PRODUCTION AND CHARACTERIZATION OF ESTERASE-LIPASE FROM *LACTOBACILLUS CASEI* SUBSPECIES

by

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NOVEL ESTERASE-LIPASE FROM *L. CASEI* SUBSPECIES

ABSTRACT

PRODUCTION AND CHARACTERIZATION OF ESTERASE-LIPASE FROM *LACTOBACILLUS CASEI* SUBSPECIES

Esterase-lipases were produced by cell lysis of *Lactobacillus casei* subspecies during the late logarithmic growth phase. The enzymes were purified to 67 fold for *L. casei-subsp-casei* LLG, and to 54 fold for *L. casei-subsp-pseudoplantarum* LE2 by ion exchange chromatography and gel filtration chromatography using the FPLC system (Pharmacia), respectively. Native-PAGE and SDS-PAGE using PhastSystem (Pharmacia) of the purified enzyme showed a single protein band with M.W. 320 K Dalton for butyrate esterase, 110 K Dalton for caproate esterase, and 40 K Dalton for capryate esterase, regardless of strains. The maximum lipolytic activity was observed at pH 7.2 and 37^{0}C. The activity was inhibited by silver and mercury ions but magnesium and calcium stimulated lipolytic activity. The kinetics of this enzyme, using p-nitrophenyl fatty acids as substrate, were determined: the Km and V_{max} values for *L. casei*-subsp-*casei* LLG were about 0.57 mM, 76 umol/min/mg of protein, whereas those for *L. casei*-subsp-*pseudoplantarum* LE2 were 1.20 mM and 90 umol/min/mg of protein, respectively. These enzymes were stable at room temperature for at least 2 days.

These novel esterase-lipases from *L. casei* subspecies were unique in their specificities characteristics splitting C_4 to C_{10} and allowing them to be efficiently incorporated directly into milk or curd to produce Enzyme-Modified Cheese (EMC) and Accelerated-Ripened Cheese (ARC).

Résumé

Production et caracterisation des estérase-lipases de sous-espèces de *Lactobacillus casei*

Les estérase-lipases ont été produits par lyse cellulaire de sous-espèces de *Lactobacillus casei* en fin de croissance logarithmique. Les enzymes ont eté purifiées 67 fois pour *L. casei*-subsp-*casei* LLG, et 54 fois pour *L. casei*-subsp-*pweudoplantarum* LE2 par chromatographie échangeuse d'ions et filtration sur gel à l'aide du systeme FPLC (Pharmacia). La determination des poids moleculaires par Native-PAGE et SDS-PAGE avec le PhastSystem (Pharmacia), ont montré une bande unique de protéine de 320 K Dalton pour l'estérase butyrate, 110 K Dalton pour l'estérase caproate, et 40 K Dalton pour l'estérase capryate, indépendemment des souches. L'activité lipolytique maximum a été observée à pH 7.2 et 37°C. L'activité a été inhibié par les ions argent et mercure mais le magnésium et le calcium ont stimulé l'activité lipolytique. Les cinétiques de ces enzymes ont été déterminées en utilisant les acides gras *p*-nitrophenyl comme substrat: les valeurs de K_m et V_{max} pour *L casei*-subsp-*casei* LLG furent de 0.57 mM, 76 umol/min/mg de protéine, tandis que celles de *L. casei*-subsp-*pseudoplantarum* LE2 furent de 1.20 mM et 90 umol/min/mg de protéine. Ces enzymes furent stable à témperature de la pièce au moins deux jours.

Ces nonvelles estérase-lipases de sour-espéces de *L. casei* se sont montrées uniques dans leurs caractéristiques specifiques à scinder les C_4 à C_{10}. Leur permettant ainsi d'être incorporées directement et efficacement dans le lait ou le caillé pour produire du fromage modifié enzymatiquement (EMC) et du fromage à maturation accéléré (ARC).

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PUBLICATIONS RESULTING FROM PRESENT STUDY

The following publications have resulted from the research done during this study :

Lee, S.Y., Lee, B.H., and Kermasha. S. 1988. Production and characterization of esterases and lipases of *Lactobacillus casei* group. Can. Inst. Food Sci. Technol. J. 21:367 (Abstract).

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Lee, S.Y. and Lee, B.H. 1989. Production and characterization of esterase-lipase from *Lactobacillus casei*-subsp-*casei* LLG. J. Food Sci. (Submitted).

Lee, S.Y. and Lee, B.H. 1989. Production and characterization of esterase-lipase from *Lactobacillus casei*-subsp-*pseudoplantarum* LE2. Biotech. Appl. Biochem. (Submitted).

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INTRODUCTION

The mechanism by which fresh curds mature from a relatively bland elastic mass to become a well bodied cheese with a developed flavour has been the subject of much interest. It is generally understood that proteases and lipases of lactic acid bacteria (streptococci, lactobacilli and pediococci) are responsible for the maturation of cheese (El Soda *et al.*, 1986b; Marshall, 1987). The specific oligopeptides and amino acids produced from the milk proteins (principally casein) and the free fatty acids released from the triglycerides impart the characteristic flavour attached to the various types of cheeses. In case of Cheddar type of cheeses, ripening normally is carried out for a period of 9-12 months to fully develop the desired Cheddar flavour. The slow microbial process incurs high cost of refrigeration and warehousing. Aging costs contribute significantly to the final product price (from 8-10 c/month/kg of cheese aged). Any development leading to the acclerated maturation of cheese therefore has a direct economic importance (Arbiege, 1986).

Almost all of the reported attempts to accelerate cheese ripening fall into one of four categories; elevated temperature (Law & Wigmore, 1982; Cromie *et al.*, 1987), enzyme addition (Sood & Kosikowski, 1979a; Law, 1984b), the use of attenuated starters in addition to normal cultures (Petersson & Sjostrom, 1975), and liquid slurry method (Krsitoffersen *et al.*, 1967; Sood & Kosikowski, 1979b). The most widely employed method is the external addition of enzymes to the cheese vats (Law, 1984b).

Although there are a number of cheap commercial food grade enzymes available for the above purpose, almost all have their accompanying limitations,

especially towards control of their addition to young cheese, and their hydrolytic action on milk components, resulting in rheologically poor final product, and often with bitter flavour.

Lipolysis is one of the important mechanism contributing to flavour development of cheese besides proteolysis (Moskowitz & Noelck, 1987). Lipases and esterases from different animal and microbial sources have been tried to develop the piquant flavour, characteristic of Provolone and Romano cheeses. The different short chained fatty acid combinations produced result in different Italian cheese flavours (Dziezak, 1986). Lipolysis also play an important role in flavour profile of enzyme modified Swiss and Cheddar cheeses. A right blend of alkanoic acids with carbon chains from C_4 to C_{10} appear to impart cheese-like flavour, whereas the release of long chained (C_{12-16}) fatty acids by microbial lipases produce "soapy" flavour, and that of short-chain acids by animal esterases produce an unclean flavour (Nelson, 1972, Law & Wigmore, 1982).

The novel lactobacilli enzymes developed in our laboratory are unique in its specificities characteristics, thus allowing them to be incorporated directly into milk curd or cream to produce desirable cheeses, and snackfood flavourings. In addition, these enzymes in conjunction with usual starter culture can significantly reduce the ripening period and therefore the manufacturing cost of the final cheese, while enhancing its quality.

The review will discuss the role of esterase-lipase in EMC (Enzyme Modified Cheese) and ARC (Accelerated-Ripened Cheese). The final section will deal with some ideas of novel esterase-lipase from *Lactobacillus casei* subspecies and the objective of this research.

LITERATURE REVIEW

A. PROPERTIES OF THE ESTERASE-LIPASE

I. Characteristics of the esterase and lipase reaction

There exists much confusion concerning the exact meaning of the terms lipases and esterases. Often these terms are used interchangeably since these enzymes catalyze the hydrolysis of carboxylic acid ester bonds and are classified among hydrolyases and comprise the enzymes belonging to the class 3.1.1 of the International Union of biochemistry classification. The term esterases assigned to enzymes capable of hydrolyzing carboxylic acid esters is rather broad. The term esterase is rather nonspecific and does include lipase. Of the esterases that hydrolyze the carboxylic esters of fatty acids and simple alcohols, there exists carboxylesterase (EC 3.1.1.1), which was formerly called aliesterase or B esterase; arylesterase (EC 3.1.1.2), formerly called A esterase, which hydrolyzes the aromatic esters, such as phenyl acetate; and lipase (EC 3.1.1.3), which hydrolyzes carboxylic esters of glycerol.

According to the IUB nomenclature, a lipase catalyzes the following hydrolytic reaction.

$$\text{Triglyceride} + H_2O \text{ <--------> } \text{diglyceride} + \text{fatty acid}$$

Essentially, the above reaction is not a complete one since the lipase action may carry the reaction to the monoglyceride or even to the glycerol stage.

By and large, the major difference between the lipases and esterases seems to be associated with the state of the substrate they act upon. While

esterases can hydrolyze soluble or fully dispersed substrates, the lipases cannot. As suggested by several workers (Krisch, 1971, Brockerhoff & Jensen .1974; Rudek, 1978), it thus appears appropriate to define lipases as enzymes which hydrolyze the esters at an oil-water interface in an insoluble or heterogeneous system.

The requirement by lipases for aggregated substrates which provide an interface for rapid catalysis was illustrated by a study of the effect of substrate concentration on lipolysis of the partially soluble triglycerides triacetin and tripropionin using the lipase from *Rhizopus arrhizus* (Semervia & Dufour, 1972) With these substrates, appreciable lipolysis was observed only above their critical micellar concentration. It was apparent that the lipase will catalyse hydrolysis of the substrates when they are present in the form of micelles, small aggregates or emulsion particles.

The natural function of lipases is to catalyse the hydrolysis of triglycerides. However, this reaction is reversible and the enzymes can be shown to catalyse the formation of glycerides from glycerol and free fatty acid. These esterification reactions are probably of no significance in the biosynthesis of lipids but can be utilized in the production of modified fats and fatty acid esters (Kilara, 1985).

II. The sources of esterase-lipase

Esterase-lipases are produced by plants, animals, and microorganisms (Shahani, 1975). Castor bean and wheat germ contain lipase, a wide variety of micro-organisms (including yeasts, moulds, and bacteria) are source of this enzyme. Animals contain pregastric esterases, lingual lipases, and pancreatic lipases as well. The plant lipases do not have commercial applications in producing modified ingredients but the microbial and animal lipases have been

used successfully in producing novel ingredients. As early as 1940 it was realized that calves could secrete a salivary lipase (Wise *et al.*, 1940) and it was confirmed to be a true lipase and was named pre-gastric esterase (Desnuelle, 1972). The occurrence of pre-gastric esterase in a number of mammalial species, including humans, has been reviewed (Nelson *et al.*, 1977). The most important sources of pre-gastric esterase are kid and lamb although calf pre-gastric esterase has also been studied.

Pancreatic lipase has been studied extensively and the enzyme is produced in the acinar cells prior to release into the duodenum, where it facilitates the intraluminar digestion and absorption of lipids. Hog pancreatic lipase is the most studied lipase and has been extensively reviewed (Desnuclle, 1972). Milk, the physiological secretion of mammals, has also been reported to contain lipase and this enzyme has been characterized and reviewed (Shahani, 1975).

Many procedures for the production of microbial lipases in submerged culture have been published in the general and patent literature. The nutrients and culture conditions used vary widely, but some general conclusions can be drawn.

Microbial lipases from the yeasts *Candida* and *Torulopsis,* moulds *Rhizopus, Penicillium, Aspergillus, Geotrichum,* and *Mucor* genera and bacteria of the genera *Pseudomonas, Achromobacter,* and *Staphylococcus* have been shown to produce lipases. The diversity of the pH optima, temperature optima, heat inactivation and the physical, chemical properties of several purified microbial lipases are presented in Table 1a & 1b. The formation of the enzyme is under feedback control of mono- and di- saccharides and glycerol in the growth medium. This phenomenon is illustrated by examing lipase production by *Geotrichum candidum* (Tsujisaka *et al.*, 1973). Similar effects of glucose on

Table 1a. Properties of various purified microbial lipases (Shahani, 1975)

Species	Molecular weight	Isoelectric point	Carbohydrate content (%)	Specific activity of purified enzyme (lipase units/mg protein)	Average hydrophobicity
Aspergillus niger[a]	25 000	4·6	10	2[illegible]	1 058
Candida cylindracae[b]	120 000	4·2	4·2	1 140	1 150
Chromobacterium viscosum[c]	30 000	7·3	0·0	5 780	986
Geotrichum candidum[d]	54 000	4·3	7 0	447	1 135
Humicola lanuginosa[e]	27 500	—	0·0	1 490	1 079
Mucor javanicus[f]	21 000	—	2·6	1 322	—
Pseudomonas fluorescens[g]	32 000	4·5	0 0	4 200	1 001
Rhizopus arrhizus[h]	43 000	6·3	6 7	9 300	1 097
Rhizopus delemar[i]	41 300	4 2	—	4 000	1 270
Torulopsis emobii[j]	42 500	2·9	15	—	1 218

[a] M P Tombs, private communication.
[b] Tomizuka *et al*, 1966
[c] Isobe & Sugiura, 1977, Horiuti & Imamura, 1977.
[d] Tsujisaka *et al*., 1973
[e] Liu *et al*, 1973*a*, *c*.
[f] Ishihara *et al*, 1975.
[g] Sugiura & Oikawa, 1977; Sugiura *et al*., 1977
[h] Semeriva *et al*, 1969, Laboureur & Labrousse, 1968.
[i] Chiba *et al*, 1973, Iwai & Tsujisaka, 1974
[j] Yoshida *et al*, 1968.

Table 1b.

Temperature and pH Optima and Heat Inactivation of Various Microbial Lipases (Macrae, 1983)

Source of lipase	pH optimum	Temperature optimum (°C)	Heat inactivation time temperature*	Reference
Penicillium chrysogenum	6 2-6 8	37	—	Ramakrishnan and Banerjee, 1952
Pseudomonas fragi	7 0-7 2	32	15.72	Nashif and Nelson, 1953
Rhizopus delemar	5 6	35	15 50	Fukumoto *et al*, 1964
Aspergillus niger	5 6	25	15 45	Fukumoto *et al*, 1963
Penicillium roqueforti	8 0	37	10 50	Eitenmiller *et al*, 1970
Staphylococcus aureus	8 5	45	30·70	Vadhera, 1974
Geotrichum candidum	8.2	37	15.60	Jensen, 1973, Tsujisaka *et al*, 1973
Achromobacter lipolyticum	7.0	37	40.99	Khan *et al*., 1967

* First figure refers to time (min), and the second figure refers to temperature (°C).

lipase formation have been observed with *Rhizopus japonicus* (Aisaka & Terada, 1980) and *Candida parolipolytica* (Sugiura & Isobe, 1975). Some microbial lipases are glycoproteins where the sugar moiety is thought to facilitate the passage of the enzyme through the microbial cell wall and into the growth medium.

Extracellular lipases are produced by most microorganisms under suitable conditions (Lawrence *et al.*, 1967; Brockerhoff & Jensen, 1974) and have been studied for four main reasons. First, lipases of pathogenic bacteria such as *Corynebacterium acnes* (Hassing, 1971), *Staphylococcus aureus* (Alford *et al.*, 1964), *Leptospira pomona* (Patel *et al.*, 1964) and *Mycoplasma* spp. (Rottem & Razin, 1964) have been implicated in the etiology of disease. Second, bacterial lipases are involved in such environmental problems as the breakdown of fat in domestic sewage (Nadkarni, 1971) or ecological aspects of river sediments (Breuil & Kushmer, 1975). Third, the spoilage of dairy products and other foods that contain fat involves lipases, in particular those of psychrotrophic bacteria (Downey, 1980; Cousin, 1982). Fourth, lipases, mainly from fungi (Iwai & Tsujisaka, 1984) have many uses in the food and other industries by virtue of their diverse substrate specificities.

Isolation, characterization, and properties of various lipase enzymes have been reviewed by several authors over the past two decades . Earlier reviews reflect concern with flavour defects caused by the action of lipolytic enzymes while later reviews reflect the intensive study of various lipase sources in relation to their potential application for controlled modification of milk fat.

III. Specificity of lipolytic enzyme

Lipolytic enzymes exhibit several types of specificities which affect their role and significance in flavour development. The most significant single factor

in the development of useful lipolytic technology was the discovery and application of a variety of lipase systems, with differing specificities for fatty acid chain length, type of glyceride molecule, and physical condition of the substrate (Kilara, 1985).

Rate of enzyme activity on various triglyceride substrates commonly is used to compare lipolytic enzymes. Khan *et al.* (1967) reported that milk lipase showed greater activity on tributyrin substrate than on triolein substrate while *Achromobacter lipolyticum* lipase exhibited opposite relative activities. Eitenmiller *et al.* (1970) also reported the *Penicillium roqueforti* lipase hydrolyzed tributyrin, tricaprylin, tricaproin, tripropionin, and triolein in decreasing order, thus confirming and expanding information on the relative substrate specificity of this enzyme while *Aspergillus niger* lipase hydrolyzed tricaprylin faster than the other substrates (Shipe, 1951). The pregastric esterase is specific for glycerides containing short chain fatty acids. (Nelson *et al.*, 1977).

Another type of substrate specificity is the relative activity of lipolytic enzymes on triglyceride versus mono- and di- glyceride substrates. Pregastric esterase showed no activity on the monobutyrin whereas pancreatic lipase maintained high activity on the mono- and di- glyceride substrates. Lamb gastric lipase hydrolyzed mono- and dibutyrins more rapidly than did lamb pregastric esterase (Richardson & Nelson, 1967; Richardson *et al.*, 1971). Stadhouders and Veringa (1973) indicated that the 'ali-esterases' from starter culture bacteria used in cheese manufacture show limited capacity to hydrolyze milk fat triglycerides but are capable of hydrolyzing fairly rapidly mono- and di-glycerides.

Selective release of specific fatty acids from substrates by various lipolytic enzymes have been studied extensively. Milk lipase and certain other

lipolytic enzymes, such as steapsin and pancreatic lipase, are broad spectrum or nonspecific in their action (Nelsen, 1972). These enzymes release free fatty acids in approximately the same proportion as they are present in the intact fat. By contrast, other lipolytic enzymes demonstrate preferential release of specific fatty acids. The relative specificity of calf esterase for release of short-chain fatty acids, particularly butyric acid, from milk fat is apparent (Nelson, 1972).

Still another characteristic of substrate specificity is related to the physical form of the substrate. Interdependence of physical form of the substrate and the specific lipolytic enzyme is an extremely important consideration in designing systems for the application of lipolytic enzymes for flavour development in dairy manufacturing (Richardson & Nelson, 1967).

Lawrence *et al.* (1967) stated that specificity of some microbial lipases appears to be related to the position of the fatty acid in the triglyceride and in other cases to the fatty acid itself and its degree of unsaturation.

IV. Detection and determination of lipolytic activity

Lipolytic activity is generally signified by an accumulation of free fatty acids and disappearance of triacylglycerols when a tissue or fluid is incubated or stored. The conditions of detection, i.e., temperature, pH, presence of cations and fatty acid acceptors, will provide information required for the design of a suitable assay system. Beyond these parameters, it is necessary to assure that the definition of a lipase is met, but this may be implicit in detection.

The factors that affect enzyme activity are dependent source of the enzyme. Also, the substrate specificity and temperature of inactivation of enzyme are effected for characterization of enzyme.

Jensen (1983) provided a thorough review of the extensive studies on the numerous lipase assay method. Several assay methods have been used to

determine lipase activity quantitatively. They included the following.

(a) Titration method. Titrimetry can be done directly on the assay mixture. This is the one of common method at the end of the incubation period which reaction is stopped by addition of solvent (alcohol or acetone) and the released fatty acids are titrated either using indicator or with a pH-stat set to an end-point (Fitz-Gerald & Deeth, 1983; Bozoglu *et al.*, 1984). Direct titration has also been carried out continuously with a recording pH stat for pancreatic lipase (Brockman, 1981) and adipocytes (Stralfors, *et al.*, 1981). The other type of titrimetry is nonaqueous titration of digestion mixture in the extraction solvent with alcoholic base (Horowitz, 1980).

(b) Copper soap extraction method. Fatty acids can be converted to Cu soaps which are measured spectrophotometrically after reaction with chromogenic reagents. The method originally developed by Duncombe (1963) has been refined and applied to plasma (Hron & Menahan, 1981) , serum (Brunk & Swanson, 1981), milk (Shipe *et al.*, 1980; Christen & Marshall, 1984) and single oat grains (Sahasrabudhe, 1982). Redding *et al.* (1983) described a semiautomated assay for free fatty acids in serum employing Cu soaps.

(c) Colorimetric assay method. Stead (1983) have developed fluorimetric lipase assays based on the hydrolysis of 4-methylumbelliferyl oleate. This assay has been modified for use with skim milk as well as skim milk powder, and whey protein concentrate (Stead, 1984). McKellar (1986 a & b) has developed a colorimetric lipase assay based on the method of Nachlas & Blackburn (1958). p-Naphthol released from the colourless ester p-naphthyl caprylate (p-NC) reacts with a diazonium salt to produce an insoluble azodye. Activity is quantitated by measuring absorbance at 540 nm after extraction with ethyl acetate. Brandl and Zizer (1973) described that the esterase activity of the soluble extract was evaluated using several o- and p- nitrophenyl derivatives of

fatty acids chromogenic substrate. Gatt *et al.* (1981) assayed lipases of rat brain microsomes, lyophilized rat bile, hog pancreas and *Rhizopus arrhizus delemar* with acylglycerol esters of trinitrophenylaminolauric acid. The free fatty acids are measured spectrophotometrically.

(d) Plate assay method. A plate assay to detect bacterial lipase in a medium containing trioleoylglycerol and the fluorescent dye rhodamine B is developed (Kouker & Jaeger, 1987). Substrate hydrolysis causes the formation of orange fluorescent halos around bacterial colonies visible upon UV irradiation. This method was suitable either to identify lipase-producing bacteria or to quantify lipase activity in culture supernatants. Modification of these assays uses Tween 80 in combination with Nile-blue (Karnetova *et al.*, 1984).

(e) Method of using radioactive fatty acids. The activities of lipases from the following sources have recently been obtained with radioassays: postheparin plasma (Baginsky, 1981) and human liver (Breckenridge & Palmer, 1982). Oleoylglycerols labeled with ^{14}C or ^{3}H were substrates and nanogram quantities of the product are detectable.

(f) Gas Liquid Chromatograpghy (GLC). GLC has been applied to the quantitation of free fatty acids in serum (Brunk & Swanson, 1981), plasma (Tserng *et al.*, 1981), and vegatable oils (Chapman, 1979). These methods were capable of measuring 100 nmol of free fatty acids in serum or plasma and 2 mg in the oils. Gas chromatographic analysis of free fatty acids has been generally accepted by researchers as one of the best analytical methods for evaluating esterase-lipase action in dairy products (Deepth *et al.*, 1983).

(g) Treatment with enzymes. Treatment of free fatty acid with various enzymes results in products which are detectable at very low levels. Mizuno *et al.* (1980) converted free fatty acid to the CoA esters with a synthetase, oxidized the acyl CoA to a *trans* enoyl CoA and H_2O_2 using an oxidase and then the

H_2O_2 with peroxidase to a chromogen. Miles *et al.* (1983) reported on the direct determination of free fatty acid in 2-5 ul of plasma eliminating extraction. The method is based on the quantitation of adenosine monophosphate produced by the formation of acyl-CoA with ATP and acyl CoA synthetase. Glycerol also released by lipolysis from human fat cells was analyzed by bioluminescence (Bjorkhem *et al.*, 1981).

(h) Immunological methods. Specific immunoassays have been developed for chicken adipose tissue lipoprotein (Cheung *et al.*, 1979) and human pancreatic lipases (Grenner *et al.*, 1982). Cheung *et al.* (1979) incubated antilipoprotein lipase immunoglobulins coupled to hydrophilic beads with the lipase and immunoglobulins labeled with ^{125}I were added. The labeled immunoglobulin was reacted with the antigen (lipase) associated with the immunoabsorbent. The quantity of lipoprotein lipase in the sample was proportional to the amount of radioactivity bound to the solid phase immunoabsorbent.

For most, a direct titration of the assay medium or of a nonaqueous extract will suffice. With the latter, a pH meter is not needed as indicators are satisfactory. The choice of procedure will also be influenced by the number of samples to be analyzed, the sensitivity desired and the availability of equipment and enzymes.

B. THE ROLE OF ESTERASE-LIPASE IN ENZYME-MODIFIED CHEESE (EMC) AND ACCELERATED-RIPENED CHEESE (ARC)

I. The role of esterase-esterase in cheeese flavouring & cheese ripening

Cheese that has been treated enzymatically to enhance the flavour profile can be called enzyme-modified cheese (Moskowitz & Noelck, 1987).

Lipolysis is an important reaction in flavour development of cheese. Cheese flavour develops as a result of the action of starter bacteria, added deliberately, and of secondary microflora whose composition varies according to the type of cheese (Chapman & Sharpe, 1981). Many of the reactions involved in flavour production are enzymatically catalysed and the addition of microbial proteinases and lipases can accelerate ripening (Law, 1984a & 1984b). Woo *et al.* (1984) found that the fatty acid composition was related to flavour quality and intensity but did not correlate with the age of the cheese. Addition of pre-gastric esterase in Italian cheese manufacture leads to the development of the much desired picant flavour in cheeses such as Romano and Provolone (Nelson *et al.*, 1977). Lamb gastric extracts in combination with lamb pre-gastric esterase results in more desirable Provolone-like flavour (Richardson & Nelson, 1967). *Mucor miehei* esterase has been used to develop desirable flavour in Fontina and Romano cheese (Peppler *et al.*, 1976). This enzyme has high activity against tributyrin and trioctanoin, but less against triglycerides with long-chain fatty acids (Moskowitz *et al.*, 1977). Inclusion of pre-gastric calf esterase and fungal esterase at equivalent lipolytic activities led to an acceptable Fontina cheese whereas in Romano cheese five-fold more fungal esterase was needed on an activity basis than pre-gastric kid esterase.

Another popular cheese flavour line is blue cheese flavour. The flavour of blue cheese is derived from lipids and involves four major enzymatic processes where in (a) free fatty acids are liberated from milk fat by lipase, (b) the free fatty acids produced are oxidized to p-keto acids, (c) the p-keto acids undergo decarboxylation to generate methyl ketones and (d) the methyl ketones are reduced to yield secondary alcohol (Hawke, 1966). Blue cheese flavours can be produced by submerged mycelial fermentation processes involving sterile milk or milk fat in its various forms. Such products are suitable

for incorporation into salad dressings, appetizers, dips, and products that can be incorporated into extended and baked snack foods.

Two lipases are secreted by *Penicillium roqueforti* but only the acid enzyme is important in cheese ripening (Menassa & Lambert, 1982). The addition of a commercial enzyme preparation from *Aspergillus* spp. improved the rate of development and quality of flavour of Blue cheese. Concentrations of total carbonyls, monocarbonyls and methyl ketones were higher than in control cheeses, made without enzyme addition (Jolly & Kosikowski, 1975b).

Compared to Italian and Blue cheese, Cheddar, Swiss and Dutch cheese undergo very low levels of lipolysis. It has been claimed that the addition of rennet paste, pre-gastric esterase, and gastric lipase improves the flavour of Cheddar cheese and several patents have been awarded for such applications. Acceleration of Cheddar cheese ripening by the addition of various protease and lipases has been reported (Kosikowski & Iwasaki, 1975).

The white surface mould of Brie and Camembert cheese (*Penicillium camemberti*) is also lipolytic and produces methyl ketones by oxidation of the fatty acids released. The major products of its action on milk lipids are 2-nonanone and 2-undecanone. Sodium laurate inhibited mycelial growth in a model system containing milk lipids but extensive synthesis of 2-undecanone occurred. Oleic acid was less inhibitory and mostly 2-heptanone and 2-nonanone were produced (Okumura & Kinsella, 1985).

Surface smear-ripened cheeses such as Limburger or Romadur develop a characteristic red-brown growth of *Brevibacterium linens* which produces both proteolytic and lipolytic changes (Chapman & Sharpe, 1981). However, lipases from yeasts are also involved in ripening of Limburger cheese, and from milk fat *Candida mycoderma* lipase produced more lauric, myristic and palmitoleic and less stearic acid than did *Debaryomyces kloeckeri* lipase

(Hosono & Tokita, 1970).

The role of lipolysis is more difficult to assess in cheeses where ripening does not involve the growth of moulds. However, volatile fatty acids are responsible for part of the characteristic flavour of hard and semi-hard cheeses (Law, 1984a) and their concentrations increase during Cheddar cheese maturation due to esterase and lipase activities of the milk flora and starter *streptococci* (Stadhouders & Veringa, 1973). Fryer *et al.* (1967) detected weak lipolytic activity in 56 strains of lactobacilli, pediococci and leuconostocs. Weak lipolytic activity has also been shown for *Streptococcus thermophilus*, *Lactobacillus helveticus, Propionibacterium shermanii* used in Swiss cheese manufacture (Paulsen *et al.*, 1980) and for *Streptococcus cremoris, Streptococcus lactis, Lactobacillus plantarum* and *Lactobacillus casei* in Cheddar cheese manufacture (Umemoto & Sato, 1975). Suarez *et al.* (1984) found that strains of *Lactobacillus plantarum* and *Lactobacillus casei* from Mahon cheese had moderate activity against p-naphthyl esters with C_4 and C_8 fatty acids, but low activity against that with C_{14} fatty acid. Addition of food grade lipase from *Candida cylindracea* increased the concentration of volatile free fatty acids and favorably increased the flavour intensity of American Cheddar cheese (Sood & Kosikowski, 1979b), although excessive lipase induced rancid off flavours. The effect of English Cheddar of incorporating lipase from *Mucor* spp. and esterase from animal sources, both with and without a flavour enhancing proteinase, was studied by Law & Wigmore (1985). The lipase and esterase were shown, by their reaction with different esters of 4-methylumbelliferone (Stead, 1983), to have specificity for different chain length fatty acids, but none, even of addition produced lipolytic rancidity (Law & Wigmore, 1985). Frick *et al.* (1984) found that addition of lipase from *Aspergillus oryzae* to Colby cheese curd resulted in typical aged Cheddar flavour. This

enzyme released longer-chain fatty acids than did another unspecified lipase (Miles 600) which produced a Romano type flavour and was less active at low temperature.

Microbial lipase from an unspecified source has been used successfully in the ripening of Feta cheese provided that its concentration was low enough to avoid excessive generation of volatile fatty acids (Mahmoud & Kosikowski, 1980). Nasr (1983) reported accelerated flavour development in Romi cheese to which a commecrcial fungal esterase-lipase preparation (Piccantase) had been added. Addition of *Mucor miehei* lipase in the manufacture of Ras cheese produced an increase in volatile free fatty acid on storage, but the effect on flavour was not reported (Soliman *et al.*, 1980). However, Hagress *et al.* (1983) added Piccantase in the manufacture of this variety and reported that increased volatile free fatty acids on ripening was accompanied by a development of rancidity.

Cheddar cheeses were organoleptically preferred when gastric lipase preparations were included in their manufacture. Increased free fatty acid production and flavour intensification resulting from addition of lipase preparations have been reported for Feta cheese (Efthymiou & Mattrick, 1958), Domiati cheese (El Neshawy *et al*, 1982), and Cephalotyre 'Ras' cheese (Abdel Baky, 1982).

II. Current application of esterase-lipase in the food industry

Strongly flavoured cheese is produced by adding protease and lipase mixtures to the scalded curds and then curing at 10-25^{o}C for 1-2 months. The ratio of esterase:lipase activities should be two or three to one. Over treatment results in excessive development of methyl ketones in the cheese, especially 2-heptanone and 2-nonanone. Concentrated cheese flavourings are produced by

the rapid modification of slurries of milk solids or casein, various fats, and emulsifiers (Godfrey, 1983). Effective from August 13, 1974, enzyme-modified cheese became an acceptable optional ingredient in processed cheese, processed cheese food, or process cheese spread.

Blue cheese is useful as an additive to salad dressings or as a cheese dip. As a cheaper alternative to the natural cheese, Jolly & Kosikowski (1975a) have developed a 'Blue cheese food'; fat in the form of pasteurized heavy cream, plastic cream or coconut fat was incubated with food grade lipase from *Aspergillus oryzae* and with spores of *Penicillum roqueforti*. A culture of *Streptococcus lactis* or *Streptococcus cremoris* was added to produce acidity and the partly lipolysed fat concentrate was then added to skim milk concentrated by ultrafiltration. The final product had a smooth, creamy texture, and also had the flavour and aroma characteristic of good quality Blue cheese.

Several processes for using microbial lipases to produce cheesy and buttery flavours for incorporation into bakery products, cereal product, confectionery and imitation dairy products have been described (Seitz, 1974; Arnold *et al.*, 1975; Dziezak, 1986). In a recent patent application process, Boudreaux (1985) used *Candida lipolytica* as a source of lipase/proteinase, and two strains of lactic acid producing bacteria to ferment different media. The final product was dried or concentrated, and used as a cheese flavour. Lipase from *Rhizopus delemar* also produced buttery flavours on incubation with fresh milk or cream (Tanabe Seiyaku Co. Ltd, 1971a) and has been used in Japanese yogurt production (Tanabe Seiyaku Co. Ltd, 1971b) both to accelerate the rate of fermentation by *Lactobacillus acidophilus* and to produce a butter-like aroma when *Lactobacillus bulgaricus* was used in the fermentation.

An exciting development in the new focus on enzymes that can be used in the mainstream of oilchemical processing. Three key areas with potential for

improvement by enzymology are fat splitting for fatty acid production, lipid synthesis via reversal of hydrolysis and lipid modification by ester interchange or interesterification (Posorske, 1984). While none of these applications are yet commercial, each currently is the focus of intense investigation.

Currently available suppliers of lipase preparations and their known properties are given in Table 2. A listing of the suppliers of flavour ingredients was also compiled to show over 60 companies in the US alone.

C. APPLICATION OF NOVEL ESTERASE-LIPASE FROM *LACTOBACILLUS CASEI* SUBSPECIES

I. Classification of Lactobacilli

The species of the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* form a supercluster within the so-called clostridia sub-branch of the Gram-positive bacteria, as shown by oligonucleotide cataloging of their 16S rRNA (Stackebrandt *et al.*, 1983). Bifidobacteria, already excluded from the family *Lactobacillaceae* in *Bergey's Manual* (Kandler & Weiss, 1984), have proved to be completely unrelated to lactobacilli. They belong to the so-called actinomycetales sub branch of the gram-positive bacteria.

The species of *Lactobacillus* are arranged into the traditional three groups resembling Orla-Jensen's three genera without designating them as formal subgeneric taxa since they do not represent phyiogenetically defined clusters. Although the majority of strains of each of the new groups agree with the original definition of thermobacteria, streptobacteria and betabacteria, many of the recently described species do not fit these definitions. Hence, the following new definitions contain neither growth temperature nor morphology, the classical characteristics of Orla-Jensen's subgenera.

Table 2. Some companies that supply lipases and characteristics of the available enzymes (Kilara, 1985)

	Company	Address	Country	Enzyme	Form supplied	pH Optimum	Temperature optimum (°C)
1	Amona Pharmaceutical Co	1-21-Chome, Nishiki, Naka Ku.	Japan	•	•	•	•
2	Chr Hansen Laboratories A/S	3-Sankt Annae Plads, DK-1250 Copnehagen	Denmark	Lipase pregastric esterase from lamb calf kid	Powder	5 7–6 6	30–37
3	Dairyland Food Laboratories	620 Progress Ave Waukesha, W1 53187	USA	Italase C (calf pregastric esterase)	Powder	5 0–5 5	5–60
				Capalase K (kid)	Powder	5 0–5 5	5–60
				Capalase L (lamb)	Powder	5 0–5 5	5–60
				Capalase KL (kid, lamb)	Powder	5 0–5 5	5–60
4	Enzyme Development Corporation	2 Penn Plaza, New York, NY 10121	USA	Enzeco	Powder		
5	Gist – Brocades NV	P O Box 1, Wateringseweg, 1 Delft-2600 MA	Holland	Piccantase (fungal lipase esterase from *Mucor miehei*)	Powder	7 5	45–50
6	Hughes and Hughes (Enz) Ltd	Elms Industrial Estate, Church Rd , Harold Wood, Romford, Essex	UK	Lipase (fungal lipase esterase from *Rhizopus arrhizus*)	Powder	7–7 5	37
7	Miles – Kali – Chemie GmbH	3 Hannover-Kleefeld, Hans-Buek er Allee 20 Posttach 690307	Federal Republic of Germany	Miles Labs subsidiary in Federal Republic of Germany	•	•	•
8	Miles Laboratories	Elkhart IN 46514	USA	Takamine pancreatic lipase	Powder	5 0–9 5	>50
				Takamine lipase glandular	Powder	5 5–9 5	>50
9	Novo Industri A/S	Novo Allee, DK-2800 Bagsvaerd	Denmark	Novozyme 206 (fungal lipase esterase *Aspergillus niger*)	Liquid	5–7	35–40
				Novozym 244 (Animal phospholipase A2)	Powder	8	50–50
10	John and E Sturge Ltd	Dennison Rd Selby North Yorkshire YO8 8EF	UK	Lipase A (fungal lipase-esterase *Aspergillus niger*)	Powder	3–8	40
11	Swiss Ferment Co	Vogewenstrasse 132, 4056 Basel, 13	Switzerland	Novo subsidiary in Switzerland	•	•	•
12	Tanabe Seiyaku	Via Siber Hegner Bendolux BV Postbus 414 Rotterdam, Westersingsgel 107	Japan & Holland	•	•	•	•
13	Rohm Enzyme	Rohm GmbH Posttach 4242, Kirschenallee, D-6100-Darmstadt 1	Federal Republic of Germany	Lipase preparation 2212F (Fungal)	Powder	4–7	35–45
				Lipase preparation 2212C (hog pancreas)	Granules	7–9	40
14	American Enzyme Corporation	Box 23441, Brown Deer WI 53223	USA	•	•	•	•
15	American Laboratories Inc	4410 S 102 St , Omaho NE 68127	USA	Lipase, Pancreatin.	•	•	•
16	Atomergic Chemetals	100 Fairchild Ave., Plainview NY 11803	USA	•	•	•	•
17	Biocon (US) Inc	2348 Palumbo Dr Lexington KY 40509	USA	•	•	•	•
18	Fermco Biochemic Inc	202 Lunt Dr Elk Grove Village, IL 60007	USA	Fermlipase (pancreatic)	Powder	•	•
19	Finn-Cal Products Inc	1140 Mission Ave San Rafael, CA 94901	USA	•	•	•	•
20	Semco Laboratories Inc	3950 N Hutton Street, Milwaukee, WI 53212	USA	•	•	•	
21	Viobin Corporation	226 W Livingston St Monticello, IL 61856	USA		•	•	•

* *Data unavailable*

In group 1, obligately homofermentative lactobacilli: hexose are fermented almost exclusively to lactic acid by the Embden-Meyerhof pathway while pentose or gluconate are not fermented. Rare reports on pentose fermentation by particular strains of members of group 1 should be reinvestigated. Fermentation balances should be determined, in order to get information on the possible fermentation mechanism of such atypical strains. The type strains of the four former species, *Lactobacillus delbrueckii, Lactobacillus bulgaricus, Lactobacillus lactis* and *Lactobacillus leichmannii*, were found to possess between each other more than 80% DNA/DNA homology (Weiss et al., 1983).

In group 2, facultatively heterofermentative lactobacilli: hexoses are fermented almost exclusively to lactic acid by the Embden-Meyerhof pathway or, at least by some species, to lactic acid, acetic acid, ethanol and formic acid under glucose limitation; pentoses are fermented to lactic acid and acetic acid via an inducible phosphoketolase. *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus curvatus,* and *Lactobacillus sake* are included in this group.

In group 3, obligately heterofermentative lactobacilli: hexoses are fermented to lactic acid, acetic acid (ethanol) and CO_2; pentoses are fermented to lactic acid and acetic acid. In general, both pathways involve phosphoketolase. However, some species which probably possess other pathways for carbohydrate breakdown but performing also a heterofermentation including the production of gas from hexoses are tentatively also included in group 3. *Lactobacillus bifermentans, Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus divergens*, and *Lactobacillus viridescens* are in this group (Kandler, 1984).

A second complex of at least three genotypes is formed by the subspecies of *Lactobacillus casei*. While the type strain and only two strains

originally designated '*L. zeae*' (Kuzetnov, 1959) are related at a DNA/DNA homology level of 80-100 %, the majority of the strains of *Lactobacillus casei*-subsp-*casei*, *Lactobacillus casei*-subsp-*pseudoplantarum* and *Lactobacillus casei*-subsp-*tolerans* form a second genotype at a homology level of 80-100 % among each other, but with only 40 % homology toward the genotype which contains the type strain. Strains of *Lactobacillus casei*-subsp-*rhamnosus* represent a third genotype which shares only 30-50 % homology with strains of the other two genotypes. Because of the low DNA/DNA homology, and distinct phenetic differences to other subspecies, *Lactobacillus casei*-subsp-*rhamnosus* is a candidate to be raised to the species status. The two other subspecies, although closely related to *Lactobacillus casei-subsp-casei* are distinctly phenotypically different from *Lactobacillus casei*-subsp-*pseudoplantarum* by forming DL-lactic acid via lactic acid racemase or from *Lactobacillus casei*-subsp-*tolerans* by heat tolerance and an extremely sparse pattern of fermented carbohydrates (Stetter and Kandler, 1973).

The differential characteristics of the group 2 species of *lactobacillus* are indicated in Table 3 (Bergey's manual, Kandler & Weiss, 1986).

II. Application of Lactobacilli in Food industry

Lactobacillus play a key role in various food fermentation processes as well as for the production of lactic acid. The preparation of cheese, yogurt, fermented beverages, cured meats, pickles, and silage are examples of processes that utilize strains of lactobacilli. In all types of cheese with ripening periods longer than about 14 days, several mesophilic lactobacilli (*L. plantarum, L. brevis, L. casei*, etc.) originating from the milk or the dairy environment, reach levels as high as 10^6-10^8/g cheese (Sharpe, 1979; Law,1982). The modification of flavour caused by the *Lactobacillus* strains used in culturing buttermilk,

Table 3. Physiological and biochemical characteristics of the facultatively heterofermentative species of the genus *Lactobacillus* (Bergey's manual, 1986)

Species	Peptidoglycan type[b]	Teichoic acid	Electrophoretic mobility[c] D LDH	Electrophoretic mobility[c] L LDH	Allosteric L LDH	Mol% G + C	Lactic acid isomer(s)[d]	Growth at 15°C	NH_3 from arginine
L. agilis	mDAP Direct	None	1.40	1.20	–	43–44	L	–	–
L. alimentarius	Lys-DAsp	None	0.80	1.10	–	36–37	L(D)	+	–
L. bavaricus	Lys-DAsp	None	–	1.60	+	41–43	L	+	–
L. casei subsp *casei*	Lys-DAsp	None	1.22[e]	0.93	+	45–47	L	+	–
L. casei subsp *pseudoplantarum*	Lys-DAsp	None	1.04	0.93	+	45–47	DL	+	–
L. casei subsp *rhamnosus*	Lys-DAsp	None	0.75	0.93	+	45–47	L	+	–
L. casei subsp *tolerans*	Lys-DAsp	None	–	0.93	+	45–47	L	+	–
L. coryniformis subsp *coryniformis*	Lys-DAsp	None	0.38	–	–	45	D(L)	+	–
L. coryniformis subsp *torquens*	Lys-DAsp	None	0.38	–	–	45	D	+	–
L. curvatus	Lys-DAsp	None	1.20	1.60	+	42–44	DL	+	–
L. homohiochii	Lys-DAsp	Glycerol	ND	ND	–	35–38	DL	+	–
L. maltaromicus	mDAP Direct	None	ND	ND	–	36	L	+	ND
L. murinus	Lys-DAsp	None	–	0.92	+	43–44	L	–	–
L. plantarum	mDAP-Direct	Ribitol or glycerol	1.44	1.28	–	44–46	DL	+	–
L. sake	Lys-DAsp	None	1.20	1.60	+	42–44	DL	+	–

acidophilus milk, or San Francisco sour dough bread are obvious examples of flavour being changed by a *Lactobacillus*. In these products, the appearance also changes as a result of bacterial action as it does with yogurt. The contribution that a fermentation may make to B-vitamin and free amino acid content is more subtle (Kilara & Treki, 1984). The levels of some essential amino acids can be significantly raised by the fermentation process (kilara & Treki, 1984; Newman & Sands,1984).

In certain cases, *Lactobacillus* are used for the production of lactic acid, although this fermentation process competes with chemical synthesis. In a region rich in biomass or where a readily available source of fermentable material is available, i.e., from a large cheese operation, fermentation processes for lactic acid are likely to be competitive with chemical synthesis (Kilara & Treki, 1984).

Anaerobic glycolysis of carbohydrates by lactobacilli results in the production of lactic acid which lowers the pH and discourages growth of other contaminating microbes upon subsequent storage (Kandler, 1984). Their use of carbohydrate substrates competitively contribute to their ability to retard spoilage (Kandler, 1984; Kilara & Treki, 1984). Some strains are known to produce bacteriocins and possibly other specific antimicrobials which may contribute to this effect (Barefoot & Klaenhammer, 1983). *Lactobacillus* starter cultures could be used to repress growth of pathogenic organisms in raw milk, extend the shelf-life of cottage cheese and other dairy foods, sausages, ground beef and deboned poultry meat (Schroder *et al.*, 1980; Lee & Simard, 1984).

Probiotic effects are far more difficult to document than the other applications. The controversy began in 1908 when Metchnikoff suggested that intestinal lactobacilli could play a role in the prolongation of life as certain lactobacilli are commensal colonizers of the human and animal gastro-intestinal

epithelium (Chassy, 1986). However, their exact role and function are hard to define. But it is believed that they can secrete enzymes such as lactase which might facilitate lactose digestion in less tolerant individuals (Goldin & Gorbach, 1984). Lactobacilli have the potential to metabolize dietary cholesterol. Strains of lactobacilli have been shown to detoxify potential carcinogens while compounds with anti-tumor activity have been isolated from others. The total effect on prolongation of life or enhancement of human health by lactobacilli is far from firmly established. It is tempting to believe that lactobacilli do us no harm, and that they may well have a positive effect on life.

III. Production and Characterization of esterase-lipase from Lactobacillus casei subspecies

Both modern and traditional methods for the manufacture of Cheddar aged longer than three months rely upon the chance presence of lactobacilli to facilitate the latter stage of ripening. Consequently it was proven (Lee *et al.*, 1986) that the lactobacilli produce greater range of enzymes than do the streptococci starter and the enzymatic apparatus (peptidase and esterases) are seen to be responsible for the development of desirable flavours. *Lactobacillus casei* groups *(L. casei* subsp. *casei, L. casei* subsp. *pseudoplantarum* and *L. casei* subsp. *rhamnosus*) were found to be more active in various peptidases and esterases activities than any other lactic acid bacteria.

Further studies in pilot scale (Laleye *et al.*, 1986) demonstrated that the use of *Lactobacilli casei* in conjunction with the starter streptococci shortened the ripening period from 10 to 4 month, with an improvement in Cheddar flavour and elimination of the gas encountered by heterofermentative lactobacilli.

Lipolysis along with lactose hydrolysis and proteolysis represents the major biochemical changes occuring during cheese ripening. Although a great

deal of attention has given to their enzymatic activities responsible for lactose and peptides degradation, very little informations are available as far as lipolytic and esterolytic systems are concerned. The proposed research involves the investigation of two key sets of enzyme, namely esterases-lipase and peptidases from selected *Lactobacillus casei* subspecies in order to develop enzyme-modified cheese (EMC) and accelerated-ripened cheese (ARC). Initial screening of about 25 strains of various starter cultures clearly exhibited that Lactobacilli possess for more numbers and higher concentrations of esterases and peptidases than the most commonly used starter strains of *Streptococcus lactis* and *Streptococcus cremoris* (Lee *et al*, 1986). Further screening of 20 strains of the *Lactobacillus casei* group using a novel method of microenzyme (APIZYM method) and synthetic substrates showed that some strains of *Lactobacillus casei*-subsp-*casei, Lactobacillus casei*-subsp-*rhamnosus,* and *Lactobacillus casei*-subsp-*pseudoplantarum* possess the highest peptidase and esterase activities. Final selection of best three strains was done by quantitative determination of concentrations of these enzymes using chromogenic substrates. Hence the crude cell-free extracts of these strains have been partially characterized for their specificities in our laboratory.

These extracts when added to young cheese slurry showed strong desirable cheddar cheese flavour within 2 days without bitterness and rancid off-flavor. Although some studied the crude enzymes (esterases and peptidases) of different lactobacilli strains (El Soda *et al.*, 1986a & 1986b), very little information is available on enzyme systems of *Lactobacillus casei* subspecies.

Traditional proteolysis by commercial enzymes leads to the generation of bitterness in the hydrolysate limiting their use and commercial value. On the other hand, a new enzyme, debitrase containing proteolytic and peptidolytic

enzyme isolated from starter cultures and lactobacilli strains allows selective hydrolysis resulting in a non-bitter product and can achieve a high degree of hydrolysis. Also the long chain (C_{12}-C_{16}) fatty acids released by most microbial lipases produce an unpleasant 'soapy' flavor defect, On the other hand lactobacilli esterase producing alkanoic acids from C_4 to C_{10} appear to impart cheese-like flavours. This is why our interest became focused particularly on these enzymes in *Lactobacillus casei* subspecies.

In light of those information at hand, Genecor and Chr. Hansen laboratory developed a blended formulation of specific lipase with a fungal protease, which is marketed as Flavor-Age-Fr. This along with starter cultures, produces a high quality Cheddar flavour with a reduction in maturation time of approx. 60%. The other companies marketing similiar formulations are Impei al Biotechnology Ltd. (U.K.), Novo Industry Ltd. (Denmark), and Miles/Marshall (U.S.A.). Imperial Biotechnology Ltd. is marketing a blend of enzyme of dairy starter; *Streptococcus lactis* strain. However, *Streptococcus lactis* is not an active producer of peptidases and esterases, as compared to *Lactobacillus casei* (Lee *et al.*, 1986).

Although *Lactobacillus casei* groups have been shown to produce strong esterase and esterase-lipase activities besides various peptidases, their utility and specificities have not been studied yet. Therefore, the immediate goal of the proposed research is to produce esterase-lipase from two selected *Lactobacillus casei* species, and to examine the properties of these enzymes.

MATERIALS AND METHODS

1. Microorganisms

The two strains investigated for this research were *Lactobacillus casei-subsp-casei LLG* and *Lactobacillus casei-subsp-pseudoplantarum LE2* These strains were obtained from Agriculture Canade Food Research Centre (St-Hyacinthe, Quebec). These strains were initially isolated from a high quality Cheddar cheese. The cultures were maintained either on lactobacilli MRS agar (Difco Laboratories) or at -30^{O}C in 20% (^{V}/v) skim milk (Difco Laboratories) solution diluted equally with growth medium. These organisms were revived by two consecutive inoculations.

2. Growth and preparation of cell extracts

A loopful of *Lactobacillus casei* subspecies was transferred from a freshly inoculated slant into 5 ml MRS broth (Difco Laboratories) and grown through successive pre-inoculum cycles. A portion (0.3 ml) of this pre-inoculum culture was then transferred to 300 ml of MRS broth (Difco Laboratories) and were grown for 16h at 30^{O}C. All incubations were conducted at 30^{O}C on a water bath shaker (Versa-bath S Model 236, Fisher scientific.) which was calibrated to 60 rpm.

Cells were harvested at the late logarithmic phase by centrifuging at 10,000 g for 15 min (Sorvall RC2-B, rotor GS, 4^{O}C) and the pellet was washed twice with 0.05 M sodium phosphate buffer (pH 7.1); it was then suspended in 10 ml of the same buffer. The cells were subsequently disrupted by three passages through a French press (14,000 to 16,000 psi, American Instrument Co. Ltd). The crude extract was centrifuged at 15,000 g for 35 min (Sorvall RC2-B, rotor SS34, 4^{O}C) and gave rise to a clear cell free extract. After

centrifugation, a portion of supernatent was passed through a P-D 10 column (Pharmacia) equilibrated with the same buffer. The cells and supernatents were maintained at 4^{o}C throughout the experimental procedure.

The growth of the culture was followed by measuring changes in turbidity of the culture. Optical density measurements were initially read on a Ultrospec 2 spectrophotometer (LKB Biochrom. Ltd.). All measurement were read optical density at 600 nm. The spectrophotometer was previously blanked with sterile medium.

3. Media

The MRS broth (Difco Laboratories) was used as described by De Man *et al.* (1960). This medium consisted of Bacto protease peptone #3, 10 g; Bacto beef extract, 10 g; Bacto dextrose, 1 g; Tween 80, 1 g; Ammonium citrate, 2g; Sodium acetate, 5 g; Magnesium sulfate, 0.1 g; Manganese sulfate, 0.05g; Dipotassium phosphate, 2 g. The pH was near 6.5 after sterilizing. To rehydrate the medium, was suspended 55 grams of MRS in 1 liter deionized water, and heated to boiling to dissolve completely and dispensed 300 ml in each 500 ml flask. Finally, It was sterilized in the autoclave for 15 minutes at 15 pounds pressure (121^{o}C).

4. Chemicals

Unless specifically listed, commercially available reagent analytical grade chemicals were used.

5. Protein assay

Protein was assayed by BCA protein assay reagent supplied with the systems (Pierce chemical Co. Rockford. Il). The Pierce BCA protein assay

reagent is a highly sensitive reagent for the spectrophotometric determination of protein concentration (Smith *et al.*, 1985). This unique system combines the well known reaction of protein with Cu^{2+} in an alkaline medium (yielding Cu^{1+}) with a highly sensitive and selective detection reagent for Cu^{1+}, namely bicinchoninic acid (BCA).

Bicinchoninic acid (BCA), in the form of its water soluble sodium salt, is a sensitive, stable and highly specific reagent for cuprous ion $(Cu^{1+})^3$. This attribute is utilized to assay uric acid and glucose-substances capable of reducing Cu^{2+} to Cu^{1+}.

The BCA protein assay reagent combined the well known biuret reaction (protein reaction with Cu^{2+} in an alkaline medium to produce Cu^{1+}) with the unique features of BCA (Fig 1a). The purple coloured reaction product which was formed due to the interaction of two molecules of BCA with one cuprous ion (Cu^{1+}), was water soluble quantitation of protein in aqueous solutions.

A set of protein standards of known concentration was prepared by diluting a stock solution of BSA (Bovine Serum Albumin) in the same diluent as testing samples. The set of protein standards should covered the range of concentrations suitable for the assay (Table 4). 0.1 ml of each standard and testing samples were pipetted into the appropriately labelled test tube. 0.1 ml of diluent was used for blanks. BCA working (2.0 ml) reagent was added to each tube and mixed well. To prepare BCA protein assay working reagent, 50 parts of reagent A (Sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.1 N NaOH) was mixed with 1 part of reagent B (4% copper sulfate solution). This working reagent was prepared fresh the day which planned to use it, but was stable for 1 week at room temperature. After

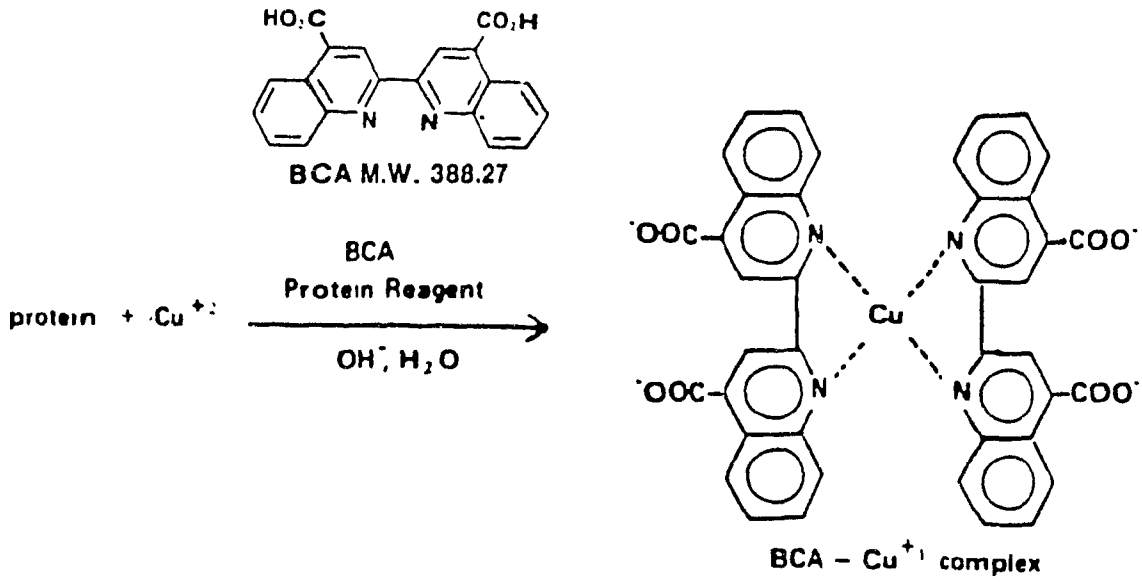


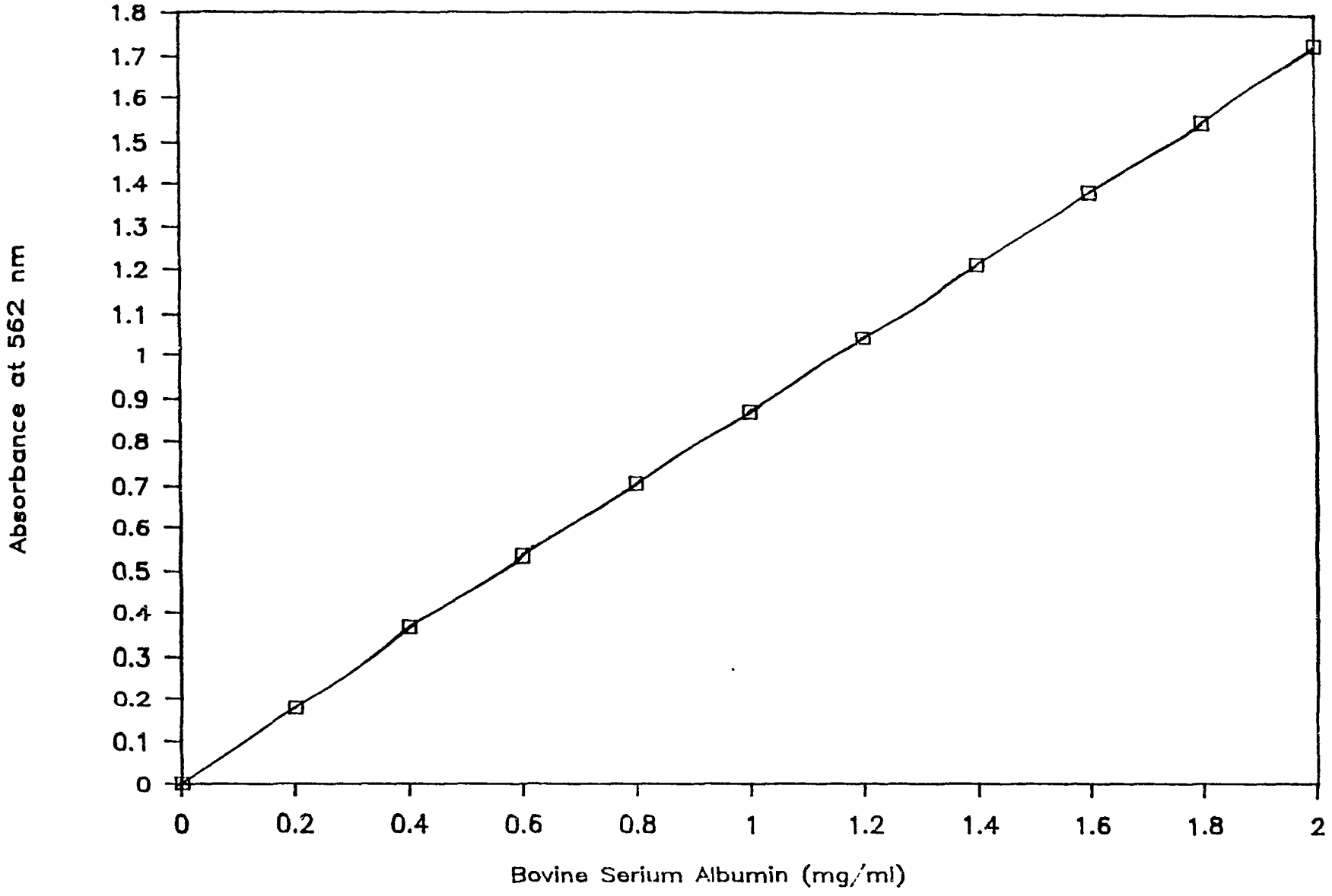
Fig 1a. The reaction scheme of BCA protein reagents

Table 4. Preparation of appropriate dilutions of the stock standard for using at 37°C for 30 min.

Volume of BSA (2mg/ml)	Volume of H_2O	Final Conc. of Calibration Std.
0.10 ml	0.90 ml	0.2 mg/ml
0.20 ml	0.80 ml	0.4 mg/ml
0.30 ml	0.70 ml	0.6 mg/ml
0.40 ml	0.60 ml	0.8 mg/ml
0.50 ml	0.50 ml	1.0 mg/ml
0.60 ml	0.40 ml	1.2 mg/ml
0.70 ml	0.30 ml	1.4 mg/ml
0.80 ml	0.20 ml	1.6 mg/ml
0.90 ml	0.10 ml	1.8 mg/ml
1.00 ml	0.00 ml	2.0 mg/ml

Fig 1b. Standard curve of Bovine Serium Albumin (BSA) for BCA protein assay

* Points are the averages of 3 replicates


Absorbance at 562 nm
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
Bovine Serium Albumin (mg/ml)

incubation (30 min at 37^{O}C), the absorbance of each tube was measured spectrophotometrically at 562 nm of each tube vs. water reference. A standard curve was then prepared by plotting the net (blank corrected) absorbance vs. protein concentration (Fig 1b). Using this standard curve, the protein concentrations for each unknown samples were determined from the standard curve.

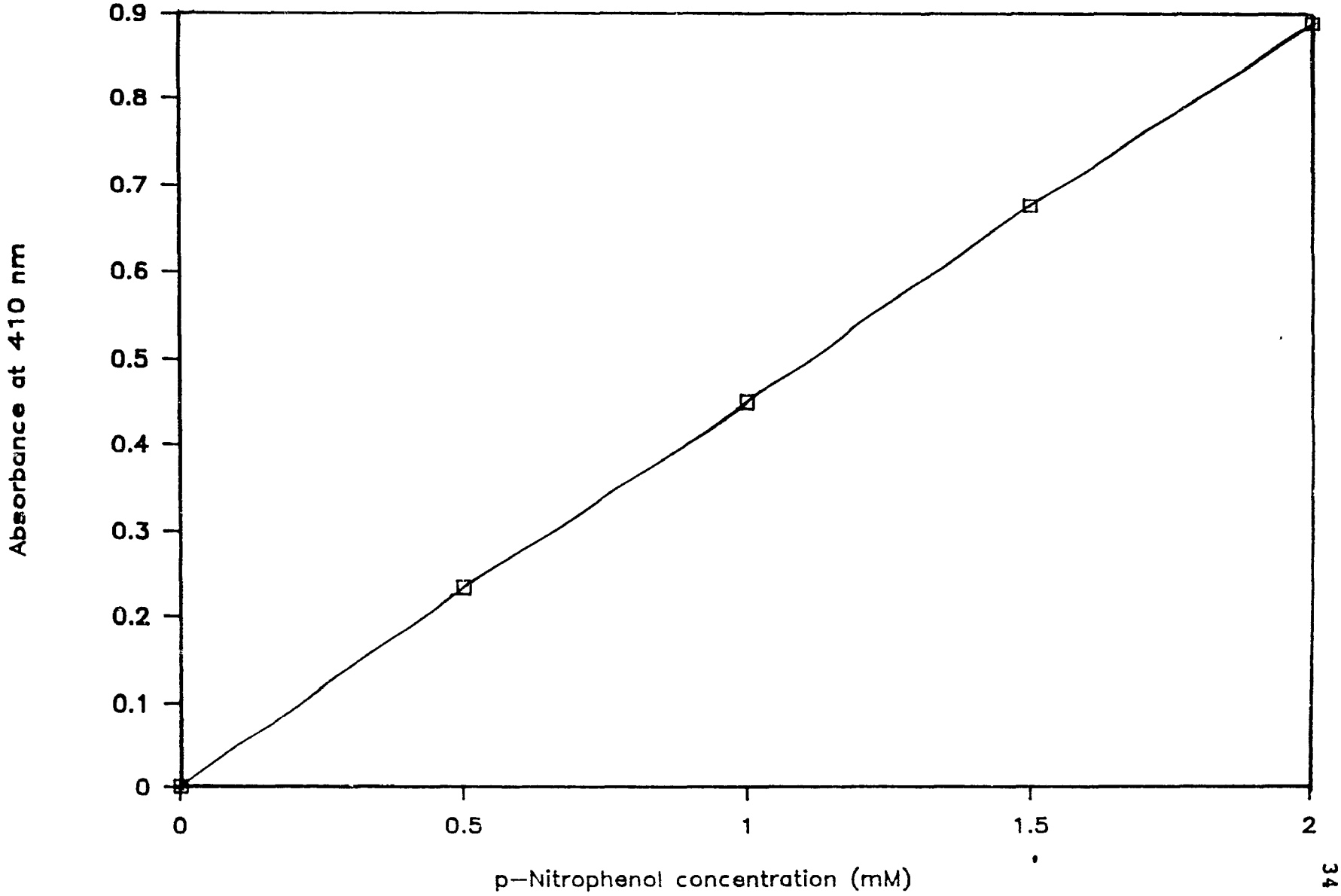
6. Enzyme assay

1. The total lipolytic activity was determined by conventional titration method (Brockerhoff & Jensen, 1974) using Tween 80 (Sigma) and Tributyrin (Sigma) as substrate. A reaction medium consisted of 0.1 ml 5×10^{-1} M KCl, 1 ml 5×10^{-3} M NaCl, 0.1 ml 1.2×10^{-3} M sodium deoxycholate, and 2 ml 1×10^{-3} M $CaCl_2$. Different concentrations of the substrates were added to the mixtures. The volume of the mixture was adjusted to 9.5 ml with distilled deionized water. The reaction was initiated by adding 0.5 ml of enzyme extract containing 20 mg protein/ml. After shaking at 180 rpm for 30 min at 37^{O}C (Versa bath S Model 236, Fisher scientific), the reaction was stopped by adding 10 ml of a 3:1 mixture of diethylether/ethanol. The free fatty acids produced by the reaction were measured by titration (Brinkmann digital buiret, Fisher scientific) against 0.01 N NaOH using phenolpthalin as an indicator. The specific activity of lipase was expressed as milliequivalents of fatty acid liberated / mg of protein / h.

2. The esterase-lipase activity was also evaluated spectrophotometrically using a mixture of p-nitrophenyl derivatives of fatty acids (C_2, C_4, C_6, C_8, C_{10}, C_{12}, C_{14}; Sigma). This photometric assay using p-nitrophenol as the substrate (Brandle & Zizer, 1973; Winkler & Stuckman, 1979) was modified as follows. The assay mixtures contained 1.8 ml 0.01 M phosphate buffer (pH 7.1), 0.1 ml 1.5 mM of p-nitrophenyl derivatives of fatty acids mixtures (C_2-C_{14}) and 0.1 ml

Fig 2. Standard curve for determination of p-nitrophenol in measurement of lipolytic activity

* Points are the averages of 3 replicates


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 410 nm
0
0.5
1
1.5
2
p-Nitrophenol concentration (mM)

enzyme solution (20 mg protein/ml) in a total volume of 2 ml. After shaking at 180 rpm for 30 min at 37^{O}C, enzyme activity was measured by absorbance at 410 nm (Ultraspec 2, LKB biochrom. Ltd). One unit of enzyme activity was defined as the enzyme necessary to release 1 umol of p-nitrophenol per min at 37^{O}C from the p-nitrophenol standard curve (Fig 2) and specific activity of the enzyme was defined as enzyme units / mg protein.

7. Enzyme purification

A. Apparatus

A Pharmacia FPLC (Fast Protein Liquid Chromatography) system was used. The FPLC system consisted of two P-500 pumps, a V-7 injection valve, UV monitor with HR flow cell, a GP-250 gradient programmer, a Frac-100 collector and a REC-482 recorder (Fig 3). In this system, the sample and eluent are not in contact with stainless steel, and no corrosion of the pump at high pH or in the presence of salts, with subsequent degradation of column performance, took place. All buffers were filtered through 0.22- m Millipore filter and degased under vacuum before use.

DEAE-Sepharose CL-6B, prepacked Superose 12 HR 10/30, Mono Q HR 5/5 columns as well as the FPLC system were from Pharmacia Fine Chemicals (Uppsala, Sweden).

B. Ion exchange chromatography

To isolate esterase-lipase from *Lactobacillus casei*-subsp-*pseudoplantarum* LE2, FPLC system was employed with a Mono Q HR 5/5 column (5cm x 0.5cm). The column was equilibrated with buffer A (5 mM phosphate buffer, pH 7.1). After running with buffer A, an elution gradient of 0-100 % buffer B (1M sodium chloride in buffer A) was performed autometically for

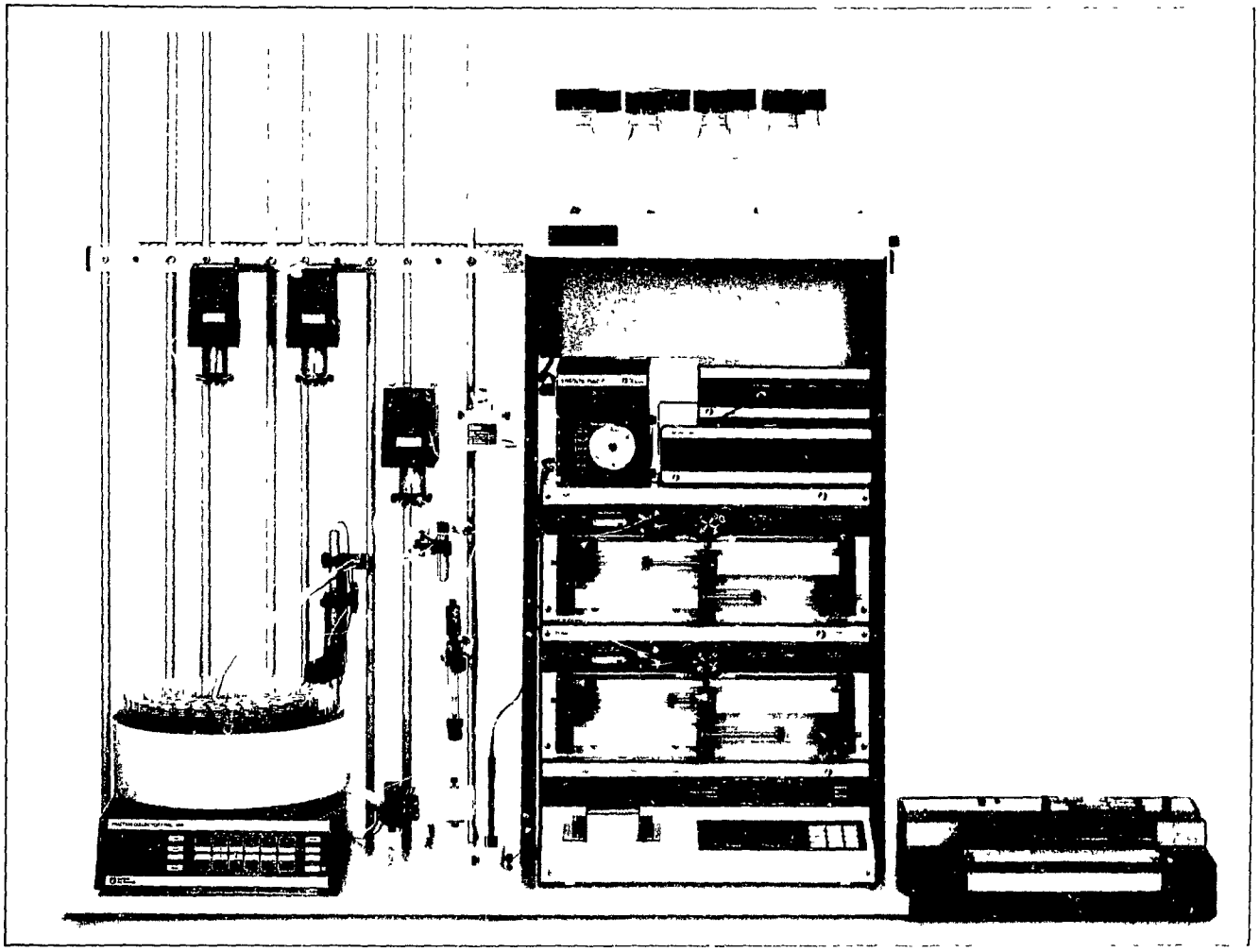


Fig 3. Photograph of the Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Sweden)

28 min, then at 100 % for 2 min. Elution was performed with a flow-rate of 1 ml/min and 1 ml fractions were collected. The crude extract (500 l) was applied three times for ion exchange chromatography step.

The DEAE-Sepharose CL-6B column (10cm x 1cm) equilibrated with buffer A (5mM phosphate buffer A, pH 7.1) was used for *Lactobacillus casei*-subsp-*casei* LLG lipolytic enzymes. After rinsing with buffer A for 10 min, the crude extract was eluted using a linear salt gradient of 0-1 M sodium chloride in buffer A for 5h, then at 100 % for 10 min. Elution was also performed with a flow-rate of 1ml/min and 1 ml fractions were collected.

C. Gel filtration chromatography

The major esterase-lipase peaks obtained by ion-exchange chromatography on the Mono Q column or DEAE Sepharose CL-6B were concentrated to 1 mg/ml by ultrafiltration CX-30 (Millipore) and were applied to a Superose 12 HR 10/30 (30cm. x 1cm) equilibrated with 5 mM phosphate buffer (pH 7.1). The fraction collector peak cut-off was also set at 10 % of full scale deflection , the flow-rate at 0.5 ml/min and the fraction size at 1.0 ml. The 100 % full deflection of the UV monitor was usually set and absorbance of 0.2. The gradient programmer facilitates reproducible separations, and the monitor and the fraction collector permit automated handling of large batches.

D. Electrophoresis

The purified enzyme fractions were subjected to polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using miniaturized "Phast" system (Pharmacia).

The Phast system consisted of a separation and control unit, a development unit, high-performence Phast gel separation media, accessories,

and a technical support package (Fig 4). The schematic diagram (Fig 5) illustrated the steps involved in producing a finished electrophoresis gel using Phast system with PhastGel separation media.

PhastGel gradient 8-25 polyacrylamide gel (Pharmacia) was used for native-PAGE and SDS-PAGE. This gel had a 13 mm stacking gel zone (4.5% T, 3% C) and a 32 mm gradient gel zone. Gel contained a continuous 8 to 25% gradient gel zone with 2% crosslinking. The buffer system in the gel was of 0.112 M acetate and 0.112 M Tris, pH 6.4. The buffer system in PhastGel native buffer strips was of 0.86 M L-alanine and 0.25 M Tris, pH 8.8. The buffer strips were made of 2% Agarose IEF. The buffer system in PhastGel SDS buffer strips was of 0.20 M tricine (trailing ion), 0.20 M tris and 0.55% SDS (analytical grade), pH 7.5. The buffer strips were made of 2% Agarose IEF.

For SDS-PAGE sample preparation, 2.5% SDS and 5.0% p-mercaptoethanol were added to the sample, heated at 100^{o}C for 5 min, and added bromphenol blue to approximately 0.01%. Any insoluble material was removed by centrifugation to prevent streaking patterns in the developed gel.

The coomassie staining technique was used for detecting proteins in PhastGel separation media using PhastGel Blue R. PhastGel Blue R is a coomassie R 350 dye in readily soluble tablet form. Staining solution was composed of 0.1% PhastGel Blue R solution in 30% methanol and 10% acetic acid in distilled water. 30% methanol and 10% acetic acid in distilled water (3:1:6) was prepared for destaining solution which was prepared at least 400 ml enough to fill the chamber three times. Preserving solution consisted of 5% glycerol and 10% acetic acid in distilled water. This solution would help to keep the gradient gels flexible and resistant to cracking. These solutions were prepared fresh the day which planned to use it and did not recycle these solutions.

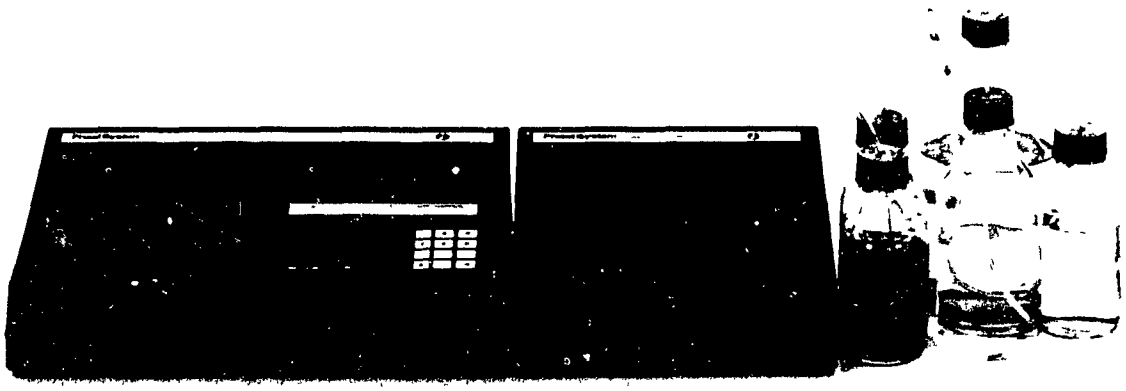


Fig 4. Photograph of the PhastSystem (Pharmacia, Sweden)

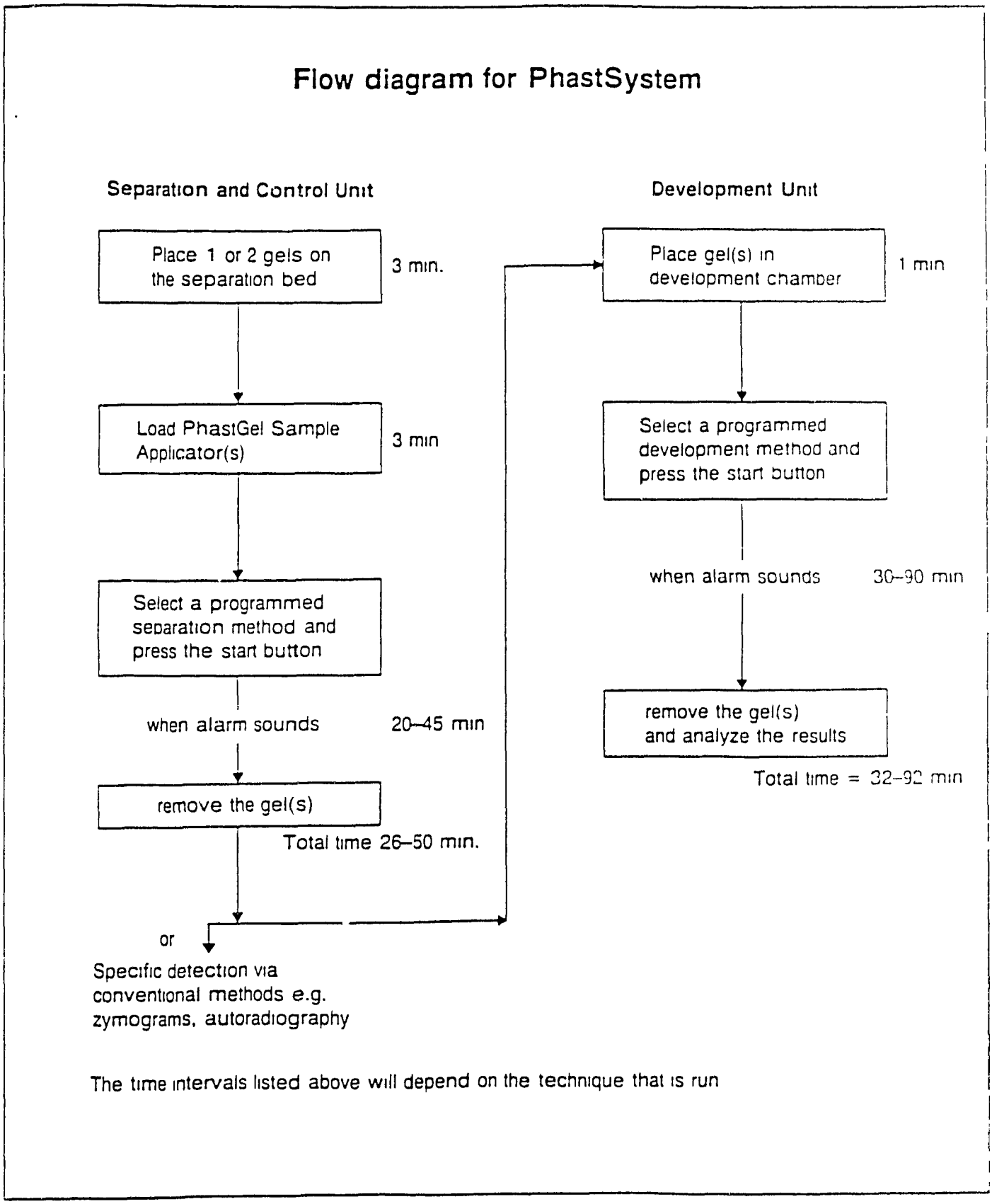


Fig 5. Flow diagram for PhastSystem

Phast system automatically adjusted the current and power so that two gels would run under the same conditions according to programmed method. Tables 5 and 6 showed the running conditions for native-PAGE and SDS-PAGE. The programmed development method shown in Table 7 was for the native-PAGE with PhastGel gradient 8-25, while the program in Table 8 showed the development method file for SDS-PAGE with PhastGel gradient 8-25. Native-PAGE gels took about 10 min longer to destain than SDS-PAGE gels.

Relative molecular weights were estimated by SDS-PAGE using high molecular weight proteins kits (Albumin, Lactate dehydrogenase, Catalase, Ferritin and Thyroglobulin; Pharmacia) as references.

The gel obtained from Phast system was scanned by a densitometer (Beckman Model CDS-200, BREA, CA), and the migration distance of the standards and the proteins of interest. Their corresponding R_f(Relative Mobility) values were then measured. After a calibration curve by plotting R_f vs log molecular weight for the calibration proteins was constructed, the molecular weights was determined by position of its Rf value on the calibration curve (Manual of calibration kits for M.W. determination using electrophoresis, Pharmacia)

Table **5**: Optimized method for native-PAGE in PhastGel gradient media to program into the separation method file of PhastSystem (given as method 1).

SAMPLE APPL	DOWN AT 1.2	0 Vh			
SAMPLE APPL	UP AT 1.2	2 Vh			
SEP 1.1	400 V	10.0 mA	2.5 W	15°C	10 Vh
SEP 1.2	400 V	1.0 mA	2.5 W	15°C	2 Vh
SEP 1.3	400 V	10.0 mA	2.5 W	15°C	268 Vh[1]

[1] This is based on runs using commercially prepared and crude extract proteins. You might have to adjust this time to suit your application.

Table **6**: Optimized method for SDS-PAGE with PhastGel Gradient 8–25 to program into the separation method file of PhastSystem (given as method 2).

SAMPLE APPL	DOWN AT 2.1	1 Vh			
SAMPLE APPL.	UP AT 2.1	10 Vh			
SEP 2.1	250 V	10.0 mA	3.0 W	15°C	65 Vh[1]
SEP 2.2	50 V	0.1 mA	0.5 W	15°C	0 Vh[2]

[1] This is based on runs using commercially prepared and crude extract proteins. You might have to adjust this time to suit your application. The run should be stopped when the tracking dye reaches the anode buffer strip.

[2] This step is optional. Its purpose is to reduce the risk of proteins migrating off the gel should you miss the alarm that marks the end of the method (after step 1).

Table 7 Coomassie staining method for native-PAGE with PhastGel gradient media to program into the development method file

Step number	Solution	IN-port	OUT-port	Time	Temperature
1	Stain	1	0	7 min	50°C
2	Destain	2	0(or 4)[1]	1 min	50°C
3	Destain	2	0(or 4)	10 min	50°C
4	Destain	2	0(or 4)	15 min	50°C
5	Preserving solution	3	0	15 min	50°C

[1] The destaining solutions in steps 2, 3, and 4 can be filtered through activated charcoal and recycled 3 to 4 times Use out-port 4 to collect the solutions for recycling

Table 8 · Coomassie staining method for SDS-PAGE with PhastGel gradient media to program into the development method file.

Step number	Solution	IN-port	OUT-port	Time	Temperature
1	Stain	1	0	8 min	50°C
2	Destain	2	0(or 4)[1]	5 min	50°C
3	Destain	2	0(or 4)	8 min	50°C
4	Destain	2	0(or 4)	10 min	50°C
5	Preserving solution	3	0	5 min	50°C

[1] The destaining solutions in steps 2, 3, and 4 can be filtered through activated charcoal and recycled 3 to 4 times Use out-port 4 to collect the solutions for recycling

RESULTS

A. Production and characterization of esterase-lipase from *Lactobacillus casei*-subsp-*casei* LLG

1. Lipase production during growth of *L. casei-subsp- casei LLG*

The time course of esterase-lipase production from *L casei-subsp-casei LLG* in Fig 6a and 6b showed that the maximal lipolytic activity was detected after 16 h of incubation at the late logarithmic phase, and it decreased when the strain entered the stationary phase. The samples were taken at 2 hour intervals, assayed for cell protein and the enzyme activities using two assay method Cells were completely disrupted by three passages through a French press, which was confirmed by cell protein assay as well as by microscope. No significant differences in lipolytic activity were observed between the titration method and the chromogenic substrate method

2. Properties of esterase-lipase

Varying quantities of partially purified cell extract, containing 20 mg protein/ml were added to assay mixtures. Free fatty acid (m Equiv.) or increased O.D at 410 nm using chromogenic synthetic substrates was not directly proportional to enzyme concentration (Fig 7a). Similar plots were seen when activities were plotted against time of incubation using constant enzyme concentration. Cell extracts and incubation times were limited to 0.02 ml (0 4 mg protein) and 30 min, respectively as the assay was linear for the activity An increase in the substrate concentration in the assay resulted in a considerable increase in the amounts of FFA equivalents released per unit time (Fig 7b)

Substrate inhibition was noted at concentration exceeding 0.1 ml of 1.0% Tween 80. The optimum pH of the enzyme at 37^{o}C was found to be around pH 7.2 but the enzyme was stable above pH 7.0 (Fig 7c and 7d). Although there was a sharp decrease in activity after 50^{o}C, the enzyme was stable at room temperature for at least 2 days.

3. Purification of esterase-lipase

A representative purification profile is presented in Table 9. Esterase-lipase from *L casei-subsp-casei LLG* was purified approximately 67 fold over the crude extract and obtained in 61% yield with ion-exchange and gel-filtration steps. The repeated cycles gave well reproducible results. The protein fractions collected from Sepharose column were resolved into four major peaks, but lipolytic activity was present in two major peaks, the most important one being the third eluted (Fig 8). It appears that esterase-lipase represents a very low proportion (less than 3%) of the proteins present in the crude preparation, but the specific activities of these enzymes were quite high. When the two major active fractions were further subjected to gel filtration column, the proteins were resolved into five major peaks (Fig 9). The enzyme activity was present in three major peaks, the most important one being specific for butyrate (C_4), and the others for caproate (C_6) and capryate (C_8), respectively. Some contaminating activities such as C_6, C_8 and C_{10} were present in the esterase activity. Although crude extracts contained the other activities on C_2 and C_{10} substrates, these activities could not be detected in any fraction. After purification esterase-lipase seems to be practically pure at this step, as only one band was detected from each fractions by both PAGE and SDS-PAGE loaded with concentrated samples (Fig 10 and 11). The molecular weights were 320 K Dalton for butyrate-esterase, 110 K Dalton for caproate-esterase, and 40

K Dalton for capryate-esterase, respectively.

4. Effect of metallic ions and some compounds on the lipolytic activity

A mixture consisting of 0.1 ml of the partially purified enzyme solution and 0.1 ml of metallic ions or some compounds (final concentrations indicated) was incubated for 30 min at room temperature, and the enzyme activity was measured under standard conditions. Ag^{+} and Hg^{2+} strongly inactivated lipase activity, while Mg^{2+}, Ca^{2+}, and mercaptoethanol stimulated enzyme activity (Table 10).

5. Effect of substrate concentration for enzyme kinetics

The effect of substrate concentration on the rate of release of p-nitrophenyl derivatives of fatty acids was investigated. The concentrations of substrate ranged from 0.3 to 3.5 mM, and the enzyme assay was done by the standard assay procedure. The apparent Michaelis-Menten constant (Km) and maximum velocity (V_{max}) for the enzyme were calculated from a Lineweaver-Burk plot to be about 76 umol/min/mg and 0.57 mM, respectively (Fig 12).

Fig 6a. Growth curve of *Lactobacillus casei*-subsp-*casei* LLG using the semilogarithmic drawing paper

* Strain LLG was grown in 300 ml of MRS broth. Cell growth was measured by optical density at 600nm

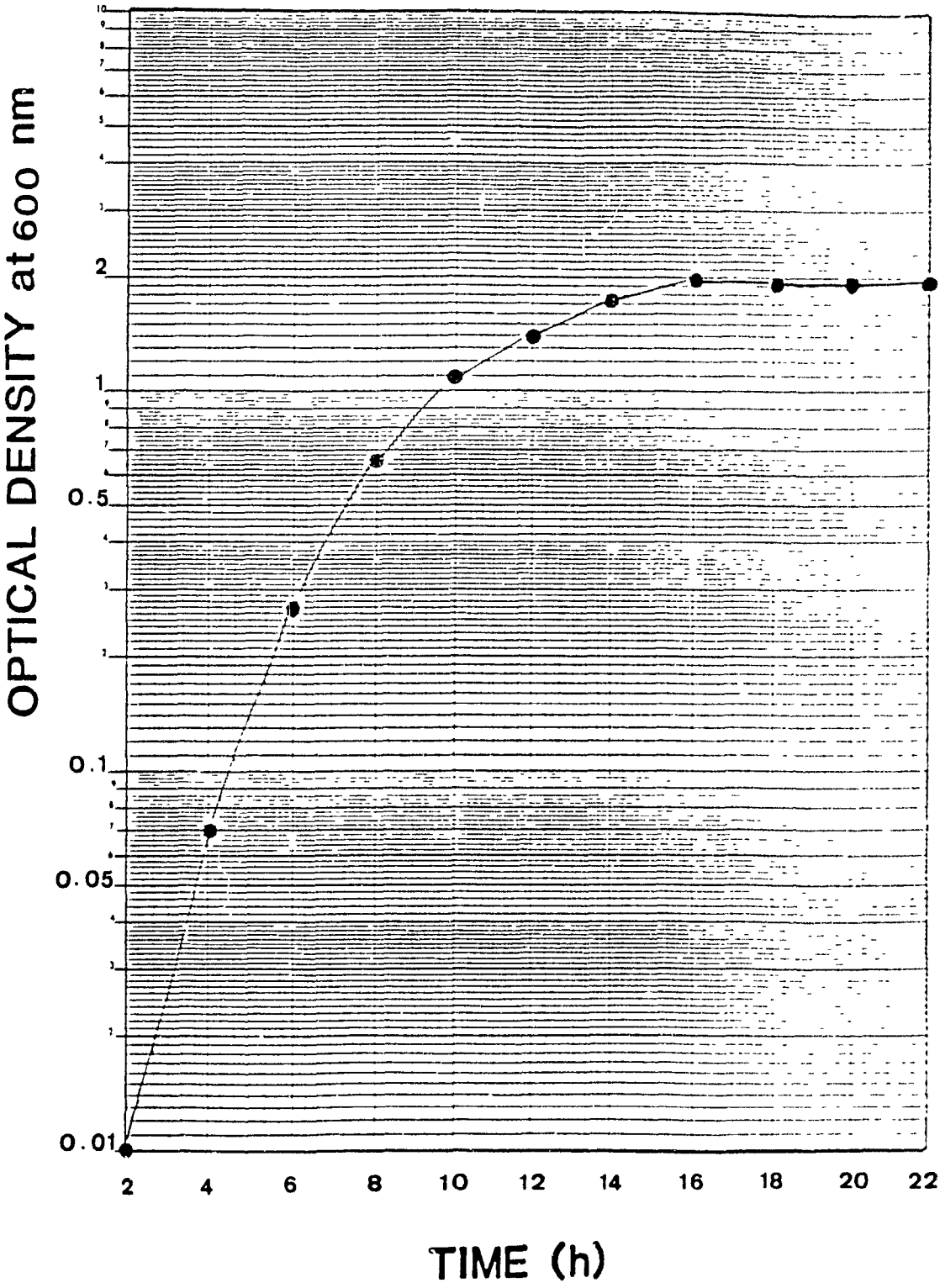

OPTICAL DENSITY at 600 nm
2
1
0.5
0.1
0.05
0.01
2
4
6
8
10
12
14
16
18
20
22
TIME (h)

Fig 6b. The time course of esterase-lipase production from *Lactobacillus casei*-subsp-*casei* LLG. Strain LLG was grown in 300ml of MRS broth. Samples are taken at 2h intervals and assayed for cell mass (■), cell protein (+), enzyme activity by titration (▲), enzyme activity by chromogenic synthetic substrate (◆)

* Protein was measured by BCA protein assay using BSA standard curve
* Enzyme activity was expressed as milliequivalents of free fatty acid liberated per ml for conventional titration method
* Enzyme activity was defined as the enzyme necessary to release 1 umol of *p*-nitrophenol per min per ml from the p-nitrophenol standard curve

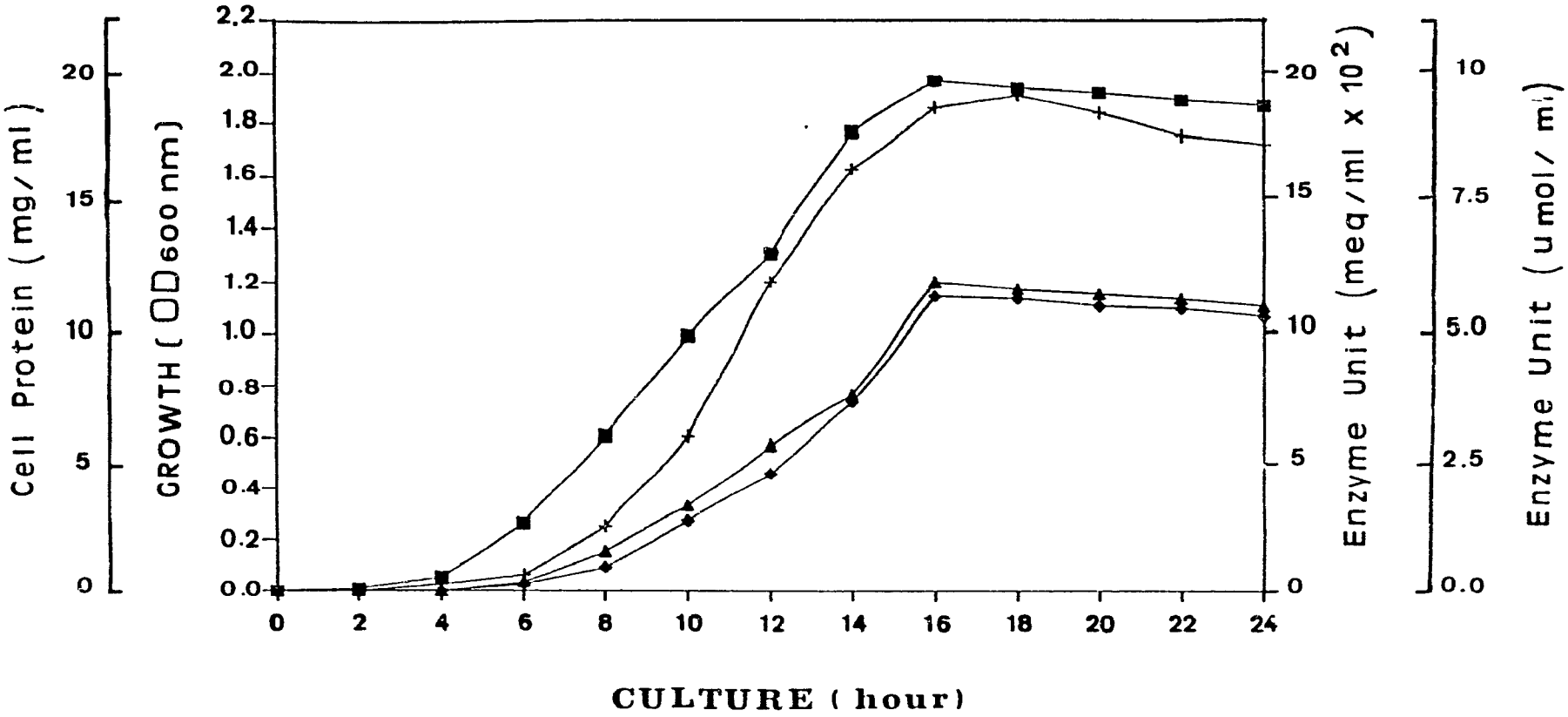

Cell Protein (mg/ml)
GROWTH (OD600 nm)
Enzyme Unit (meq/ml x 10²)
Enzyme Unit (umol/ ml)
CULTURE (hour)
0
2
4
6
8
10
12
14
16
18
20
22
24
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
0
5
10
15
20
0.0
2.5
5.0
7.5
10

Fig 7a. **The effect of enzyme concentration on esterase-lipase activity with 1.5 mM *p*-nitrophenol hydrolysis from *Lactobacillus casei-subsp-casei* LLG**

* Partially purified enzyme extracts containing 20 mg protein/ml added to assay mixture (0-4 mg of protein/0-0.2 ml of cell extract)
* One unit of enzyme is defined as the enzyme necessary to release 1 uMol of p-nitrophenol per min at 37^{o}C from p-nitrophenol standard curve
* Points are the averages of 3 replicates

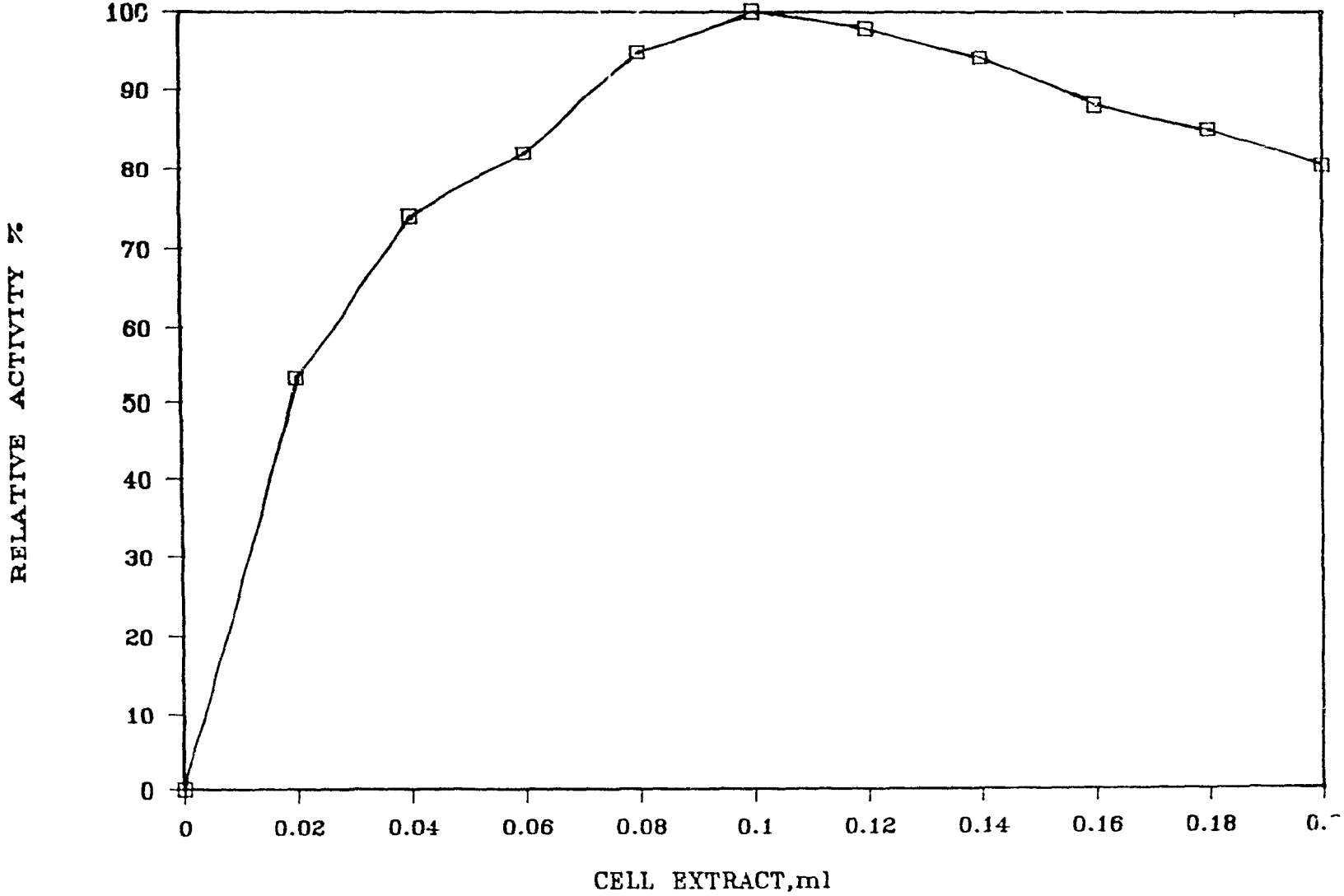

RELATIVE ACTIVITY %
100
90
80
70
60
50
40
30
20
10
0
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
0.18
CELL EXTRACT,ml

Fig 7b. The effect of substrate concentration on esterase-lipase activity from *Lactobacillus casei*-subsp-*casei* LLG

* Partially purified enzyme extracts containing 20 mg protein/ml added to assay mixture
* 0 to 0.2 ml of 1.5 mM-p-nitrophenol added to assay mixture
* One unit of enzyme is defined as the enzyme necessary to release 1 uMol of p-nitrophenol per min at $37^{o}C$ from p-nitrophenol standard curve
* Points are the averages of 3 replicates

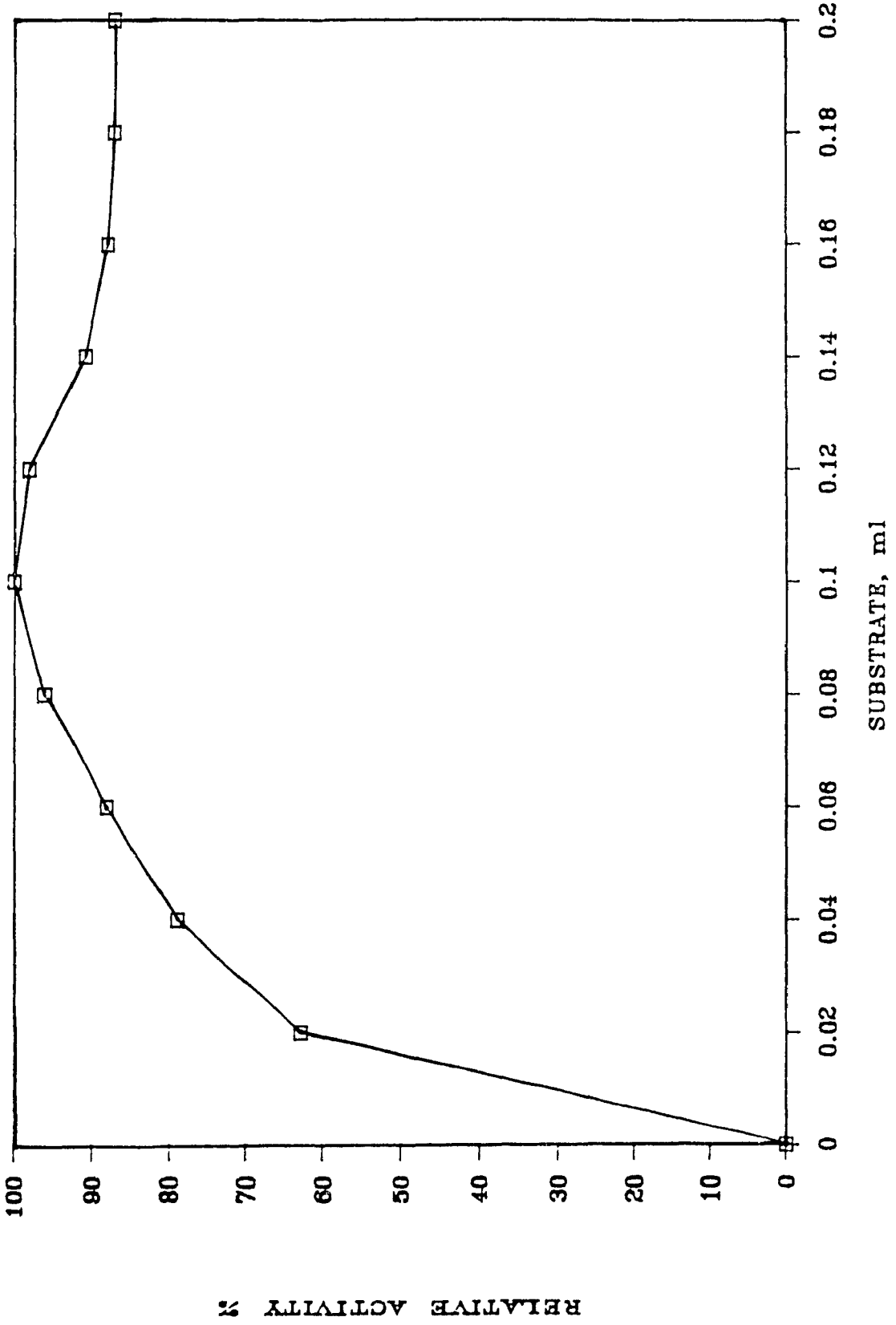

RELATIVE ACTIVITY %
100
90
80
70
60
50
40
30
20
10
0
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
0.18
0.2
SUBSTRATE, ml

Fig 7c. The effect of temperature on esterase-lipase activity from *Lactobacillus casei*-subsp-*casei* LLG

* Partially purified enzyme extracts containing 20 mg protein/ml added to assay mixture
* 1.5 mM p-nitrophenol added to assay mixture
* One unit of enzyme is defined as the enzyme necessary to release 1 uMol of p-nitrophenol per min from p-nitrophenol standard curve
* Points are the averages of 3 replicates

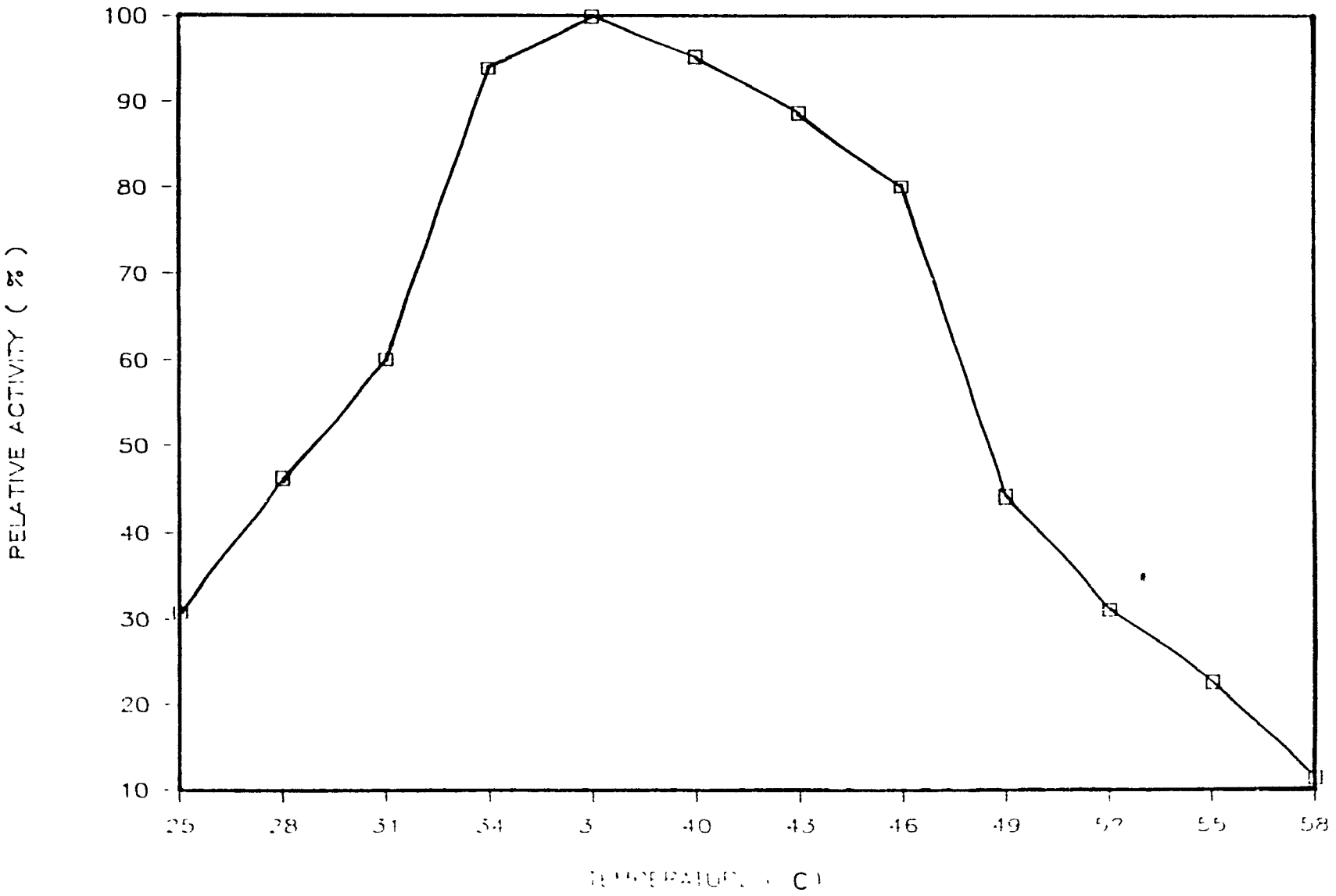

100
90
80
70
60
50
40
30
20
10
RELATIVE ACTIVITY (%)
25
28
31
34
37
40
43
46
49
52
55
58
TEMPERATURE (C)

Fig 7d. The effect of pH on esterase-lipase activity from *Lactobacillus casei*-subsp-*casei* LLG

* Partially purified enzyme extracts containing 20 mg protein/ml added to assay mixture
* 1.5 mM p-nitrophenol added to assay mixture
* One unit of enzyme is defined as the enzyme necessary to release 1 uMol of p-nitrophenol per min at 37^{o}C from p-nitrophenol standard curve
* Points are the averages of 3 replicates

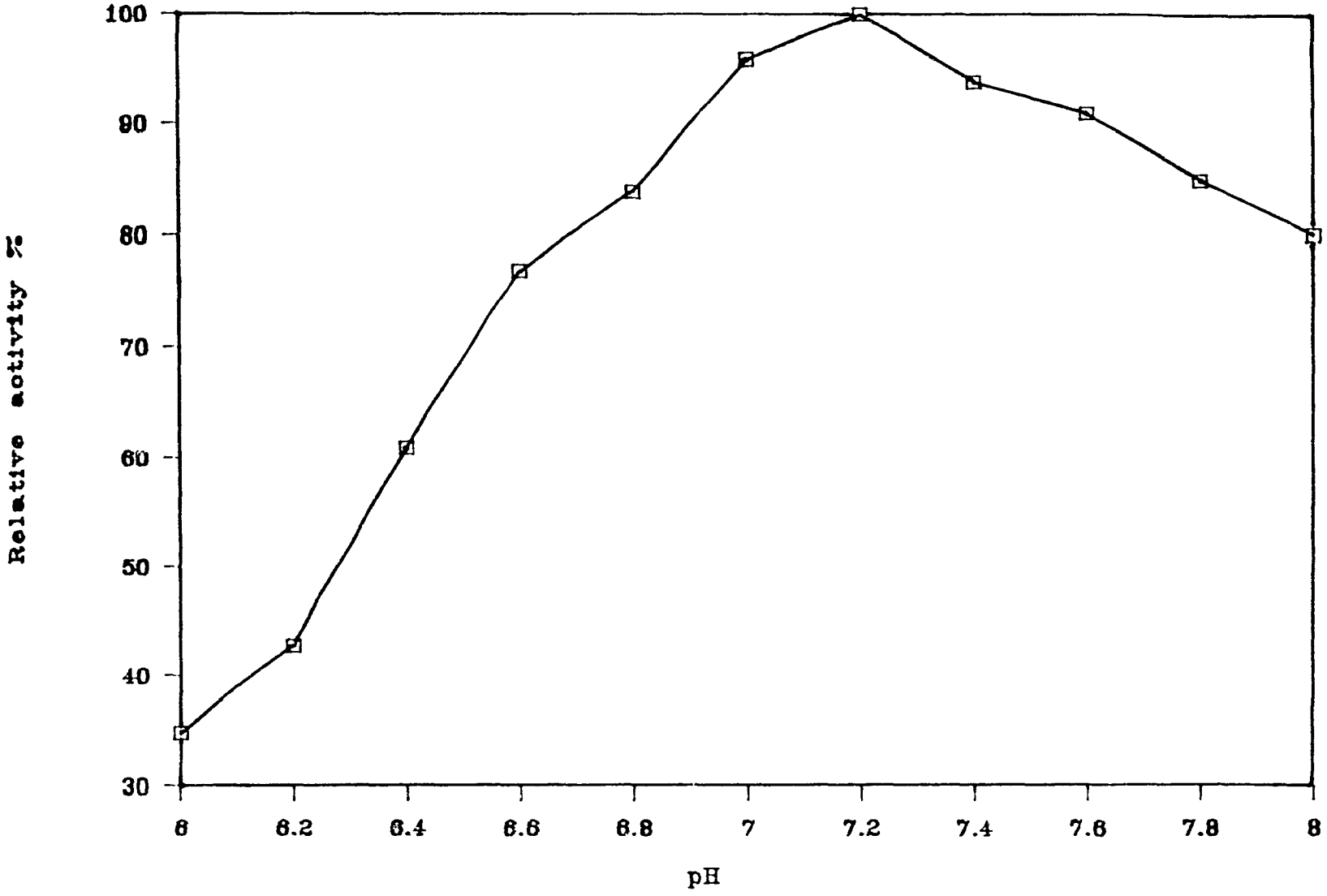

Relative activity %
100
90
80
70
60
50
40
30
6
6.2
6.4
6.6
6.8
7
7.2
7.4
7.6
7.8
8
pH

Table 9. Summary of the procedure for the purification of esterase-lipase of L. casei-subsp-casei LLG

FRACTION	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (UNITS)	SPECIFIC ACTIVITY (UNITS)	YIELD (%)	FOLD PURIFI CATION
1.CRUDE EXTRACT	40.0	1088.0	27.2	100.0	1.0
2.SEPHADEX G-25	39.4	1129.6	28.2	104.0	1.1
3.ION EXCH.					
1	16.1	53.2	3.3	4.9	0.1
2	1.4	766.2	547.3	70.2	19.9
3	2.6	-	-	-	-
4.GEL FILTRATION					
2A	0.5	412.0	824.1	37.7	31.9
2B	0.6	97.2	162.0	8.9	6.0
2C	0.2	157.4	787.1	14.5	29.2
TOTAL				61.1	67.1

* One unit of enzyme is defined as the enzyme necessary to release 1 uMol of p-nitrophenol per min at 37^{o}C from p-nitrophenyl standard curve.
* Specific activity is defined as enzyme units per mg proteins.

Fig 8. The profile of Ion Exchange Chromatograph on a Sepharose CL-6B column with a gradient 1 M sodium chloride in 0.05 M phosphate buffer (pH 7.1)

* Lipolytic activity : . . . . . .

* Elution gradient of 1 M NaCl : —— - —— - ——-

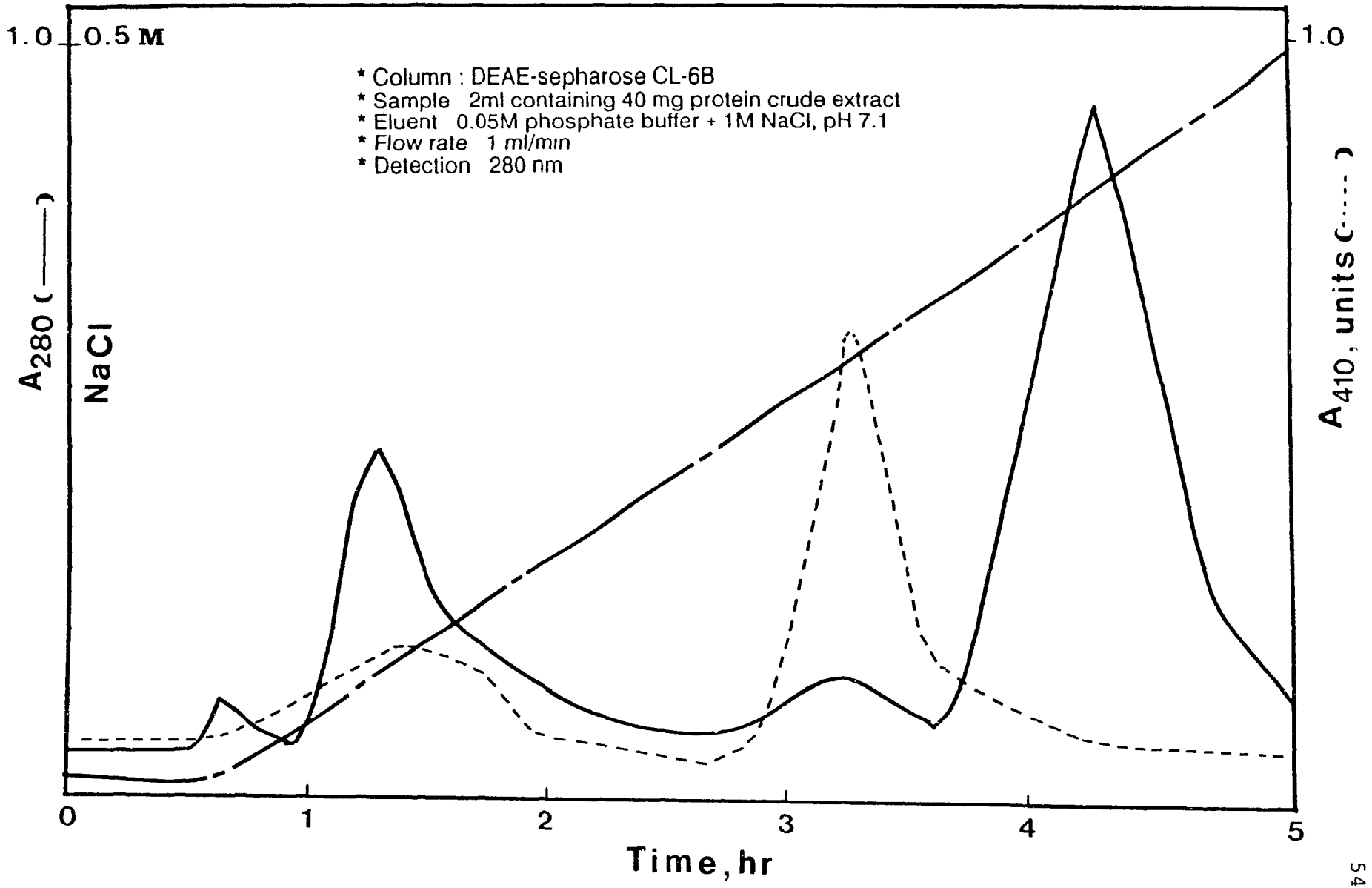

1.0
0.5 M
* Column : DEAE-sepharose CL-6B
* Sample 2ml containing 40 mg protein crude extract
* Eluent 0.05M phosphate buffer + 1M NaCl, pH 7.1
* Flow rate 1 ml/min
* Detection 280 nm
A280 (——)
NaCl
A410, units (----)
1.0
0
1
2
3
4
5
Time, hr

Fig 9. The profile of Gel Filtration Chromatograph on a Superose 12 HR 10/30 column with 0.05 M phosphate buffer (pH 7.1)

* lipolytic activity : ----------

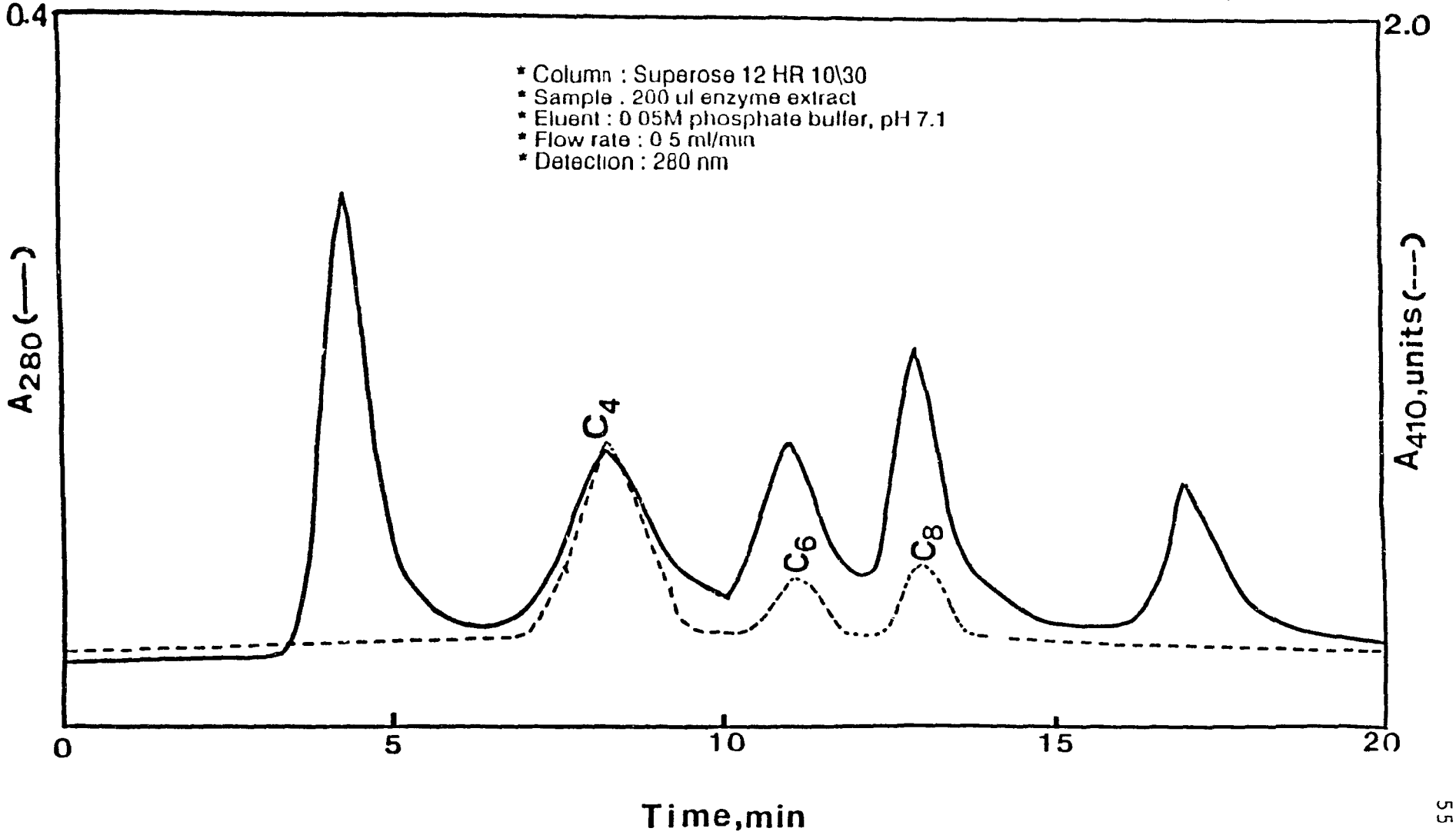

0.4
2.0
* Column : Superose 12 HR 10\30
* Sample . 200 ul enzyme extract
* Eluent : 0 05M phosphate buffer, pH 7.1
* Flow rate : 0 5 ml/min
* Detection : 280 nm
A280 (——)
A410,units (---)
C4
C6
C8
0
5
10
15
20
Time,min

Fig 10. The polyacrylamide gel electrogram of purification steps; 1. High molecular weight standards; 2 & 3. Different concentration of crude extracts; 4. Peak fraction after ion exchange chromatography; 5 & 6 & 7. Each specific peak fraction after gel filtration chromatography

* PhastGel gradient 8-25 polyacrylamide gel was used for native-PAGE and the coomassie staining technique was used for detecting proteins in PhastGel separation media using PhastGel Blue R

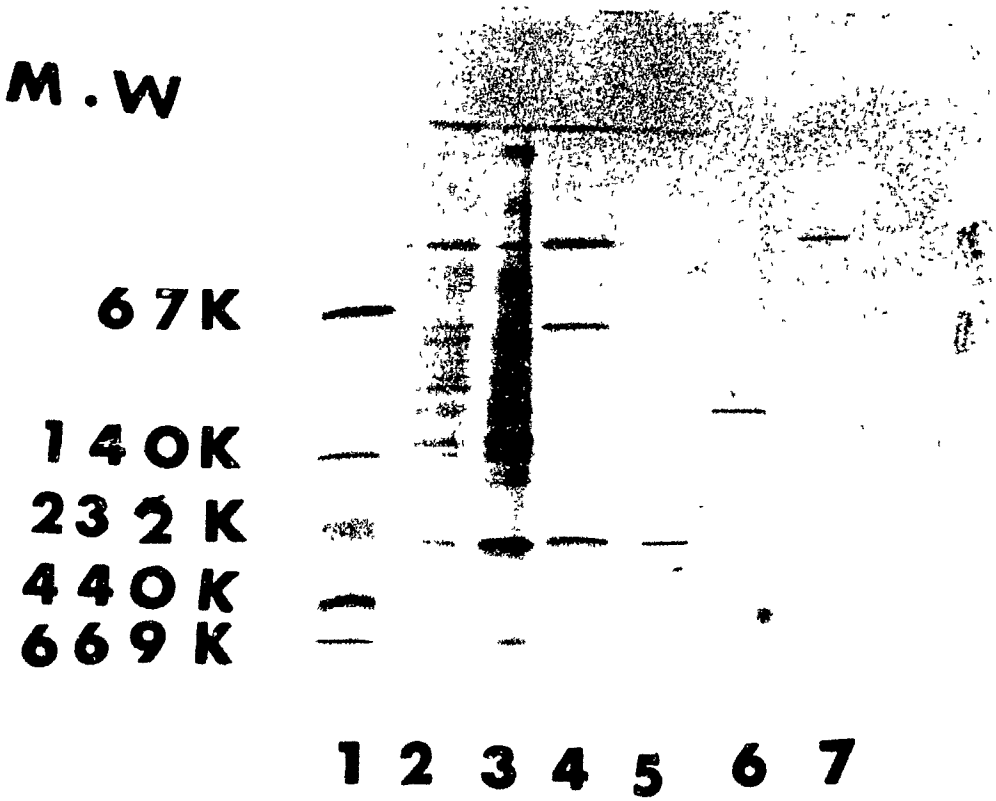

M.W
67K
140K
232K
440K
669K
1 2 3 4 5 6 7

Fig 11. Molecular weight and localization of esterase on SDS-PAGE; 1. Butyrate esterase; 2. Caproate esterase; 3. Capryate esterase

* PhastGel gradient 8-25 polyacrylamide gel was used for SDS-PAGE and the coomassie staining technique was used for detecting proteins in PhastGel separation media using PhastGel Blue R

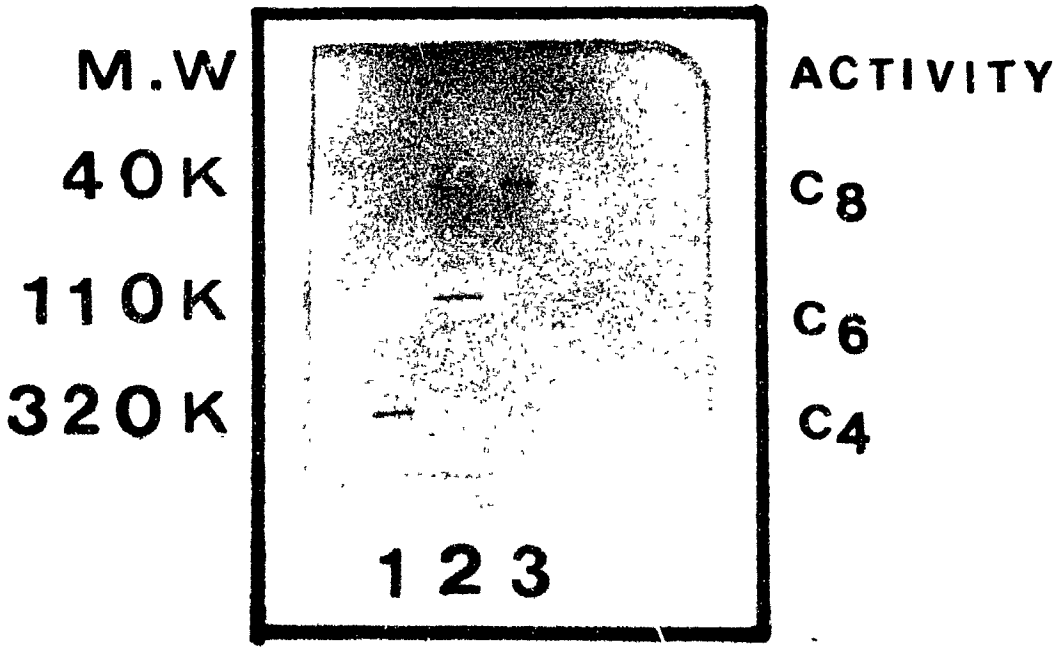

M.W
40K
110K
320K
ACTIVITY
C8
C6
C4
1 2 3

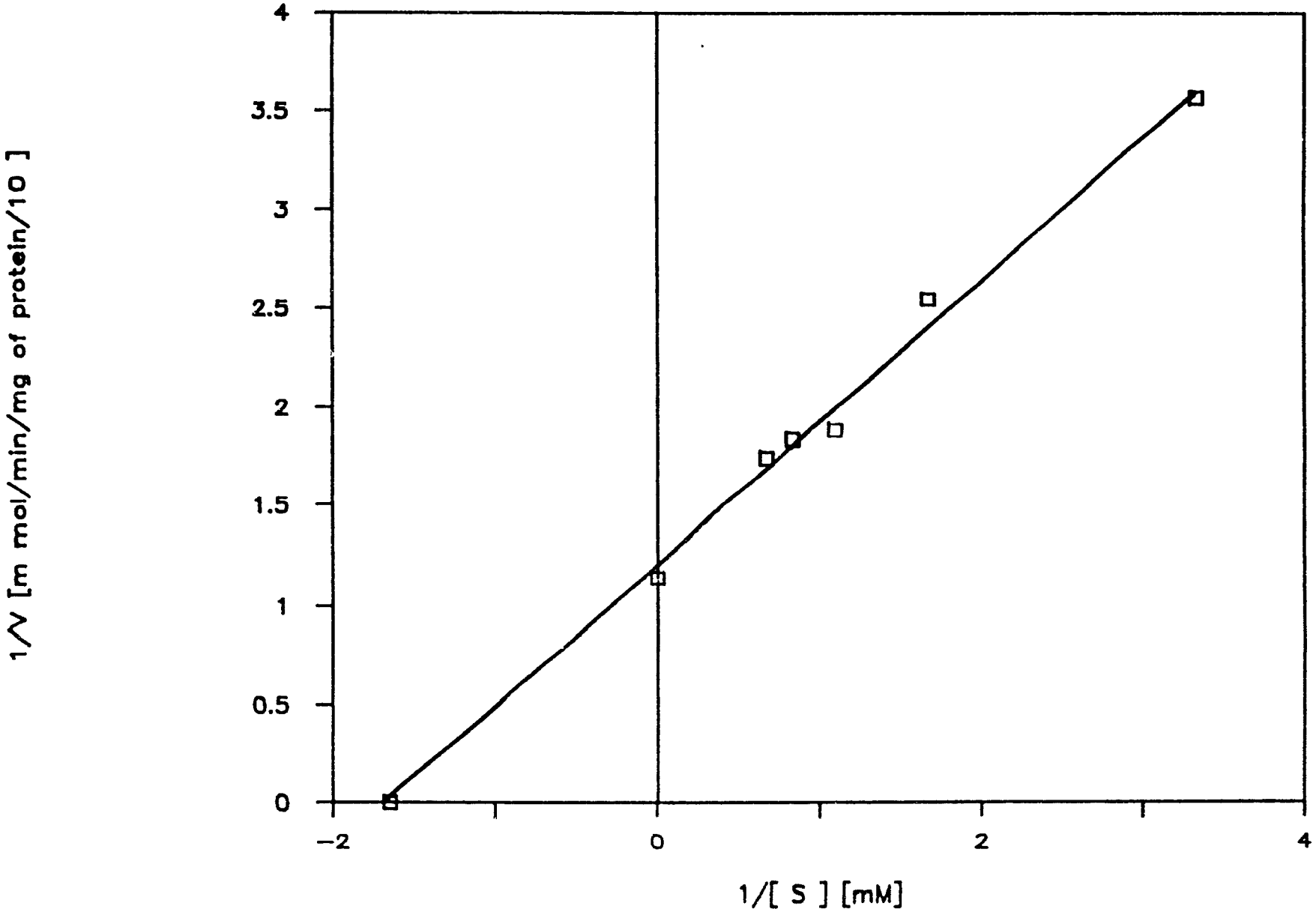
Table 10. Effect of metallic ions and some compounds on the lipolytic activity of L.casei-subsp-casei LLG

Compound	Concentration(mM)	Relative activity(%)
Control	1	100.0
$AgNO_3$	1	15.4
$HgCl_2$	1	41.1
EDTA	1	84.5
$CuSO_4$	1	101.0
$ZnCl_2$	1	102.5
$FeSO_4$	1	108.1
$FeCl_3$	1	111.2
Cysteine	1	118.9
Mercaptoethanol	1	122.0
$MgSO_4$	1	125.2
$CaCl_2$	1	126.3
$CoCl_2$	1	155.9

* Mean of three replicate determinations.

Fig 12. **Double reciprocal plot of esterase velocity versus concentration of *p*-nitrophenyl chromogenic substrate in 0.05 M phosphate buffer (pH 7.1)**

* The apparent Michaelis-Menten constant (Km) and maximum velocity (V_{max}) for the enzyme were calculated from a Lineweaver-Burk plot


1/V [m mol/min/mg of protein/10]
4
3.5
3
2.5
2
1.5
1
0.5
0
−2
0
2
4
1/[S] [mM]

B. Production and chracterization of esterase-lipase from *Lactobacillus casei*-subsp-*pseudoplantarum* LE2

1. Esterase-lipase production during growth of *L. casei-subsp-pseudoplantarum* LE2

A gradual increase occurred in production of the esterase-lipase during growth of *L. casei-subsp-pseudoplantarum* LE2 which was maximum at the late logarithmic phase followed by a slight decrease in the specific activity of lipolytic enzyme in stationary phase (Fig 13a and 13b). To assay cell protein and enzyme activities, the samples were taken at 2h intervals. There was no significant differences in lipase activities between the titration method and the chromogenic substrate method.

2. Properties of esterase-lipase

In order to determine the optimum conditions for esterase-lipase assay, The effects of enzyme concentration, substrate concentration, temperature and pH were studied. Partially purified enzyme extracts containing 18.5 mg protein/ml were added to standard assay mixtures. The effect of different concentration of enzymes on p-nitrophenol hydrolysis in Fig 14a shows that activity increased in proportion to enzyme addition up to 0.08 ml enzyme. The response of the enzyme to increasing concentration of the substrate is shown in Fig 14b. Maximum activities were obtained at 0.1 ml (1.5 mM). The lipolytic activity in the standard assay mixture was maximal at 37^{O}C (Fig 14c) which was used with the standard assay conditions for all further studies. Although there was a sharp decrease in activity after 55^{O}C (Fig 14c), the enzyme was stable at room temperature for at least 2 days. The optimal reaction for esterase-lipase

was near pH 7.1 and the enzyme appeared to be stable above pH 7.0 (Fig 14d).

3. Purification of esterase-lipase

A typical procedure for purifying esterase-lipase from culture supernatants of *L. casei-subsp-pseudoplantarum* LE2 is summarized in Table 11. This table shows that the enzyme was purified approximately 54 fold with a yield of 65 % from the crude cell extract. The repeated cycles gave well reproducible results. The protein fractions collected from Mono Q HR column were resolved into various peaks (Fig 15), but lipolytic activity was present in only the second and third major peaks. It appears that esterase-lipase represents a low proportion of the proteins present in the crude preparation, but the specific activity of this enzyme was quite high. When the two major active fractions were further subjected to gel filtration column, the proteins were resolved into three major peaks (Fig 16). The enzyme activity was present in two major peaks, the most important one being specific for butyrate (C_4), and the others for caproate (C_6) and capryate (C_8), respectively. However second major peak showed mixed caproate (C_6) and capryate (C_8) esterase activities. This fraction was further applied to a Mono Q HR 5/5 column and eluted with an NaCl gradient. Unlike the first eluate, this eluate gave the better resolution (Fig 17). PAGE and SDS-PAGE using the "Phast" system of the purified enzyme fraction obtained showed a single protein band (Fig 18 and 19) with M.W 320 K Dalton for butyrate-esterase, 110 K Dalton for caproate esterase, and 40 K Dalton for the capryate esterase, respectively.

4. Effect of metallic ions and some compounds on the lipolytic activity

Quantitative assessment of lipolytic activity effect was carried out by

addition of metallic ions or some compounds in final concentration of 1 mM to enzyme assay mixture and incubated for 30 min. Enzyme activity was assayed under the standard conditions. The enzyme was inhibited by metallic ions such as Ag^{2+} and Hg^{2+}, while Mg^{2+}, Ca^{2+}, Co^{2+} and mercaptoethanol stimulated enzyme activity (Table 12).

5. Effect of substrate concentration for enzyme kinetics

The kinetics of the release of p-nitrophenyl derivative of fatty acids were studied on this partially purified enzyme. Incremental substrate concentration from 0.3 to 3.5 mM in 0.05 M phosphate buffer were incubated in 37^{o}C for 30 min as described in section of Materials and Methods. Fig 20 shows the reaction velocity expressed as moles of fatty acids liberated per min plotted against substrate concentration. The Km value was obtained according to a Lineweaver-Burke plot. The apparent Michaelies-Menten constant (Km) and maximum velocity (V_{max}) for this enzyme were calculated to be about 90 umol/min/mg and 1.2 mM, respectively.

Fig 13a. Growth curve of *Lactobacillus casei*-subsp-*pseudoplantarum* LE2 using the semilogarithmic drawing paper

* Strain LE2 was grown in 300 ml of MRS broth. Cell growth was measured by optical density at 600 nm

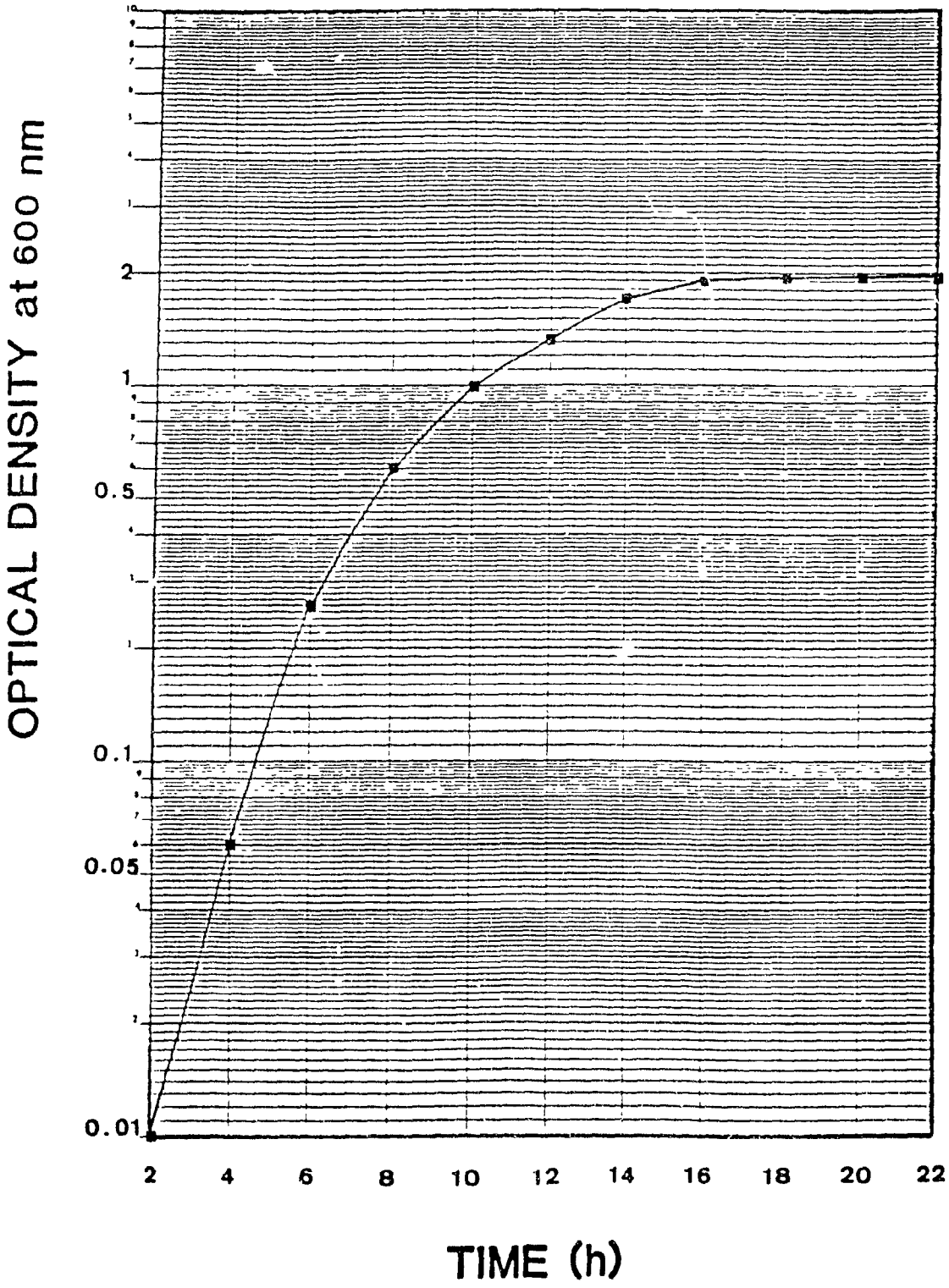

OPTICAL DENSITY at 600 nm
2
1
0.5
0.1
0.05
0.01
2
4
6
8
10
12
14
16
18
20
22
TIME (h)

Fig 13b. The time course of esterase-lipase production from *Lactobacillus casei*-**subsp**-*pseudoplantarum* **LE2. Strain LE2 was grown in 300 ml of MRS broth. Samples are taken at 2h intervals and assayed for cell mass (■), cell protein (+), enzyme activity by titration (▲), enzyme activity by chromogenic synthetic substrate (◆)**

* Protein was measured by BCA protein assay using BSA standard curve
* Enzyme activity was expressed as milliequivalents of free fatty acid liberated per ml for conventional titration method
* Enzyme activity was defined as the enzyme necessary to release 1 umole of *p*-nitrophenol per min per ml from the *p*-nitrophenol standard curve

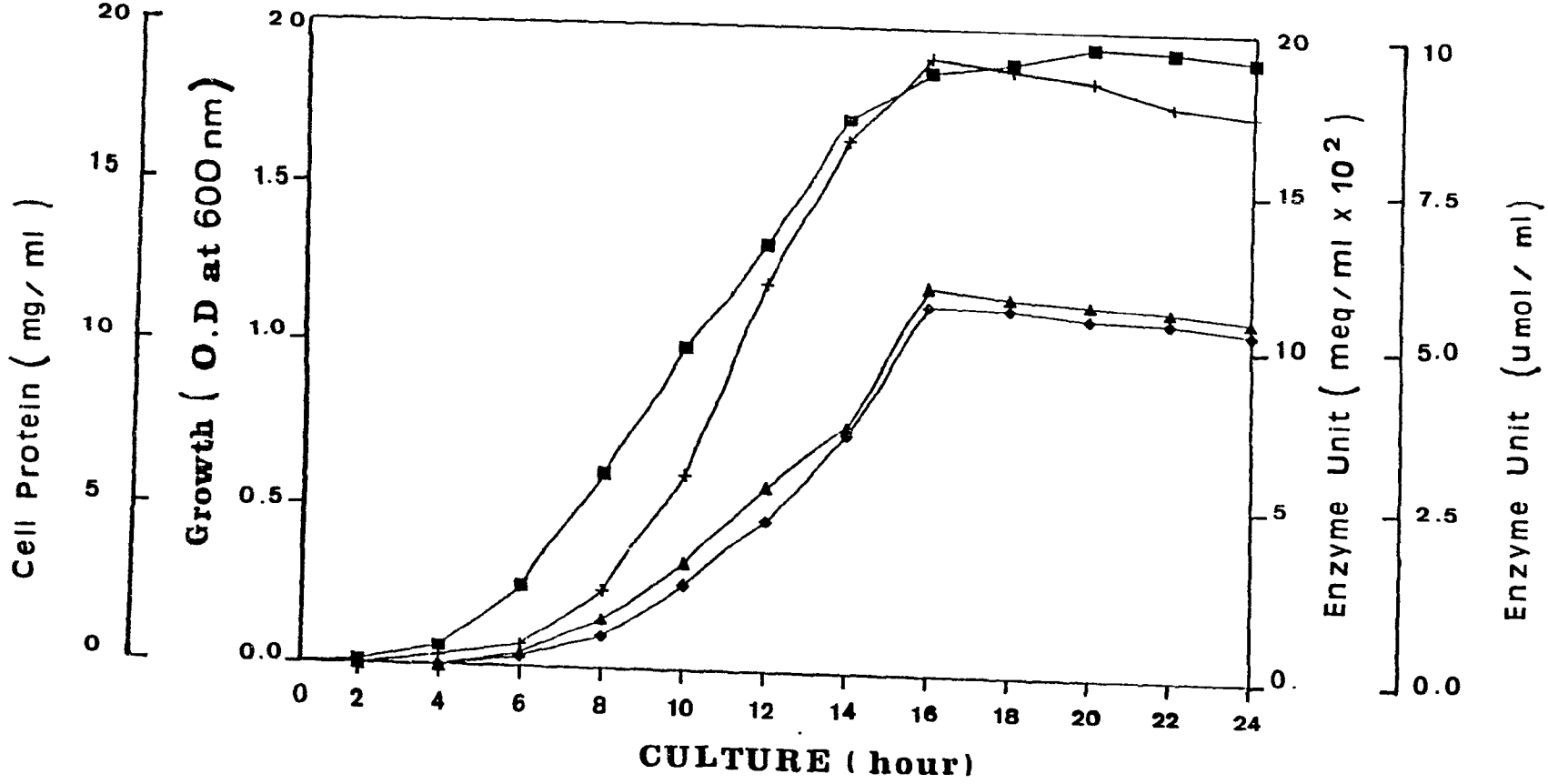

Cell Protein (mg / ml)
Growth (O.D at 600 nm)
Enzyme Unit (meq / ml x 10 2)
Enzyme Unit (umol / ml)
CULTURE (hour)
0
2
4
6
8
10
12
14
16
18
20
22
24
0.0
0.5
1.0
1.5
2.0
0
5
10
15
20
0.0
2.5
5.0
7.5
10

Fig 14a. The effect of enzyme concentration on esterase-lipase activity with 1.5 mM *p*-nitrophenol hydrolysis from *Lactobacillus casei*-subsp-*pseudoplantarum* LE2

* Partially purified enzyme extracts containing 18.5 mg protein/ml added to assay mixture (0-3.7 mg of protein/ 0-0.2 ml of cell extract)

* One unit of enzyme is defined as the enzyme necessary to release 1 uMol of p-nitrophenol per min at $37^{O}C$ from p-nitrophenol standard curve

* Points are the averages of 3 replicates

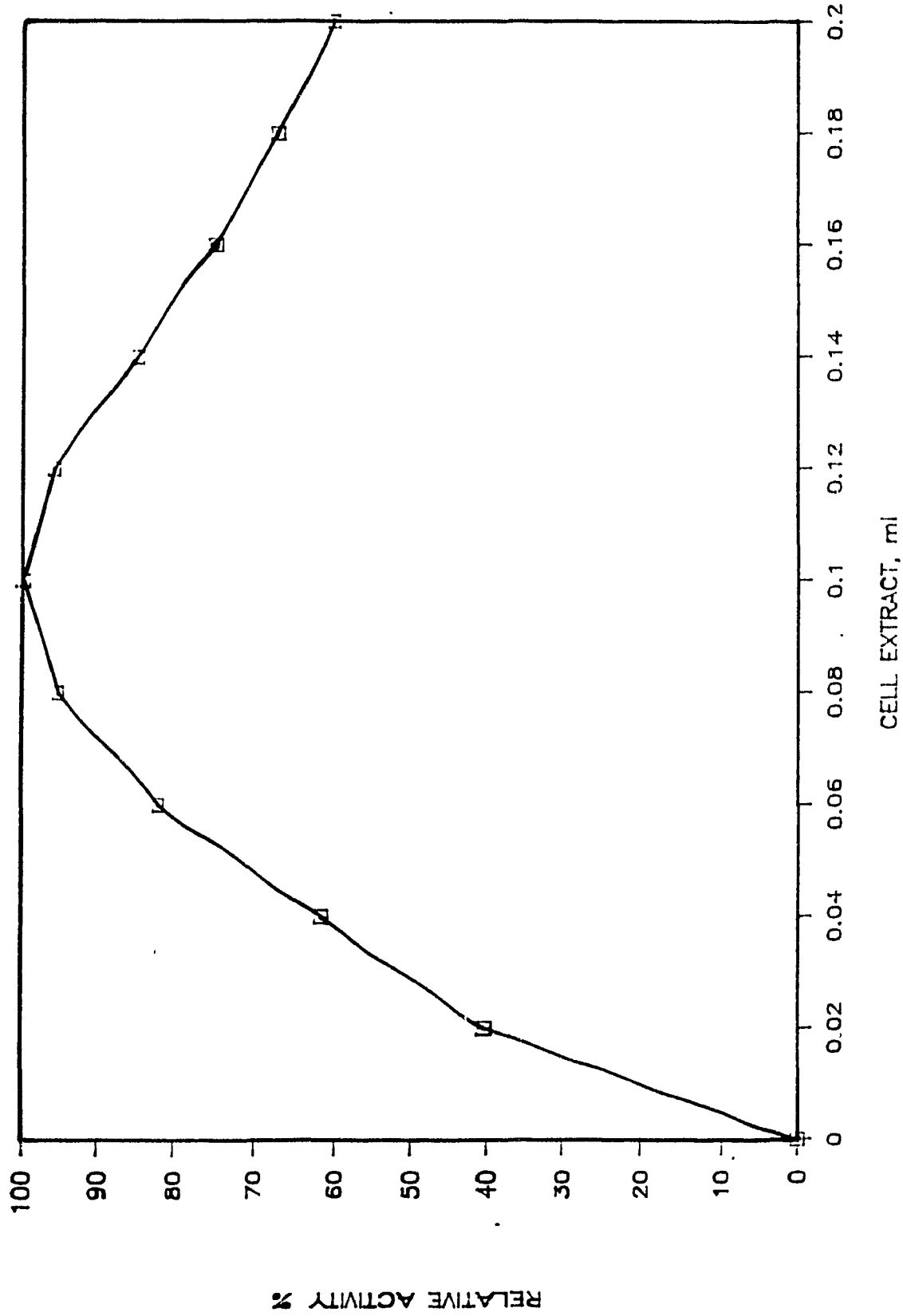

RELATIVE ACTIVITY %
100
90
80
70
60
50
40
30
20
10
0
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
0.18
0.2
CELL EXTRACT, ml

Fig 14b. The effect of substrate concentration on esterase-lipase activity from *Lactobacillus casei*-subsp-*pseudoplantarum* LE2

* Partially purified enzyme extracts containing 18.5 mg protein/ml added to assay mixture

* 0 to 0.2 ml of 1.5 mM-p-nitrophenyl substrates added to assay mixture

* One unit of enzyme is defined as the enzyme necessary to release 1 uMol of p-nitrophenol per min at 37^{O}C from p-nitrophenol standard curve

* Points are the averages of 3 replicates

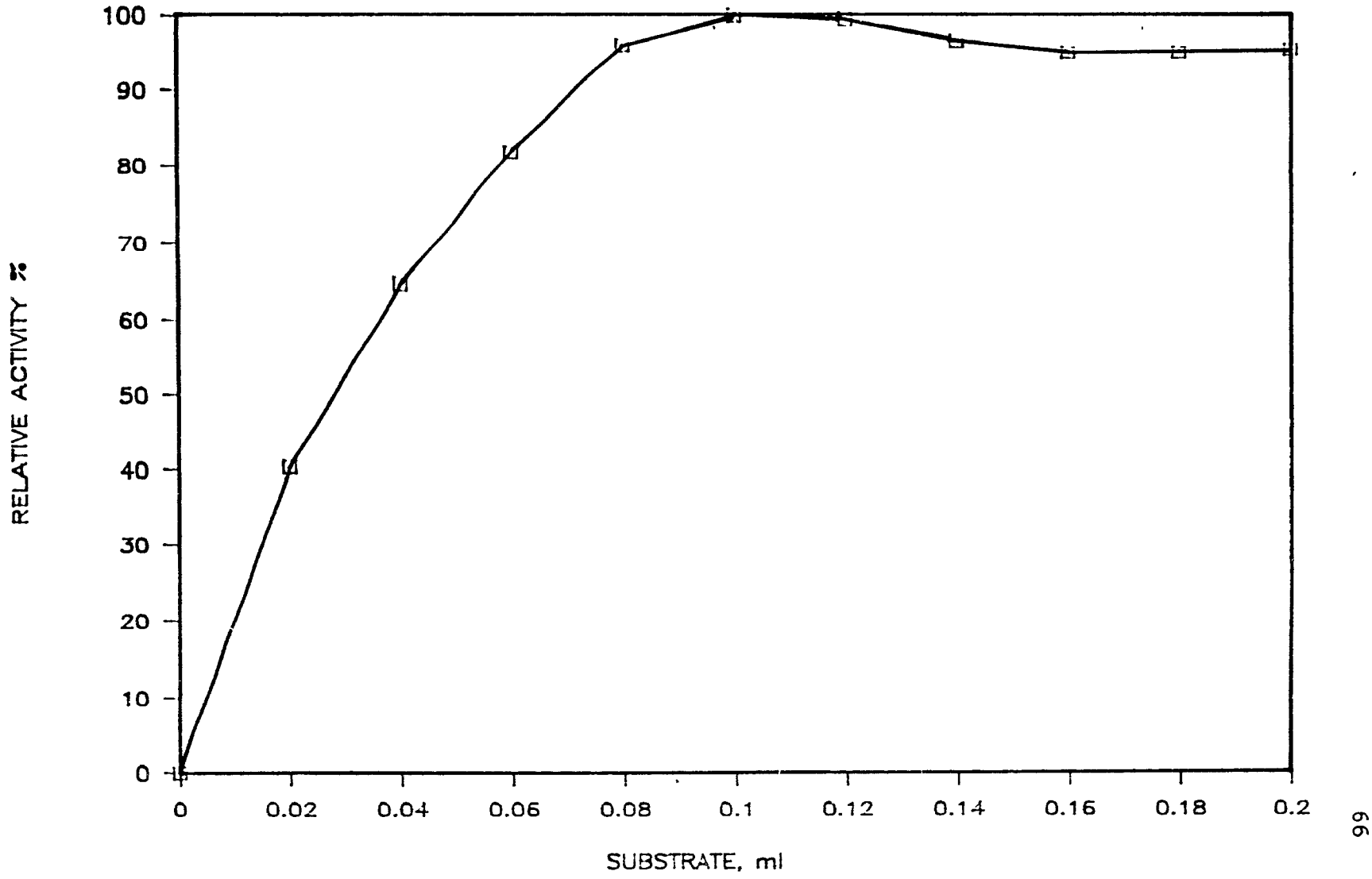

RELATIVE ACTIVITY %
100
90
80
70
60
50
40
30
20
10
0
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
0.18
0.2
SUBSTRATE, ml

Fig 14c. The effect of temperature on esterase-lipase activity from *Lactobacillus casei*-subsp-*pseudoplantarum* LE2

* Partially purified enzyme extracts containing 18.5 mg protein/ml added to assay mixture

* 1.5 mM of p-nitrophenyl substrates added to assay mixture

* One unit of enzyme is defined as the enzyme necessary to release 1 uMol of p-nitrophenol per min at from p-nitrophenol standard curve

* Points are the averages of 3 replicates

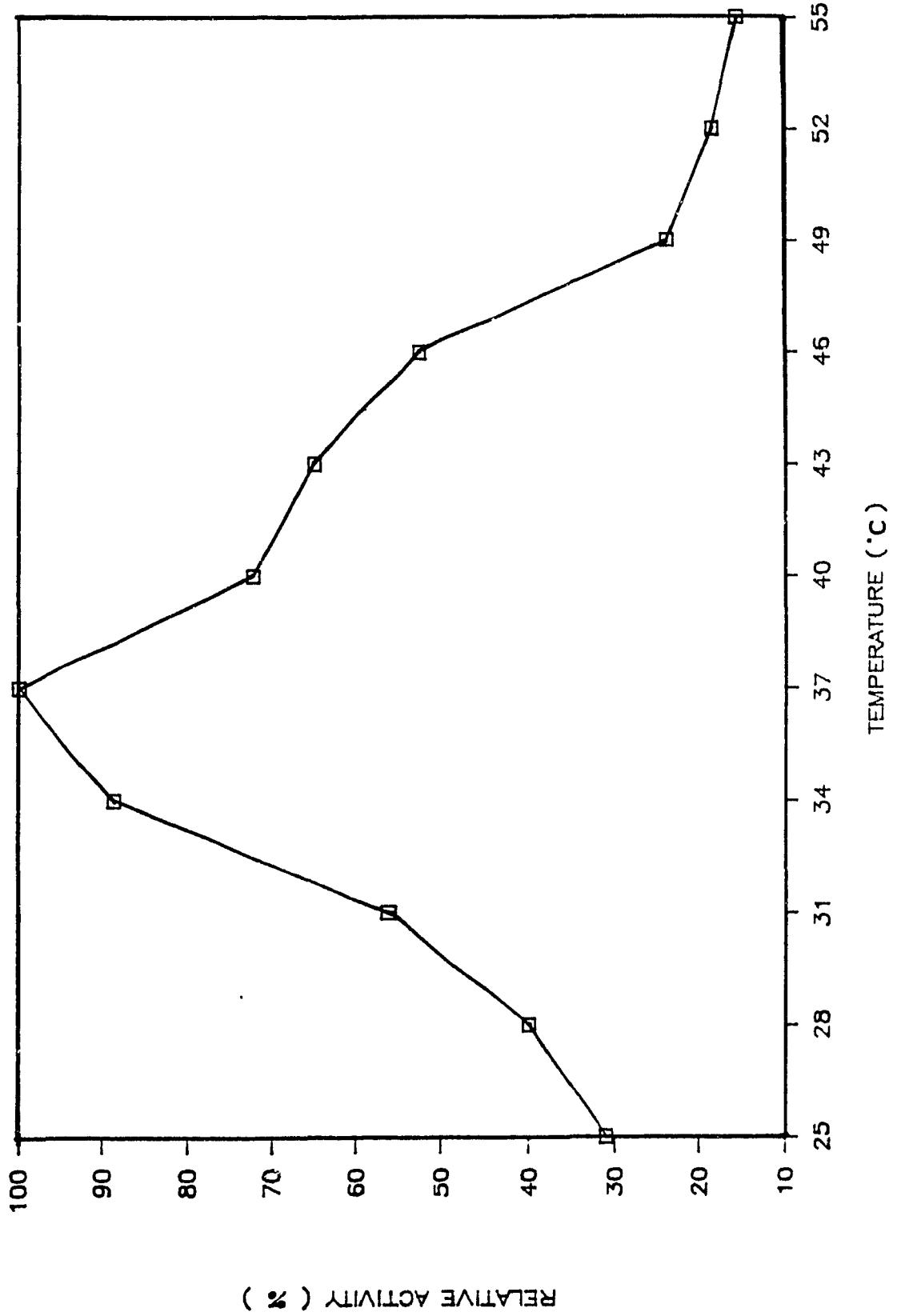

RELATIVE ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
25
28
31
34
37
40
43
46
49
52
55
TEMPERATURE (°C)

Fig 14d. The effect of pH on esterase-lipase activity from *Lactobacillus casei*-subsp-*pseudoplantarum* LE2

* Partially purified enzyme extracts containing 18.5 mg protein/ml added to assay mixture

* 1.5 mM of p-nitrophenyl substrates added to assay mixture

* One unit of enzyme is defined as the enzyme necessary to release 1 uMol of p-nitrophenol per min at 37^{O}C from p-nitrophenol standard curve

* Points are the averages of 3 replicates

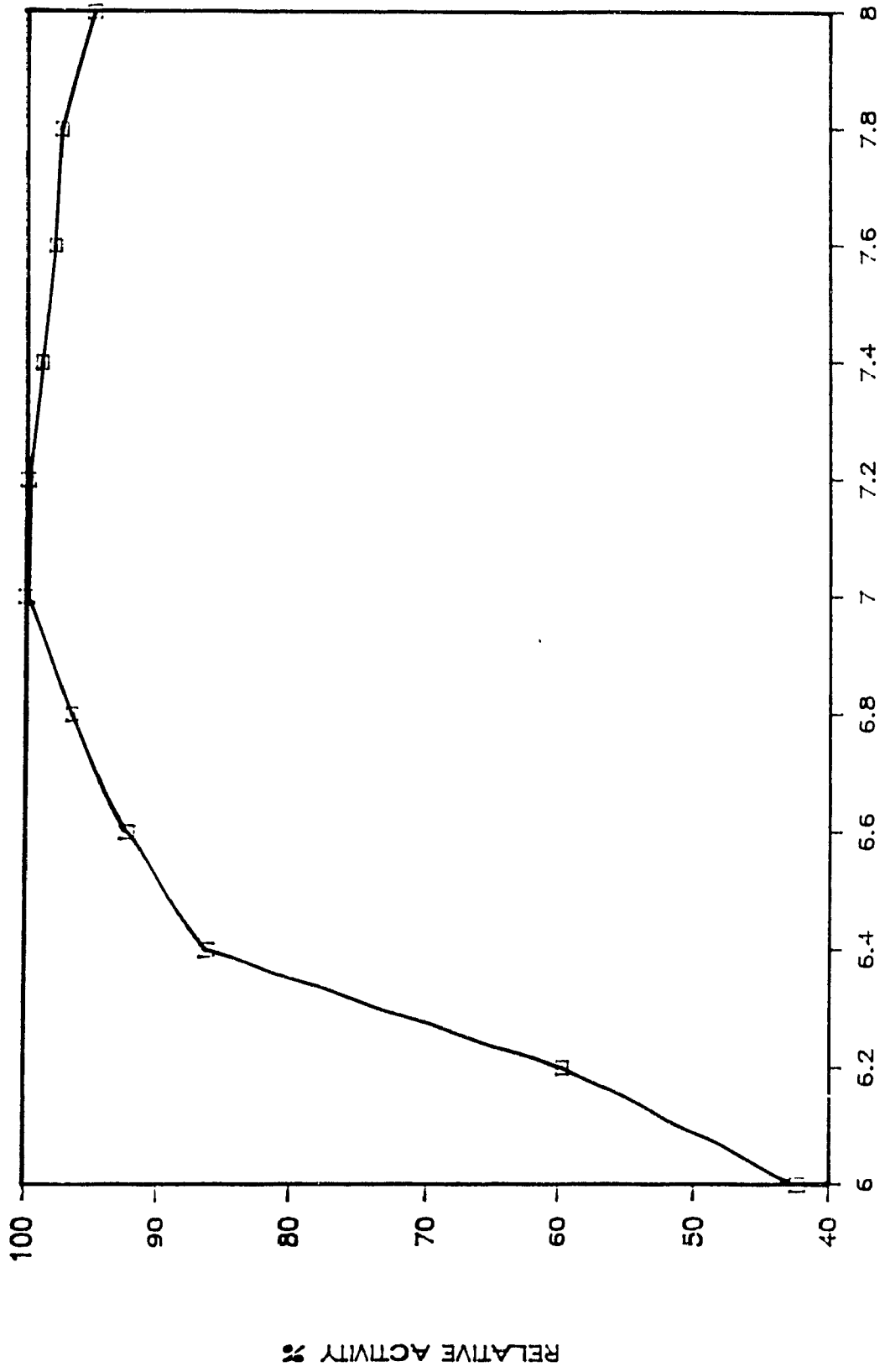

RELATIVE ACTIVITY %
100
90
80
70
60
50
40
6
6.2
6.4
6.6
6.8
7
7.2
7.4
7.6
7.8
8
pH

Table 11. Summary of the procedure for the purification of esterase-lipase of L.casei-subsp-pseudoplantarum LE2

FRACTION	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (UNITS)	SPECIFIC ACTIVITY (UNITS)	YIELD (%)	FOLD PURIFICATION
1.CRUDE EXTRACT	26.43	689.81	26.10	100.00	1.00
2.SEPHADEX G-25	25.96	726.85	27.99	105.37	1.07
3.ION EXCH.					
1	8.42	-	-	-	-
2	3.20	620.37	193.87	89.87	7.41
3	6.47	-	-	-	-
4.GEL FILTRATION					
2A	0.30	171.30	570.98	24.83	21.83
2B	0.45	122.69	272.63	17.79	10.42
2C	0.47	97.22	206.86	14.09	7.91
2D	0.90	39.35	43.73	5.70	1.67
5.ION EXCH. 3=2B+2C					
3A	0.29	136.57	470.94	19.80	18.00
3B	0.31	118.06	380.82	17.11	14.56
TOTAL 2A+3A+3B				61.74	54.39

* One unit of enzyme is defined as the enzyme necessary to release 1 uMol of p-nitrophenol per min at 37°C from p-nitrophenol standard curve.
* Specific activity is defined as enzyme units per mg proteins.

Fig 15. The profile of Ion Exchange Chromatograph on a Mono Q HR 5/5 column with a gradient 1 M sodium chloride in 0.05M phosphate buffer (pH 7.1)

* Lipolytic activity : - - - - - - - - -
* Elution gradient of 1 M NaCl : ——— – ——— – ———

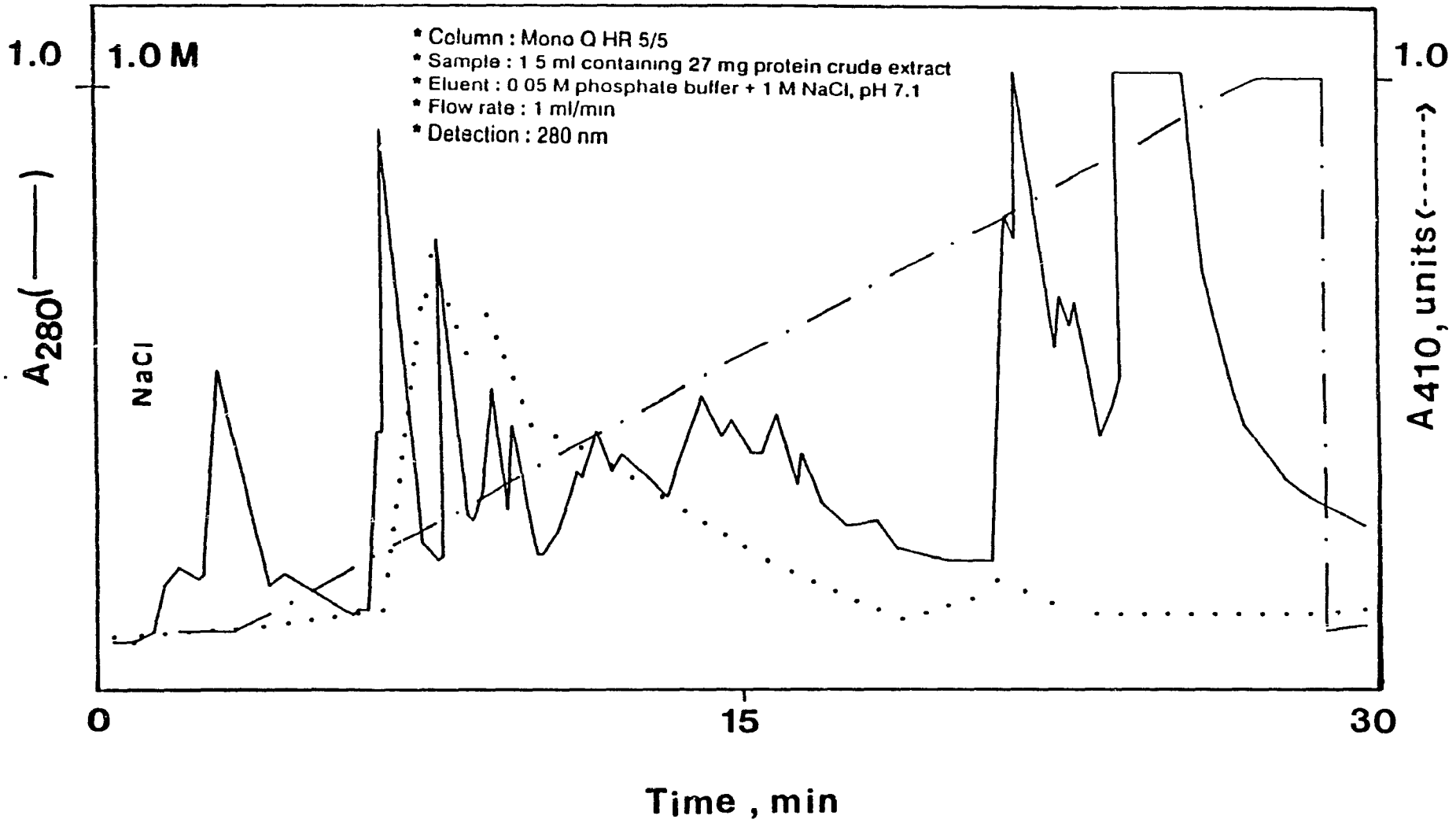

* Column : Mono Q HR 5/5
* Sample : 1 5 ml containing 27 mg protein crude extract
* Eluent : 0 05 M phosphate buffer + 1 M NaCl, pH 7.1
* Flow rate : 1 ml/min
* Detection : 280 nm
1.0
1.0 M
NaCl
A280 (——)
1.0
A410, units (------)
0
15
30
Time , min

Fig 16. The profile of Gel Filtration Chromatograph on a Superose 12 HR 10/30 column with 0.05 M phosphate buffer (pH 7.1)

* lipolytic activity : - - - - - - - - - -

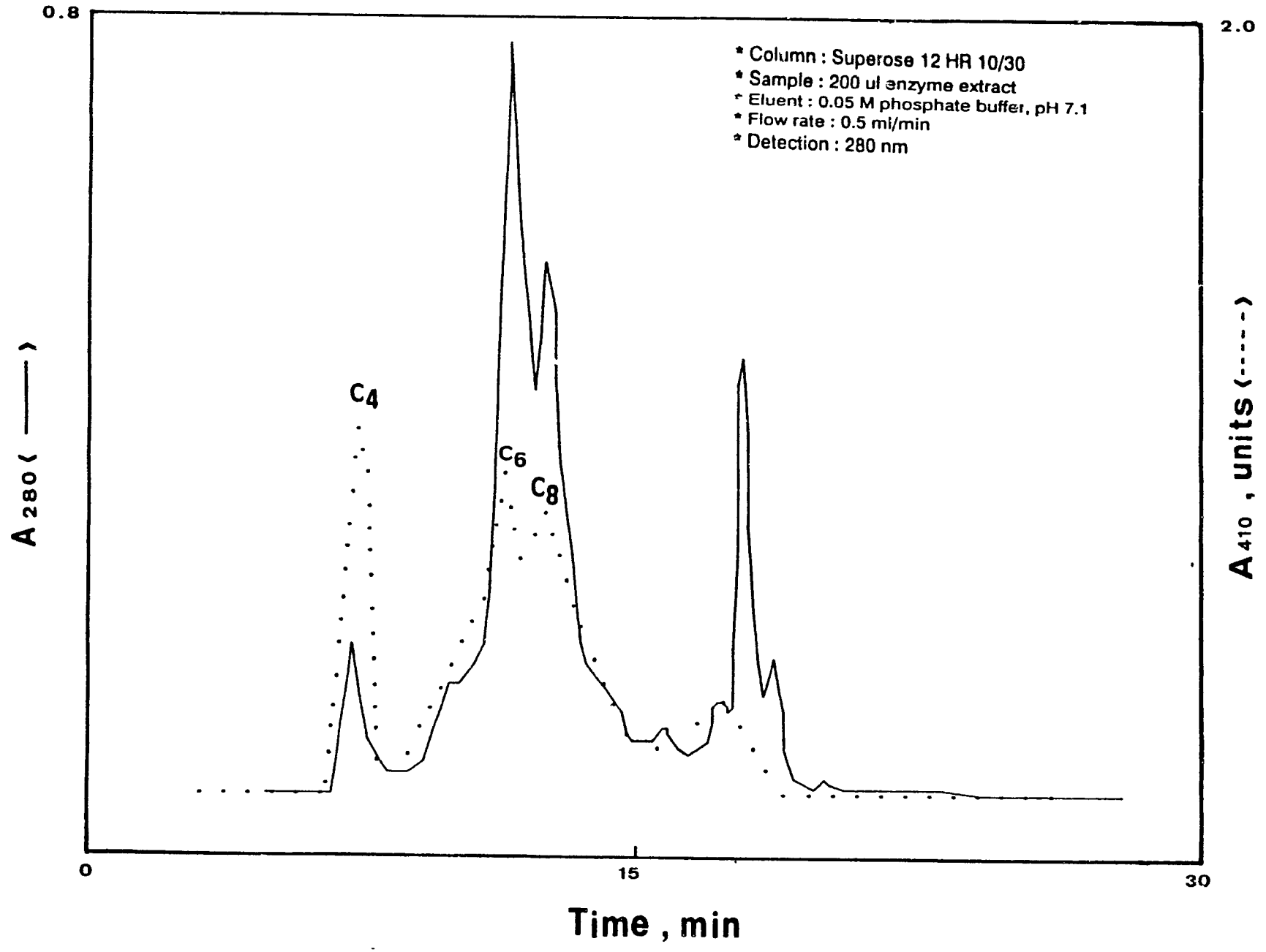

0.8
2.0
* Column : Superose 12 HR 10/30
* Sample : 200 ul enzyme extract
* Eluent : 0.05 M phosphate buffer, pH 7.1
* Flow rate : 0.5 ml/min
* Detection : 280 nm
C4
C6
C8
A280 (——)
A410 , units (- - - -)
0
15
30
Time , min

Fig 17. The profile of Re-Ion Exchange Chromatography for separation caproate activity and capryate activity on a Mono Q HR 5/5 column with a gradient 1 M sodium chloride in 0.05 M phosphate buffer at pH 7.1

* Lipolytic activity : - - - - - - - - - - -
* Elution gradient of 1 M NaCl : —— - ——— - ——— -

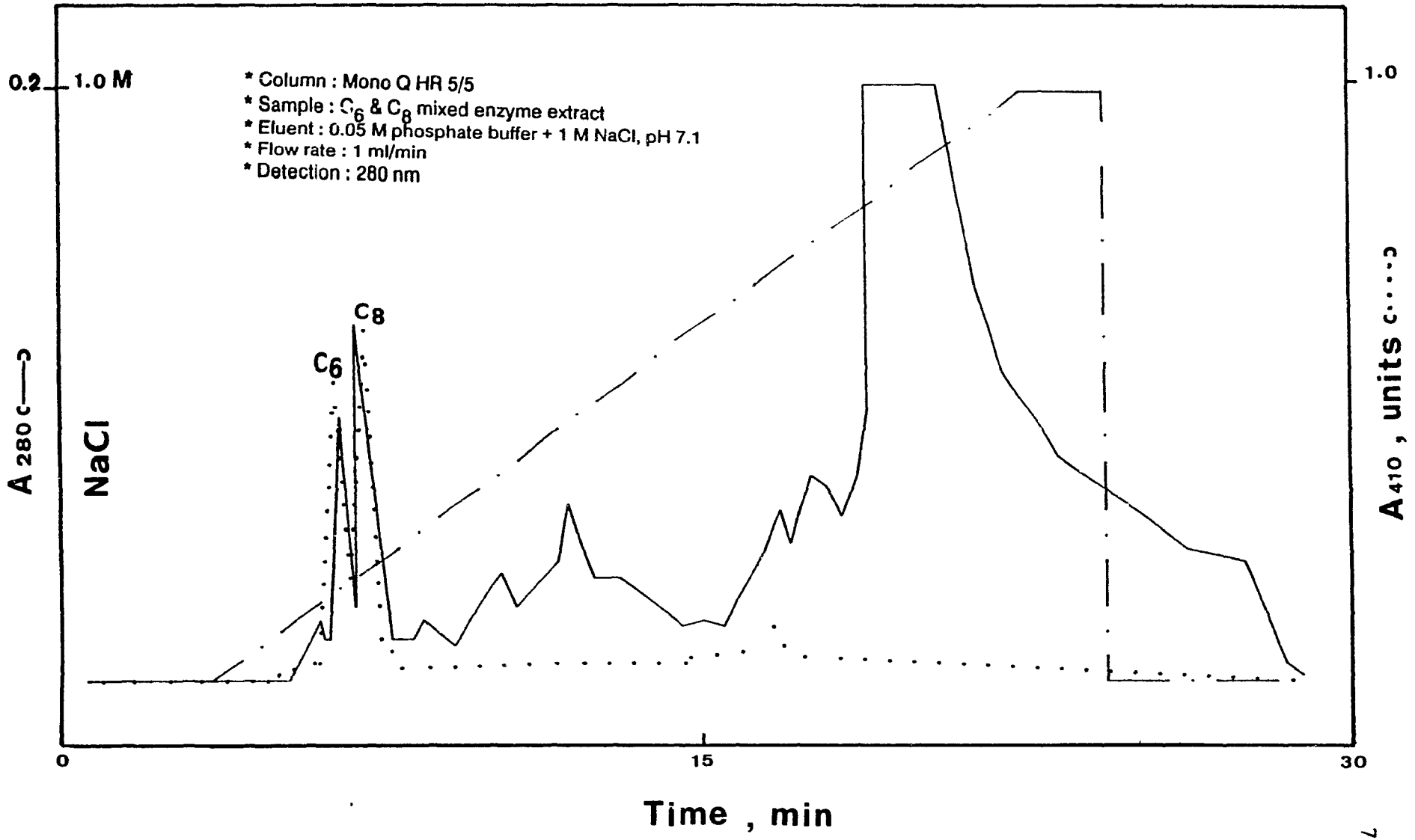

0.2
1.0 M
* Column : Mono Q HR 5/5
* Sample : C_6 & C_8 mixed enzyme extract
* Eluent : 0.05 M phosphate buffer + 1 M NaCl, pH 7.1
* Flow rate : 1 ml/min
* Detection : 280 nm
C_8
C_6
NaCl
A_{280}
1.0
A_{410}, units
0
15
30
Time , min

Fig 18. The polyacrylamide gel electrogram of purification steps; 1. High molecular weight standards; 2. Peak fraction after ion exchange chromatography; 3 & 4 & 5. Peak fraction after gel filtration chromatography

* PhastGel gradient 8-25 polyacrylamide gel was used for native-PAGE and the coomassie staining technique was used for detecting proteins in PhastGel separation media using PhastGel Blue R

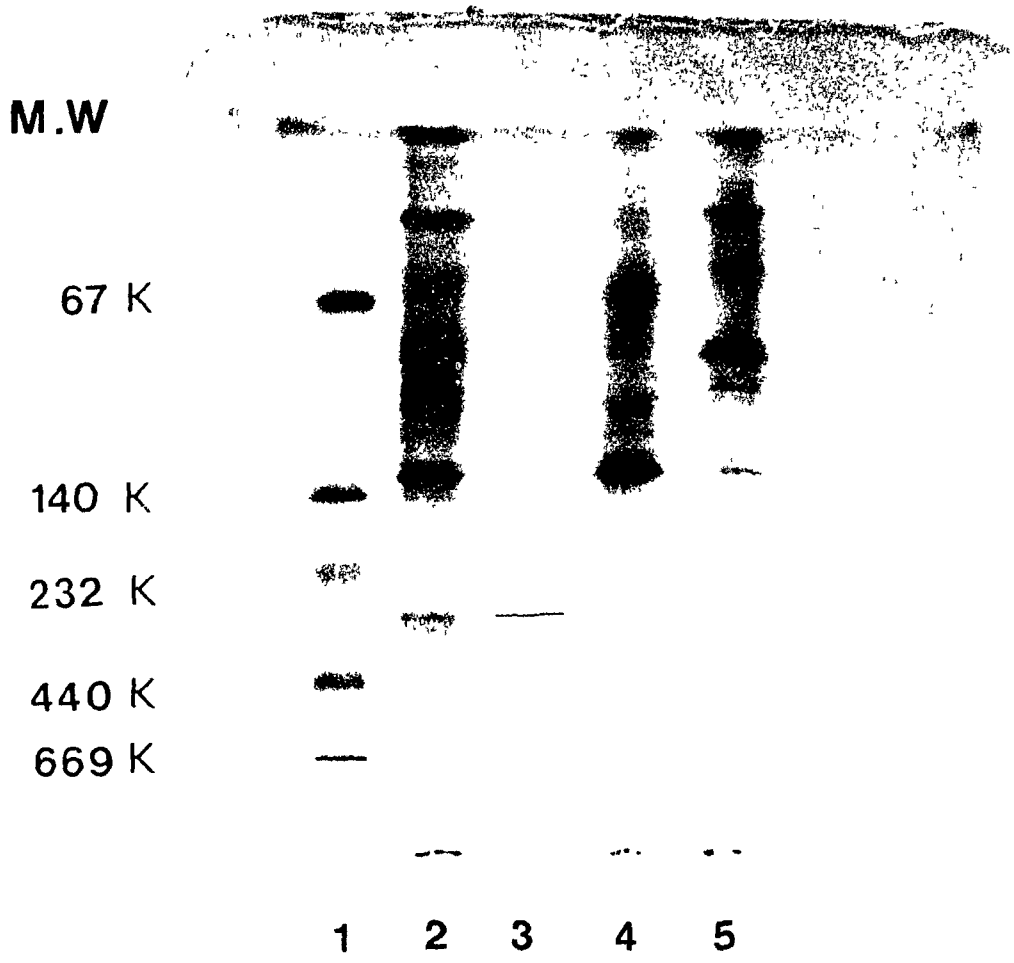

M.W
67 K
140 K
232 K
440 K
669 K
1
2
3
4
5

Fig 19. Molecular weight and localization of esterase on SDS-PAGE;

1.Butyrate esterase; 2. Caproate esterase; 3. Capryate esterase

* PhastGel gradient 8-25 polyacrylamide gel was used for SDS-PAGE and the coomassie staining technique was used for detecting proteins in PhastGel separation media using PhastGel Blue R

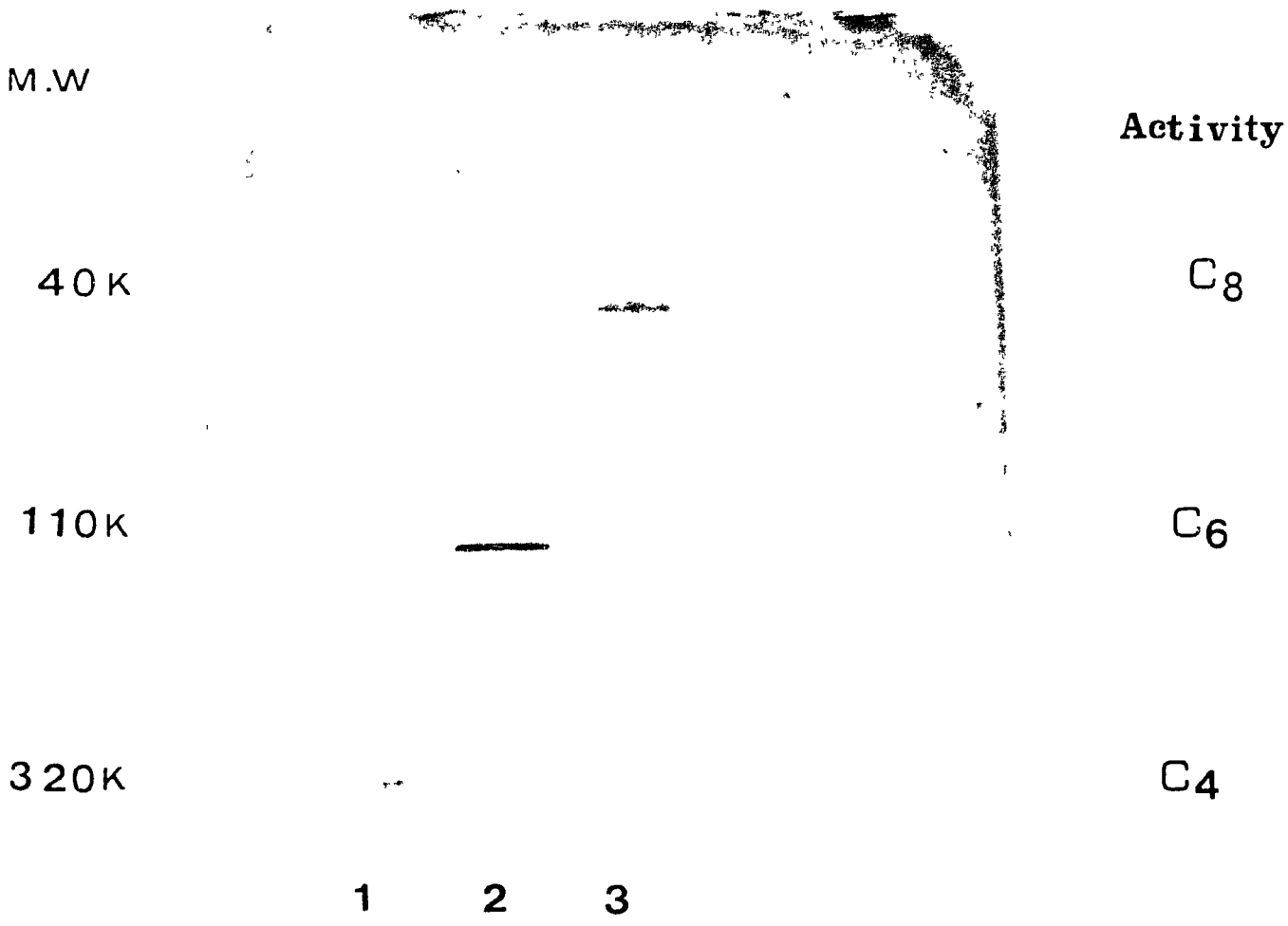

M.W
Activity
40K
C8
110K
C6
320K
C4
1
2
3

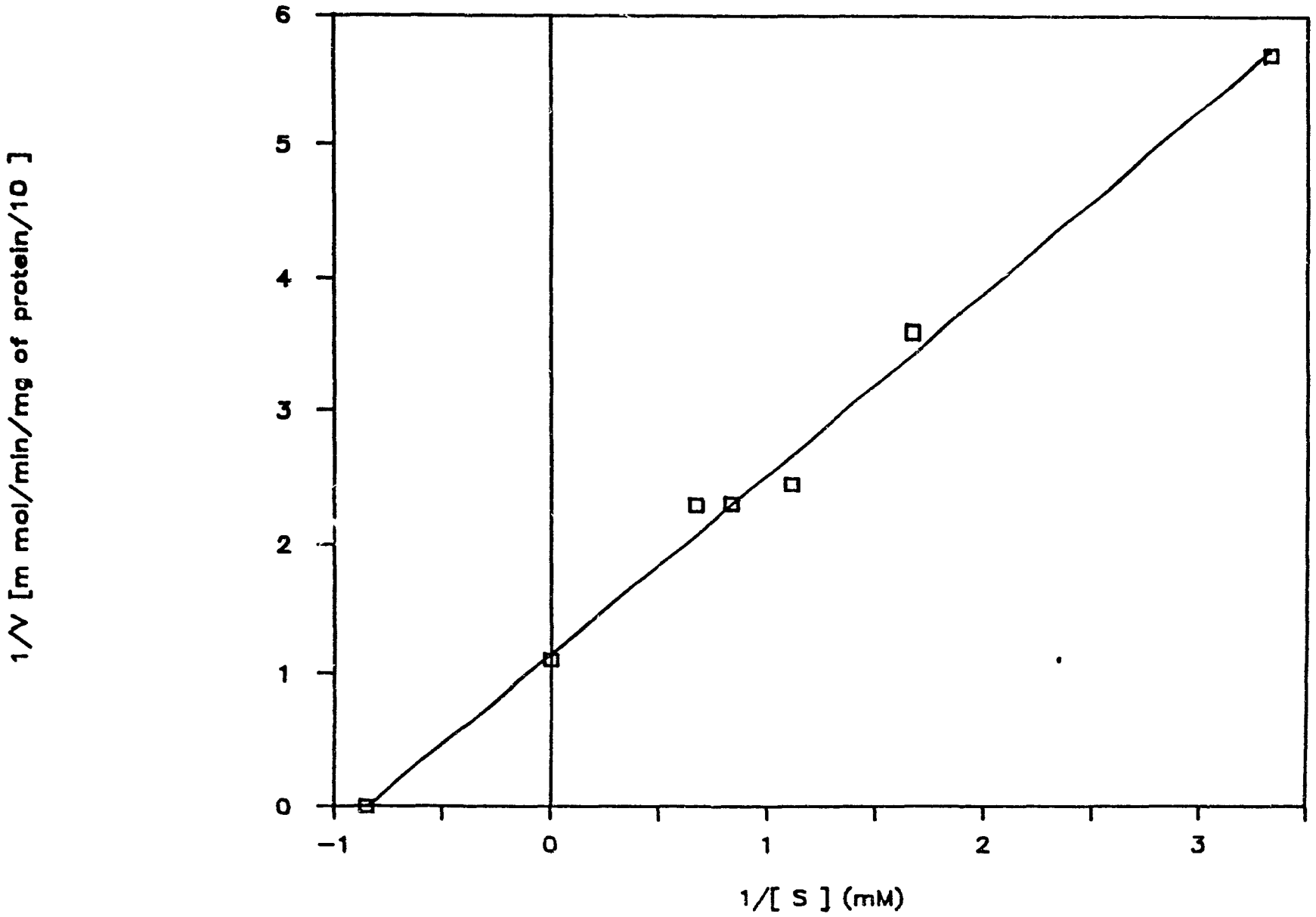
Table 12. Effect of metallic ions and some reagents on the activity of L. casei-subsp-pseudoplantarum LE2

Compound	Concentration(mM)	Relative Activity (%)
Control	1	100.0
$HgCl_2$	1	12.3
$AgNO_3$	1	29.7
EDTA	1	83.6
Cysteine	1	100.8
$ZnCl_2$	1	105.9
$CuSO_4$	1	106.4
$FeSO_4$	1	109.7
$FeCl_3$	1	112.0
Mercaptoethanol	1	118.4
$CoCl_2$	1	119.1
$MgSO_4$	1	129.3
$CaCl_2$	1	130.1

* Mean of three replicate determinations

Fig 20. Double reciprocal plot of esterase velocity versus concentration of *p*-nitrophenyl chromogenic substrate in 0.05 M phosphate buffer (pH 7.1)

* The apparent Michaelis-Menten constant (Km) and maximum velocity (V_{max}) for the enzyme were calculated from a Lineweaver-Burk plot


1/V [m mol/min/mg of protein/10]
6
5
4
3
2
1
0
−1
0
1
2
3
1/[S] (mM)

DISCUSSION

High intensity cheese flavour can be produced within days, by careful addition of selective enzymes to young cheese curd or melted cheese, which not only cuts the aging time and hence the production cost, but also significantly improves cheese quality. However, the commercial use of EMC and ARC is limited, as the various attempts made to date by different commercial food grade enzymes often develop bitterness and other off-flavours, generally attributed to accumulation of bitter peptides, and due to excessive proteolysis and lipolysis.

Weak lipolysis by lactic flora, particularly lactobacilli appears to be an important mechanism contributing to flavour development of cheese (El soda *et al.*, 1986a and 1986b; Lee *et al.*, 1986). However, very little information is available on the purification and characterization of enzyme system from lactobacilli. The investigated research dealed with the production, characterization of esterase-lipase from selected *Lactobacillus casei* subspecies, for their use in the manufacture of dairy products having intense and unique flavour profiles such as enzyme-modified cheese (EMC) and accelerated-ripened cheese (ARC).

It was proven by Lee *et al.* (1986) that the lactobacilli produce a greater range of enzymes than do the streptococci starter and the enzyme apparatus (peptidases and esterase) are seen to be responsible for the development of desirable flavour. *Lactobacillus casei* group were found to be more active in various peptidase and esterase activities than any other lactic acid bacteria. In preliminary experiment (Arora *et al*, 1989), various *Lactobacillus casei* were

screened for enzyme profiles by APIZYM procedure. *Lactobacillus casei* strains, though vary in enzyme concentration, showed the typical enzyme profile of *Lactobacillus casei* subgroup. The strains exhibited many desirable characteristics for its suitability towards cheese flavouring for EMC and ARC. These bacteria possessed weak proteinases, good galactosidases (& ß), and strong peptidase and esterase-lipase activities. The method was further explored for localization of their enzymes in the cell. All aminopeptidases and most carbohydrases were intracellular in nature. No extracellular enzyme was located and phosphatases were found to be bound to membrane. Since mechanical disintegration may result in dislodging loosely bound membrane enzymes, some enzymes exhibited activity both in cytoplasm and in membrane fraction (Arora *et al.*, 1988). Lipolytic activity of 20 strains divided into three groups; *Lactobacillus casei*-subsp-*casei*, *Lactobacillus casei*-subsp-*pseudoplantarum* and *Lactobacillus casei*-subsp-*rhamnosus*. Among these group, *Lactobacillus casei*-subsp-*casei* LLG, *Lactobacillus casei*-subsp-*pseudoplantarum* LE2 and *Lactobacillus casei*-subsp-*rhamnosus* S95 showed the highest activity in their respective group. Ultimately, LLG and LE2 were selected for this study.

Esterase-lipase production by microorganism depends greatly on the growth phase and the incubation condition. In this study, the esterase-lipase from *Lactobacillus casei* subspecies showed maximum activity during the late logarithmic phase. The observation of similar growth rates for each strain of lactobacilli during logarithmic growth phase is in keeping with the concept that the production of enzymes is advantageous to microorganisms only when nutrients become limiting, i.e. during late logarithmic phase or early stationary phase (Lawrence *et al.*, 1967). As reported in this study for *Lactobacillus casei*

subspecies, lipase production by *Brochothrix thermosphacta* (Papon & Talon, 1988), *Acinetobacter* (Breuil & Kushmer, 1975), *Pseudomonas fragi* (Nadkarni, 1970) and *Pseudomonas aeruginosa* (Umemoto & Sato, 1975) is maximal during the logarithmic phase of growth and then decreases. However, lipolytic activity of *Lactobacillus casei* subspecies were intracellular while lipolytic enzymes of lactic acid bacteria are generally cell associated (Oterholm *et al.*, 1970; Singh *et al.*, 1973). However, Oi *et al* (1967) and Iwai & Tsujisaka (1974) mentioned respectively three kinds of lipases in the culture supernatent fluids of *Pennicillium crustosum* and *Rhizopus delemer*.

The partially purified esterase-lipase from *Lactobacillus casei* subspecies was most highly active on either Tween 80, Tributyrin or chromogenic synthetic substrate between 30 and 37^{O}C, and within a broad pH range from 6 to 9. Tributyrlglycerol or Tributyrin is a convenient substrate because it can be dispersed in water by shaking or stirring without the addition of emulsifiers (Jensen, 1983). The Tweens (esterified polyoxyethylene derivatives of sorbitan) also were substrate for various microorganism (Nagata & Murata, 1972; Rudek, 1978). However, many investigators had employed chromogenic substrate. Esterase releases an alcohol (phenol, eosin, umbelliferone, naphthol, etc) from the chromogenic substrate which is determined directly by fluorimetry. This method using p-nitrophenol as substrate is very sensitive and many applications were reported in the literatures (Downey & Andrew, 1965; Brandle & Zizer, 1973; El soda *et al.*, 1986b; Kamely *et al.*, 1988). The optimal temperatures for esterase-lipase production vary from species to species but are often close to the optimal temperature for growth (Chander *et al*, 1977). However, with respect to temperature "optimum" most lipases are optimally active between 30^{O}C - 40^{O}C (Shahani, 1975). The pH optima of most microbial

lipases lie between 7 and 9 (Shahani, 1975; Law *et al.*, 1976; Stead, 1986) with few exceptions, which was confirmed in this study. Phosphate, NH_4OH-HCl and Tris have been the traditional buffers for esterase-lipase. The Good series, a group of zwitterionic buffers, are suitable for pH range of 6-8 (Good *et al.*, 1966) whereas Ferguson *et al.* (1980) mentioned 5 new zwitterionic buffers with pKa between 6.9-7.9, which appeared to be equal to or better than other buffers available. These optima of lipolytic enzymes from *Lactobacillus casei* subspecies agreed well with those obtained by *Streptococcus lactis* (Kamaly *et al.*, 1988), *Lactobacillus* (El soda *et al.*, 1986b), and *Micrococcus* (Lawrence *et al.*, 1967). The esterase-lipase from *Lactobacillus casei* subspecies was stable at room temperature and resistant to moderate heating. This has also been reported for other bacterial lipases (Chander *et al.*, 1977), making them easy to handle during experiments and suitable for industrial applications.

The ion-exchange Mono Q HR 5/5 or DEAE-sepharose (CL-6B) and gel filtration (Superose 12 HR 10/30) columns using the FPLC system provided a rapid and reproducible procedure for the separation of different components of esterase-lipase complex of *Lactobacillus casei* subspecies. This study demonstrated the advantages of using FPLC to achieve enhanced resolution, reproducibility and processing time in the separation of different components of esterase-lipase complex. The use of conventional chromatography is time consuming and requires large amounts of material and is therefore less pratical for analytical work. No reports of the FPLC technique have yet been for separation of esterase-lipase complex. As shown by the results in Table 1 and 3, excellent recovery and purification of esterase can be obtained by this FPLC technique. Previously, the FPLC technique has successfully been used for isolation of other enzymes (Markey, 1984; Buchwaldt *et al.*, 1986; Johansen *et*

al., 1987).

The number of components from the extracts that give esterase-lipase activity was unclear, but the data from the profiles of purification and activity showed the existance of three distinct enzyme proteins, thougth low C_2 and C_{10} activities could not be purified. This might be explained by a deactivation of the enzyme during purification steps.

The miniaturized "Phast" system also made it possible to achieve high resolution and reproducible results with a minimum time and effort. In case of SDS-PAGE, it took approximately 1h from sample to result. The molecular weights of the esterase-lipase were estimated to be at 320 K Dalton (Butyrate), 110 K Dalton (Caproate), and 40 K Dalton (Capryate). The molecular weights of caproate-esterase and capryate-esterase were in agreement with those of other lipolytic enzyme from *Staphylococcus aureus* (Kotting *et al.*, 1983; Rollof *et al.*, 1987). The molecular weights of butyrate-esterase weights from *Lactobacillus casei* subspecies were remarkably higher than those reported for other lipolytic enzymes (Stuer *et al.*, 1986). It may be dimer or trimer because of relatively high molecular weights. But our experimental condition did not show subunits of this enzyme. Lee *et al.* (1980) estimated molecular weights 172 K Dalton for calf esterase, 168 K Dalton for kid esterase, and 150 K Dalton for lamb esterase and Tomizuka *et al.* (1966) showed 120 K Dalton for *Candida cylindracae* lipase and *Staphlococcus aureus* lipase appeared 110 K Dalton, respectively. Those of carboxy-esterases from horse, pig, and ox liver or pig kidney, which ranged between 150 K Dalton and 168 K Dalton (Krisch, 1971). These results are agreed well with caproate-esterase molecular weight (110 K Dalton) in this study. Caprylate-esterase (40 K Dalton) from *Lactobacillu casei* subspecies agreed very well with estimates for lipase of *Rhizopus delemar* (Iwai & Tsujisaka, 1974), *Torulopsis ernobii* (Yoshida *et al.*, 1968), rat adipose tissue

(Downey & Andrews, 1965), and 42 K Dalton for pig pancreatic (Downey & Andrews, 1965). Although the profiles of the enzyme purification steps of the two *Lactobacillus casei* subspeices differed, the molecular weights of the purified enzyme showed the similar results. This might indicate that there is no significant difference in profiles and evolution of esterase-lipase at the subspecies level though the intensity of enzyme activity may differ.

Esterase-lipase from *Lactobacillus casei* subspecies were inhibited by heavy metal ions such as silver and mercury ions while EDTA caused some inhibition of esterase-lipase activity. This is in agreement with the results from other lipolytic enzyme (Nadkarni, 1971; Chopra *et al.*, 1982; Deeth & Fitz-Gerald, 1983). The inhibition by silver ions may be attributed to their reaction with the carboxyl group or histidine residue, and that by mercury ion may be attributed to their binding of the thiol group of the enzyme or sulphydryl groups which may be in the active center of the enzyme (Chopra *et al.*, 1982). This enzyme was activated by calcium and magnesium ions, which are also in accordance with data from other lipases (Nadkarni, 1971; Sugiura *et al.*, 1977; Deeth & Fitz-Gerald, 1983). Stimulatory action of calcium ions may be attributed to promotion of alignment of the enzyme on the substrate molecule (Chopra *et al.*, 1982). Otherwise, it is most likely due to stabilization of the active configuration of the enzyme (Oi *et al.*, 1967). Some of metal ions had slightly activated on esterase-lipase activity in this study.

This study clearly indicates that two *Lactobacillus casei* subspecies screened from many *Lactobacillus casei* species contain very active esterase-lipase complex system for hydrolyzing fats which were proven by previously APIZYM test and current purification steps. The specificity of these enzymes

shows that they preferentially hydrolyze esters containing short chain fatty acids up to C_{10} which may have an important role in cheese flavour development. Peterson *et al.* (1949) reported that volatile fatty acids, particularly acetic and butyric acids, were substantially higher in raw milk cheese than in pasteurized milk cheese. The presence of free butyric, caproic, caprylic acids in aged cheese was attributed largely to the action of intracellular bacterial lipases on cheese fats. Quantitative analyses of free fatty acids in cheese have been reported by many others (Billis & Day, 1964; Anderson & Day, 1965; Langler & Day, 1966). Review on cheese flavour by Day (1967) and Law (1984b) have alluded to the importance of lipolytic release of free fatty acids in development of flavour in aged cheeses. Woo *et al.* (1984) also summarized free fatty acids concentrations of various cheeses.

Seitz (1974) reviewed the industrial application of microbial enzymes, with specific mention of the role of lipases of raw milk microorganisms such as *Achromobacter lipolyticum, Pseudomonas fragi, Pseudomonas fluorescens,* and *Achromobacter lipids* in development of cheese flavour. Microbial lipases from *Candida cylindracae, Candida lipolytica,* and *Rhizopus* sp. were used for foods and beverages in Japen. (Hass, 1976). *Candida lipolytica* was used in the manufacture of Blue cheese (Peters & Nelson, 1965). The flavour of Pecorino cheese was successfully duplicated by using lipolytic enzyme derived from microorganisms obtained from the abomasum of lambs (Salvadori, 1961). Jolly and Kosikowski (1975b) reported the use of microbial lipases in manufacture of good quality Blue cheese. *Rhizopus* lipase has been applied to development of artificial butter flavours and in yogurt manufacture (Stadhouders & Veringa, 1973).

In a preliminary experiment, various enzyme combinations of esterase

and peptidases from *Lactobacillus casei* subspecies were incubated with pasteurized cheese slurry at 37°C and 12°C for varying period of time according to the procedure of Novo Laboratories (Quebec). *Lactobacillus casei* developed an intense Cheddar cheese like flavour without bitterness and off-flavour (unpublished data). The release of fatty acids between C_4 and C_8 from milk fat by *Lactobacillus casei* esterase-lipase appears to produce right blend of flavour precursors as seen in this study. These enzymes also can be added to produce ARC by direct addition of enzymes to cheese milk 15 minutes prior to the addition of rennet, and incubated for 1,2,3, and 4 months to develop desired flavour at different temperatures. Other combinations of lactobacilli enzymes and fungal proteases and lipases (Novo) may also be possible to observe their concerted action for flavour development which is underway in our laboratories.

Chromatographic analysis of free amino acids and volatile fatty acids from EMC and ARC samples will definitely determined the usefulness of these enzyme from *Lactobacillus casei*. Organoleptic properties of EMC and ARC should also be evaluated along with the chemical analysis data.

From the foregoing discussion, one may conclude that the novel esterase-lipase from two *Lactobacillus casei* subspecies are unique in its specificities characteristics, allowing it to be efficiently incorporated directly into milk or curd to produce EMC for cheeses and snackfood flavouring as well as ARC. When this enzyme in conjunction with a defined fungal protease and lipase is used with the starter culture, aging time and therefore manufacturing costs can also be significantly reduced while enhancing cheese quality.

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