THE PURIFICATION AND KINETICS OF WHEAT GERM LIPASE

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By

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Approval of the Graduate School of Natural and Applied Sciences.

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ABSTRACT

THE PURIFICATION AND KINETICS OF WHEAT GERM LIPASE

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The commercial wheat germ lipase was purified by the methods of gel filtration and ion-exchange chromatography. The activity of isolated fractions was tested with p-nitrophenyl acetate and dimercaptopropanol tributyrate, as substrate. Three active enzymes were obtained. A lipase catalyzes the hydrolysis of both p-nitrophenyl acetate and dimercaptopropanol-tributyrate, a tributyrinase catalyzes the hydrolysis of dimercaptoprapanol-tributyrate and an esterase catalyzes the hydrolysis of p-nitrophenyl acetate, only. The hydrolysis of p-nitrophenyl acetate by commercial enzyme gives a nonlinear Lineweaver-Burk plot whereas a linear plot was obtained with DMP-tributyrate. However heat inactivation with DMP-tributyrate as substrate was nonlinear.p-Nitrophenyl acetate hydrolysis by isolated esterase fraction obeys Michealis-Menten kinetics.With lipase fraction,the hydrolysis of p-nitrophenyl acetate deviates from Michaelis-Menten kinetics.

The results were discussed in terms of the catalytic properties of the enzymes.

Key words : Wheat germ, Lipase, Kinetics, Purification

ÖZET

BUGDAY ÖZÜ LİPAZININ SAFLASTIRILMASI VE KİNETİĞİ

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Ticari buğday özü lipazı, jel filtrasyonu ve iyon değiştirme kromatografisi metodları ile saflaştırıldı. Aktif fraksiyonlar sübstrat olarak p-nitrofenil asetat ve dimerkaptopropanol tributirat kullanılarak belirlendi. Aktif üç enzim bulundu : p-nitrofenil asetat ve dimerkaptopropanol tribütiratı hidroliz eden lipaz, dimerkaptopropanol tribütiratı hidroliz eden tribütirinaz ve sadece p-nitrofenil asetatı hidroliz eden esteraz. p-Nitrofenil asetatın ticari enzimle hidrolizi doğrusal olmayan bir Lineweaver-Burk çizimi verirken DMP-tribütirat hidrolizi için lineer bir çizim elde edildi. DMP-tribütiratın sübstrat olarak kullanıldığı ısı inaktivasyonu çalışmasında ise bir eğri elde edildi. Saflaştırılan esteraz fraksiyonunun p-nitrofenil asetatı hidrolizi Michaelis-Menten kinetiğine uygun bulundu. Lipaz fraksiyonu ile p-nitrofenil asetatın hidrolizi Michaelis-Menten kinetiğinden sapma gösterdi.

Bulunan sonuçlar, enzimlerin katalitik özellikleri açısından tartışıldı.

Anahtar kelimeler : Buğday özü, Lipaz, Kinetik, Saflaştırma

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To my family

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ABBREVIATIONS

BAL	British antilewisite
DDT	1,1-Bis(4-chlorophenyl)-2,2,2-trichloroethane
DEAE	Diethylaminoethyl
DFP	Diisopropyl phosphorofluoridate
DMP	2,3-Dimercaptopropan-1-ol
DTNB	5-5'-Dithiobis(2-nitrobenzoic acid)
EC	Enzyme commission
EDTA	Ethylenediamine-N,N,N',N'-tetra acetic acid
KP	Potassium phosphate
PAGE	Polyacrylamide gel electrophoresis
PCMB	p-Chloromercuri benzoate
PNPA	p-Nitrophenyl acetate
SDS	Sodium dodecyl sulfate
Tris	2-Amino-2(hydroxymethyl)-1,3-propandiol

CHAPTER I

INTRODUCTION

Lipolytic enzymes are widely distributed, being found in many tissues, fluids, cells, seeds, and organs. The enzymes are important metabolically to produce the free fatty acids and partial glycerides necessary for the transport of fatty acids through membranes and to release acids for oxidation and resynthesis into triglycerides and phospholipids. For these reasons and because of improved methodology, lipolytic enzymes have recently received much attention.

There has been much difficulty in defining a lipase, because,

(a) Impure, nonphysiological, non-emulsified, often water-soluble substrates have been used;

(b) there are variety of methods for assaying lipase activity; and

(c) investigators have reported activity in many different ways.

To avoid this confusion, the following definition is rigorously applied. Lipases can be defined as "enzymes

hydrolyzing the esters from emulsified glycerides at an oil-water interface" [1]. Water-soluble substrates or substrates which produce water-soluble intermediates should be avoided.

The International Union of Biochemistry recommends that lipases be called glycerol-ester hydrolases (Triacylglycerol acyl hydrolase, EC.3.1.1.3)[2], a very general systematic name.

I.1. Physiological Significance

A large part of the living matter on earth consists of lipids, and lipolytic enzymes play an important role in their biological turnover.

Lipids can be classified into four major groups :

(1)neutral esters of glycerol,especially triglycerides,

(2)cholesterol and its relatives,

(3)phospholipids, and

(4)glycolipids

Table 1 shows the classification of some major lipolytic enzymes according to these categories.

The enzymes are important because none of the fatty acid esters-triglycerides, cholesterol esters, or phospholipids can be used for energy generation or in other metabolic reactions without prior enzymatic hydrolysis. Furthermore,the lipids cannot pass through the biological food chains except after hydrolysis; animals must hydrolyze fats to digest them. This fact makes lipolytic enzymes interesting for the

Table 1

Classification of Lipolytic Enzymes [3]

Triglycerides :	Cholesterol Esters :
Lipases	Cholesterol esterase
Microbial	Phospholipids :
Plant	Phospholipase A_1
Milk	Phospholipase A_2
Pancreatic	Phospholipase B
Lipoprotein	Lysophospholipase
Hormone-sensitive	

nutritionist. In addition, many practical problems in food processing involve lipolytic enzymes.

I.2. Microbial Lipases

Most foods contain significant amounts of fat which can be hydrolyzed giving rise to quality changes [4]. Most microorganisms are able to produce fat hydrolyzing enzymes, lipases, which contribute to fat degradation and quality changes in fatty foods [5,6,7]. A number of microorganisms have been reported to produce inducible lipases when fat is present in the growth medium [8,9]. Even constitutive lipases have been found. However, fat supplemented media can also

result in decreased production of lipase [8,10,11].

Some microbial lipases, especially from Staphylococcus aureus, show very low specificity [12], while most of them are specific to the position, carbon chain length or degree of unsaturation of the fatty acids.

Some of the released fatty acids can either be taken up and metabolized by the organism or broken down into volatile compounds. The uptake of free fatty acids by bacteria is independent of lipolytic activity [13].

Few studies have been made with Rhizopus oligosporus to examine the control of lipase production. R.oligosporus is a fungus used to produce tempeh, a fermented soybean food product. This fermentation is reported to enhance the digestibility of soybean and the lipolytic activity of the fungus contributes to imparting desirable flavours [14].

Molecular weights of lipase from two microbial species Rhizopus delemar [15] and Penicillium cyclopium [16] were found to be 45,000 and 110,000, respectively. The molecular weights were determined by the method of gel filtration and the latter enzyme was a dimer.

The substrates and optimum pH's of some microbial lipases are summarized in Table 2.

I.2.1. The Use of Microbial Lipases

Lipases, especially from psychotrophic bacteria, can be remarkably heat resistant and such a lipase from Pseudomonas fluorescens can survive pasteurization or even sterilization processes and may therefore cause quality changes in

Table 2

pH Optimum of Lipases from Microbial Sources

Microorganism	Substrate	Opt. pH	Reference
G.candidum	Oleic acid	8.1-8.5	13,17
P.fragi	Glycerol esters of fatty acids	8.6-8.7	17
S.aureus	Unsaturated oils	8.3	17
P.cyclopium	p-Nitrophenyl laurate	6.0	16
P.fluorescens 27	Butter oil	8.0	5
H.lanuginosa*	Olive oil	8.0	18

* Immobilized enzyme; Amberlite XAD-7 with glutaraldehyde cross-linking.

otherwise microbially stable foods when stored at temperatures where such lipases are active [13].

Most higher fatty acids do not themselves contribute to flavor, but can be precursors to the formation of volatile compounds through oxidation. Hydrolysis of triglycerides and oxidation of lipids produce volatile components including acids, alcohols, esters, aldehydes, ketones, lactones and

furans usually cause off-flavor [19]. In certain ripening processes, however, such as, cheese making and production of dry, fermented sausages, the presence of free fatty acids and their oxidation products is considered positive to flavor.

Recently, it was reported that the lipase from C.rugosa is being used to hydrolyze oils for the production of soaps and that the enzymatic method yielded products with a better odor and color and was a cheaper overall process than the conventional uncatalyzed splitting method [7].

The most important industrial application of microbial lipase is the reactions to alter the composition of acyl moieties of triacylglycerols naturally occuring in fats and oils in order to modify their physical and chemical properties. One of the processes commonly used to alter the physical properties of triacylglycerols by manipulating the composition of their acyl moieties is interesterification. The classical interesterification is characterized by a randomization in the distribution of acyl moieties in the triacylglycerol molecule by applying a chemical catalyst such as sodium alkoxide [20].

The use of lipases to catalyze interesterification reactions has received considerable attention lately, because of certain advantages over chemical catalysts. By using a sn-1,3-specific lipase, for example, the exchange of acyl moieties is confined to the sn-1-and sn-3-positions giving rise to products with characteristics that cannot be obtained by chemical interesterification. Lipase catalyzed inter-

esterification reactions are widely used for the preparation of a wide variety of acylglycerols and alkyl esters of fatty acids that are useful as food additives and agrochemicals. Melting behavior of the triacylglycerols formed by interesterification reveals their potential use in food and dietetic products [20, 21, 22].

It also appears quite feasible that triacylglycerols having unusual structures seldom occuring in nature can be prepared by interesterification of common fats and oils using sn-1,3-specific lipases. For example, triacylglycerols containing a high level of linoleoyl moieties at the sn-1,3-positions and exclusively medium-chain acyl moieties at the sn-2-position, which rarely occur in nature and are difficult to prepare by chemical synthesis, can be easily obtained by interesterification of medium chain triacylglycerols. Such triacylglycerols could be of interest as dietetic products, since the linoleoyl moieties at the sn-1,3-positions would be rapidly released by the pancreatic lipases of most mammalian organisms and the linoleic acid would be rapidly available to the organism as an essential fatty acid.

I.2.2. Immobilization of Microbial Lipases

Interest on the microbial lipase include lipase catalyzed triglyceride hydrolysis and esterification, as energy saving processes. However, as the applications increased, the requirement for bulk amounts of enzymes became a limiting factor. Today, immobilization of a lipase is

expected to allow reuse of the enzyme, and longer usage might be possible.

An important characteristic of an immobilized enzyme preparation is, in general, the preservation of its catalytic activity through the immobilization procedure and the retention of this activity over prolonged periods of time. Generally, the immobilized lipase preparations initially showed about 85% to 90% of the activity used for immobilization. This activity is retained for several weeks, both in dry state and in the presence of the organic subtrate solution. The properties of immobilized enzymes have been studied under controlled conditions [18, 20, 21, 22].

Table 3 gives the materials used for immobilization of lipases from different microbial sources.

The method of adsorption on Amberlite XAD-7 with subsequent treatment with glutaraldehyde was superior as to several points. Firstly, the optimal temperature was about 10°C higher than that for the free enzyme, which is vital for solid fat hydrolysis. Secondly, good activity and stability were retained on repeated batch hydrolysis in both shaking and recycling systems. Thirdly, the technique employed was simple and there was a relatively high activity yield. Finally, a purified or partially purified enzyme was not necessary [18].

I.3. Milk Lipase

The milk lipase that is activated by foaming and causes

Table 3

Immobilized Microbial Lipases

Microbial Source	Immobilization Material	Reference
R.arrhizus	Celite(BDH,30-80 mesh,GLC-grade)	21
C.cylindraccea	Celite	22
H.lanuginosa	Amberlite XAD-7	18
H.lanuginosa	Controlled Pore Glass*	18
M.miehei	Commercial immobilized enzyme(Nova)	20

*Improved by either glutaraldehyde or 1-cyclohexyl-(2-morpholinethyl) carbodiimide.

the rancidity of milk is a glycoprotein or a family of glycoproteins. It is inhibited by diisopropylfluorophosphate and is specific for primary ester bonds. Since in milk fat both butyric and caproic acids are located largely in the sn-3-position and milk lipase is specific for primary esters, relatively large quantities of butyric and caproic acids are released. When the right concentration is reached a pungent and altogether frightful flavor can be detected which renders the milk unmarketable not to mention unpalatable. Similar to many lipases, the milk enzyme has an optimum pH range of 8-9,

an optimum temperature of 35-40°C and requires Ca^{2+} [17].

Milk lipases and lipolysis of milk fat have been the subject of the various studies [23,24]. In addition to lipases, milk contains a lipoprotein lipase and several esterases [25,26].

I.3.1. Metabolic Action of Milk Lipase

Fat is the main energy source of the newborn infant. In addition to providing 40% to 50% of the total calories in human milk or formula, fats are essential to normal development because they provide fatty acids necessary for brain development, are an integral part of all cell membranes, and are the sole vehicle for fat-soluble vitamin and hormones in milk. Furthermore, these energy-rich lipids can be stored in the body in nearly unlimited amounts in constrast to the limited storage capacity for carbohydrates and proteins.

Mature human milk has a fat content of 3.5% to 4.5%. The fat in milk is contained within membrane-enclosed milk fat globules. The core of the globules consists of triglycerides (98% to 99% of total milk fat), whereas the globule membrane is composed mainly of phospholipids,cholesterol,and proteins. Fat digestion requires adequate lipase activity and bile salt levels, the former for the breakdown of triglyceride, the latter for emulsification of fat prior to and during lipolysis.

In the newborn, and especially the premature infant,

pancreatic lipase and bile acid levels (the major components of intestinal fat digestion) are low. The efficient fat absorption in the newborn depends on alternate mechanisms for the digestion of dietary fat [24]. Of special importance is intragastric lipolysis, in which lingual and gastric lipases compensate for low levels of pancreatic lipase [23], whereas the products of lipolysis, fatty acids, and monoglycerides, compensate for low bile salt levels by emulsifying the lipid mixture [27]. Initial hydrolysis of the fat within the core of the milk fat globule by lingual lipase, probably facilitates the subsequent action of pancreatic lipase and of the bile-salt-stimulated lipase of human milk.

Free fatty acids and monoglycerides, the products of intragastric lipolysis, are relatively polar, and they can locate in the surface layer, dislocating phospholipids and proteins and thereby making the core triglyceride more accessible to pancreatic lipase and human milk bile-salt stimulated lipase. The latter hydrolyzes milk fat at pH 7.0 to 8.0, in the presence of bile salts, thus acting in the intestine to complete the digestive process initiated in the stomach by lingual and gastric lipases. The combined action of intragastic lipolysis and intestinal hydrolysis of fat by the bile-salt-stimulated lipase of human milk can effectively substitute for low pancreatic lipase activity and low levels of bile salts in the newborn [23].

I.3.2. Milk Lipase in Food Processing

Lipases and proteases hydrolyze casein and milk fat, affecting flavor and rheological properties of cheese. It is doubtful that hydrolytic enzymes naturally occuring in milk (catalase, peroxidase, xanthine oxidase, alkaline and acid phosphatases, amylases, proteases, lipases, aldolase) contribute greatly to ripening of cheese varieties such as cheddar since only a small fraction of the total activity has an optimum in the pH range of cheese while it is curing. Proteolytic and lipolytic activities of added and adventitious microorganisms contribute significantly to the characteristics of many varieties of cheese, especially the molded-ripened and surface-ripened cheeses. Esterases isolated from the oral glands of calves, kids and lambs produce the desired lipolysis and flavor in italian varieties of cheese such as Romano and Provolone [28].

I.4. Human Lipase

Two pancreatic lipases of different molecular weight were found in human pancreatic juice. The low molecular weight lipase was found in pure human pancreatic juice and the high molecular weight lipase in human pancreatic juice mixed with bile. The two enzymes were immunologically identical. The high molecular weight lipase seems to be a low molecular weight lipase-phospholipid complex [29], (Table 4).

pH optimum of pancreatic lipase is merely 8.5. But, in

the presence of bile salts and colipase, pH optimum is shifted to 6, which is the pH of the upper small intestine. The major site of lipid digestion is the small intestine.

Table 4

Lipases from Human Pancreatic Juice [29]

Fraction	Molecular weight	Method	Opt.pH
Lipase-S	35,000	SDS-PAGE	8.6
Lipase-F	800,000	Gel filtration	6.0-10.0

Colipase is a polypeptide with a molecular weight of 10,000 Daltons present in pancreatic juice and forms 1:1 complex with lipase [30].

The bulk of dietary lipid is composed of triacylglycerols. In the gastrointestinal tract, these are emulsified by the detergent action of the bile salts and hydrolyzed by lipases. The resulting products are a mixture of fatty acids and monoacylglycerols. The fatty acids and glycerides are absorbed by the cells of small intestine and within the intestinal cells are resynthesized into triacylglycerols.

The function of the bile is to promote emulsification and solubilization of lipids through the powerful detergent

action of bile salts. Bile salts also serve to solubilize the products of lipid digestion, thus promoting their absorption. Since lipids are essentially insoluble in water, enzymatic hydrolysis occurs only at the interface between the lipid droplet and the aqueous phase. High bile salt concentrations markedly reduce the activity of pancreatic lipase on triglycerides [31]. A recent paper suggests that the bile salts are acting not simply as detergents but as specific ligands [32]. The purified bovine pancreatic lipase exhibited a pH optimum of 8.8, an isoelectric point near pH 5.5 and an optimum temperature of 37°C [33].

Porcine pancreatic lipase catalyzes transesterification reactions between a number of sugar alcohols and various plant and animal oils in dry pyridine which can dissolve sugars in a synthetically acceptable concentration range and in which some lipases still can function as catalysts. The products of this process have been identified as primary monoesters of sugar alcohols and fatty acids. These enzymatically prepared sugar alcohol esters have been found to be excellent surfactants in terms of their ability to reduce interfacial and surface tensions and to stabilize emulsions [34]. Biological surfactants possess a number of potential advantages over their chemically manufactured counterparts, including lower toxicity, biodegradability, a wide variety of possible structures, and ease of synthesis from inexpensive, renewable feed stocks.

Extensive work has been devoted to the effects of

dietary fibers from cereals on lipid metabolism in animals and humans [35]. Some beneficial effects of fiber-enriched diets were observed, such as lowering of fasting [36] or post-prandial triglyceride values [37], decrease in hepatic triglyceride storage and favorable changes in lipoprotein lipid patterns [38].

Human serum lipase has been found to be a useful index, like serum amylase for the diagnosis of pancreatic diseases. Elevated serum lipase was detected in patients with acute, chronic pancreatitis, pancreatic carcinoma, and renal insufficiency. National and international recommendations for the standardization of the analytical and the diagnostic procedures for this enzyme are still lacking.

Recently, a variant form of lipase linked to immunoglobulin-G-lambda is reported with a molecular weight of >200,000, called as "Macro lipase" in human serum of a patient suffering from a malignant non-Hodgkin Lymphoma [39].

The isoelectrofocusing profiles of serum lipase showed the presence of three kinds of lipases with pI 7.4, 6.8 or/and 6.4. A lipase with properties similar to those of the serum lipase was found to be present in human pancreatic juice [40].

The liver of many species, on the other hand, contains an extracellularly located lipase termed the hepatic lipase that is capable of degrading triacylglycerol and phospholipids in addition to its preferred substrate, monoacylglycerol. The hepatic enzyme also catalyzes a series

of transacylation reactions; for example, it will catalyze the conversion of two monoacylglycerols to diacylglycerol plus free glycerol [41]. Liver lipase seems to play an important role in lipoprotein metabolism [38,42]. Inhibition of liver lipase causes a decrease in the cholesterol synthesizing capacity of the tissue [42].

Transesterification, esterification, aminolysis, acyl exchange, thiotransesterification and oximolysis reactions of lipases take place in organic solvents [34, 43, 44].In water, these reactions are suppressed by hydrolysis and therefore do not occur to any appreciable extent [44]. It appears that enzymatic activity exhibited in organic solvents is the full inherent catalytic power of enzymes not just a small fraction of it [44, 45].

I.5. Plant Lipases

Information on lipolytic enzymes in higher plants is important in understanding their physiological roles as well as their action in agricultural products during storage. In post-germination of oil-seeds, the mobilization of oil reserves is essential in providing energy and carbon skeletons for embryonic growth. Lipolytic enzymes catalyze the initial steps during lipid mobilization, and thus may be rate-controlling in germination and post-germinative growth [46].

In agriculture, the crushing or storage of seeds or other agricultural products may lead to an increase in

lipolytic activity. This increase results in an accumulation of free fatty acids in oil seeds, and the removal of fatty acids from food oil products add extra costs. Also, lipolytic activities during seed storage may cause a loss of seed vigor or ability of the seeds to germinate rapidly. Furthermore, lipolytic activities cause rancidity which renders agricultural products unsuitable for human consumption. It is apparent that the study of plant lipolytic activities is important. However our knowledge in this area is still very limited, especially when compared with information on mammalian lipases.

This confusion may arise due to,

(1) the nomenclature for plant lipolytic enzymes, especially with reference to substrate specificity. Different workers may assign various names to apparently very similar enzymes without realizing the close similarities.

(2) the use of improper substrates. Acyl hydrolases may have very general or restricted substrate specificity, and using an artificial substrate which is utilized by most lipolytic enzymes would lead to confusion .

(3) the use of commercial substrates of low or unknown purity and containing unspecified contaminants.

True lipases (EC 3.1.1.3), which attack the fatty acyl linkage of water-insoluble triacyglycerols, are known to occur in oil seeds and cereals. Most of the efforts in this area have been devoted to seed lipases.

I.5.1. Lipases in Oil Seeds

Oil seeds generally contain 20–50% of their dry weight as storage triacyglycerols. In post-germinative growth, the oil reserve is rapidly mobilized to provide energy and carbon skeletons for the growth of the embryo [47]. Within a few days, most of the oil reserve is consumed. Highly active lipases are found to catalyze the hydrolysis of reserve triacylglycerols. The triacylglycerols are localized in subcellular organelles called lipid bodies which are bound by a half-unit membrane [48]. Depending on the plant species, the lipase may be localized in the membrane of the lipid bodies, or in other subcellular compartments. With few exceptions, lipase activity is absent in ungerminated seeds and increases rapidly in post-germination [49]. Crushing or storage generally activates dormant lipases in a seed, and the resulting accumulation of free fatty acids can cause an industrially important oil to become unacceptable or to require additional processing to remove the acids. Nevertheless, investigators have neglected the lipases in most of the important food fat oil seeds, e.g, soybean, cottonseed, corn, coconut, sesame, peanut, as well as other industrial seeds such as tung. Very few reports on the lipases of these seeds are available. Most attention has been paid to seed lipases that exhibit some exotic properties [50].

Clearly, castor bean is unigue among the oil seeds

examined. Only castor bean lipid bodies, isolated from either ungerminated or germinated seeds, can undergo autolysis. The castor bean acid lipase can release all three fatty acids from one triacylglycerol. Like most lipases, the acid lipase preferentially hydrolyzes triacylglycerols of shorter fatty acid chain length [51, 52]. The enzyme is already present in an active state in ungerminated seeds. It was observed that the decrease in enzyme activity in post-germination preceded the disappearance of the storage lipids [52].

A second acyl hydrolase, the alkaline lipase, appears in the endosperm after germination has begun and is the only known enzymically active integral membrane protein in the glyoxysomes.

In peanut, a lipase was partially purified from an acetone powder of maturing seeds, using tributyrin as the substrate [53]. The enzyme activity increases during seed maturation. It has a higher activity on tributyrin than corn oil.

A lipase was partially purified from ungerminated seeds of Vernonia anthelmintica [54], an oilseed in which the major storage lipid is trivernolin. It hydrolyzes both primary and secondary ester bonds of triacylglycerols and is equally active on saturated (palmitate, stearate) and unsaturated (oleate) triacylglycerols.

In acetone powder of apple seeds, two lipase activities are present. The activities are low in ungerminated or dormant seeds and increase in post-germination. The lipase

activity declines as the storage lipids are exhausted. The intracellular location of lipase activity has been determined for a few species and is found to vary considerably. It is found with the lipid bodies of castor bean, cotton, corn and oilseed rape, the glyoxysomes of soybean, peanut and castor bean, and the light microsomal membranes of oilseed rape [55]. The only plant lipases to have been purified to date are the lipid body lipase of corn scutellum [56], and the glyoxysomal lipase of castor bean [57].

Optimal activity for castor bean acid lipase, and castor bean glyoxysomal lipase was obtained at about pH 4.1 [46, 51] and 9.0 [57] respectively. However lipase purified from ungerminated seeds of Vernonia anthelmintica has an optimal activity at pH 7.5-8.0 [54].

The molecular weights of lipases from various plant sources are given in Table 5.

I.5.2. Lipases in Cereal Grains

I.5.2.1. Wheat Germ

Wheat, like other cereal grains, produces dry, one-seeded fruits which do not split open to shed the seed at maturity.

The seed consists of germ, or embryo, and endosperm enclosed by a nucellar epidermis and a seed coat. A fruit coat, or pericarp surrounds the seed and adheres closely to the seed coat. This type of fruit, commonly called a kernel or grain, is known to the botanist as a caryopsis.

Table 5

The Molecular Weights of Lipases from Plant Sources

Lipase	Molecular Weight	Method	Reference
Vernonia anthelmintica	>200,000	Gel filtration	50,54
Castor Bean Glyoxysomal	62,000	SDS-PAGE	55
Peanut Glyoxysomal	62,000	SDS-PAGE	55
Corn Scutellum	270,000*	Density Gradient Centrifugation	56

* Tetrametric enzyme

The composition of the three parts differs markedly, both quantitatively and qualitatively (Table 6).

Wheat germ contains relatively high proportions of carbohydrate (app.50%) and protein (app.32%) but its unique feature is its high fat content. In flour milling, the germ must be separated from the endosperm without contaminating the flour with germ fat. High fat content of flour shortens its shelf-life [58]. Germ, if left in flour, has an adverse effect on the flour, and on breadmaking quality. Flour may not store well, because the presence of large amounts of germ

causes development of rancidity and spoilage. Furthermore, germ increases the ash content and impairs flour color [59]. The wheat germ is a unique highly concentrated source of nutrients. Wheat germ proteins have been classed with superior animal proteins. The presence of large amounts of fats and sugars makes the wheat germ highly palatable. In addition to oilseed meals, wheat germ, as a by product of the

Table 6

Composition of Whole Grain, Endosperm, Bran and Germ [58]

Constituent	Grain (%)	Endosperm (%)	Bran (%)	Germ (%)
Dry matter	100	(82)*	(15)	(3)
Carbohydrates	82.7	86.4 (85)	70.0(13)	50.6 (2)
Protein (Nx5.7)	12.8	11.2 (72)	16.7(20)	32.4 (8)
Fat	2.5	1.6 (52)	5.4(32)	11.9(16)
Minerals	2.0	0.8 (34)	7.4(58)	5.1 (8)

* Percentage of total in the grain

flour milling industry, is one of the potential sources of much needed protein, calories and vitamins.

Wheat germ oil is one of the richest natural sources of

vitamin E and is used as a component of health foods, cosmetics and toiletries. The separation of oil from wheat germ by extracting with supercritical carbondioxide (CO_2) was studied [60]. Oil extracted with supercritical CO_2 was lighter in color and contained less phosphorus than that extracted with hexane.

Wheat germ was obtained in two forms; conventional flaked germ that had passed through reduction rolls at the flour-mill and a granular form, from a germ separator that removed a germ stream before the reduction stages of milling [59].

The hydrolysis of triacylglycerols was the main source of fatty acid released during storage and that the bran fraction appeared to have greater triacylglycerol lipase activity than did the germ fraction. The activity in bran was around 10-fold greater than that in germ by using [^{14}C] trioleoylglycerol as substrate. On the other hand, the lipoxygenase activity (Enzyme catalyzes the O_2 -dependent oxidation of 18:2 and 18:3 free fatty acids) in these fractions was greater (3 to 8 fold) in the germ than in the bran [61].

An enzyme from wheat germ hydrolyzing monobutyrin and designated as a lipase was originally studied by Singer and Hofstee [62], with the activity being determined manometrically. Today, there is considerable doubt that the activity was a lipase. It was actually an esterase, since it hydrolyzes triacetin and tributyrin but is inactive on long-

chain triacylglycerols. It seems likely that Singer and Hofstee studied an esterase rather than a lipase.

However subsequent investigations by Stauffer and Glass [63] established that wheat germ does contain a true lipolytic enzyme as well as an esterase and a tributyrinase. The above "tributyrinase" that hydrolyzes emulsions of short-chain triacylglycerols (triacetin, tributyrin), an esterase that hydrolyzes water-soluble esters, and a hydrolase that is active on monoolein emulsified with olive oil.The last enzyme is inactive on tributyrin and has an optimal activity at pH 8.0, whether it hydrolyzes long-chain triacylglycerols is unknown.

With olive oil as the substrate, true lipase activity in the embryo of ungerminated seeds is very low and increases rapidly in post-germination [64, 65]. This lipase activity is highest at alkaline pHs (7-9) and requires Ca^{2+} for maximal activity. It preferentially hydrolyzes fatty acids from the 1 and 3 positions of triacylglycerols.

I.5.2.2. Oat

Oat lipase has been studied using tributyrin or triolein as the substrate. The partially purified enzyme has an optimal activity at pH 7.4 [66]. Di- and monobutyrins were not digested; the authors interpreted this as specificity for the triglyceride. A more likely explanation is that the increased solubility of the di- and monobutyrins in water relative to tributyrin caused them to be unavailable to the

lipase [50]. Berner and Hammond [67] further examined the specificity of oat lipase. The lipase releases linoleic acid and palmitic acid, but no stearic acid, from cacao butter (triacylglycerols containing mostly linoleic acid, palmitic acid, and stearic acid in roughly equal proportion) in its native state or having the positions of fatty acyl group attachment randomized chemically. It exerts no preference on the primary or secondary position of triacylglycerols.

I.5.2.3. Rice

Most of the lipase activities in rice are present in the outer layers (bran) of the grains. Three rice-bran lipases have been separated from one another by ion exchange chromatography and gel filtration [68, 69]. Two of them have been purified to homogeneity and their properties studied. Lipase I has a molecular weight of 40,000 [68, 70, 71] and is activated by Ca^{2+} at low concentrations (0.5 mM). The N-terminal amino acid is glutamic acid and the C-terminal amino acids are glycine and serine. However, a lipase with a molecular weight of 40,000 isolated by a different laboratory has an N-terminus that is blocked by an acetyl group and the C-terminus is phenylalanine [72].

Lipase II has a molecular weight of 33,000, consisting of unknown numbers of subunits having molecular weights of 14,000 and 3900 and held together by disulfide bonds [73]. Both lipase I and lipase II have an optimal activity at pH 7.5-8.0 with tributyrin as the substrate.

Both hydrolyze short- and long-chain triacylglycerols, although the activity is much higher on short chain (C_2 -C_6) triacylglycerols. Although these rice-bran lipases have been studied rather extensively, their role in causing rancidity is still unclear. Rice bran contains other lipolytic enzymes that may be involved in generating rancidity.

The substrates and pH optima of lipases from various cereal grains are given in Table 7.

Table 7

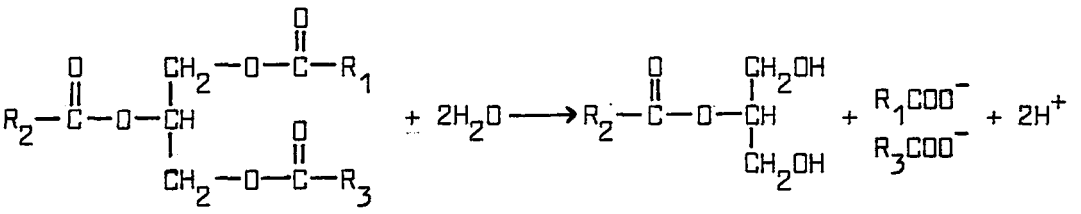
pH Optimum of Lipases from Cereal Grains

Lipase	Substrate	Opt. pH	Reference
Wheat germ	4-methyl umbelliferone butyrate	7.5	74
Wheat germ	Fluorescein dibutyrate	8.0	75
Oat grains	Tributyrin	7.4	66
Rice bran	Tributyrin	7.5	68
	Ethyl acetate	5.5	72
	Triglycerides	7.4-7.6	72

I.6. Catalytic Properties of Lipases

I.6.1. Substrates and Assay Methods

Lipases are hydrolytic enzymes which catalyze the reaction,



Pancreatic lipase removes 1- and 3- acyl groups from triacylglycerols, on the other hand, hepatic lipase demostrates specificity toward the acyl group present on monoacylglycerols [41]. Since the activity of the enzyme in the reaction mixture is greatly influenced by the interface concentrations of substrates, it was difficult to measure the lipase activity with high sensitivity and reproducibility using unstable emulsified olive oil as a substrate. Therefore a number of artificial chromogenic and fluorogenic substrates have been tested to overcome these drawbacks (Table 8).

Lipases were assayed potentiometrically using tributyrin [31,34,35], olive-oil emulsion [6], tripropionin [15], methyl oleate [16], butter oil emulsion [33] as substrate. Michaelis-Menten constant, Km, was found as 2.53 mM in the presence of 12 nM colipase for the pancreatic lipase catalyzed hydrolysis of tributyrin [31]. By using tripropionin as substrate Km was estimated as 3.8 mM with Rhizopus delemar lipase [15].

Table 8

Substrates for Lipase Assay

	Reference
Chromogenic substrates :	
2,3-Dimercaptopropan-1-ol tributyrate *	40,76
p-Nitrophenyl Laurate	16
Glycerol esters **	57
Fluorogenic substrates :	
N-methyl indoxyl myristate	78,79
Diacetyl fluorescein	75
4-methyl umbelliferone butyrate	74
4 methyl umbelliferone myristate	79
4-methyl umbelliferone oleate	77
4-methyl umbelliferone heptanoate	80,81
4-methyl umbelliferone octanoate	80

* in combination with a chromogenic SH-reagent, DTNB

** free fatty acids as copper soaps using
1,5 diphenylcarbazide

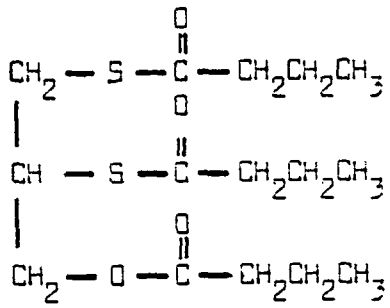
Wheat germ lipase catalyzed hydrolysis of 4-methyl umbelliferone butyrate gave a Km value of 0.031 mM [74] and

4-methyl umbelliferone heptanoate hydrolysis with porcine pancreas, castor bean, wheat germ lipases gave Km values of 7.3 μM [80], 10 μM [81], and 2.7 μM [81], respectively.

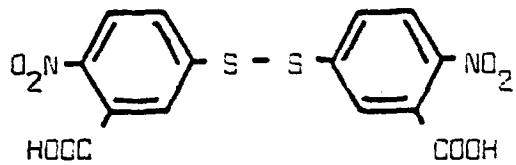
A colorimetric method was tried by using various glycerol esters as substrates [57] . The free fatty acids released were measured as copper soaps, using 1,5-diphenylcarbazide. Lipases catalyze the hydrolysis of some S-fatty acyl thiol compounds [76]. 2,3-Dimercaptopropan-1-ol tributyrate (DMP-tributyrate) using DTNB as a chromogenic reagent, was hydrolyzed by pancreatic lipase [40, 76]. The substrate DMP-tributyrate is also called as BAL-tributyrate, since the substance 1-propanol-2,3-dithiol acts as an antidote in arsenic poisoning arising from sources such as lewisite, and other war gases containing this element. The substance BAL(British antilewisite) is effective probably because of the presence of SH groups, which form complex salts with arsenic,making it nontoxic to the animal organism.

Structural formulae of some lipase substrates and related substances are given in Figure 1.

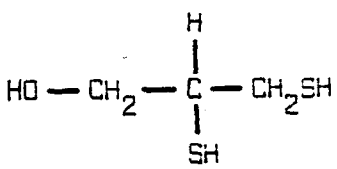
Some of the substrates used for the assay of lipase was found to catalyze the hydrolysis of various carboxylic esters. For example, 4-methyl umbelliferone heptanoate hydrolysis was appreciably effected by horse serum cholinesterase and β-chymotrypsin, in addition to porcine pancreatic lipase [80]. When DMP-tributyrate was used as a substrate for lipase, a potent inhibitor,phenylmethylsulfonyl fluoride was used to inhibit the esterase activity [76].



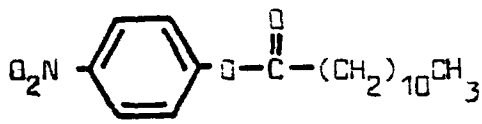
2,3-Dimercaptopropan-1-ol tributyrate (DMP-tributyrate)



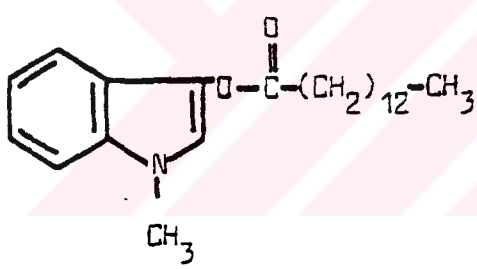
5-5'-Dithiobis(2-nitrobenzoic acid) (DTNB)



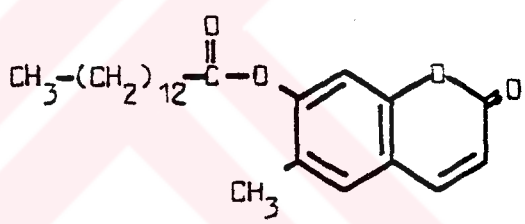
1-propanol-2,3-dithiol (BAL)



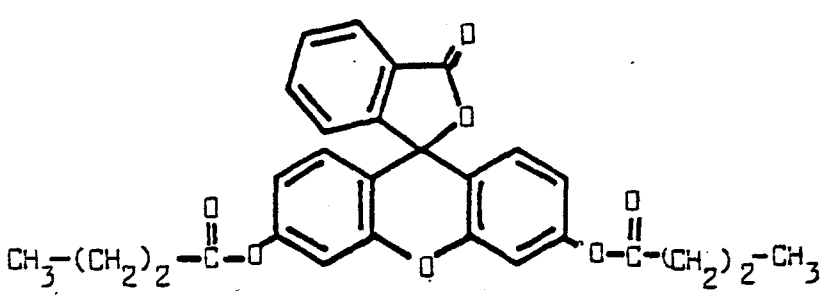
p-Nitrophenyl Laurate



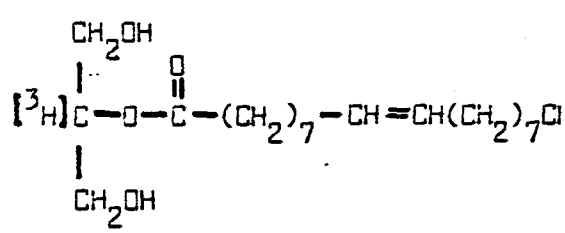
N-Methylindoxyl myristate



4-Methylumbelliferone myristate



Dibutyrylfluorescein



[2—^{3}H] — monoolein

Figure 1. Structural Formulae of Some Lipase Substrates and Related Substances

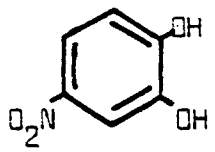
Due to the impure enzyme preparations and the absence of a specific substrate for lipase assay, there is limited number of studies on the kinetic properties of lipases. Most of the kinetic parameters obtained was doubtful with respect to the substrate concentration ranges because of the limited solubility of the substrates or for some other reasons.Within the substrate concentration range of 0.56 to 3.18 mM tripropionin, Km value was estimated as 3.8 mM [15]. The substrate concentration range was not stated in various reports [74, 81].

I.6.2. Inhibition and Activation

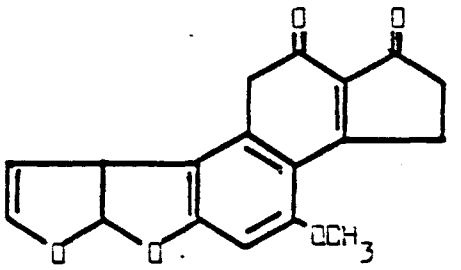
4-Nitrocatechol, aflatoxin, fluoride, PCMB, DFP p-aminophenylarsine, o-iodobenzoate, DDT, EDTA were reported as inhibitors for lipases of different sources in addition to several ions such as CN^-, Cu^{2+}, Fe^{3+}, S^{2-} [74,82]. SDS and DDT lipase complex of 1:9 ratio activates lipase activity [15]. A glycoprotein activator of human pancreatic lipase with a molecular weight of 13,000 and a proteinic inhibitor extracted from cereals, wheat bran, wheat germ of the same enzyme was also reported [35, 83]. The structural formulae of some lipase inhibitors are given in Figure 2.

I.7. Aim of This Study

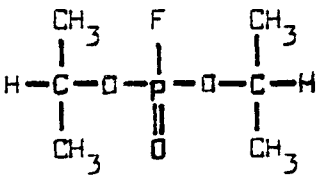
In recent years, remarkable progress has been made in studies of enzymes, especially in connection with their purification and analysis of the molecular constituents.



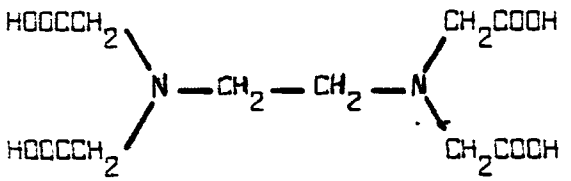
4-Nitrocatechol



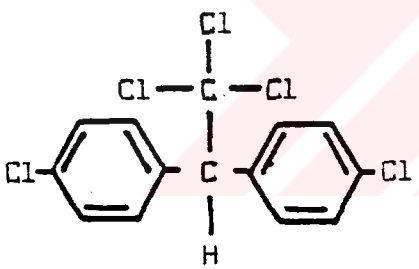
Aflatoxin B_1



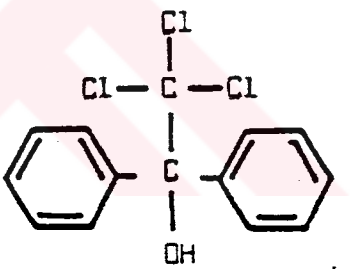
Diisopropylphosphorofluoridate
(DFP)



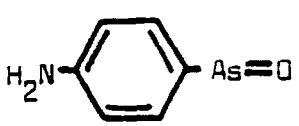
Ethylenediamine-NNN'N'-tetra acetic acid
(EDTA)



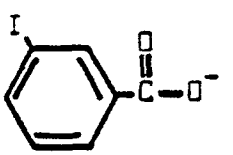
1,1-Bis(4-chlorophenyl)-2,2,2-trichloroethane
(DDT)



1,1-Bis(4-chlorophenyl)-2,2,2-trichloroethanol
(kelthane)



p-aminophenyl arsine oxide



o-Iodo benzoate

Figure 2. Structural Formulae of Some Lipase Inhibitors

However, there is still many problems concerning the optimum assay conditions, especially with the enzymes acting upon water-insoluble substrates. In addition, kinetics of enzyme catalyzed reactions depend on various factors such as pH, temperature, ionic strength, concentration of buffer, and solvent, nature of buffer and solvent and most importantly on the type of substrate used.

The commercial preparations of some enzymes with different levels of purity are now available. But commercial wheat germ lipase contains several esterase and other enzymic activities, together with the lipase activity [84].

The aim of the present work was to isolate the enzymatic activities and to study the kinetics of esterase and lipase activities present in wheat germ lipase. In order to check the presence of different activities, some common purification methods such as gel filtration and ion exchange chromatography were used. Substrates choosen for the assay of esterase and lipase activities are p-nitrophenyl acetate and DMP-tributyrate, respectively.

CHAPTER II

MATERIALS AND METHODS

II. 1. Enzyme and Substrates

Lipase (Triacylglycerol acyl-hydrolase EC.3.1.1.3) from wheat germ (Type I, specific activity of 7 units/mg solid) was obtained from Sigma Chemical Company, U.S.A. A stock solution of 10 mg solid/ml was prepared in water and diluted to the desired concentration with buffer in the cell prior to assay. When stock was kept frozen, reproducible results were observed for at least one month.

DMP-tributyrate and p-nitrophenyl acetate were also purchased from Sigma. DMP-tributyrate stock solution was prepared freshly in 100 % distilled ethanol. The stock solution of PNPA was prepared either in 100 % acetonitrile or 100 % distilled methyl glycol.

II.2. Column Materials and Other Reagents

Gel filtration material, Sephadex G-100 was purchased from Pharmacia, Sweden. Ion exchange materials, DEAE-Sephadex A-50 and Cellulose phosphate were obtained from Sigma.

Chromogenic SH reagent, DTNB (5-5'-dithiobis (2-nitro-benzoic acid)) was obtained from Sigma and the stock solution was prepared freshly in buffer.

The common reagents used were all of reagent grade (Merck or Riedel de Haen) and all solutions were prepared using triple-distilled water.

II.3. Activity Measurements

Unless otherwise stated all of the activity measurements were done at 25°C. A Beckman model 24 double-beam spectrophotometer with a Beckman recorder model 24-25 AC was used for activity measurements and the temperature of cell compartment was maintained with Haake circulator KT 33. The initial rates of reactions were taken from the slopes of the linear progress curves generated on the recorder.

In all measurements, the volume of the assay mixture was 2 ml and the sample was assayed against a reference which contained all components except enzyme.

When PNPA was used as substrate, p-nitrophenolate ion released was followed at 405 nm in an assay mixture containing 5% solvent (v/v), 0.5 mg enzyme/ml and 100 mM buffer. The substrate concentrations ranged from 0.01 to 1.00 mM.When DMP-tributyrate was used as substrate,in the presence of DTNB, thionitrobenzoate dianion produced was followed at 412 nm in an assay mixture containing 0.3 mM DTNB, 5% ethanol (v/v) and 100 mM buffer.The enzyme concentration was 2 mg/ml. Substrate concentrations ranged from 0.003 to 0.1 mM. To

ascertain whether DTNB concentration was rate limiting factor or not, various DTNB concentrations were tested, but no effect was observed based on increasing reagent concentration.

Molar extinction coefficients under assay conditions, (1 cm cells,25°C, pH 8.5) were determined with both of the substrates, and the values were estimated by taking the average of at least five measurements.

During purification, the active fractions were determined by the two substrates. PNPA concentration was held constant at 1 mM and DMP-tributyrate concentration was 0.05 mM. The specific activities were calculated in terms of micromoles or nanomoles product formed per mg protein per hr.

The kinetics of hydrolysis by active fractions was done and results were compared with that of obtained by commercial enzyme.

II.4. Protein Measurement

Protein concentration was measured at 280 nm. An absorbance index value was estimated by using a plot of A_{280} against concentration of wheat germ lipase. Enzyme concentration range was 0.1-1.0 mg solid/ml.

II.5. Heat Inactivation

The commercial enzyme was incubated in 100 mM Tris-HCl buffer, pH 8.5 at 40 and 50°C. After appropriate times of preincubation, the samples were rapidly cooled and the

precipitates were removed by centrifugation. After equilibration to 25°C, the residual activities were assayed by using DMP-tributyrate as substrate. The residual activity was plotted against time .

II.6. Enzyme Purification

II.6.1. Gel Filtration

Gel filtration was carried out by using Sephadex G-100. A 4 g. of material was swollen overnight in 25 mM KP buffer, pH 7.5. A pharmacia column (0.9x26 cm) was packed and equilibrated with 25 mM KP buffer pH 7.5, by washing two or three times of the bed volume. A 4 ml of 10 mg/ml of commercial enzyme was loaded to the column and 1.3 ml fractions were collected by the use of a Pharmacia peristaltic pump model P-3 (Fig.3). Flow rate was 26 ml/hr. Elution buffer was 25 mM KP buffer, pH 7.5. Protein concentration and activity of collected fractions were determined in 0.1 M Tris-HCl, pH 8.5 at 25°C. The substrates used for the activity measurements were p-nitrophenyl acetate and DMP-tributyrate. The fractions which show maximum activity were pooled and kept frozen for further experiments.

II.6.2. Ion-Exchange Chromatography

Ion-exchange chromatography was tried by two types of column materials : An anion exchanger, DEAE-Sephadex A-50, and a cation exchanger, cellulose phosphate.

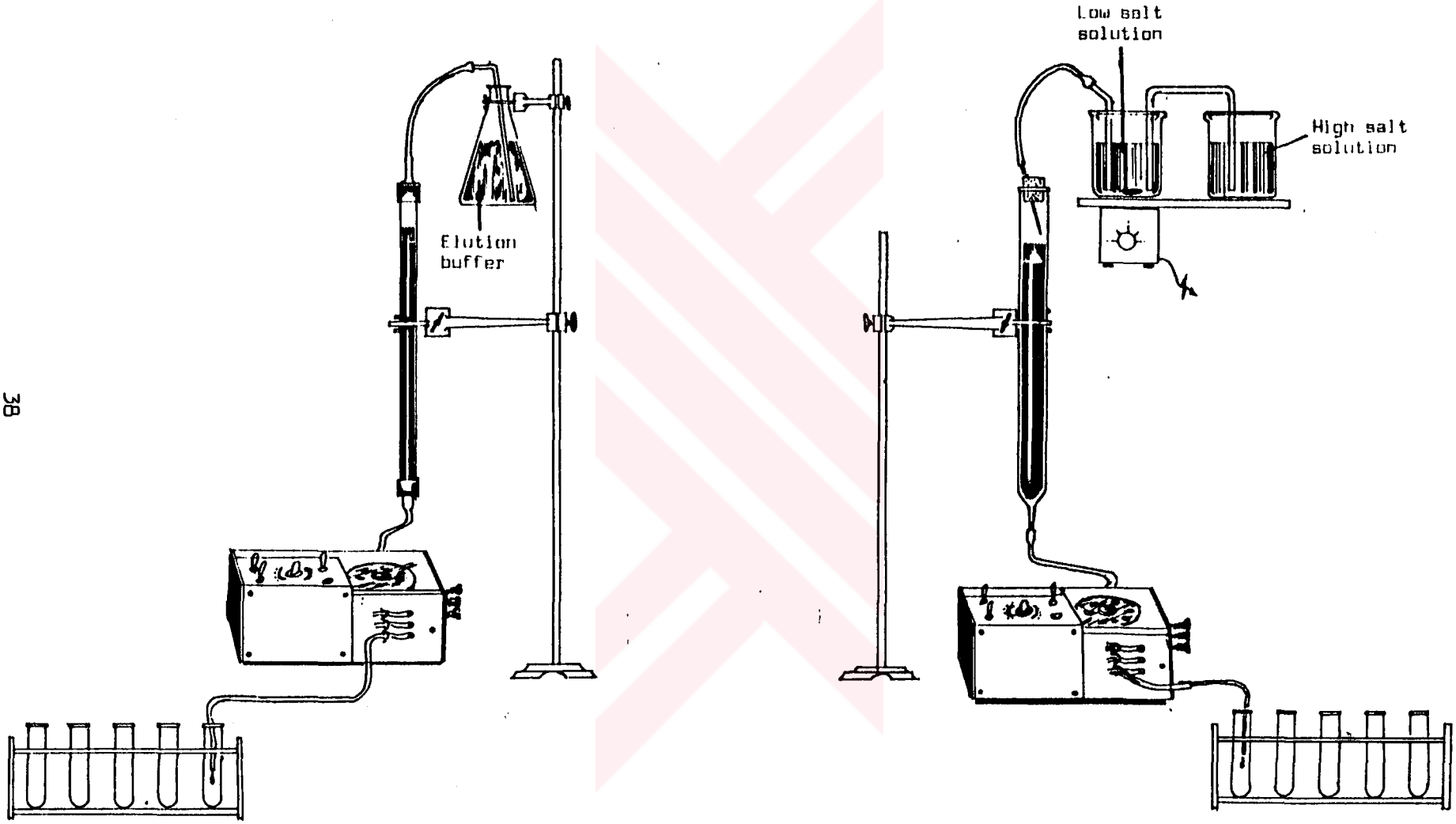


Figure 3. Gel Filtration (left), and Ion-Exchange Chromatography (right) Systems

II.6.2.1. DEAE-Sephadex A-50

A 3 g. of material swollen in 100 ml of 25 mM Tris-HCl buffer, pH 7.5, overnight was used for packing of column (1.4x21 cm). A 6 ml of 10 mg solid/ml was applied to column which had already been equilibrated with 25 mM Tris-HCl, pH 7.5. The enzyme was eluted with 100 ml of 0.0-0.6 M NaCl in 25 mM Tris buffer (Fig.3). The flowrate was 40 ml/hr. 2-ml fractions were collected and fractions were assayed for protein concentration and activity in 0.1 M Tris-HCl, pH 8.5 at 25°C. The activity of fractions was determined by using PNPA and DMP-tributyrate. The active fractions were pooled and reserved.

II.6.2.2. Cellulose Phosphate

A 4 g. material swollen in 100 ml of 25 mM acetate buffer, pH 4.5, overnight was used for packing of column (1.4x20 cm). The column was washed two or three times of its bed volume with the same buffer for equilibration. A 6 ml of 10 mg/ml enzyme was injected into the column and a total of 100 ml of 0.0-0.5 M NaCl in 25 mM acetate buffer, pH 4.5, was used for elution (Fig.3). Flowrate was adjusted as 30 ml/hr. 2-ml fractions were collected and protein concentration and activity were determined in 0.1 M Tris-HCl, pH 8.5 at 25°C. For the activity measurements the substrates were PNPA and DMP-tributyrate. The fractions with maximum activity were pooled and kept frozen for further experiments.

A stepwise purification was also done by the use of

cellulose phosphate. The active fractions pooled from gel filtration were applied to the cellulose phosphate column. Elution was done with a linear gradient of 0.0–0.5 M NaCl in acetate buffer, pH 4.5. Fractions containing maximum specific activity were combined and used for kinetic studies.

CHAPTER III

RESULTS

III.1. p-Nitrophenyl Acetate Hydrolysis

The kinetics of p-nitrophenyl acetate hydrolysis was studied in the 0.01 to 1.00 mM substrate concentration range. The limited solubility of the substrate was observed with both methyl glycol and acetonitrile. On the other hand, the rate of hydrolysis was found to be dependent on the identity and concentration of solvent used. The rate in methyl glycol was about twice compared to the rate obtained in acetonitrile (Fig. 4,5). An increase of methyl glycol from 5% to 10% causes about 50% increase in activity at substrate concentrations near saturation. In the light of these findings, the solvent concentration was held constant, and the highest substrate concentration studied was 1 mM at 25°C, in 100 mM KP buffer, pH 7.4, containing 5% (v/v) solvent. The Lineweaver-Burk plot obtained was nonlinear (Fig.6), indicating that the commercial enzyme preparation may contain two or more enzymes for the hydrolysis of PNPA.

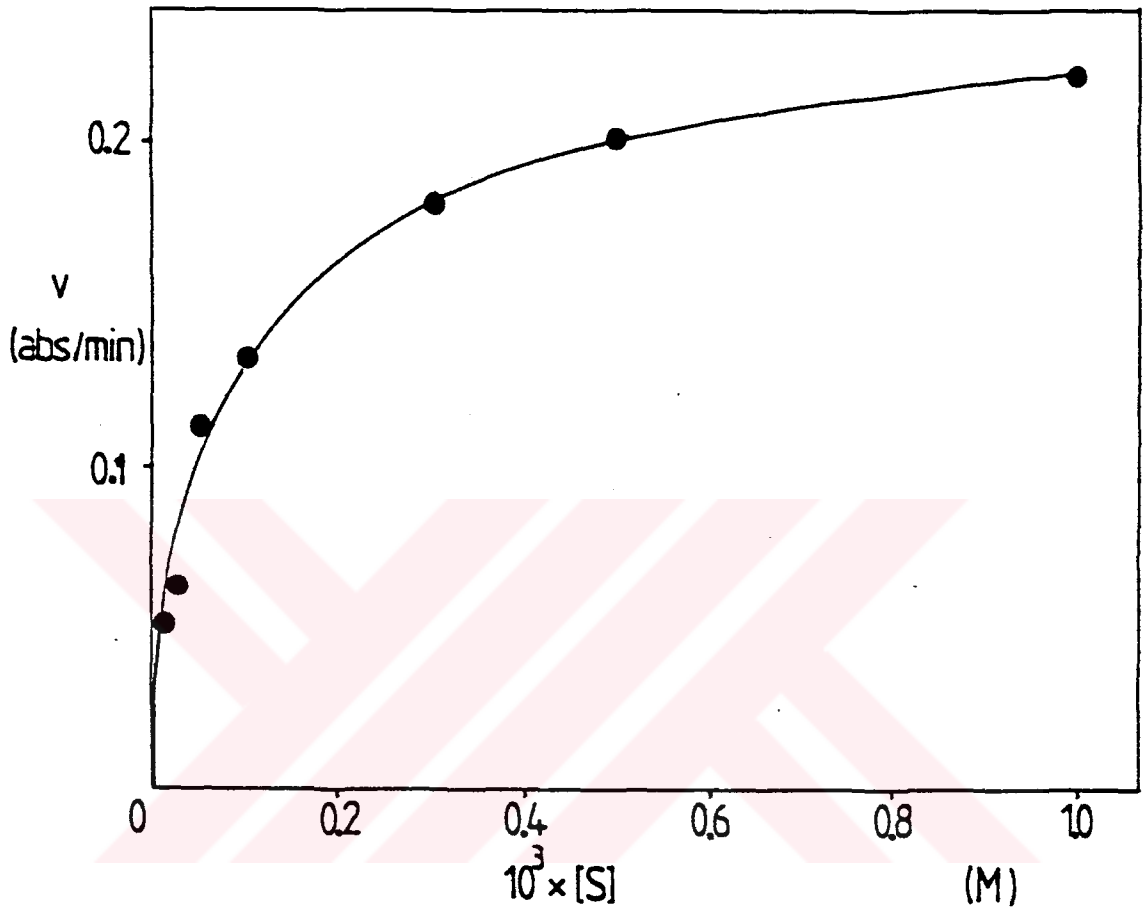


Figure 4. PNPA Hydrolysis in Methyl Glycol. Buffer is 100 mM KP, pH 7.4.

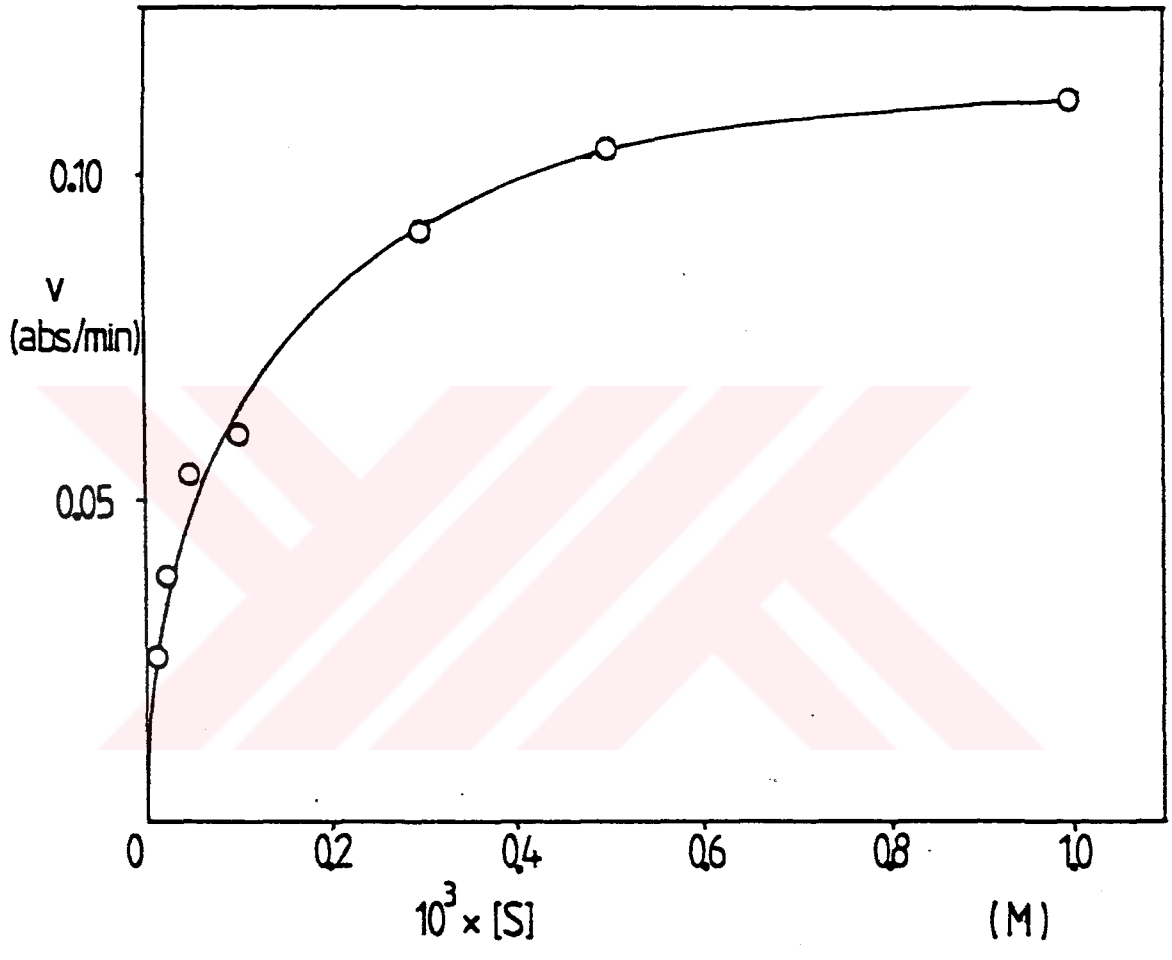


Figure 5. PNPA Hydrolysis in Acetonitrile. Buffer is 100 mM KP, pH 7.4.

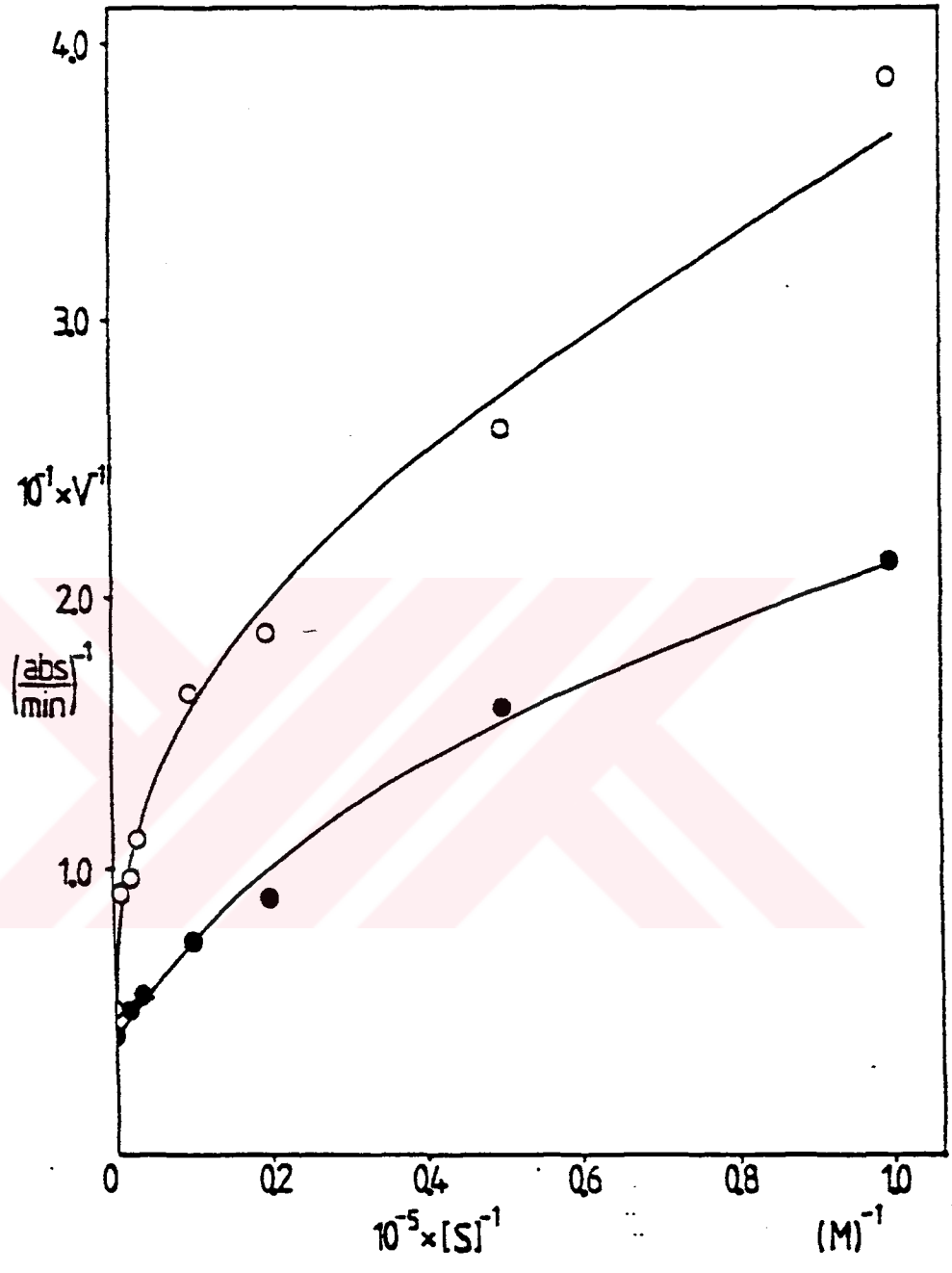


Figure 6. Lineweaver–Burk Plot for PNPA Hydrolysis : Solvent Effect. (O) in acetonitrile ; (●) in methyl glycol

If this is the case, the rate of hydrolysis at any substrate concentration is the sum of the velocities contributed by each enzyme. For two enzymes the velocity is given by :

$$v = \frac{[S]\ Vmax_1}{Km_1 + [S]} + \frac{[S]\ Vmax_2}{Km_2 + [S]} \qquad [85]$$

From the Lineweaver-Burk plot for PNPA hydrolysis in 100 mM Tris-HCl buffer, pH 8.5 (Fig.7), the presence of at least two esterases with low-Km and high-Km was suggested. Km_1 and Km_2 were estimated as 17 µM and 2.3 mM, and $Vmax_1$ and $Vmax_2$ as 0.23 abs/min. and 0.50 abs/min., respectively.

III.2. Dimercaptopropanol Tributyrate Hydrolysis

III.2.1. Kinetics

The hydrolysis of DMP-tributyrate was studied in 100 mM Tris-HCl buffer, pH 8.5, containing 5% (v/v) ethanol,at 25°C, in the 3 to 100 µM substrate concentration range. The linear Lineweaver-Burk plot indicates that, the reaction obeys Michealis-Menten kinetics (Fig.8). The Km and Vmax values were estimated as 6.7 µM and 0.05 abs/min., respectively. The rate of hydrolysis versus enzyme concentration was found linear (Fig.9), showing that the system is in steady state. During rate measurements, DTNB with a concentration of 0.3 mM was not the limiting reactant. The Hill plot was constructed

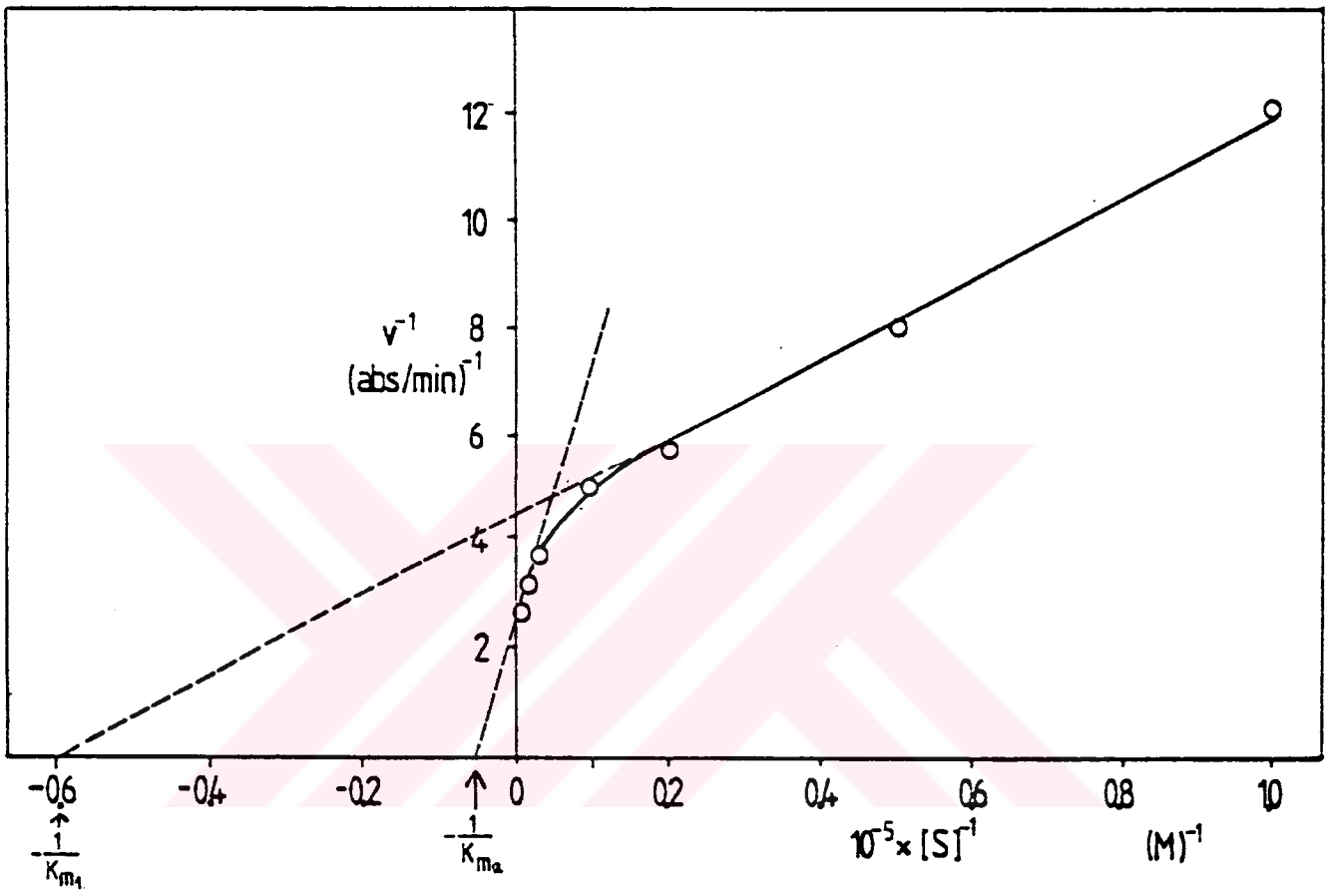


Figure 7. Lineweaver-Burk Plot for PNPA Hydrolysis in Tris HCl Buffer. Solvent is Acetonitrile and Buffer is 100 mM Tris HCl, pH 8.5.

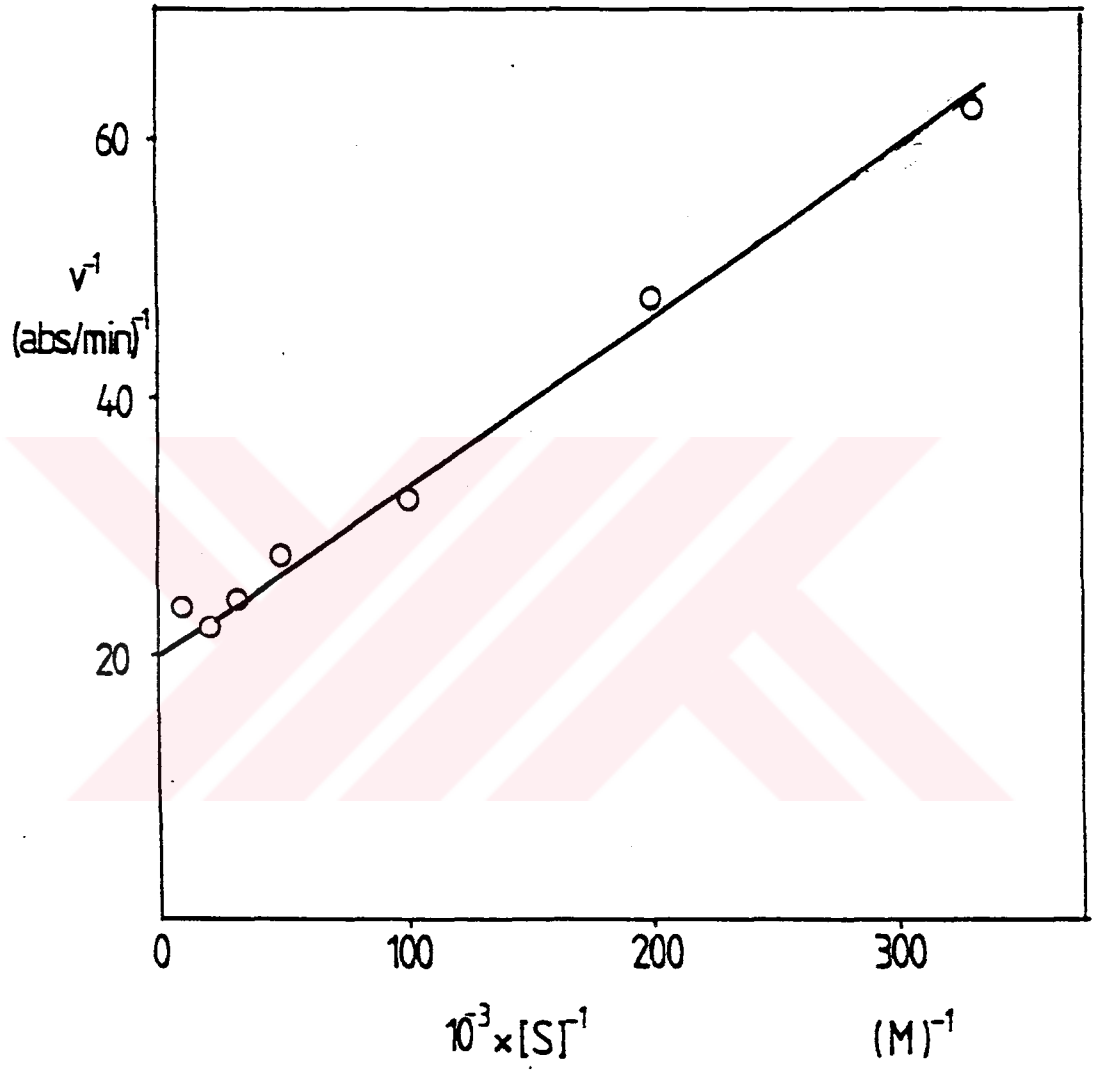


Figure 8. Lineweaver–Burk Plot for DMP–tributyrate Hydrolysis.

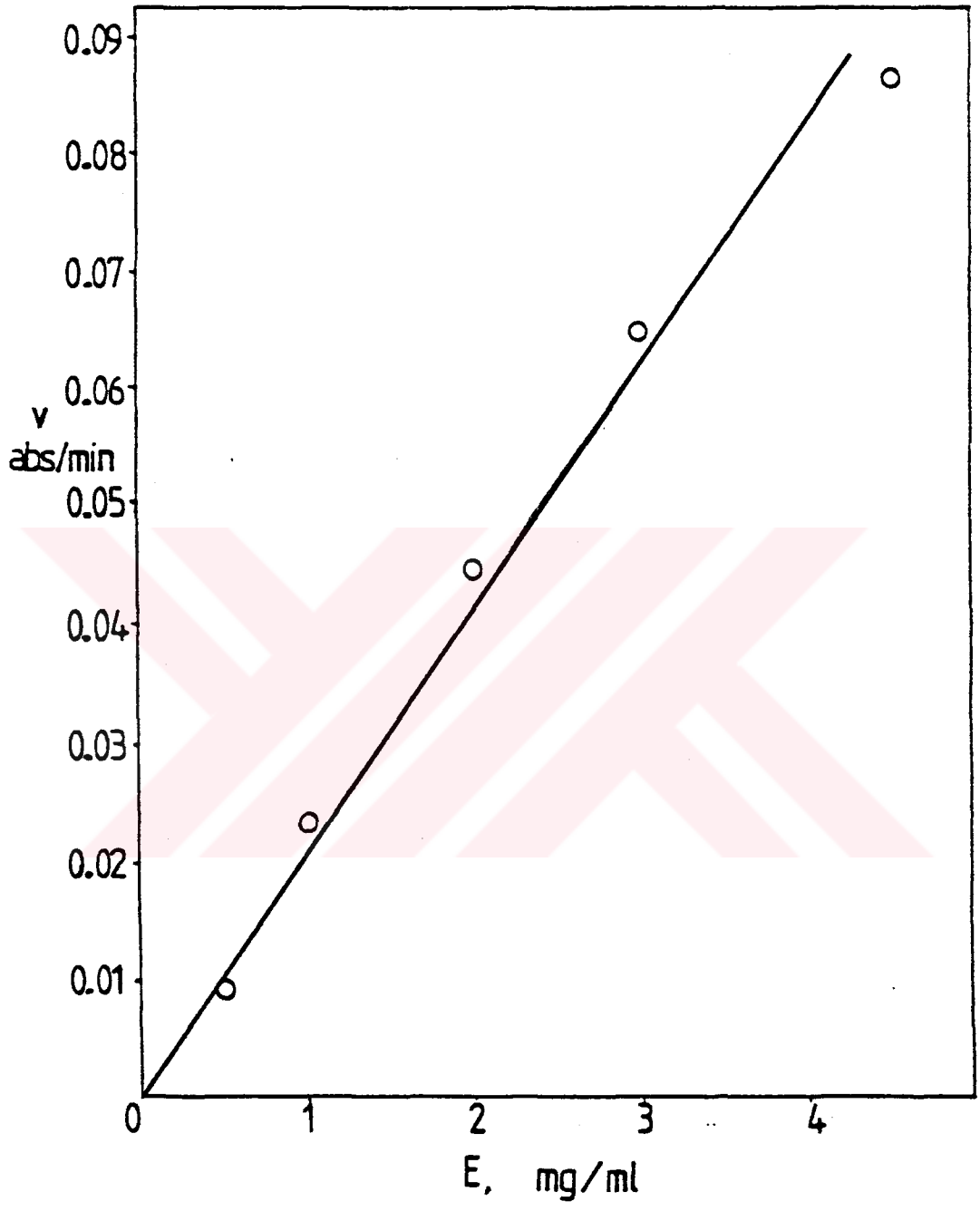


Figure 9. Dependence of Activity on Enzyme Concentration. Substrate is DMP-tributyrate at a Concentration of 0.1 mM.

by plotting log v/(Vmax-v) versus log [S] on a linear scale (Fig.10). The slope, n, estimated from Hill plot was unity, indicating the presence of one substrate binding site per molecule of enzyme component which catalyzes the hydrolysis of DMP-tributyrate.

III.2.2. Heat Inactivation

Heat inactivation with DMP-tributyrate was studied at 40 and 50°C. Substrate and enzyme concentrations were 0.5 µM and 2 mg/ml,in the assay mixture,respectively.The semilogarithmic plot of residual activity versus preincubation time was nonlinear (Fig.11). This was an unexpected result since the Lineweaver-Burk plot was linear.

III.3. Purification of Commercial Enzyme

Due to the presence of multiple components in the commercial wheat germ lipase, purification was required to isolate the esterase and tributyrinase fractions.

III.3.1. Gel Filtration of Commercial Enzyme

Gel filtration was done by the use of Sephadex G-100 (fractionation range is 1-100 kDa). A broad peak was obtained containing both esterase and tributyrinase activities at the high-molecular weight part of the peak. Nearly half of the protein was not active when PNPA and DMP-tributyrate was used as substrate (Fig.12). The fractions which exhibit highest activity with both substrates were combined and the result of

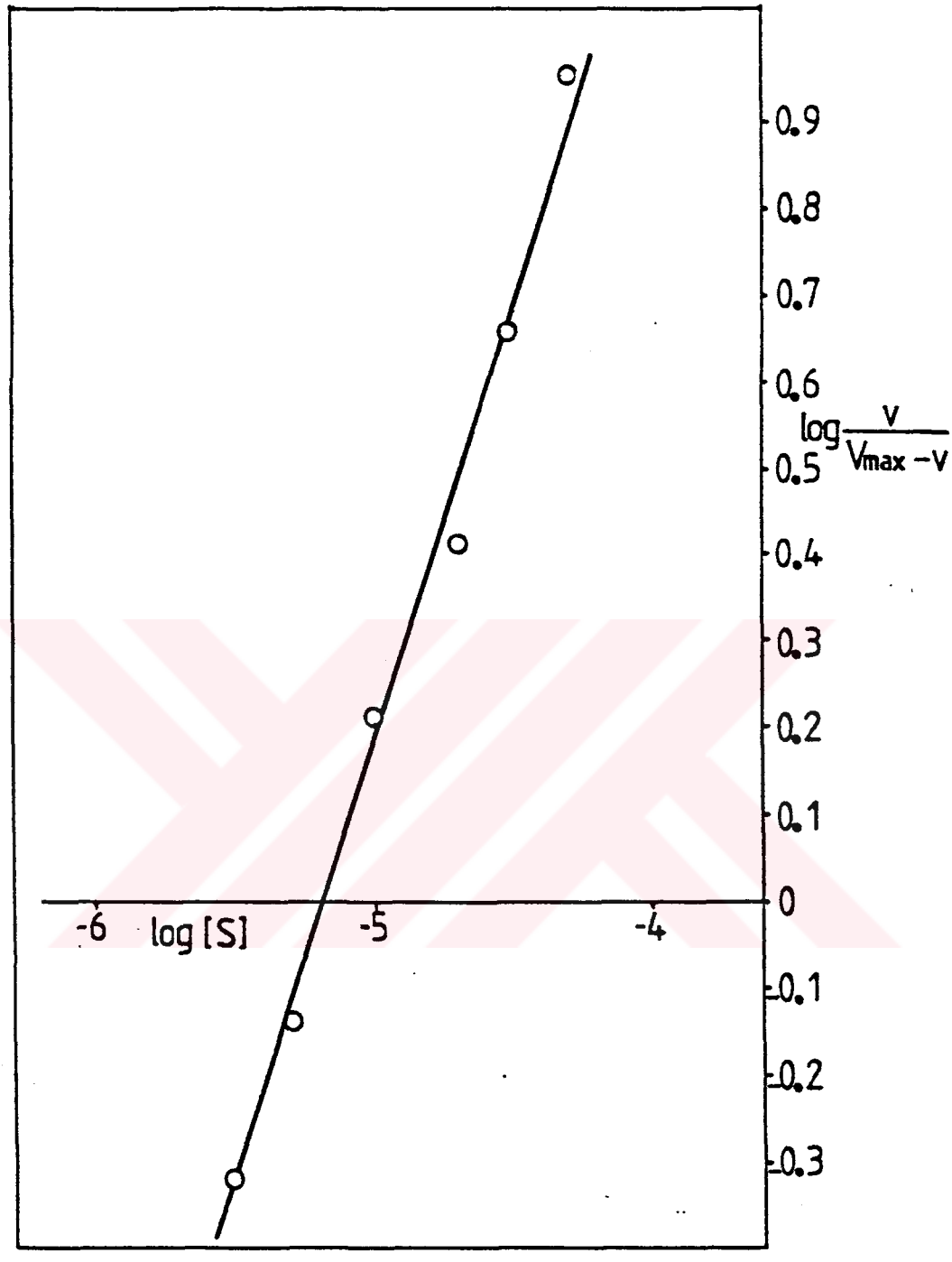


Figure 10. Hill Plot for DMP-tributyrate Hydrolysis.

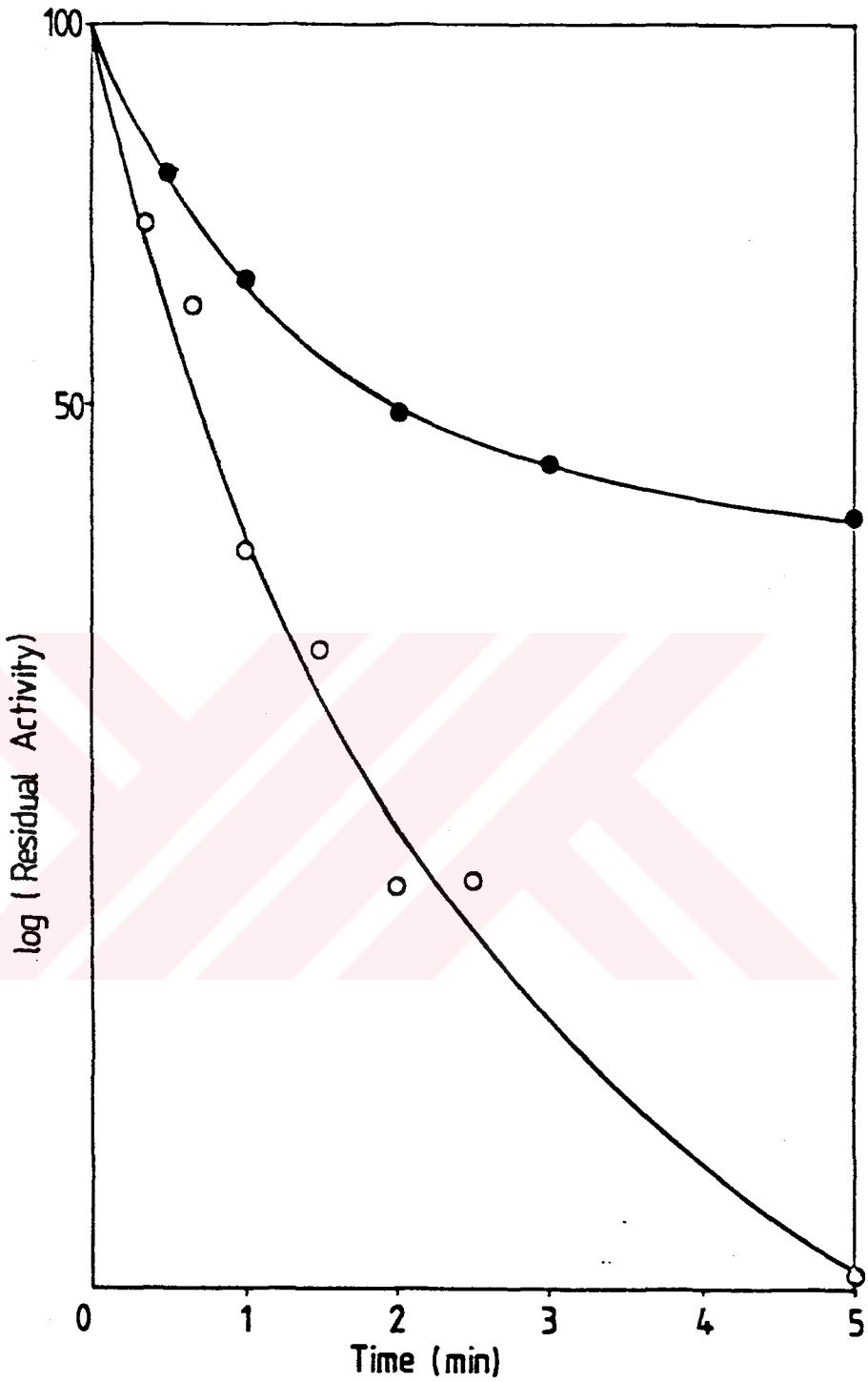


Figure 11. Heat Inactivation. Preincubation at (●) 40°C ; (○) 50°C

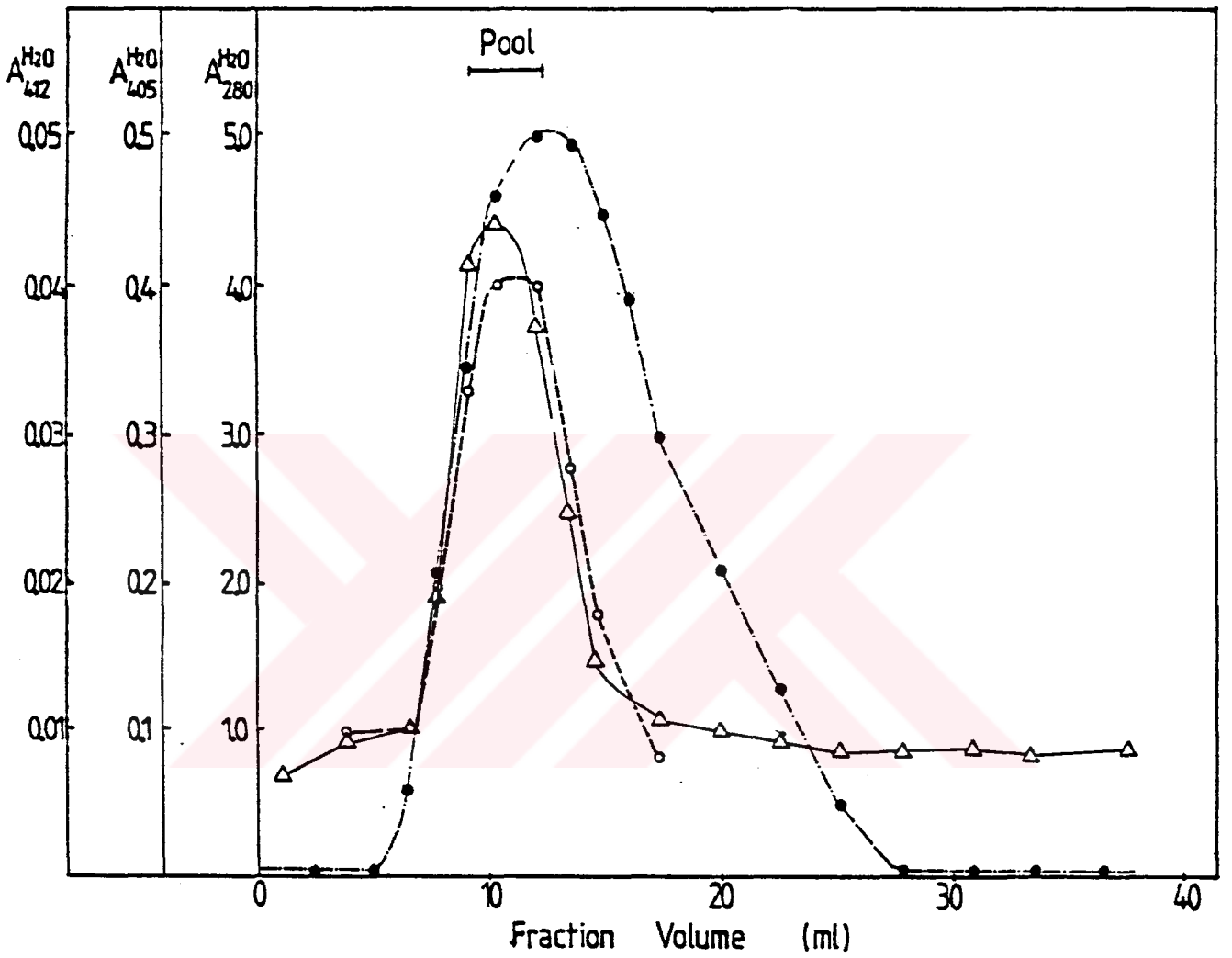


Figure 12. Gel Filtration of Commercial Wheat Germ Lipase
(●——●) Absorbance at 280 nm;
(△——△) Activity with 1 mM PNPA;
(o——o) Activity with 0.05 mM DMP-tributyrate.

separation was evaluated (Table 9).

Table 9

Gel Filtration of Commercial Enzyme*

	Input	Output (7-9)
Protein (mg)	36.1	12.0
Activity (µmol/hr)		
PNPA	202	137
DMP-tributyrate	3.18	2.26
Specific activity		
PNPA (µmol/mg hr)	5.6	11.4
DMP-tributyrate (nmol/mg hr)	88	188

*Commercial enzyme loaded is 40 mg

The protein content in mg was calculated by using 1.64 per mg per cm as absorbance index. The value was obtained by measuring the absorbance at 280 nm against wheat germ lipase concentrations (Fig.13).Molar extinction coefficient for PNPA hydrolysis was found as 13,360 per M per cm, which is in good agreement with literature value [86].For DMP-tributyrate, molar extinction coefficient was 14,200 per M per cm [87].

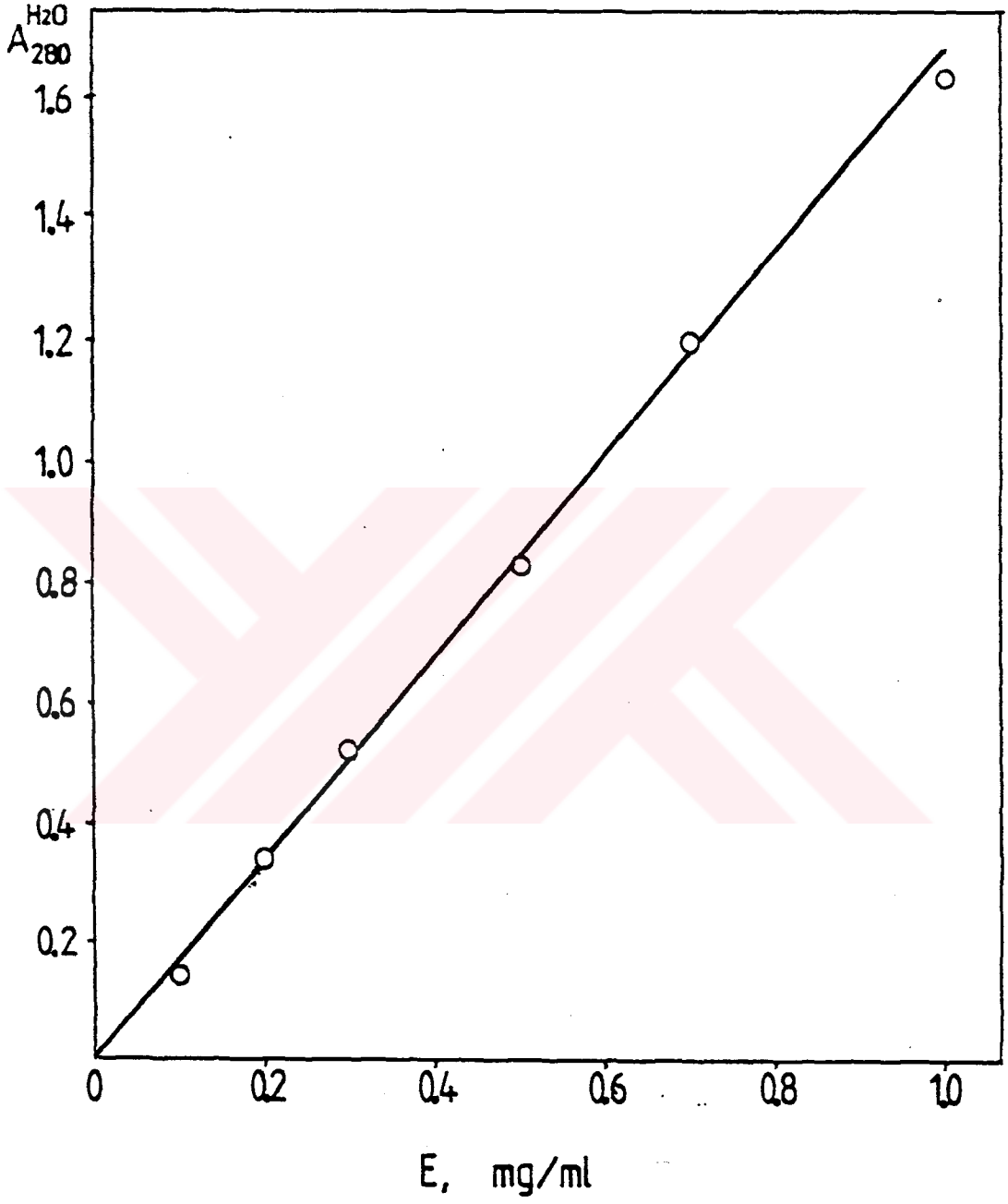


Figure 13. Determination of Absorbance Index at 280 nm. Absorbance was Measured Against Concentrations of Wheat Germ Lipase. Absorbance Index = 1.64 per mg per cm.

These values were used for the calculation of product released, in mole basis.

III.3.2. Ion-Exchange Chromatography of Commercial Enzyme

Since the isoelectric pH of wheat germ lipase was unknown, the ion-exchange chromatography was done by using an anion exchanger at pH 7.5 and a cation exchanger at pH 4.5.

III.3.2.1. DEAE-Sephadex A-50

Ion-exchange chromatography was first tried by using DEAE Sephadex A-50 at pH 7.5. A fraction both catalyzes the hydrolysis of PNPA and DMP-tributyrate came with the void volume (Fig.14). There were no isolation of the active fractions.

III.3.2.2. Cellulose Phosphate

Ion exchange chromatography by using cellulose phosphate was done at pH 4.5. A protein peak came first at about 0.2 M NaCl concentration. No activity was obtained with PNPA and DMP-tributyrate.At higher salt concentrations, three distinct activites were observed. The first active peak (37-41) hydrolyzes both of the substrates, the second active peak (45) hydrolyzes only DMP-tributyrate and the third active peak (47-53) hydrolyzes PNPA (Fig.15). The active fractions were collected and dialyzed overnight against 25 mM Tris-HCl buffer, pH 8.5. After dialyzes, the protein content and activities were measured. The activities were the same after

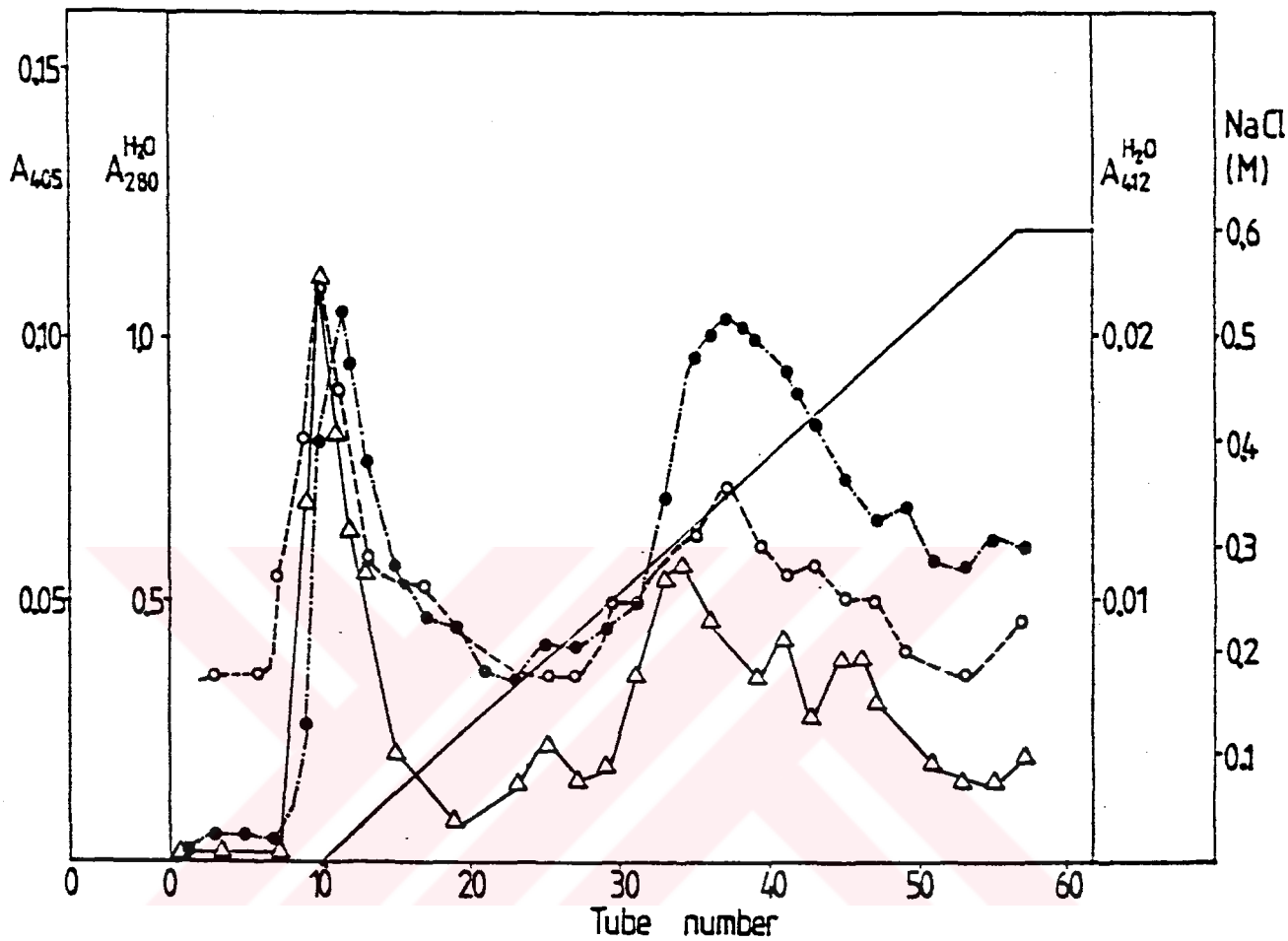


Figure 14. Ion-Exchange Chromatography by Using Sephadex A-50
(●——●) Absorbance at 280 nm ;
(Δ——Δ) Activity with 1 mM PNPA ;
(o---o) Activity with 0.05 mM DMP-tributyrate.

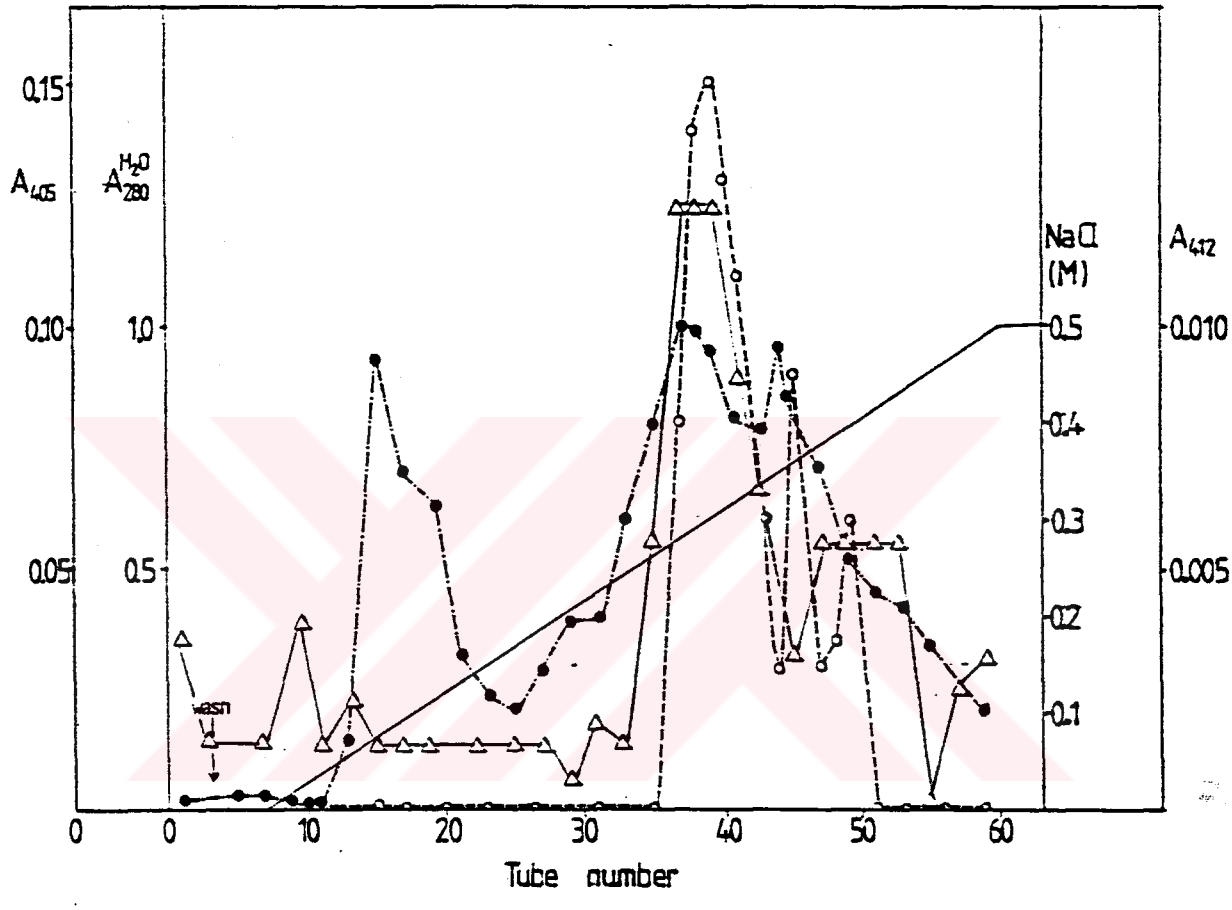


Figure 15. Ion-Exchange Chromatograpy of Commercial Wheat Germ Lipase by Using Cellulose Phosphate
(●-·-·-●) Absorbance at 280 nm ;
(△——△) Activity with 1 mM PNPA ;
(○- - -○) Activity with 0.05 mM DMP-tributyrate.

dialysis. The results were given in Table 10.

III.3.2.3. Stepwise Purification of Commercial Enzyme

A 40 grams of commercial wheat germ lipase was applied to gel filtration column two times. The fractions which exhibit maximum activity of the two trials were combined and applied to the cellulose phosphate column. Three active fractions were obtained (Fig.16). First peak catalyzes the hydrolysis of PNPA and DMP-tributyrate, second peak catalyzes only the hydrolysis of DMP-tributyrate, third peak catalyzes the hydrolysis of PNPA. The fractions {22-26},{31-32} and {40-44} were collected and reserved for kinetic studies. Specific activities were calculated (Table 11).

III.4. Kinetics of Purified Fractions

The hydrolysis of PNPA was studied by the pooled fractions {22-26} and {40-44} (Fig.17). The results were compared with the data obtained by the commercial enzyme (Fig.7). The kinetic behavior of the fractions was quite different from one another. Pool {40-44} gave a linear Lineweaver-Burk plot whereas a curvature was obtained at high substrate concentrations with the pool{22-26}. The Km and Vmax values from the linear Lineweaver-Burk plot were estimated as 10 µM and 0.106 abs/min., respectively .

Table 10

Ion-Exchange Chromatography of Commercial Enzyme by Cellulose Phosphate

Steps	Protein	Activity	Specific Activity	Recovery	Purification factor
	mg	µmol/hr		%	-fold
Input	54.2	303 (4.77)	5.6(88)	100	1
Ion-exchange chromatography					
Pool (37-41)	5.4	97 (0.98)	18(183)	32(21)	3.2(2.1)
Pool (45)	0.9	- (0.14)	-(158)	-(3)	-(1.8)
Pool (47-53)	4.2	66 (-)	16(-)	22(-)	2.9(-)

Commercial enzyme loaded is 60 mg .

Specific activity in µmol/mg hr with PNPA ;

Specific activity in nmol/mg hr with DMP-tributyrate (values in parenthesis).

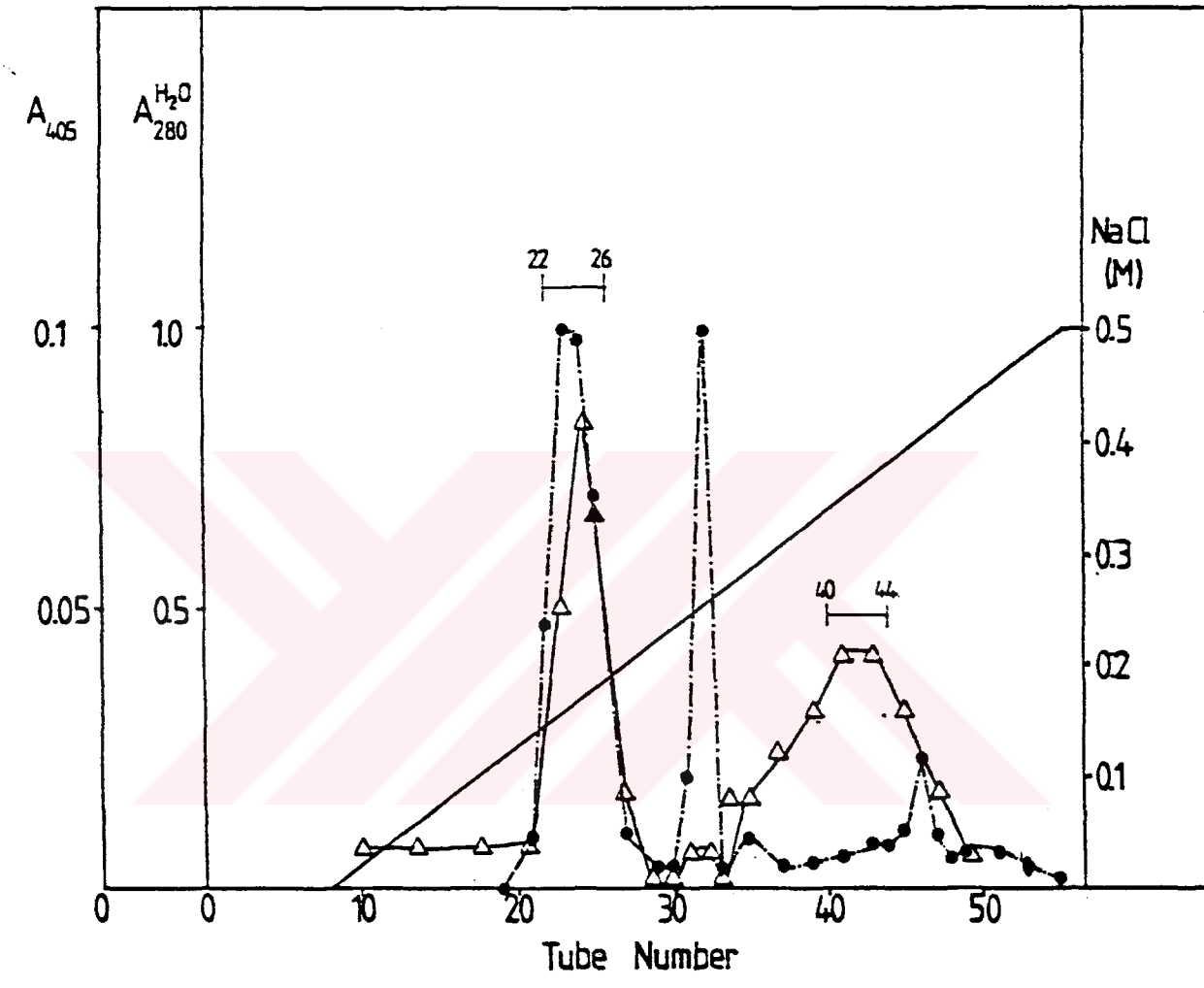


Figure 16. Ion-Exchange Chromatography of the Pool from Gel Filtration, by Using Cellulose Phosphate.
(●——●) Absorbance at 280 nm ;
(Δ——Δ) Activity with 1 mM PNPA.

Table 11

Stepwise Purification of Commercial Wheat Germ Lipase

Steps	Protein	Activity	Specific Activity	Recovery	Purification factor
	mg	µmol/hr		%	-fold
Input	72.2	404(6.36)	5.6(88)	100	1
Gel filtration	24.0	274(4.52)	11.4(188)	68(71)	2.0(2.1)
Ion-exchange chromatography					
Pool (22-26)	3.70	70(0.38)	19(104)	17(6)	3.4(1.2)
Pool (31-32)	1.02	- (0.26)	-(258)	-(4)	-(2.9)
Pool (40-44)	1.34	46(-)	34(-)	11(-)	6.1(-)

Commercial enzyme loaded is 80 mg.

Specific activity in µmol/mg hr with PNPA

Specific activity in nmol/mg hr with DMP-tributyrate (values in parenthesis)

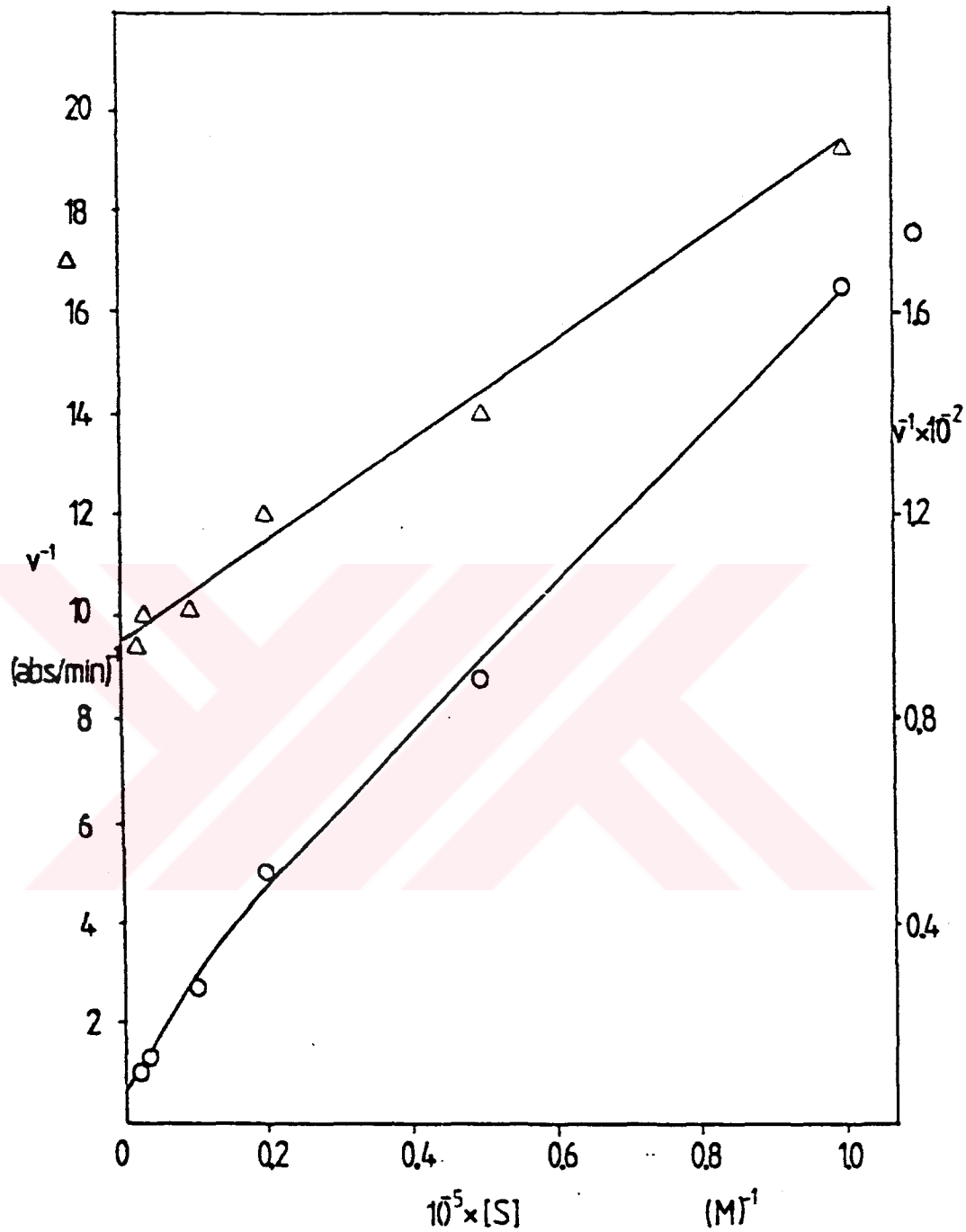


Figure 17. Lineweaver–Burk Plots for PNPA Hydrolysis by Pooled Fractions from Ion-Exchange Chromatography.
(o) Pooled Fractions (22–26)
(Δ) Pooled Fractions (40–44)

CHAPTER IV

DISCUSSION

The results showed that the commercial wheat germ lipase catalyzes the hydrolysis of PNPA and DMP-tributyrate. When PNPA is the substrate, the rate of hydrolysis was highest. A nonlinear double reciprocal plot was obtained with PNPA (Fig.7). There might be three reasons for the curved reciprocal plots: the presence of multiple enzymes ; the presence of multisite enzyme that has substrate binding sites of different affinities or displays negative cooperativity. The presence of an esterase in the commercial wheat germ lipase was previously reported [84], and lipase itself catalyzes the hydrolysis of PNPA readily, like other simple acetyl esters [84]. Hence, the presence of two enzymes that catalyzes the hydrolysis of PNPA was thought and the kinetic parameters were calculated. $Vmax_1$ and $Vmax_2$ were found as 0.23 abs/min. and 0.50 abs/min. and Km_1 and Km_2 as 17 µM and 2.3 mM, respectively. It appears that the enzyme with lower Vmax has the lower Km and the reciprocal plot bends downward close to the vertical axis due to the contribution of the high-Km enzyme to the total velocity at high substrate concentrations.

On the other hand, when the substrate is DMP-

tributyrate, the Lineweaver-Burk plot obtained was linear (Fig.8). The kinetic parameters, Km and Vmax were estimated as 6.7 µM and 0.05 abs/min., respectively. DMP-tributyrate seemed to be a better substrate compared to PNPA. The Hill coefficient was unity at 25°C (Fig.10). But, heat inactivation at 40°C and 50°C did not follow first-order kinetics (Fig.11). A mixture of enzymes should have given rise to curvature in the semilogarithmic plot of activity versus time. A probable explanation for the linearity demonstrated in Lineweaver-Burk plot is that the substrate concentration range was only 33 fold (0.45 Km to 15 Km) due to the limited solubility and the plot could distinguish only one of the enzyme component which were responsible for the hydrolysis of DMP-tributyrate. The observed curvature during heat inactivation gave evidence for the presence of more than one tributyrinase activity.

The purification of commercial enzyme was done by the methods of gel filtration and ion-exchange chromatography. Two-fold purification was achieved by gel filtration using Sephadex G-100 (Fig.12 and Table 9). The commercial enzyme and the pooled fraction (7-9) had identical PNPA : DMP-tributyrate activity ratios. About 33 % of the protein and 70% of the activity against PNPA and DMP-tributyrate were recovered in the pool (7-9).

Wheat germ lipase was fractionated on cellulose phosphate by using salt gradient. The first peak was active against PNPA and DMP-tributyrate. The purification was 3-fold

with respect to PNPA and 2-fold with respect to DMP-tributyrate (Table 10). About one-third of the activity against the latter substrate was lost.

The second and third active peaks catalyzed the hydrolysis of DMP-tributyrate and PNPA, respectively. The data at hand, could not exclude the possibility of these fractions not being the charge isomers of the enzyme present in the first peak, that is the lipase fraction. From the substrate specificity, the pool {31-32} and {40-44} were called as tributyrinase and esterase,respectively (Table 11). Stauffer and Glass [63] separated the aqueous extracts of wheat germ into three fractions : Lipase, tributyrinase and esterase. The isolated fractions in the present study agreed well with the literature except the substrate specificity of the lipase fraction, saying that the enzyme did not hydrolyze tributyrin.

The kinetics of hydrolysis of PNPA was studied by two ester hydrolase fractions : Esterase and lipase. Pool {40-44} gave a linear double reciprocal plot with Km and Vmax values of 10 µM and 0.106 abs/min., respectively (Fig. 17). Pool {22-26} was the fraction that catalyzes the hydrolysis of both PNPA and DMP-tributyrate. The Lineweaver-Burk plot of this fraction was nonlinear. The probable explanation was that the enzyme might be a multisite enzyme that has inherent substrate binding sites of different affinities for PNPA or exhibits negative cooperativity. The mechanism of action remains to be elucidated.

CHAPTER V

CONCLUSION

1. Commercial wheat germ lipase catalyzes the hydrolysis of PNPA. The rate of hydrolysis is dependent on the identity and concentration of the buffer and solvent used.

2. The kinetic data of PNPA hydrolysis fits well to a rate equation derived assuming the presence of two enzymes which process the same substrate with different affinities, and each of which is obeying Michaelis-Menten kinetics.

3. Commercial enzyme also catalyzes DMP-tributyrate hydrolysis. Compared to PNPA, DMP-tributyrate was found to be a better substrate for wheat germ lipase.

4. Purification by gel filtration gives a broad peak which is active against PNPA and DMP-tributyrate.

5. Purification by ion-exchange chromatography yields three

active peaks that are identified as lipase, tributyrinase, and esterase with respect to substrate specificity. Lipase catalyzes the hydrolysis of PNPA and DMP-tributyrate, tributyrinase hydrolyzes only DMP-tributyrate, esterase hydrolyzes PNPA.

6. The hydrolysis of PNPA by esterase fraction gives a linear Lineweaver-Burk plot whereas the plot is nonlinear by lipase fraction. The possible mechanism of action with the latter enzyme remains to be established.

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