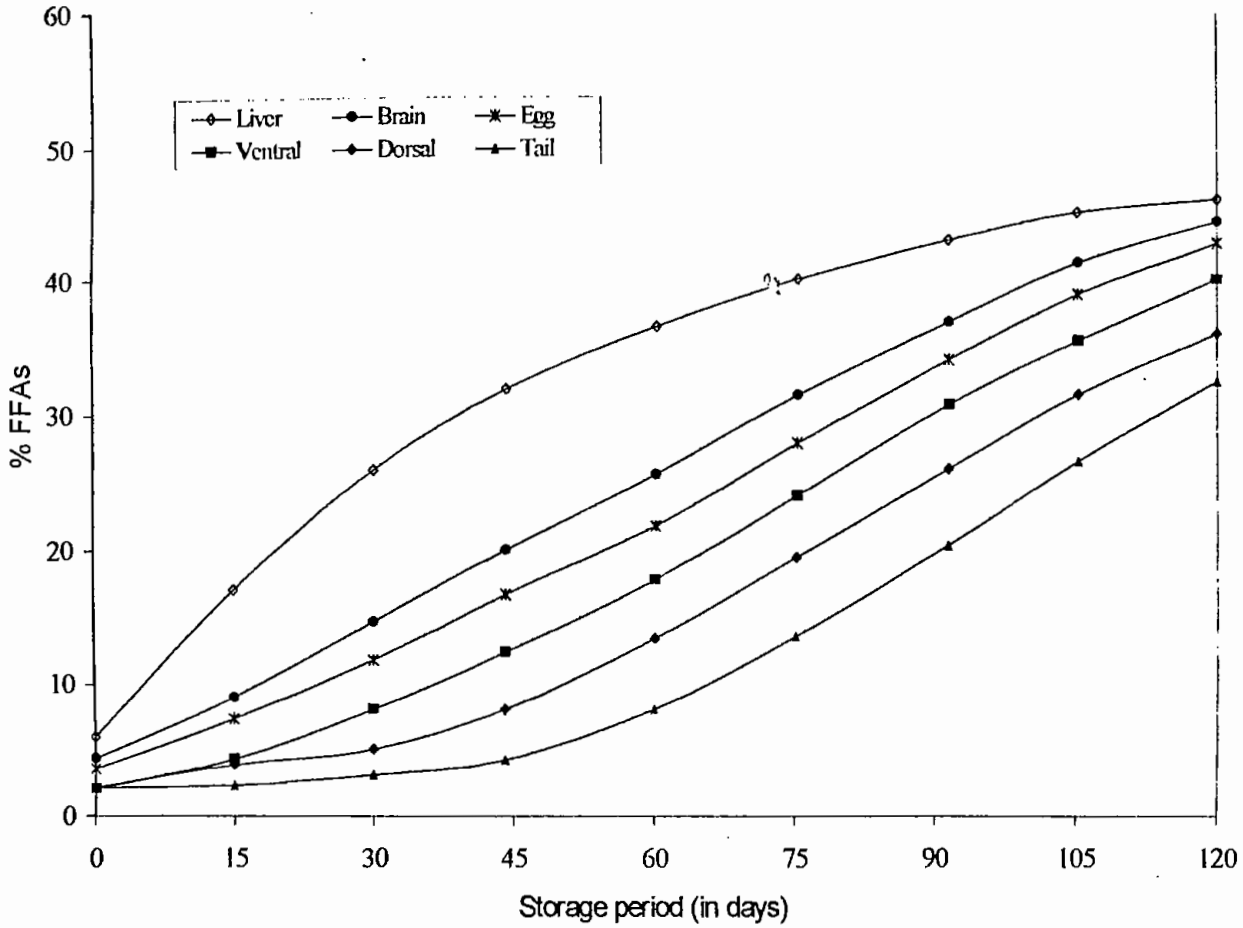


Figure-5: Changes in %FFA content after storage of hilsa fish oil at very low temperature (-10° C)

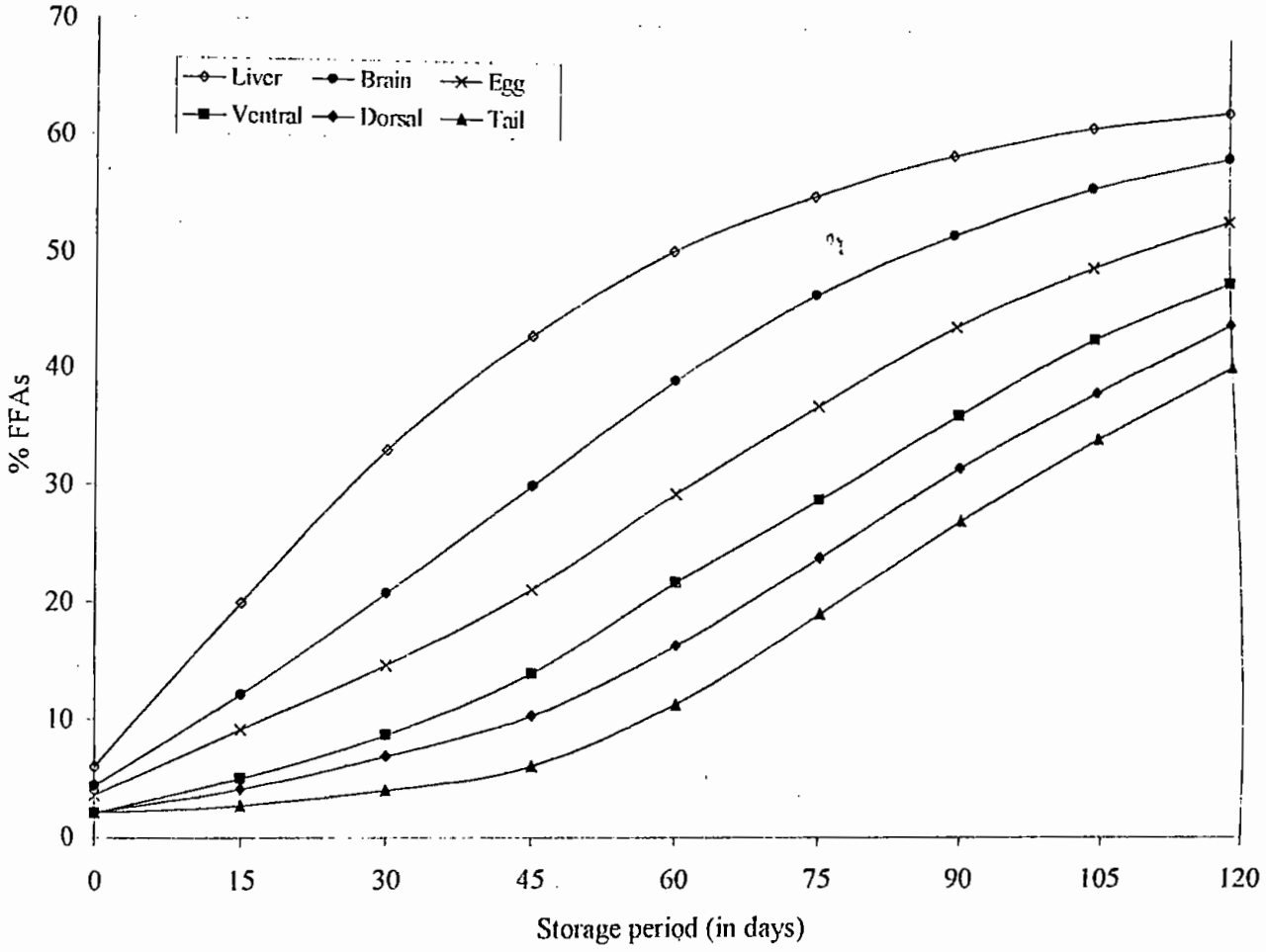


Figure-6: Changes in %FFA content after storage of hilsa fish oil at low temperature (0°C)

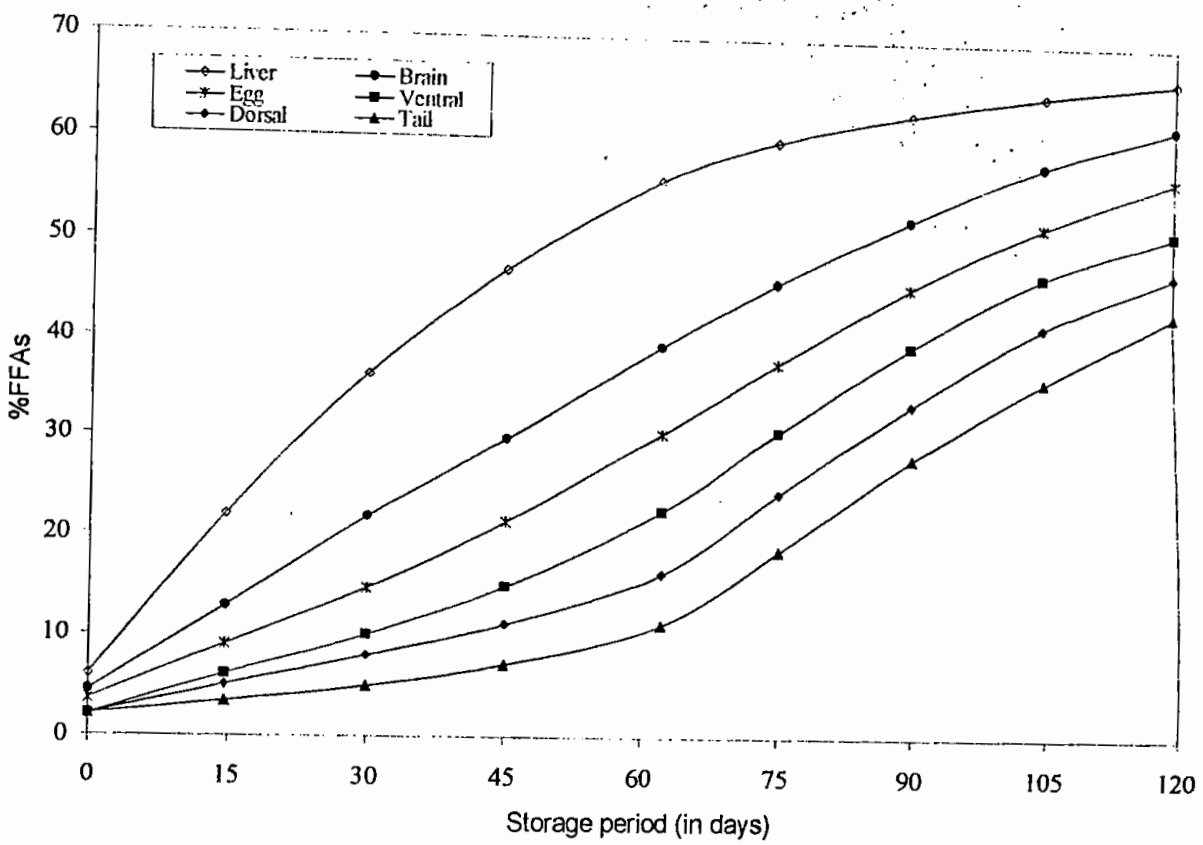


Figure-7: Changes in %FFA content after storage of hilsa fish oil at room temperature (25°C- 28°C)

CHAPTER - 4

EXTRACTION AND PURIFICATION OF LIPASES FROM DORSAL PART OF HILSA FISH

INTRODUCTION

Lipases are widely distributed in various animals, plants and microorganisms (133). During recent years considerable attention has been devoted to lipases of botanical origin and microorganisms (134-138). Several lipases of animal origin have been purified and their chemical and enzymatic properties were elucidated in details (139, 140). Lipases have been purified from several different sources by using several different methodologies (141-145).

Most of the purification procedures for lipases reported involve series of non-specific techniques, such as ammonium sulphate precipitation, gel filtration and ion-exchange chromatography. In recent years, affinity chromatographic techniques have become frequently used, decreasing the number of steps necessary for lipase purification.

Most recently, reversed-micellar and aqueous two phase systems, ultrafiltration membranes and immunopurification have also been applied to purify some lipases, mainly of microbial origin. In the present study two lipases were purified from dorsal portion of hilsa fish using ion exchange chromatography on DEAE- and CM- cellulose followed by gel filtration on Sephadex G- 50.

MATERIALS:

Dorsal part of hilsa fish was used for purification of lipase as it contained the highest enzymatic activity.

METHODS:

Preparation of Acetone Powder of Hilsa Fish:

The hilsa fish (623 g) was taken in a mortar and grinded well with pestle to make paste. Then this paste was homogenized well with twice volume of ice cold acetone. The suspension was filtered through double layer of coarse cloth and quickly washed with successive portion of acetone, acetone-ether (1:1, v/v), diethyl ether and then air dried. The dry powder is called the acetone powder which can be stored in a refrigerator for a long time (146).

Preparation of Crude Extract:

Immediately before use the dry acetone powder (160 g) was suspended in (160X4=640ml) of ice cold water. After occasional gentle stirring for three hours at 4°C, the suspension was filtered through coarse cloth. The filtrate was collected and centrifuged in a refrigerated centrifuge at 5000 g for 15 minutes. The supernatant was used as "crude extract".

Ammonium Sulphate Fractionation:

The crude extract was saturated to 85% by addition of solid ammonium sulphate under constant and gentle stirring at 4°C (0.65 g ammonium sulphate was required per ml of crude extract). The resulting precipitate was collected by centrifugation (6000 g, 10 minutes), dissolved in minimum volume of pre-cooled distilled water and dialyzed against distilled water for 24 hours at 4°C. The dialyzed solution was then centrifuged at 6000 g for 10 minutes to remove the insoluble materials. The clear supernatant thus obtained was designated as "crude enzyme solution".

DEAE- CELLULOSE CHROMATOGRAPHY:

Procedure:

(a) **Activation of DEAE- Cellulose Powder**: The DEAE-cellulose powder was suspended in 0.2 M HCl in a beaker and left to swell for a few hours. During swelling it was stirred at short intervals to prevent formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water for several times until its pH reached near to neutrality. The suspension was then transferred to another beaker containing 0.2 M NaOH and left for a few hours with slow stirring. It was again washed with distilled water to reach at neutral pH.

(b) **Packing the Column**: A column of desired length was packed in a proper way. If the column is not packed properly, accurate result can never be expected, because improperly packed column gives rise to uneven flow rates and resolution is also lost. Activated DEAE-cellulose suspension was taken in a filtering flask and deaerated by vacuum pump, otherwise it would affect the flow rate of the column after packing. The column was mounted in a laboratory stand and its narrow end was fitted with an outlet tube. It was ensured that there was no bubble in the dead space of bed support. This was easily achieved by filling approximately $1/4^{th}$ of the column including the outlet tube with distilled water. When the dead space was properly filled, the outlet tube was close by regulator and the gel suspension from the gel reservoir was added gently to the column. In order to avoid trapping of any bubble the gel suspension was

poured to the inner wall of the column. In this way, a column of desired length was packed uniformly.

(c) **Equilibration of the Column**: After completion of the column packing, it was equilibrated with 10 mM Tris-HCl buffer, pH 8.4. That is, this buffer was passed through the column until the pH of the eluate became equal with that of the eluant buffer.

(d) **Loading and Elution**: The column was placed in cold chamber at 4°C. Before loading the crude enzyme solution was dialyzed against 10 mM Tris-HCl buffer, pH 8.4 for 24 hours and the dialyzed sample (20-25 ml) was loaded onto the DEAE-cellulose column at 4°C. Now the buffer was passed through the column and the eluant was collected by fraction collector. The fraction collector was set in such way that 3 ml eluant was collected in each tube and the elution was carried out stepwisely with the buffer containing NaCl.

(e) **Detection**: The O.D (optical density or absorbance) of each of the eluant fractions was measured in UV spectrophotometer at 280 nm to detect the presence of protein. Concentration of the protein in each tube was also estimated by Lowry method (147) and the activity of lipase was also detected.

CM - CELLULOSE CHROMATOGRAPHY

Procedure:

(a) **Activation of CM-Cellulose Powder:** The CM- cellulose powder was suspended in 0.2 M NaOH in a beaker and left it to swell for a few hours. During swelling it was stirred gently at short intervals to prevent

formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water for several times until its pH reached near neutrality. This suspension was then transferred to another beaker containing 0.2 M HCl and left for a few hours. It was again washed with distilled water to neutralize its pH.

(b) **Packing the Column:** The activated CM-cellulose suspension was taken in a filtering flask and deaerated by vacuum pump. A column of desired length was packed with CM-cellulose suspension. Precaution was taken to avoid trapping of air bubbles during packing. After packing the column was equilibrated with 5 mM sodium phosphate buffer pH 6.5. The buffer was passed through the column until the pH of eluate became equal to that of eluant buffer.

(c) **Application of Sample and elution:** Before loading the sample obtained from DEAE cellulose chromatography was dialyzed against distilled water for 12 hours and against 5 mM sodium phosphate buffer pH 6.5 for 12 hours at 4°C. The dialyzed sample (15 ml) was loaded onto the CM-cellulose column at 4°C. Now the buffer was passed through the column and the eluant was collected by automatic fraction collector. The elution was performed stepwisely by the buffer containing NaCl.

(d) **Detection:** The O.D of each of the eluate fractions was measured in spectrophotometer at 280 nm wavelength to detect the presence of protein. The protein concentration in each tube was also estimated by the Lowry method and the activity of lipase was also detected.

GEL FILTRATION

Procedure:

Activation of the Gel Powder: Sephadex G-50 powder was suspended in acetic acid containing 1 M sodium chloride (1 mole of NaCl was dissolved in one litre of 10% acetic acid) in a beaker and left it to swell for overnight. Then it was transferred to a filtering funnel and washed with distilled water for several times until pH reached to neutrality.

(a) **Packing of the Column:** This is a very critical step. The column was packed in the same procedure as described earlier.

(b) **Equilibration of the Column:** After column packing it was equilibrated with 10 mM Tris-HCl buffer pH 8.4. The buffer was continued to run through the column until the pH of the eluate became same as that of the eluant buffer.

(e) **Application of the Sample:** The column was placed in cold chamber. Before loading the sample the outlet tube of the column was opened and the eluant buffer from the top of the gel bed was allowed to diffuse into the gel. The enzyme active fraction obtained by CM-cellulose chromatography was concentrated by using sucrose and then loaded on the top of the bed. After diffusion of the sample, about 1 ml of eluant buffer was poured on the top of the gel bed and allowed to diffuse. Then additional amount of buffer was added, so that the space about 3- 4 cm above the gel bed was filled with eluant. The buffer was then allowed to flow continuously through the column and 3 ml fractions of the eluate were collected by automatic fraction collector.

(f) **Detection:** Absorbances of eluate fractions were measured at 280 nm as well as protein concentration was estimated by the Lowry method. The activity of lipase was detected following the method of Sugihara *et al.*

TEST OF PURITY

SODIUM DODECYL SULPHATE- POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS- PAGE)

Slab gel electrophoresis in the presence of SDS separates proteins almost exclusively on the basis of mass, with smaller polypeptides migrating more rapidly. The protein's purity as well as their MW was determined by SDS-PAGE using 10% gel according to the method of Laemmli (148).

Reagents and Solutions:

1) **30% acrylamide solution:** 33.3 g of acrylamide and 0.9 gm of N, N-methylene bis acrylamide were dissolved in 70 ml of distilled water in a 100 ml beaker by heating in a hot water bath and the final volume was made upto 100 ml by adding distilled water.
2) **1.5 M Tris- HCl buffer (pH 8.8) (Separating gel buffer):** 18.7 g of Tris base was dissolved in 70 ml of distilled water in a 100 ml beaker. The pH of the solution was adjusted to 8.8 by adding 6N HCl drop by drop. The final volume was made 100 ml with distilled water.
3) **0.5 M Tris-HCl buffer (pH 6.8) (Stacking gel buffer):** 6 g of Tris base was dissolved in 70 ml of distilled water in a beaker. The pH of the

solution was adjusted to 6.8 by adding 6N HCl drop by drop. The final volume was made 100 ml with distilled water.

4) **10% SDS solution:** 10% SDS solution was prepared by dissolving 5 g of SDS in 40 ml distilled water in a beaker. Then final volume was made 50 ml with distilled water.

5) **10% ammonium per sulphate (APS) solution:** 10% APS solution was prepared by dissolving 0.5 g APS in 4 ml distilled water. The final volume was made 5 ml by distilled water. This solution should be prepared freshly.

6) **TEMED:** The commercially available preparation from Sigma Chemical Co. USA, was used without modification.

7) **4% SDS solution:** This is prepared by dissolving 2 g of SDS in 40 ml distilled water in a 50 ml beaker. The final volume was made 50 ml by distilled water.

8) **Bromophenol blue (BPB) solution:** This was prepared by mixing the following components and stored at 4°C.

Bromophenol blue	10 mg.
Glycerol	2 ml
Stacking gel buffer	0.2 ml
Distilled water	10 ml

9) **Sample solution:** Sample solution was prepared by mixing the following components and stored in refrigerator.

4% SDS solution	13 ml
Glycerol	5 ml

Stacking gel buffer	7 ml

10) **Electrophoretic buffer (Chamber buffer):** 6 g of Tris base, 28 g of glycine and 1 g of SDS were dissolved in distilled water making the final volume of 1000 ml and store in refrigerator.

11) **Staining solution:** This solution was prepared by mixing the following components-

Coomassie brilliant blue	1 g
Glacial acetic acid	15 ml
Ethanol	100 ml
Distilled water	85 ml

12) **Destaining solution–1:** This solution contains the following components-

Staining solution	10 ml
Ethanol	10 ml
Glacial acetic acid	10 ml
Distilled water	70 ml

13) **Destaining solution- 2:** This solution contains the following components-

Acetic acid	45 ml
Ethanol	30 ml
Distilled water	125 ml

14) **Preparation of sample:** 100 µl sample was mixed with 100 µl of sample solution in an eppendorf tube. Then the mixture was heated at 100°C for 3 minutes.

Procedure:

Clean and dry plates (7 cm X 10 cm) were assembled with a spacer (1.5 mm thick) and were hold together on a gel-casting stand. The assembly was checked for leakage.

a) **Preparation of separating or running gel for slab gel electrophoresis**: The following solutions were taken in a 50 ml conical flask. Then the flask was swirled gently for mixing the contents. To avoid instantaneous polymerization, the flask was kept in an ice bath. The solution was used immediately.

Components	Amounts
30% acrylamide solution	2.5 ml
Separating gel buffer	3.75 ml
10% SDS solution	37.5 µl
Distilled water	1.16 ml
TEMED	11.25 µl
10% APS solution	30 µl

b) The separating gel solution was applied to the sandwich.

c) The top of the gel was covered slowly with a layer of water. It was then allowed to polymerize the gel solution for about one hour at room temperature.

d) The water layer was removed.

e) **Preparation of stacking gel:** The following solutions were taken in a 50 ml conical flask kept in an ice bath and swirled gently to mix the content.

Components	Amounts
30% acrylamide solution	0.625 ml
Stacking gel buffer	1.87 ml
10% SDS solution	18.75 µl
Distilled water	1.21 ml
TEMED	5.62 µl
10% APS solution	15 µl

f) The stacking gel was poured on the separating gel. Then the Teflon comb was inserted immediately into the layer of stacking gel solution. The gel solution was allowed to polymerize for about 30 minutes.

g) The Teflon comb was then carefully removed without tearing the edges of the polyacrylamide pockets. After removing the comb the pockets were rinsed with electrophoretic buffer to remove unpolymerized monomers. Then the gel pockets were filled by different samples to be tested for purity.

h) The gel sandwich was then attached to buffer chambers. The lower chamber was filled with the recommended amount of electrophoretic

buffer and the upper chamber was partially filled with the same buffer so that the top of the gel sandwich was sunk into the buffer.

i) Power supply: Both chambers were connected to the power supply. Initially 25 mA current was applied. When the blue mark reached at the interface between separating gel and stacking gel, then the current supply was raised to 40 mA. The power supply was disconnected when BPB dye reached at the mark point on the bottom of the gel.

j) Recovery of the gel: The gel sandwich was removed from the chamber and kept on a petridish containing distilled water. Upper plate was removed carefully and thus the gel was removed.

k) Staining the gel: The gel was kept on a petridish, the staining solution was added slowly and stained for two hours at room temperature.

l) Destaining the gel: After two hours staining solution was removed and destaining solution –1 was added. Then the petridish was placed on a rotary shaker and shaken slowly (30-40 rpm) for 8 to 12 hours. Then the destaining solution –1 was removed and destaining solution –2 was added and was shaken for about one hour. Then the destaining solution-2 was changed by another portion of same solution. In this way the destaining solution-2 was changed at intervals until the solution became clear.

ENZYME ASSAY

Lipase activity was assayed as described by Sugihara *et al* (149) using olive oil as substrate. The lipase activity was measured by estimating the release of free fatty acids. One unit of lipase activity is defined as the amount that

liberates one micromole of fatty acid under the specified conditions. Specific activity of lipase is expressed as the enzyme unit per mg of protein.

Reagents:

a) Olive oil

b) 50 mM acetate buffer, pH 5.6

c) 100 mM $CaCl_2$

d) Ethanol

e) 50 mM KOH solution

f) Phenolphthalein indicator (1% in ethanol)

Procedure:

The standard assay mixture contained 1 ml of olive oil, 4.5 ml of 50 mM acetate buffer, 0.5 ml of 100 mM $CaCl_2$ and 1 ml of enzyme solution. The mixture was incubated for 30 minutes at 35° C with slow stirring and the lipase reaction was stopped by adding 7 ml of ethanol. Then a few drops of phenolphthalein indicator were added. The amount of free fatty acids released during the incubation was estimated by titrating the mixture with 50 mM KOH solution.

RESULTS AND DISCUSSION

PURIFICATION OF LIPASES:

DEAE-Cellulose Chromatography:

The crude enzyme solution prepared from 85% ammonium sulphate saturation of hilsa fish acetone extract was dialyzed against 10 mM Tris-HCl buffer, pH 8.4 for 24 hours and was applied to a DEAE- cellulose column at 4° C, which was previously equilibrated with the same buffer and eluted by stepwise elution of NaCl in the same buffer.

As shown in Figure-8, the proteins of crude enzyme solution were separated as two major peaks, F-1 and F-2, and one minor peak, F-3. Of these fractions F-1 was eluted by buffer only while F-2 and F-3 were eluted by the buffer containing 0.1 M NaCl and 0.2M NaCl respectively. Further, it was found that only F-1 fraction contained lipase activity. So, this fraction was pooled together and used for further purification by CM-cellulose chromatography.

CM-Cellulose Chromatography of F-1 Fraction:

The enzyme active fraction, F-1 was dialyzed against distilled water for 12 hours and against the eluting buffer (5 mM sodium phosphate buffer, pH 6.5) for 12 hours at 4° C. After centrifugation the clear supernatant was applied to the CM-cellulose column, which was previously equilibrated with the same buffer at 4° C and eluted with the same buffer also. As shown in Figure-9, the components in F-1 was separated into two sharp peaks F-1a and F-1c, and one minor broad peak, F-1b. Of these fractions F-1a was

eluted by the buffer only while F-1b and F-1c were eluted by the buffer containing 0.1M and 0.2M NaCl respectively.

Further it was found that the peaks, F-1a and F-1c contained lipase activities while F-1b possessed no such activity. The purity of these fractions i.e., F-1a and F-1c, was checked by SDS- PAGE (electrophoresis).

SDS-PAGE:

The photographic representation of the electrophoretic patterns of F-1a, F-1c was presented in Fig-11. From the result it might be concluded that the fraction F-1a was not pure as it gave more than one band on the gel. On the other hand, the fractions F-1c must be contained pure protein as they gave single band on the gel.

The fraction, F-1a was then further purified using gel filtration.

Gel Filtration:

The fraction F-1a was dialyzed against distilled water for 12 hours and then against 10 mM Tris- HCl buffer, pH 8.4 for 12 hours. After concentration by sucrose, this fraction was applied to a Sephadex G-50 column at 4° C, which was previously equilibrated with the same buffer. As shown in figure-10, the components present in this fraction was eluted as one major sharp peak, F-1a′ and one minor peak F-1a″. It was also found that only the fraction F-1a′ contained the lipase activity while F-1a″ contained no lipase activity. The active enzyme fraction, as indicated by solid bars was pooled and its homogeneity was checked by SDS-PAGE.

DEAE-CELLULOSE CHROMATOGRAPHY OF CRUDE ENZYME SOLUTION

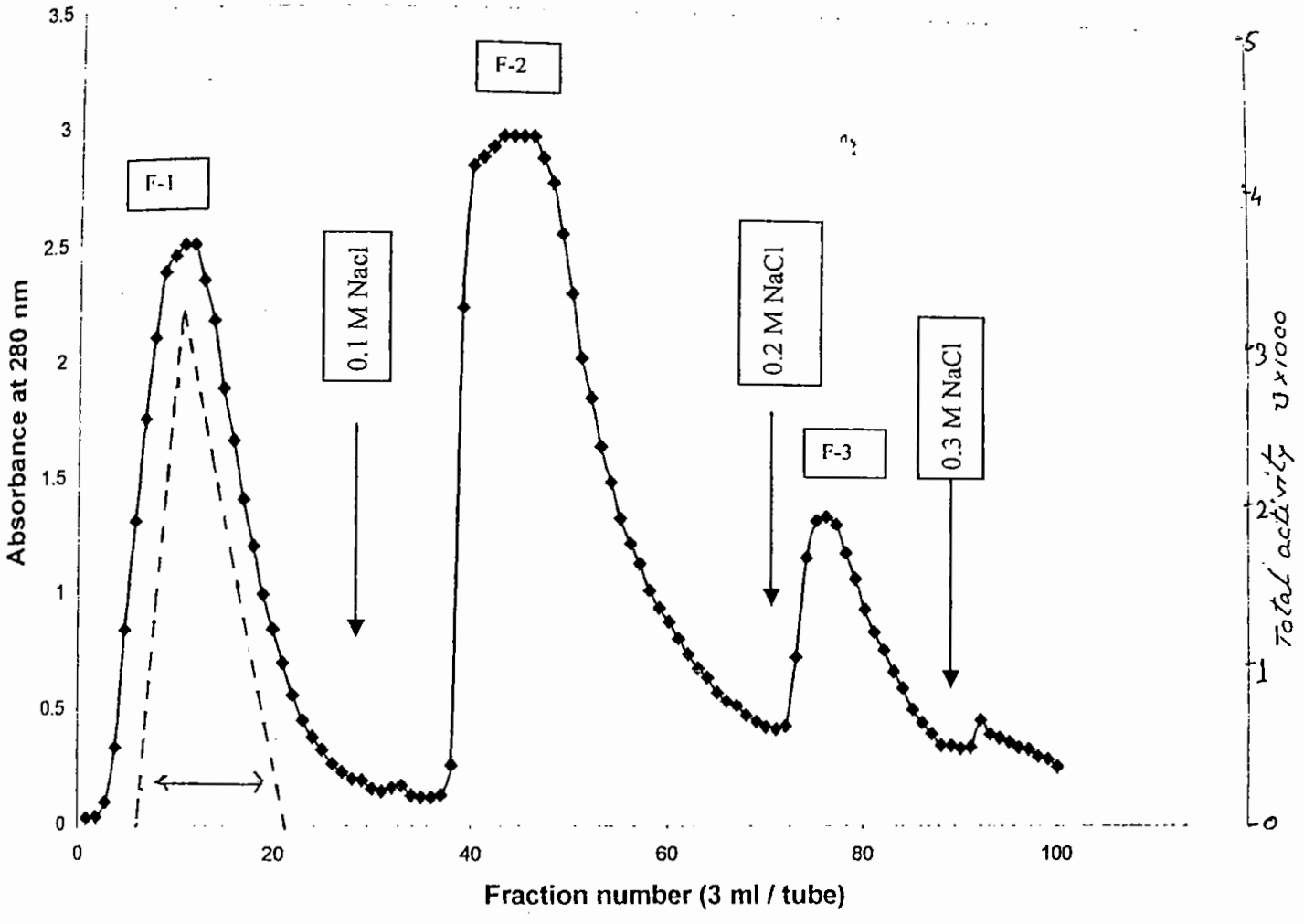


Figure-8: Ion exchange chromatography of crude enzyme solution on DEAE-cellulose. The crude solution (95 mg) was applied to the column (2.1 X 25 cm) pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.4 at 4° C and eluted by stepwise increases of NaCl concentration in same buffer. Flow rate: 45 ml/ hour.

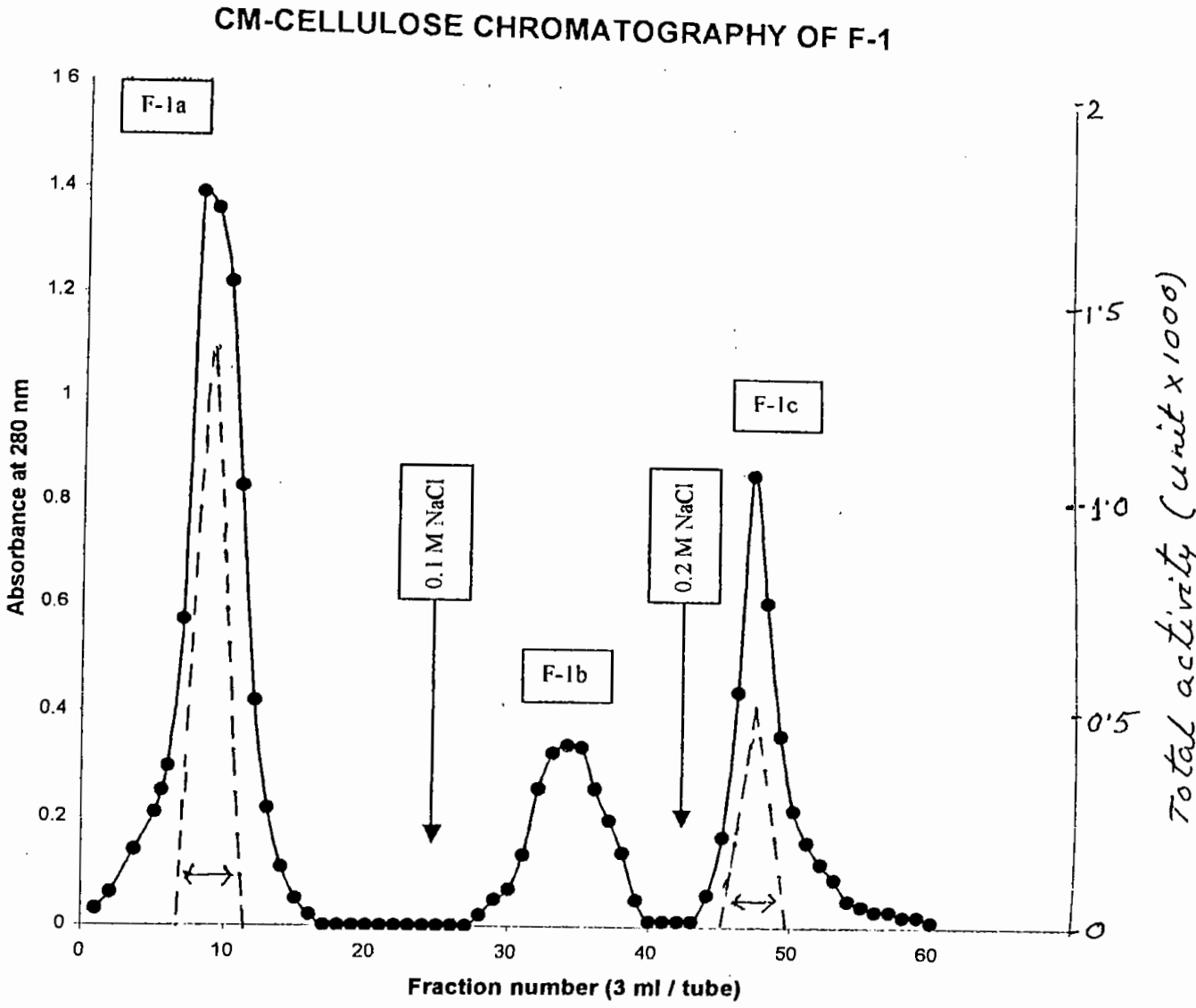


Figure-9: CM- cellulose chromatography of F-1 fraction obtained from ion exchange chromatography on DEAE- cellulose. Fraction F-1a (25 mg) was applied to the column (1.5 X 15 cm) pre-washed with 5 mM sodium phosphate buffer, pH 6.5 at 4° C and eluted by stepwise increases of NaCl in the same buffer. Flow rate was 45 ml/hour.

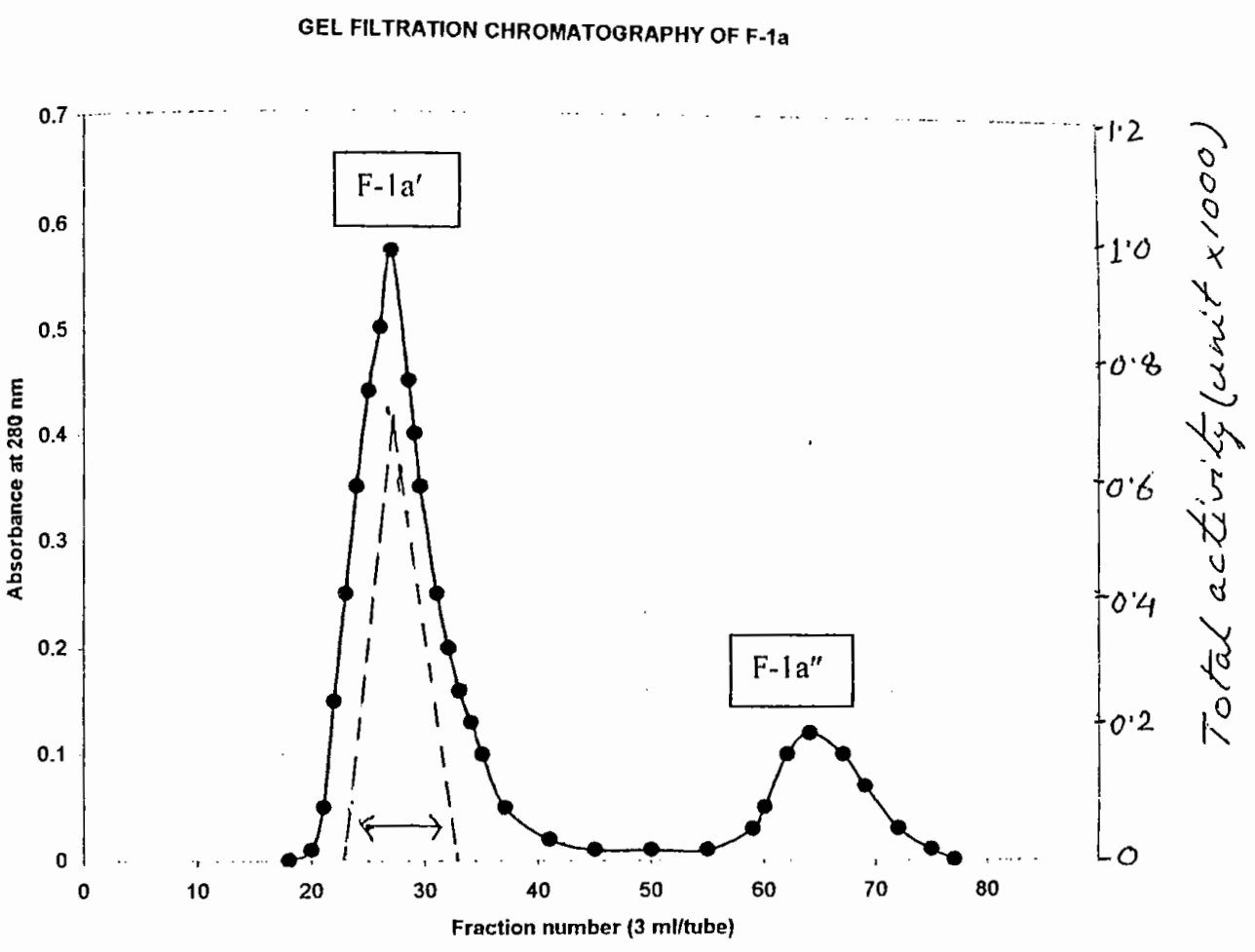


Figure-10: Gel filtration of F-1a fraction obtained from CM-cellulose chromatography on Sephadex G-50. F-1a fraction (12 mg) was applied to the column (3 X 120 cm) pre-washed with 10 mM Tris- HCl buffer, pH 8.4 at 4° C and eluted by the same buffer. Flow rate: 25 ml/ hour.

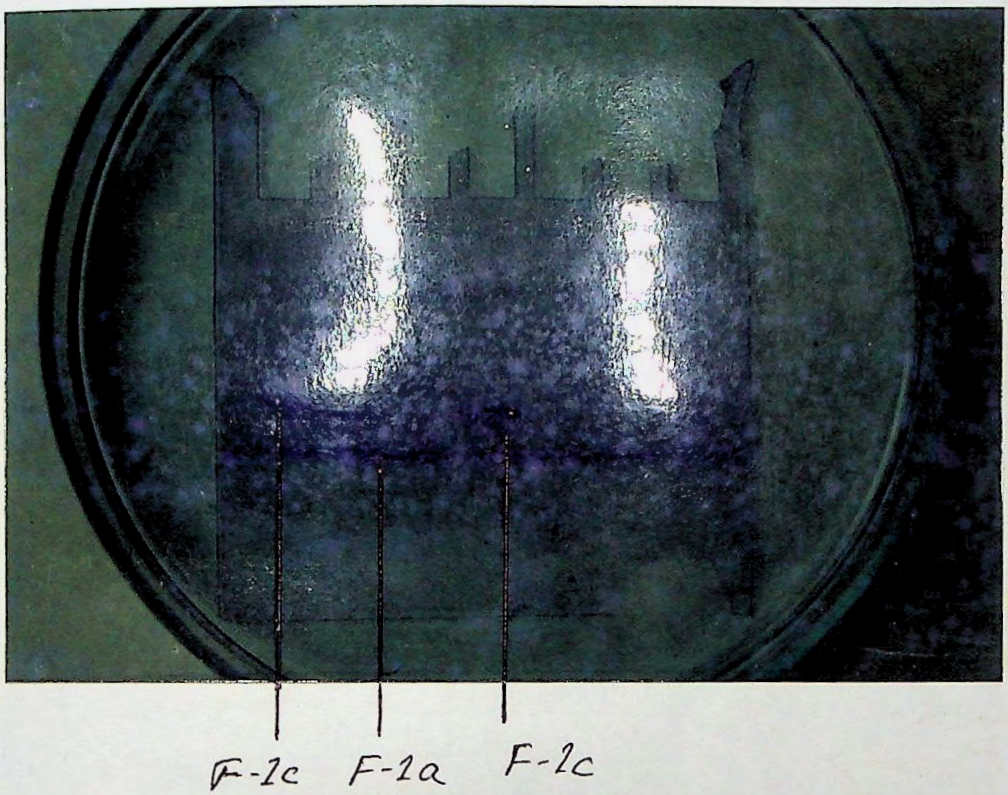


Figure- 11: Electrophoretic pattern of the fractions F- 1a and F- 1c.

The photographic representation of the electrophoretic patterns of F-1a′ was presented in Fig-12. The fraction F-1a′ must be contained pure protein as they gave single band on the gel.

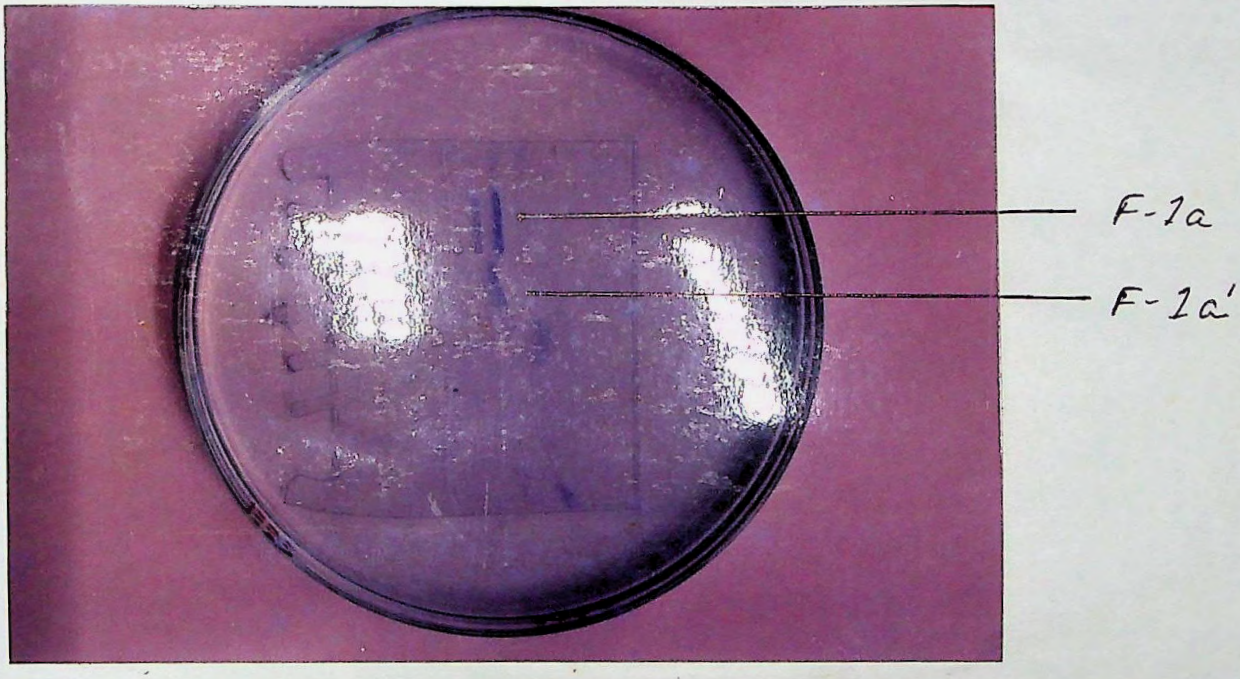
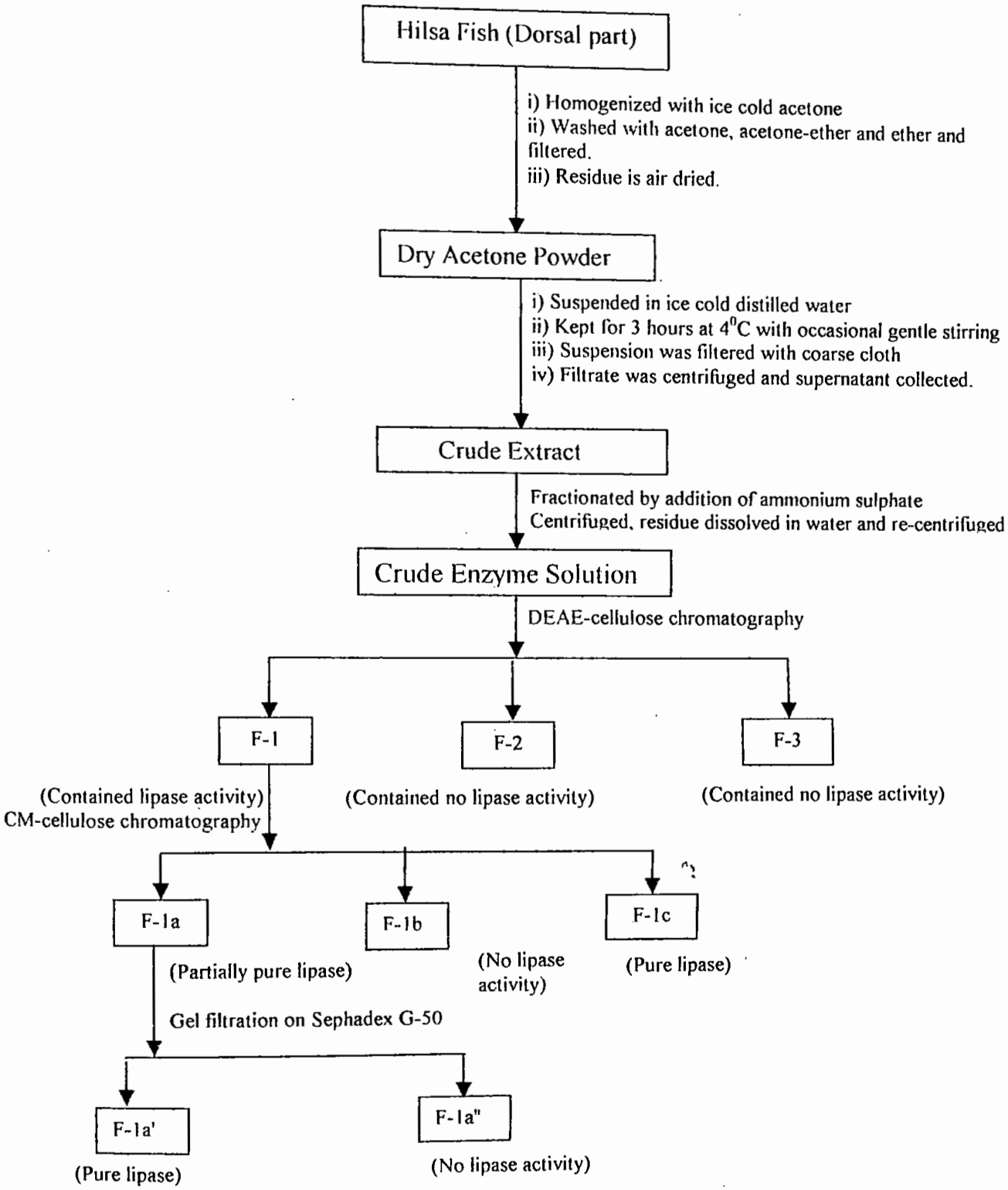


Figure- 12: Electrophoretic pattern of F-1a and F-1a′.

Table- 9: Lipase activities in the course of purification of Hilsa fish lipase.

Steps of purification		Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)	Purification fold
Crude extract		4984.7	42049.6	8.43	100	1
85% Ammonium sulphate precipitation (Crude enzyme)		1051	15599.7	14.84	37.10	1.76
DEAE-cellulose fractions	F-1	59.52	4544.90	76.36	10.80	9.06
CM-cellulose fractions	F-1a	13.32	1897.5	142.45	4.51	16.9
	F-1c	7.80	1259.5	161.47	3.00	19.15
Gel filtration on Sephadex G-50	F-1a'	4.08	989.8	242.6	2.25	28.77

Table 9 summarizes the data pertaining to the purification of two lipases from dorsal part of hilsa fish. The fraction, F-1a' showed maximum lipase activity with a purification fold of 28.77 fold, while F-1c showed 19.15 fold increases in enzymatic activity. Although the yield of lipases were found to be decreased by each subsequent purification step and % of activities were destroyed during the whole purification processes, but the purification fold of the lipases were increased in different steps. This low yield may be due to denaturation of the lipases during the lengthy purification steps.



Flow diagram of the purification steps of lipases.

CHAPTER - 5

CHARACTERIZATION OF LIPASES PURIFIED FROM DORSAL PART OF HILSA FISH

METHODS

Molecular Weight Determination:

(a) By Gel Filtration:

The MW of hilsa fish lipase was determined by gel filtration following the procedure as described by Andrews (150).

Procedure:

1) **Packing of the column:** A column of desired length was packed with Sephadex G-150 gel suspension following the procedure as described before.
2) **Equilibration of the column**: After preparation of the column it was equilibrated with the eluant buffer, 10 mM Tris- HCl, pH 8.2. The buffer was continued to run through the column until the pH of the eluate became same as that of eluant buffer.
3) **Application of the sample and elution**: The standard proteins and the purified lipases were applied to the column. During gel filtration identical conditions were maintained in each time. The buffer was finally allowed to flow continuously through the column at a flow rate of 15 ml per hour and 3 ml fractions of the eluate were collected by an automatic fraction collector.
4) **Detection**: Absorbance of each fraction was measured at 280 nm. The MW of the protein was determined from a standard curve, which was constructed by plotting the elution volume against log of MW of standard proteins.

(b) Determination of MW by SDS-PAGE Method:

1) **Reagents and Solutions:** Same as described before.
2) **Preparation of Marker Protein mixture:** Marker protein mixture was prepared by mixing β- galactosidase (MW 116 kd), bovine serum albumin (MW 66 kd), ovalbumine (MW 63 kd), carbonic anhydrase (MW 29 kd), trypsin inhibitor (MW 20 kd) and lysozyme (MW 14 kd), with equal volume of sample solution. The mixture was heated for 2 minutes at 100° C and then one drop of BPB solution was added.
3) **Method:** 20 µl of known protein mixture and sample protein were carefully applied at different pockets of gel. The upper buffer chamber and lower chamber were filled with electrophoretic buffer. Then chambers were connected to power supply unit and electrophoresis was carried out as described before.

Determination of Sub-unit of protein by SDS-PAGE:

1) **Reagents and solutions:** Same as described before.
2) **Preparation of sample:** The protein sample was prepared by mixing 100 µl of protein (lipase) with equal volume of sample solution and 20 µl of 2- mercapto ethanol. After heating at 100° C for 5 minutes one drop of BPB was added to the mixture.
3) **Procedure:** Same as described before.

Determination of Protein Concentration:

Protein concentration was determined following the method of Lowry *et al* (147) using BSA as a standard.

Reagents:

a) 2% Na_2CO_3 solution in 0.1 N NaOH.

b) 0.5% copper sulphate in 1% sodium potassium tartarate.

c) Folin Ciocalteau's reagent (F.C.R)

d) Standard protein solution: 10 mg per 100 ml of distilled water.

Procedure:

Reagents (a) and (b) were mixed in the ratio of 50:1 and reagent (c) was diluted with twice the volume of water.

For the construction of standard curve 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of the standard protein solution were taken in different test tubes and made upto 1 ml with distilled water. The enzyme solution (1 ml) was also taken in duplicate in different test tubes and 5 ml of a:b mixture was added to each of the test tube. After 10 minutes 0.5 ml of FCR was added to each test tube, mixed well and kept for another 30 minutes. The absorbances of solutions contained in each test tube were measured at 650 nm. A graph was constructed by plotting concentrations against absorbances. From the graph concentration of protein was calculated.

Determination of Optical Density (O.D at 280 nm) vs Protein Concentration Relation:

The absorbance (O.D) of lipases were measured at 280 nm for different concentrations. The respective concentration for absorbance of 1.0 was estimated by Lowry method.

Measurement of Km

Michaelis constant (Km) was determined by Lineweaver-Burk double reciprocal plot (151).

The initial velocity (V_i) is equal to the amount of product formed per unit time. The initial velocity (V_i) was determined quantitatively by measuring the amount of one of the products at various time intervals.

Reagents:

i) Olive oil

ii) 50 mM sodium acetate-HCl buffer, pH 4.6-5.0

iii) 100 mM $CaCl_2$

iv) Ethanol

Procedure:

0.1, 0.2,0.3,0.4, and 0.5 ml of olive oil were taken in different test tubes:

(a) For control-1 no. (b) For blank-1 no. and (c) For experiment-2 no.'s of test tubes were taken and 4.5 ml of 50 mM sodium acetate-HCl buffer, pH 4.7 for Lip-I and pH 4.9 for Lip-II were added in each test tubes. Then 0.5 ml of 100 mM $CaCl_2$ was added to all the test tubes. The mixtures were incubated in a water bath at 35^0C for 30 minutes. Then 1 ml of enzyme extract with slow stirring was added to the experimental and control tubes whereas 1 ml of distilled water was added to the blank. Immediately after the addition of enzyme, 7 ml of ethanol was added to the control to stop reaction.

The rest of the tubes were incubated at 35^0C for various times (5 min., 10 min., 20 min., 30 min. and 50 min.) with slow stirring in the water bath. At appropriate time intervals the reaction was stopped by the addition of 20 ml of ethanol. Then the amount of fatty acids released during the incubation was estimated by titrating the mixture with 50 mM KOH.

Test of Glycoprotein and Estimation of percentage of Sugar:

Phenol in the presence of sulphuric acid can be used for quantitative colorimetric microdetermination of sugars and their methyl derivatives, oligosaccharides and polysaccharides as described by Dubois *et al*(152). The method was also employed for detecting the presence of sugar in protein.

Reagents:

1. 5% phenol (in water).
2. Concentrated sulphuric acid.
3. Protein solution.
4. Standard glucose solution.

Procedure:

The protein solution (0.3 ml) was taken in a test tube and made upto 2 ml by distilled water. Then 1 ml of 5% phenol solution was added to it and 5 ml concentrated sulphuric acid was added rapidly. To ensure good mixing the stream of acid was directed against the liquid surface rather than against the side of the test tube. The tube was allowed to stand for 10 minutes and kept

in dark at 25 – 30° C for 20 minutes after shaking. The absorbance of the solution was measured at 490 nm.

Preparation of standard curve:

A standard glucose solution (0.1 mg/ml) was prepared. Then 0.0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ml of this solution were taken into different test tubes and made upto 2 ml with distilled water. A standard graph of glucose was constructed by plotting concentration of glucose against the absorbance. From the graph, the concentration of sugar in protein was calculated.

Substrate Specificity

Activity of hilsa fish lipases was determined using different substrates, e.g., castor oil, neem oil, peanut oil, sesame oil, rapeseed oil, olive oil, rice bran oil, cottonseed oil and soybean oil. The procedure was followed as described before.

Ultraviolet Absorption Spectra

The ultraviolet absorption spectra of the enzymes were recorded in aqueous solution with a double beam spectrophotometer (Shimadzu, model UV-180) at room temperature. Enzyme solutions were taken in cuvette and absorbances were recorded at various wavelengths ranging from 240 nm to 380nm.

RESULTS AND DISCUSSION

MW of Lipases:

The MW of the lipases were determined by gel filtration on Sephadex G-150 using β- galactosidase, bovine serum albumin, α- amylase, ovalbumin, trypsin inhibitor and lysozyme as standard proteins. The MW of purified lipases were estimated to be 47,500 for F-1a′ (Lip-I) and 41,800 for F-1c (Lip-II) (Fig-13). Iwai *et al* (153) purified two lipases from *Penicillium cyclopium* having MW of 27000 and 36000, while Dowhey and Andrews (154) reported the MW of a triacetin- hydrolyzing wheat germ enzyme to be 51000 and pancreatic lipase to be 42000 by gel filtration.

Determination of MW by SDS- PAGE:

The MW of the enzymes were also determined by SDS- PAGE using the marker protein containing carbonic anhydrase.

MW of Lip-I and Lip-II were found to be 47000 and 41000 respectively (Fig-14). Further, the MW of the enzymes were found to be unchanged in the presence or absence of β-mercaptoethanol indicating that both the lipases contained only one subunit.

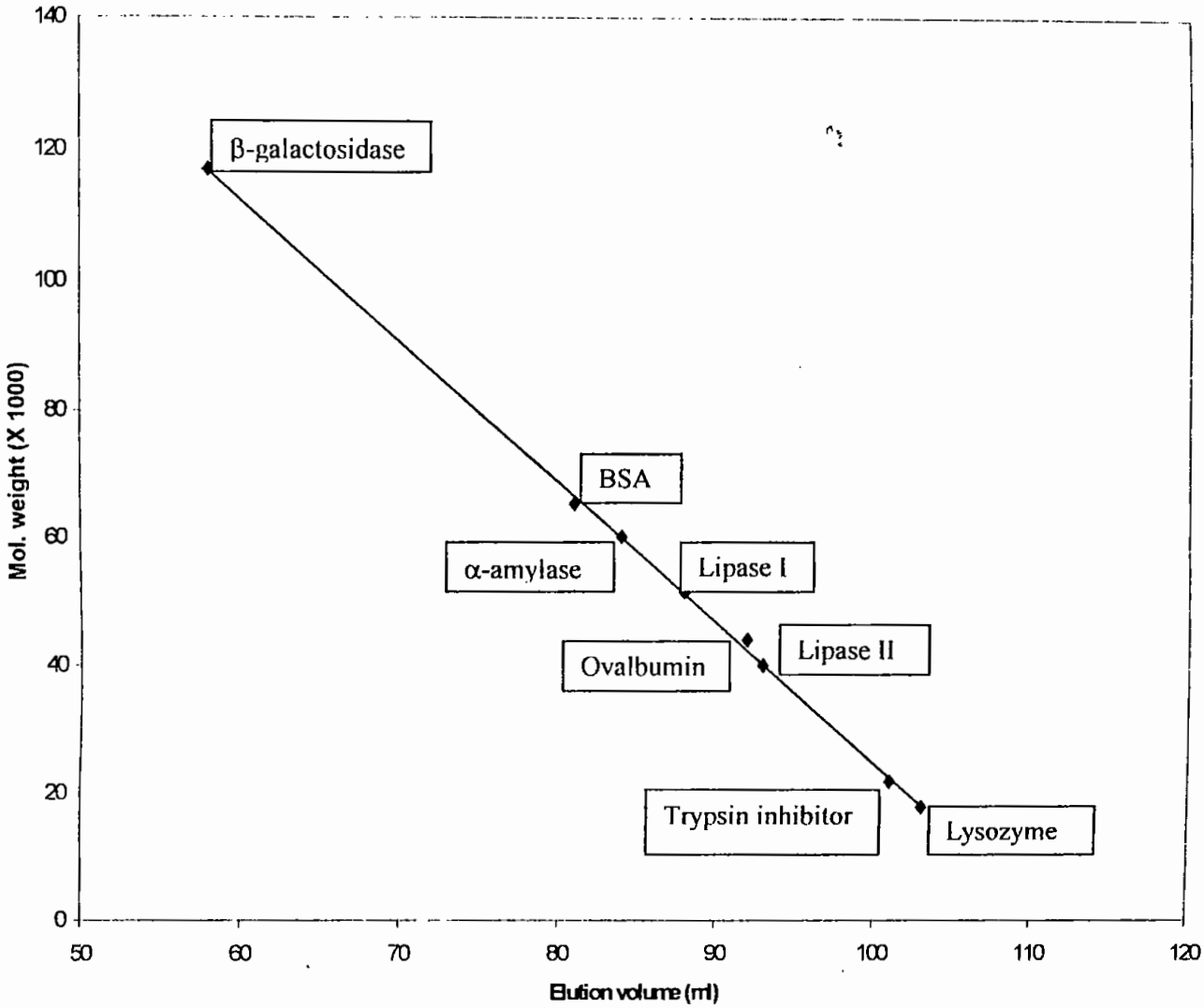


Figure-13: Standard curve for the determination of molecular weight of Lipase- I and Lipase-II by gel filtration on Sephadex G- 150. Size of column: 1.8 X 90 cm. Buffer: 10 mM Tris- HCl, pH 8.2 and flow rate: 15 ml / hour.

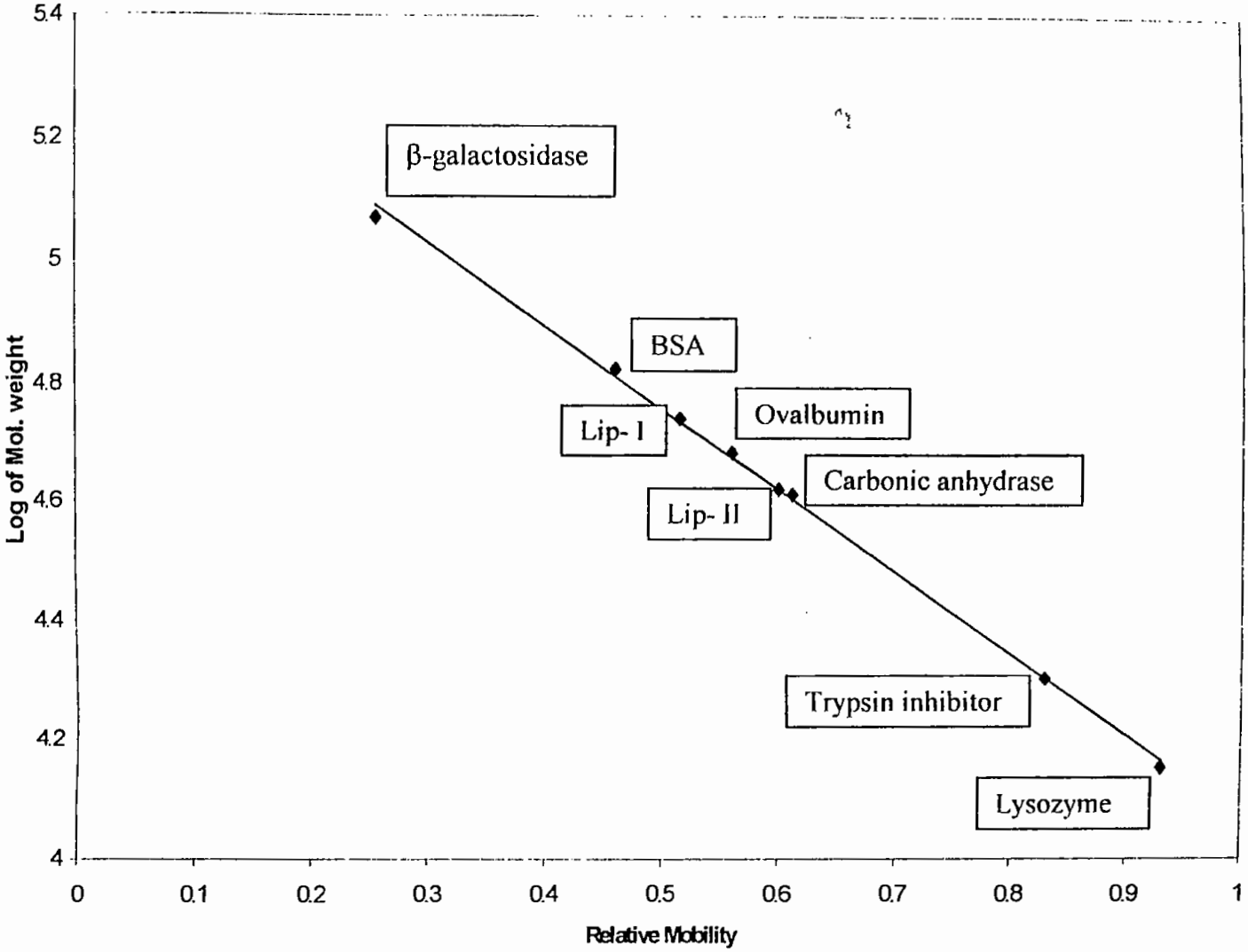


Figure-14: Standard curve for the determination of molecular weight of Lip-I and Lip-II by SDS- PAGE .

Optical density vs. concentration relation of the purified enzymes:

The absorbance of 1.0 at 280 nm for Lip-I and Lip-II were found to be equal to 0.71 and 0.68 mg of proteins respectively, as determined by the Lowry method using BSA as standard.

Determination of K_m:

Lipase activity was tested using different concentrations of olive oil as substrate and the extrapolated K_m values were calculated to be 0.11 mM and 0.175 mM for Lip-I and Lip-II respectively (Fig-15)

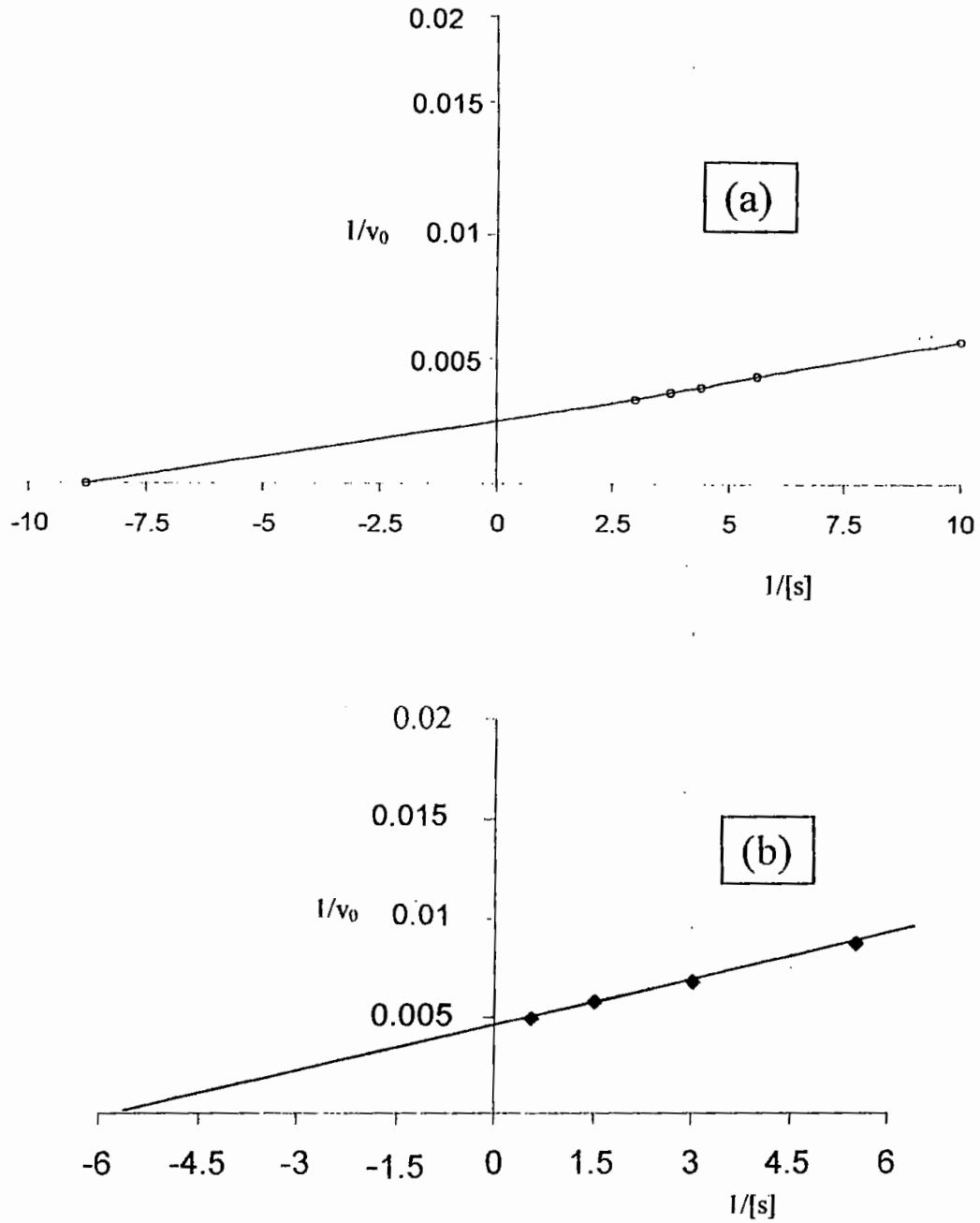


Figure-15: Lineweaver-Burk double reciprocal plots for the determination of Km of the purified Lip-I and Lip-II enzymes.

a) Lineweaver-Burk double reciprocal plot for Lip-I

b) Lineweaver-Burk double reciprocal plot for Lip-II

AMOUNT OF SUGAR PRESENT IN ENZYME SOLUTION:

Both the enzymes Lip-I and Lip-II gave yellow orange colour in the presence of phenol sulfuric acid indicating that the enzymes are glycoprotein in nature. For Lip-I amount of sugar was found 8.59% and for Lip-II it was found 5.18%.

SUBSTRATE SPECIFICITY:

The activity of Lip-I and Lip-II were determined using various substrates (oils) and the following results was obtained.

Table-10 : Specific activities of lipases against different substrates:

Substrates	Specific activity (mU/mg)	
	Lip-I	Lip-II
Castor oil	1953.12	2962.96
Neem oil	781.25	1481.5
Peanut oil	1562.5	2222.22
Sesame oil	1171.87	1481.5
Rapeseed oil	781.25	1111.11
Olive oil	390.6	740.74
Rice bran oil	1171.87	1481.5
Cottonseed oil	1562.5	1851.85
Soybean oil	1171.87	1481.5

Although the enzymes catalyzed the hydrolysis of many vegetable oils but the extent of hydrolysis varied from one another according to the constituent of fatty acids. Castor oil, peanut oil, sesame oil and cottonseed oil containing mainly unsaturated fatty acids of eighteen carbon atoms, were hydrolyzed more rapidly while neem oil, olive oil and rapeseed oil containing more saturated fatty acids underwent hydrolysis to a smaller extent. Rice bran oil and soybean oil are hydrolyzed moderately as these oils contained medium amount of unsaturated fatty acids.

Ultraviolet Absorption Spectra:

The purified lipases, Lip-I and Lip-II in aqueous solution gave absorption maxima around 270 and 275 nm, and minima around 250 and 240 nm respectively (Figure 16 and 17). These values are very similar to that reported by Funatsu *et al* (155).

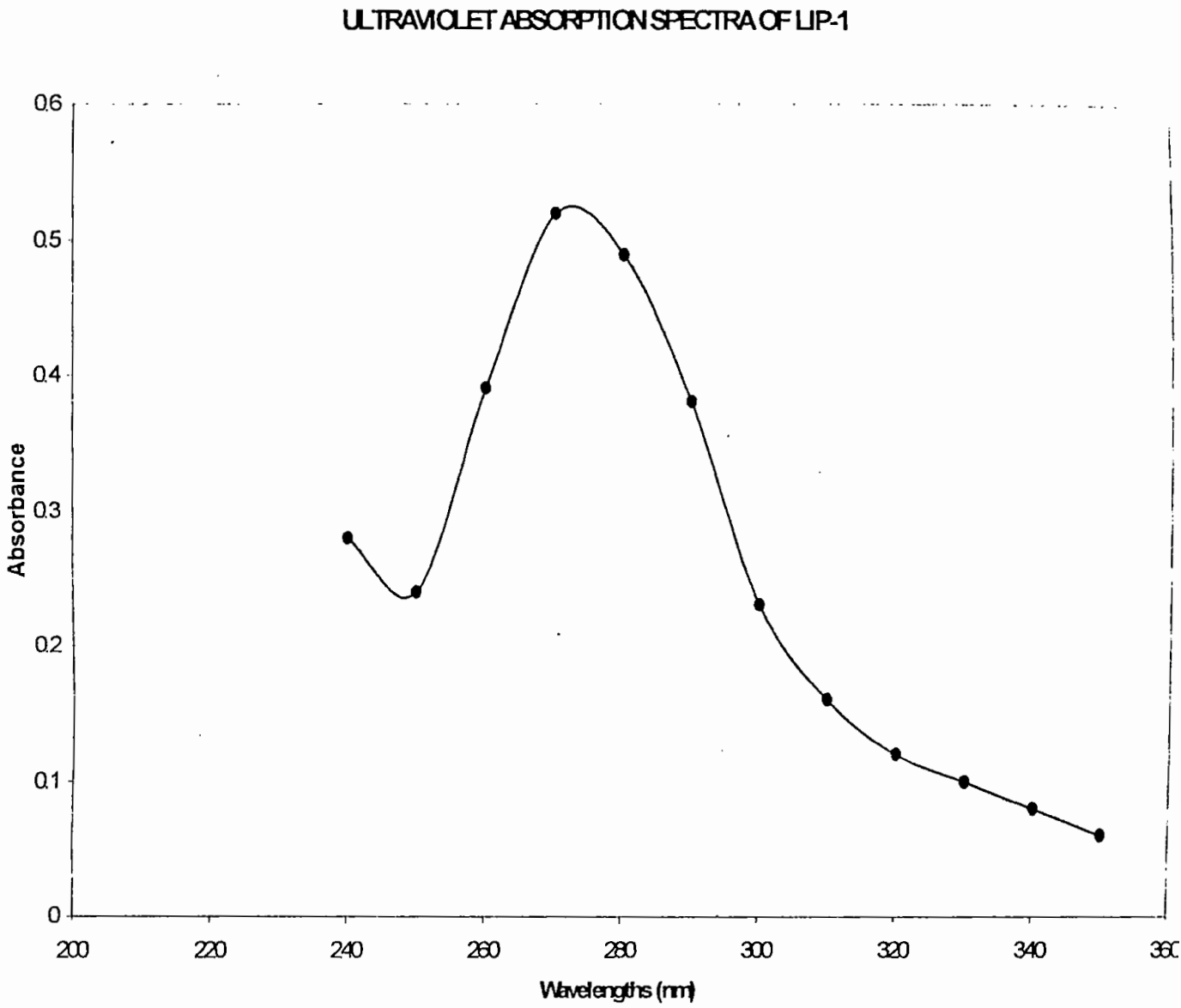


Figure 16 : Ultraviolet absorption spectrum of Lip-I.

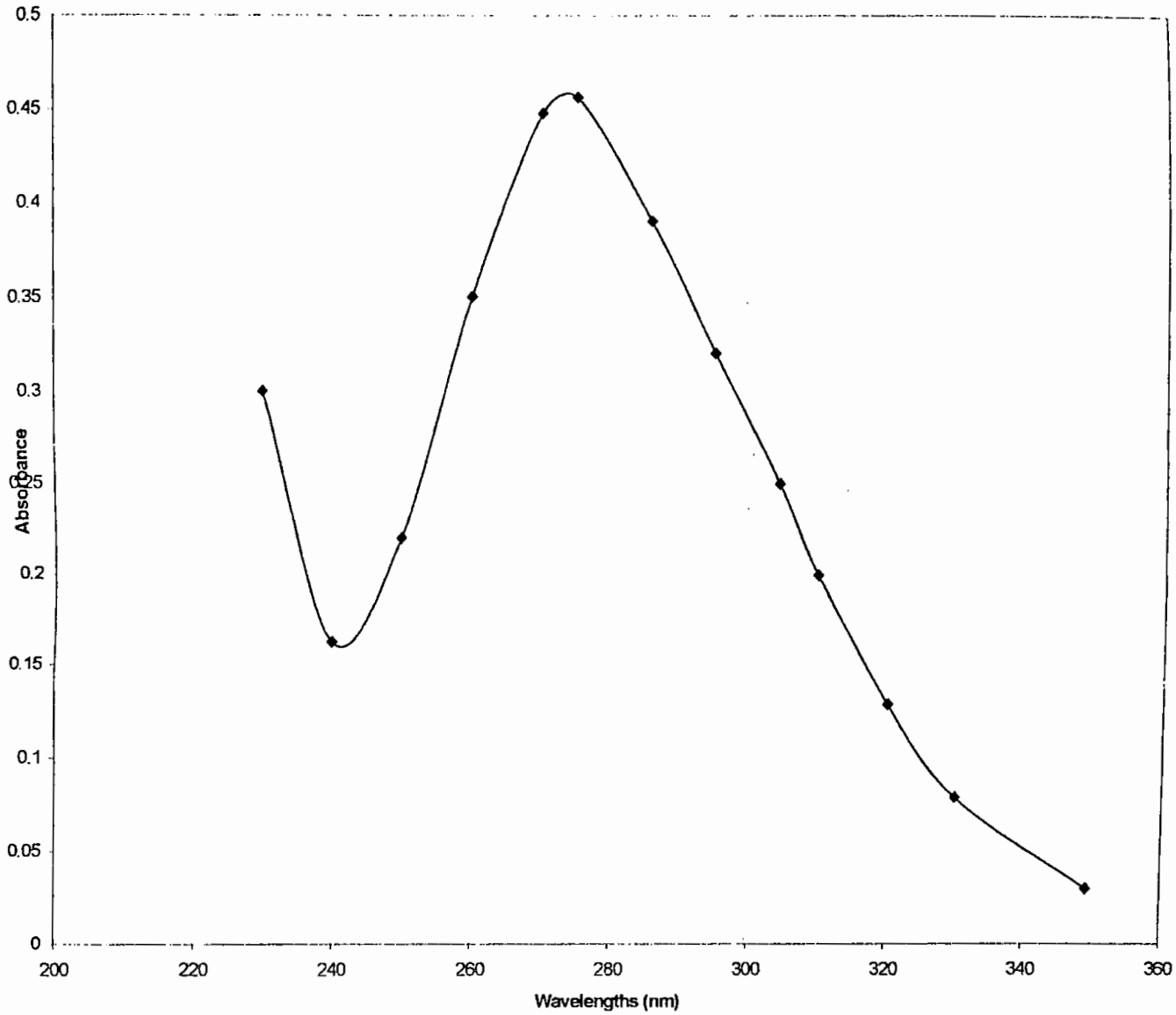


Figure 17 : Ultraviolet absorption spectrum of Lip-I

CHAPTER-6

EFFECT OF PHYSICO-CHEMICAL TREATMENTS ON THE ACTIVITIES OF LIPASES

Introduction:

With recent advances in biotechnology and genetic engineering, many new and interesting ideas to produce oleochemicals from fats have been investigated (156-158). Among the most promising chemical routes of industrial interests the hydrolysis, ester synthesis and interesterification reactions of lipids are brought about by lipases (159). Lipases selectivity lowers the activation energies of the chemical reactions they catalyze (160). With these enzymes, one can achieve much higher specificities and major enhancements in reaction rates relative to non-enzymatic reactions (161, 162). The technical and economic feasibility of the enzymatic splitting of fats under mild conditions (163- 165) relative to the uncatalyzed high pressure counter- current steam splitting process employed in industrial practice is being investigated (166). The application of lipases for the modification of fats and oils by interesterification reactions is expected to become a parallel route to that involving in uses of oil seed plants to produce oils and fats with desired characteristics (167). For this reason, the properties of lipases and sources of lipases have been extensively reviewed (168- 170).

In this chapter, the lipases purified from hilsa fish have been subjected to various physical and chemical treatments and their effects on the lipolytic activities have been investigated. The study is expected to provide in formulation regarding some of the physico-chemical properties such as pH optimum, temperature optimum, the stability of lipases towards denaturing agents etc. The experimental results will also enable us to compare the relative stability of the lipases.

Methods:

Effect of pH on Lipase Activity (Optimum pH):

To study the effect of pH on enzyme activity, the enzyme solutions (0.5-0.6%) were dialyzed against 50 mM buffer of different pHs for 24 hours with frequent changes of buffers. After necessary adjustment of pH values by addition of 0.2 N HCl or 0.2 N NaOH, the enzyme activities were assayed at room temperature (25- 28° C) using olive oil as substrate.

Effect of Temperature on Lipase Activity (Optimum Temperature):

In order to determine optimum temperature, the enzyme solutions (0.5%) in 50 mM sodium acetate-HCl buffer, pH 4.7 for Lip-I and pH 4.9 for Lip-II, were incubated at various temperatures for 30 minutes in temperature controlled bath and the activities remaining were assayed at room temperature using olive oil as substrate.

Effect of Metallic Salts and Denaturants on Lipase Activity:

Metallic salts of different concentrations were added to the enzyme solutions (0.5%) in 50 mM sodium acetate-HCl buffer, pH 4.7 for Lip-I and pH 4.9 for Lip-II and after 30 minutes incubation at room temperature the lipase activities were assayed.

RESULTS AND DISCUSSION

Optimum pH:

The enzymatic activities of lipases against olive oil as substrate were assayed at various pH values varying from 3- 10. The buffers used were as follows:

Table-11: pH ranges of different buffers:

Buffers	pH
AcONa-HCl	3.0- 4.0
AcONa-CH_3COOH	4.5- 5.5
NaH_2PO_4-Na_2HPO_4	6.0- 8.0
$Na_2B_4O_7$-HCl	8.5- 9.0
$Na_2B_4O_7$-Na_2CO_3	9.5- 10.5

As demonstrated in Figure-18, the activities of the lipases were greatly influenced by pH changes. Lip-I and Lip-II gave maximum activity in pH ranges of 4.6- 4.8 and 4.8- 5.0 respectively. Above and below this pH ranges the activities of lipases decreased abruptly. The optimum pH calculated for Lip-I and Lip-II were 4.6 and 5.0 respectively. From this result it may be concluded that the two lipases isolated from hilsa fish belong to be category of acidic lipase. Similar type of acidic lipase was reported from castor bean by Ory *et al,* (171) while Sanders and Pattee reported that peanut contained alkaline type of lipase (172).

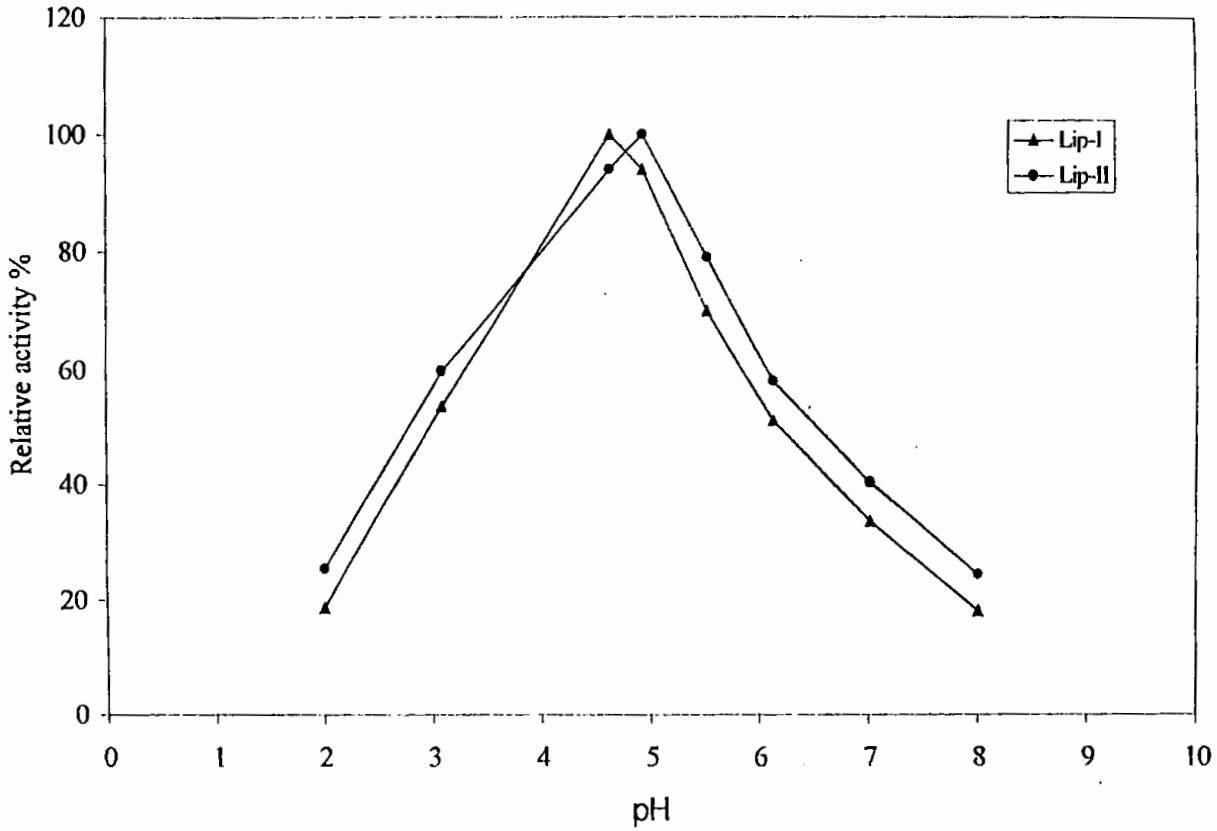


Figure-18: Effect of pH on the activities of hilsa fish lipases.

Optimum Temperature:

The effects of temperature on the activities of Lip-I and Lip-II were examined in the range of 10° C to 60° C. Lipolytic activities of hilsa fish lipases were found to be profoundly affected by temperature. As shown in Figure-19 the activities of lipases were increased gradually with rise in temperature and the maximum activity was observed around 34° C and 35° C for Lip-I and Lip-II respectively. With further rise in temperature the

activities were decreased abruptly and the enzymes lost almost 100% of their activity at 60° C and 70° C respectively.

Hilsa lipases are found to be highly active in the temperature range from 30° C to 35° C. Lipases purified from other sources were found to be highly active in the temperature range from 30° C to 40° C. The optimum temperature for bovine milk lipase was reported to be 30° C (173) while those of two lipases from *Penicillium cyclopium* were 35° C and 40° C (174) and those of three lipases from *R. delemer* were ranged from 30° C to 35° C (175).

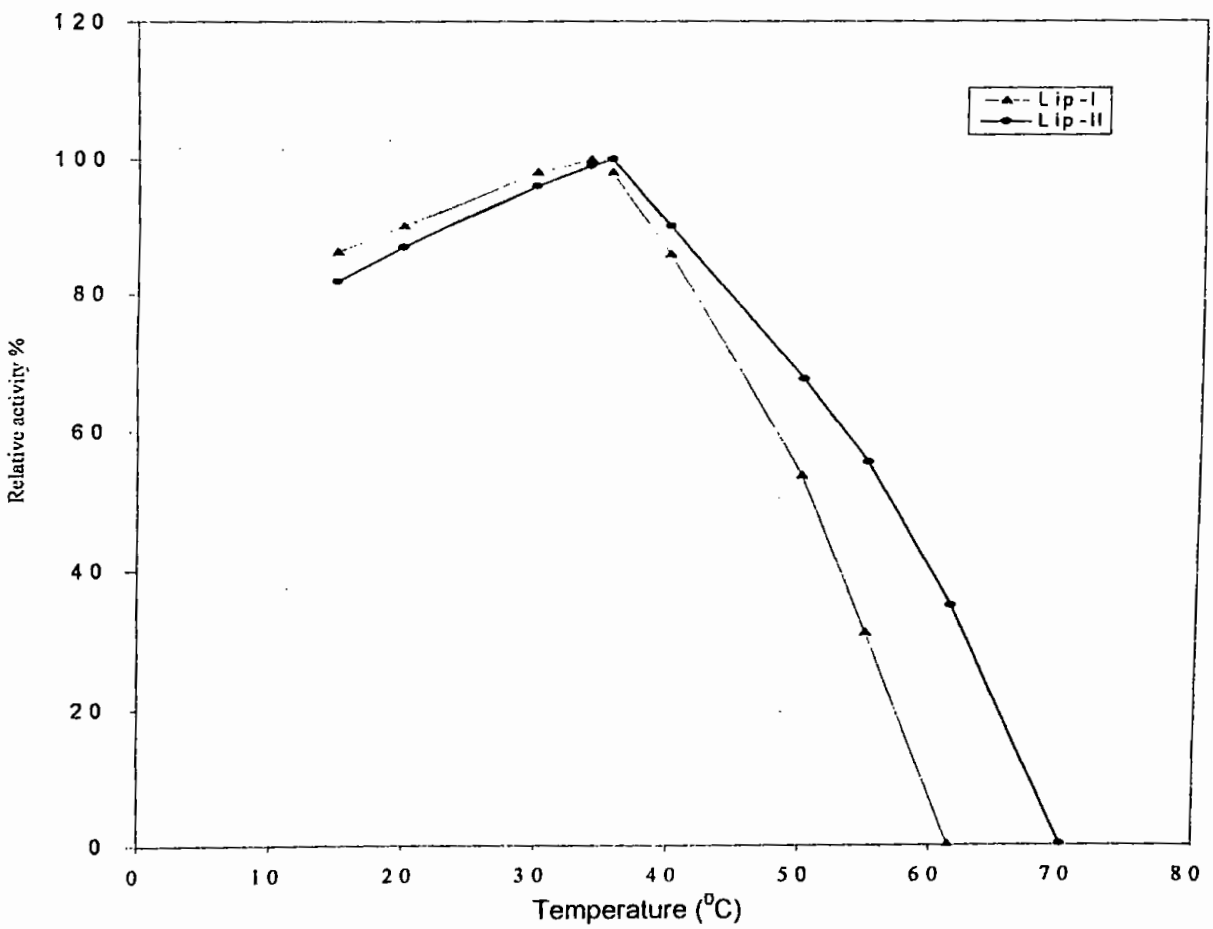


Figure-19: Effect of temperature on the activities of hilsa fish lipases.

Effect of Metallic Salts on Lipase Activity:

Table-12: Effect of metallic salts on the activities of hilsa fish lipases:

Salts added	Concentration (Molar)	Relative activity	
		Lip-I (%)	Lip-II (%)
None	-	100	100
EDTA	0.001	70	80
	0.003	50	60
	0.005	20	30
$CuCl_2$	0.001	90	85
	0.003	85	80
	0.005	70	60
$CaCl_2$	0.001	118.8	110.2
	0.003	102.5	95.68
	0.005	86	78
$CdCl_2$	0.001	70	60
	0.003	60	50
	0.005	40	30
$HgCl_2$	0.001	50	55
	0.003	35	40
	0.005	25	30
$ZnCl_2$	0.001	55	50
	0.003	35	30
	0.005	25	21

Table-12 represents the effect of various metal ions on the activities of hilsa fish lipases.

From the Table, it is evident that activities of hilsa fish lipases inhibited by heavy metals such as Cd^{++}, Zn^{++} and Hg^{++} while mild inhibitory activity was observed in the presence of Cu^{++}. The presence of Zn^{2+}, Hg^{+} and Cd^{++} potently inhibited lipolytic activities of the lipases from hilsa fish. Wheat germ lipase showed similar requirements (176). Mercury is known to bind

to thiol groups of proteins forming stable complexes. So, –SH group may be located at the active site of hilsa fish lipases. Mercury has also been shown to inhibit the lipase of rat adipose tissue (177). It has been shown conclusively that some heavy metals inhibit lipase activity (178).

Calcium ions carry out distinct role in the lipase action. The activities of all the lipases were found to be increased slightly in presence of lower concentration of Ca^{2+} but at higher concentration of Ca^{2+}, the activities were decreased slightly which is consistent with the results reported elsewhere (179). Like pancreatic lipase (180) hilsa fish lipases were stimulated in the presence of calcium ions at certain concentrations. The primary role of Ca^{++} seems to be to remove the released fatty acid as its calcium salt. The activating effect of calcium on a lipase derived from *Humicola lanuginosa* was explained by the removal of free fatty acids from the interface (181). In a calcium free system, the lipase can not adsorb at the water –fat interface, and consequently no lipolytic activity occurs. Possibly, the calcium ions compensate for the electrostatic repulsion created between the enzyme and the substrate (182).

In the presence of EDTA, a metal chelator, the activities of hilsa fish lipases were decreased significantly. The decrease in activity may be due to the removal of metal ions located on or near the active site.

CHAPTER – 7

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