Journal of Microbiology and Biotechnology
Vol. 4, No. 2, 85-94, 1994

Review

Detection and Determination of Lipase Activity

LEE, SEOUNG-YONG AND JOON-SHICK RHEE*

Department of Biotechnology, Korea Advanced Institute of Science and Technology, 373-1, Kusong-Dong, Yusong-Ku, Taejon305-701, Korea

Lipase (triacylglycerol hydrolase, EC 3.1.1.3) is able to catalyze the hydrolysis of ester bonds of triacylglycerols at the interface between aqueous phase and organic phase containing substrate. With the rapid development of lipid biotechnology, lipase-catalyzed hydrolysis of lipids has a great concern from the industrial point of view. Owing to the reversible nature of the lipase, the reactions are also applied for glyceride synthesis,interesterification and resolution of racemic mixtures into optically active alcohols or acids. For all applications of the lipases, a reliable method for the determination of enzyme activity is required. Precise quantitative determination of its activity is essential as the basis of research and development of the bioprocess involving the enzyme. This article reviews the existing literature on the detection and determination of lipase activity from microbial, mammalian and plant sources.

Lipases (EC 3.1.1.3) have been defined as the enzymes catalyzing hydrolysis of tri-, di-, and monoglyceride. Although such definition is valid, there still exists a fundamental confusion as to the exact meaning of the terms: lipases and esterases. Often these terms are used interchangeably since both enzymes catalyze the hydrolysis of carboxylic acid ester bonds and are classified among hydrolases and comprise the enzymes belonging to the class 3.1.1 of the International Union of Biochemistry Classification. Although lipases can act on soluble monomeric substrate, the practical utilization of lipase-catalyzed reactions is restricted almost exclusively to situations where the overall substrate concentration is higher than its solubility in water. This characteristic restriction provides a criterion for differentiating lipases from conventional esterases, which ordinarily act only on soluble monomeric substrates (13).

The natural function of lipases is to catalyze the hydrolysis of triglycerides. However, in anhydrous water-immiscible organic solvents, lipases can be shown to catalyze the formation of glycerides from glycerol and free fatty acid. Owing to the reversible nature of the catalysis, the reactions are applied for glyceride synthesis, interesterification and resolution of racemic mixtures into optically active alcohols or acids (37, 87, 88). Recently, extensive studies have been devoted to the application of lipase to the field of organic synthesis, on the base that the enzymes also catalyze ester bonds other than carboxylic bond (40, 70, 75). These characteristics of lipases determine the biotechnological importance of lipases. Particularly, interest in microbial lipases has markedly increased in the last two decades. The availability of relatively large quantities of microbial enzyme through bioprocess has broadened the search for the potential industrial use of bacterial and fungal lipases (69, 83). Microbial lipases are widely diversified in their enzymatic properties and substrate specificities, which are allowed to industrial applications. A large number of lipases have been screened for application as food additives, industrial reagents and detergents as well as for medical applications (7, 106).

For all applications of lipases, reliable methods for the analysis of activity are prerequisites for monitoring purification and identification of specificity. The determination of lipase activity has been studied intensively for a long time by many researchers. Jensen (49) provided a review of the extensive studies on numerous lipase assay methods. Basically, the rate of lipase reaction can be measured by determining either the rate of disappearance of the substrates or the rate of production of the fatty acids. The conditions of detection, *i.e.*, temperature, pH, presence of cations and fatty acid acceptors, will provide the information required for the design of a suitable assay system. The factors affecting enzyme activity are dependent on the source of the enzyme. Also, the substrate specificity and temperature of inactivation of enzyme are affected for characterization of enzyme.

This article reviews the existing literature on the detection and determination of lipase activity from different microbial, mammalian and plant sources.

THE SOURCES OF LIPASE

Lipases are produced by plants, animals, and microorganisms (5). As early as 1940's, it was realized that calves could secrete a salivary lipase which was later confirmed to be a true lipase and was named pre-gastric esterase (28). The occurrence of pre-gastric esterase in a number of mammalian species, including humans, has been reviewed (78).

Pancreatic lipase has been studied extensively. The

*Corresponding author

enzyme is produced in the acinar cells prior to being released into the duodenum, where it facilitates the intraluminal digestion and absorption of lipids (112). Hog pancreatic lipase is the most extensively studied lipase and has been reviewed (35). Complete and reproducible purification of mammalian lipases allowed that the primary amino acid sequence could be determined for several lipases such as pancreatic and gastric lipases (106). Further crystallographic and X-ray studies enabled the assessment of a three-dimensional structure of human pancreatic lipase (18, 39), porcine pancreatic lipase-colipase complex (15) and the gastric lipase of human, rabbit and other species (1).

Although it is known that lipase activity exists in several plant tissues (wheat, oats, corn and palm fruit), only a few studies have been made so far on the distribution of lipases in whole plants. Lipases in the castor bean seed (8), the rice bean(34), the faba bean (30) and french bean (55) have been studied. Castor bean lipase has been one of the plant lipases most extensively investigated because of its ready isolation and high activity (28). Crude lipase preparations were obtained by acetone or buffer solution extractions from easily growing seedlings of rape (*Brassica napus*), mustard (*Sinapis alba*) and cotyledons of lupine (*Lupinus albus*) (5). In 1989, Piazza *et al.* (81) applied a new lipase from oat seeds (*Avena sativa L.*) for the lipolysis of olive oil and tallow in a two-phase system. This was the first time that a lipase from a higher plant has been applied to a biotechnological process. Also, oat lipase was characterized to develop an enzymatic process for the hydrolysis of rice bean oil (52).

Currently, microorganisms give a wide spectrum of the choice of lipases, which show different characteristics for substrate specificity, reaction rate, thermal stability, range of optimum pH, and so on (50, 100). Therefore, microbial lipases are most appropriate to apply in the large scale processes. Many procedures for the production of microbial lipases in submerged culture have been published in the general and patent literature.

Microbial lipases from yeasts *Candida* and *Torulopsis*, molds *Rhizopus, Penicillium, Aspergillus, Geotrichum*, and *Mucor* genera; and bacteria of genera *Pseudomonas, Achromobacter*, and *Staphylococcus* have been shown to produce lipases. Isolation, characterization and properties of various lipase enzymes have been reviewed by several authors (5, 106). Most of the microbial lipases are extracellular, excreted through the external membrane into the culture medium. Optimization of the fermentation conditions for microbial lipases is of great importance, since culture conditions influence the properties of the enzyme producer as well as the ratio of extracellular to intracellular lipases. In general, the amount of lipase produced by each bacterial strain is dependent on environmental factors, such as cultivation temperature, composition of the nitrogen, carbon and lipid sources, concentrations of inorganic salts, and the availability of oxygen (105). However, common optimum conditions for lipase production have not yet been identified. Extracellular lipases have been studied for four main reasons. First, lipases of pathogenic bacteria such as *Corynebacterium acnes (41)* and *Staphylococcus aureus* (110) have been implicated in the etiology of disease. Second, bacterial lipases are involved in environmental problems such as the breakdown of fat in domestic sewage (77, 94), or in the ecological aspects of river sediments (12). Third, the spoilage of dairy products and other foods that contain fat involves lipases, in particular, those of psychrotrophic bacteria (26, 101). Finally, various lipases have many uses in the food and other industries by virtue of the diversity of their substrate specificities (46, 57).

Earlier reviews reflect concern with flavor defects caused by the action of lipolytic enzymes whereas the latter reviews reflect the intensive study of various lipase sources in relation to their potential application for controlled modifications of fats and oils.

ACTIVATION OF LIPASES AT INTERFACES

The major factor in controlling the lipase activity in the case of substrate emulsions is the amount of substrate molecules at the interface, which is proportional to the surface area of the emulsion. This characteristics makes it difficult to develop a reliable assay method for the lipase activity. The observed rates of lipase-catalyzed reactions are strongly influenced by the available interfacial area (56). Therefore, theoretical interpretations of the activation of lipases by interfaces have been attempted by a number of authors. The pioneering studies of Desnuelle (28), who introduced the term "heterogeneous catalysis", led to a number of proposals with respect to the molecular basis of interfacial activation, including a steep substrate concentration gradient at the interface, better orientation of the scissile ester bond, reduction in the water shell around the substrate ester molecules and the conformational change of the enzyme. These interpretations can be divided into two major groups (70). First interpretation is that the substrates are activated by the presence of an oil/water interface. Second interpretation is that the lipase is assumed to undergo a change to an activated form by the contact with an oil/water interface. The first interpretation involves higher concentrations of substrates in the vicinity of the interface than in the bulk of oil, more suitable

conformations or orientations of the lipid molecules for enzymatic reaction, and poor hydration of lipid molecules, thereby avoiding ester bond shielding in the neighboring oil/water interfaces. The latter interpretation involves the existence of separate adsorption and catalytic sites for the lipase such that the lipase becomes catalytically active only after a conformational change of the lipase by approaching the oil/water interface resulted from the high degree of order in such regions, or the reorientation of the short one-turn α-helix that covers the active sites.

It was suggested that the rate of lipolysis might depend on the available surface area of a substrate since the lipase-catalyzed reaction occurs at the interface. Pronk and Riet (85) described the interfacial behavior of lipase by studying the adsorption of and hydrolysis by lipase as a function of the amount of enzyme in different systems using the Lewis cell type reactor. Also, the kinetic and thermodynamic aspects of lipase-catalyzed reactions in anhydrous organic solvents (58) and in biphasic systems composed of aqueous and organic solvents (108, 118) have been reviewed.

SUBSTRATES OF LIPASES

The assay methods of hydrolytic activity of lipases are broadly classified into two with respect to the kind of substrate used, the one using water-soluble substrates and the other using water-insoluble substrates (69). As water-soluble substrates, triacetin (triacetylglycerol), tributyrin (tributyrylglycerol) and S-acyl compound (tributyryl-1,2-dithiogylcerol) are generally used. Tributyrylglycerol is a convenient substrate because it can be dispersed in water by shaking or stirring without emulsifiers (16). It is also useful in lipase screening tests and in the quantitation of lipolytic activity by continuous automatic titration. However, it is generally accepted that the assay using a water-soluble substrate may not give its real lipase activity as the oil-water interphase is required for the full exhibition of the lipase activity.

Olive oil, triolein (trioleoylglycerol) and chromogenic substrates such as the fatty acid esters of 4-methylcoumarin and tris[12-(2,4,6-trinitroanilino) lauroyl]glycerol are often used as water-insoluble substrates (13). Triolein is an ideal substrate for lipases because it is liquid at the usual assay temperatures and easily emulsified (48). However, commercial olive oil which contains more than 80% oleic acid is widely used as an excellent cheap substitute for triolein because pure triolein is currently too expensive.

The determination method using olive oil can be used with using a surfactant or an emulsifier or without using any surfactant at all (119). As the surfactant or emulsifier, various compounds such as poly vinyl alcohol (PVA), bile salts, bovine serum albumin, gum arabic, sodium dodecyl sulfate, phospholipids, Triton X-100 *etc* have been reported. However, when a lipase is used in the fats and oils industry, the surfactant non-addition method is more desirable because surfactant would not be used in a large scale biotechnological process. Nonetheless, this system still has major drawbacks of narrower proportionality and less precision caused by the fact that the oil phase is dispersed only by agitation with stirring.

On the other hand, in order to determine the selectivity of lipases, isopropylideneglycerols or their esters, alkylglycerols, alkylacylglycerols and dialkylacylglycerols have been reported as substitute of triacylglycerol (99, 100).

Recently, monomolecular film technique was developed by Ransac *et al.*, (86). Lipid monolayers have been used as substrates for lipolytic enzymes for over 50 years. This highly sensitive technique allows one to monitor independently several characteristics of monomolecular films. The most important advantage of this technique is that it is possible to vary the "quality of the interface". Thus one can alter the conformation of the lipid, the molecular and charge density, and water structure.

DETECTION AND DETERMINATION OF LIPASE ACTIVITY

Methods for the quantitation of lipase activity can be divided into four groups: 1) assays monitoring the disappearance of the substrate ester, 2) assays based on the physical changes in the reaction system during ester hydrolysis, 3) assays determining the amount of liberated alcohol during ester hydrolysis and 4) assays based on the quantitation of the free fatty acid formed. Currently, most of the assays used are based on the monomolecular film technique or on the fatty acid titration during hydrolysis of triglycerides.

Titration Method

As an AOAC standard method, the most widely used technique is titration method (AOAC 28.029) (45). This is one of the common methods at which the reaction is stopped by the addition of a solvent (alcohol or acetone) and the released fatty acids are titrated either by using an phenolphthalein indicator or with a pH-stat set to an end-point (9, 23, 33, 93). Some investigators have used a glass pH electrode instead of pH indicators. Development of the automatic buret and pH stat have led to methods in which continuous titration is carried out simultaneously with the enzymatic hydrolysis reaction in the same vessel. Direct titration has also been

carried out continuously with a recording pH stat for bacterial lipases (66) and adipocytes lipases (104). Taylor (107) developed a flow-through pH stat method which combines the simplicity and rapidity of continuous pH stat methods with the flexibility. This is the first continuous pH stat method in which the condition for lipase-catalyzed reaction and the titration of fatty acid products are separately controlled. For titration methods, one unit of lipase activity is defined as micromoles of fatty acid equivalent released per minute at the specified condition.

Cupric Acetate Method

Copper soap colorimetry method measures the color after the fatty acids have converted to copper soaps with chromogenic reagents. The method originally developed by Duncombe (29) has been modified and improved by many workers. The modified method was developed by Koops and Klomp (59) to estimate the intensity of lipolyzed flavor. In this method, the free fatty acids are converted to copper soaps with a color reagent. Shipe *et al.* (97) also modified the copper soap solvent extraction method for measuring free fatty acids in milk. This method can be semi automatic with an automatic sampler and a spectrophotometer with a flow through cell to test up to 200 samples per hour. Lowery and Tinsley (68) developed a rapid colorimetric method which determines fatty acids with a good sensitivity and reproducibility using cupric acetate-pyridine. This method was again modified by replacing benzene with hexane for determination of free fatty acids formed in the enzymatic hydrolysis of olive oil in the solvent system. Also, Kwon and Rhee (63) further simplified Lowery and Tinsley method for determining free fatty acids by eliminating the solvent evaporation and centrifugation steps for lipase assay. Therefore, the method takes only a few minutes to determine the free fatty acids in contrast to the conventional methods of 20 min. Redding *et al.* (89) also described a semiautomated assay for free fatty acids in serum employing Cu soaps. This cupric acetate method is readily suitable for the determination of lipase activity especially when the lipase-catalyzed reaction takes place in an organic solvent.

Colorimetric Assay Method.

Owing to their simplicity in comparison to the titration techniques, various colorimetric methods have been developed. Especially, this method can be applied for the tracing of lipase activity conveniently during the lipase purification steps. For colorimetric assay, various substrates have been used: *p*-nitrophenyl esters (19, 98, 117), ω-trinitrophenylaminolauryl esters of glycerol (32, 36), 2,3-dimercaptopropan-1-ol tributyrate (54, 62)and long chain fatty thioesters of glycerol or glycerol analogues in combination with thiol trapping agents (90). McKellar (71) has developed a colorimetric lipase assay based on the method of Nachlas and Blackburn (76). *p*-Naphthol released from the colorless ester *p*-naphthyl caprylate reacts with a diazonium salt to produce an insoluble azo dye. Brandle and Zizer (10) described that the esterase activity of the soluble extract was evaluated by using several *o*- and *p*-nitrophenyl derivatives of fatty acid chromogenic substrate. Lee and Lee (67) modified this spectrophotometric assay by using a mixture of *p*-nitrophenyl derivatives of fatty acids. Stead (103) has 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 (102).

However, most spectrophotometric methods for determining lipase activity are restricted to water soluble substrates or to assay system that contains additional amount of solubilizer or organic solvent. To overcome these drawbacks, Walde (114) developed a new colorimetric assay of fatty acids using reverse micelle system. This method utilizes phenol red as the fatty acid indicator, which is solubilized in reverse micelles formed by AOT (Bis (2-ethylhexyl) sodiumsulfosuccinate) in isooctane. Mosmuller *et al.* (74) developed a spectrophotometric method using 2,4-dinitrophenyl butyrate as the substrate. This method is based on the continuous spectrophotometric assay monitoring the increase in absorbance at 360 nm due to the formation of 2,4-dinitrophenol during hydrolysis. Vorderwulbecke *et al.* (113) compared numerous types of lipase using different chromogenic artificial substrates such as *p*-nitrophenyl palmitate (pNPP), *p*-nitrophenylbutyrate (pNPB), S,O,O-tributyryl-1-thioglycerin (TBTG) and 1,2-O-dilauryl-rac-glycero-3-glutaric acid-resorufin ester (DGGR). The ability to form esters was monitored via detection of ester formed from myristic acid and various alcohols as substrates in isooctane.

Plate Assay Method

To detect bacterial lipase directly, plate agar assay method using tributyrin as substrates was developed (2, 25, 65). The inoculated agar plates were incubated for 2-3 days and the diameter of the clearing zones was measured. Modification of this assay was performed by Kametova *et al* (51) using Tween 80 in combination with Nile blue. Kugimiya *et al.* (61) slightly modified this tributyrin diffusion agar method to find colonies with activities of lipolytic enzyme. A plate assay to detect bacterial lipase in a medium containing trioleoylglycerol and the fluorescent dye rhodamine B was developed (60). Substrate hydrolysis caused the formation of orange fluorescent halos around bacterial colonies visible upon UV irradiation. This method was suitable for

identifying lipase producing bacteria and also for quantifying lipase activity in culture supernatants (66). Fungal lipase also could be visualized when the focusing gel was overlaid by an agar gel containing trioleoylglycerol and rhodamine B after ultrathin-layer isoelectric focusing (44). These methods are excellent candidates for the screening of lipase.

Chromatography Assay Method

Chromatographic techniques such as thin laye chromatography (TLC) in conjunction with gas chromatography (GC) or high performance liquid chromatography (HPLC) are commonly used to assay the product of lipase-catalyzed hydrolysis, interesterification and esterification of lipids. Privett *et al.* (84) described the analysis of mixtures of mono-, di- and triglycerides by molecular distillation ¨nd thin-layer chromatography. Jensen (49) also explained the lipase screening method using thin layer chromatography. Currently, thin layer chromatography with flame ionization detection (FID) is being widely used for quantification of fatty acids and their glycerides (120). Gas liquid chromatography (GLC) has been applied to the quantitation of free fatty acids in serum (14), plasma (109), vegetable oils (17) and milk products (27). Recently, Kawase *et al.* (53) described the gas chromatography-mass spectrometry analysis based on the formation of an acyl-enzyme intermediate in the reaction mechanism through the observed incorporation of ^{18}O into oleic acid. This technique can be applied to elucidate the active site and reaction mechanism of lipases. Kwon *et al.* (64) recently developed a rapid gas chromatography method using a short capillary column without derivatization steps. This method can be applied not only for compositional analysis of fats and oils but also for monitoring lipase reaction. In lipase-catalyzed reaction, this GC assay made it possible to study enzyme kinetics because the amount of intermediates can be determined quantitatively. Lipid class can be also found through high pressure liquid chromatography (24, 91). Ergan and Andre (31) described a simple high pressure liquid chromatography method which easily obtains free fatty acid profiles, triglyceride profiles and lipid class profiles without any previous modification or preparation of the sample. This method can be applied to monitor lipase reaction, including not only for substrates and products of the reaction but also for intermediate products of the reaction. This HPLC method can be used to monitor bench scale and pilot plant scale reactions involving hydrolysis and synthesis of fats using lipases. Recently, Allenmark and Ohlsson (3) described a novel method for determining lipase activity in terms of rate and enantioselectivity of hydrolysis of a chiral ester substrate by using a chiral reve.sed-phase liquid chromatography.

Radiochemical Assay Method

A complex radioisotope assay was developed by Sahasrabudhe (92) for determining lipase activity in oat flour suspensions and soluble extracts. The activities of lipases from postheparin plasma ·) and human liver (11) were determined by using radiochemical assays. Oleoylglycerols labeled with ^{14}C or ^{3}H were substrates and nanogram quantities of the produc¨ were detectable. Recently, a rapid and accurate radiocl·emical method have become available for the assay of lipase activity in hydrolysis reactions (42) and for monitoring lipase catalyzed esterification and interesterification reaction (96). These techniques involve the use of radioactively labeled substrates in various reactions and the analysis of the reaction products by TLC or liquid scintillation counting (LSC) (95). The radioc: .mical techniques also can be used to determine the positional specificity of various lipase-catalyzed reactions. In such rea ions, positional specificity of lipases may be ass.yed using triacylglycerols with radioactive acyl moieties exclusively at the *sn*-1 and *sn*-3 positions. Perrella *et al.* (80) also measured phosphatidylinositol (PI) specific phospholipase C activity by using [^{3}H]PI as the substrate in the presence of 0.08% deoxycholate. nese radiochemical tec.-niques are highly suitable for a simple and sensitive assay of rates and positional specificity of lipase-catalyzed reaction such as hydrolysis, interesterification and esterification of lipids.

Coupled Enzymatic Assay Method

Treatment of free fatty acid with various enzymes results in products which are detectable at very low levels. Mizuno *et al.* (73) converted free fatty acid to the CoA esters with a synthetase, oxidized the acyl CoA to a *trans* enoyl CoA a·.d H_2O_2 by using an oxidase and then oxidized the H_2O_2 with peroxidase to a chromogen. Miles *et al.* (72) described the direct determination of free fatty acid in 2~5 μl of plasma eliminating extraction. The method was based on the quantitation of adenosine monophosphate produced by the formation of acyl-CoA ·vith ATP and acyl CoA synthetase. Hochkoeppler and Palmieri (43) developed a new lipase activity assay using the reverse micelle system. This system was based on the c .ipled enzyme lipase and lipoxygenase: the acid released by the hydrolysis of lipase-catalyzed glycerides is rapidly oxidized by lipoxygenase and monitored spectroscopically. This method was reported to be useful for detailed kinetic studies of lipase.

Immunological Assay Method

Numerous specific immunoassays were described for chicken adipose tissue lipoprotein (22), lipoprotein lipase activator in milk (4) and human pancreatic lipases (38). Anderson (4) presented an enzyme-linked immunoassay to determine the distribution and relative con-

centration in milk of lipoprotein lipase activator. Cheung *et al.* (22) 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. Grenner *et al.* (38) also described an enzyme linked immunoabsorbent assay for human pancreatic lipase in plasma. In this reaction, lipase can be detected by the spectrophotometric assay of peroxidase with H_2O_2 and o-phenylenediamine. Recently, a high degree of heterogeneity of the lipase from *Geotrichum candidum* has been demonstrated by immunoelectrophoretic techniques (47).

On-line Determination Assay Method

Poch *et al.* (82) described the application of an automatic analyzer to the on-line monitoring of lipase production. This device is based on a turbidimetric procedure that measures theturbidity change of a triolein emulsion. This method makes it very suitable for determination lipase concentration in fermentation processes. Chemnitius *et al.* (21) also reported the on-line detection of lipase activity by flow injection zymography during the enzyme purification procedure. For the on-line measurement of lipases, flow injection analysis (FIA) offers excellent features like high sample throughput, adaptation of the dynamic range by applying different reaction modes, and low consumption of reagents. Chemnitius *et al.* (20) introduced FIA system for the on-line measurement of lipases connected with fast protein liquid chromatography (FPLC) system. The connection of the FIA with a FPLC system permitted the automatization of lipase purification by monitoring protein content, salinity, and enzyme activity of the effluent from column chromatography. Its good reproducibility and capability to perform the analysis in less time than the classical titrimetric method make it more appropriate for determining on-line the lipase concentration in fermentation processes.

Infrared Spectroscopy Assay Method

Walde and Luisi (116) described a novel continuous assay for lipases in reverse micelles based on fourier transform infrared spectroscopy (FTIR). The course of hydrolysis has been followed by recording the fourier transform infrared spectrum of the entire reaction mixture as a function of time. And the kinetics of hydrolysis reaction can be followed at different regions of the spectrum. As long as the catalyst can be solubilized in reverse micelles and as long as the catalyst remains active, the FTIR method can, in principle, be applied for other reactions, involving water-insoluble substrates that can be dissolved in concentrations that are detectable with infrared spectroscopy. They (115) also described that the hydrolytic activity of lipase in reverse micelles could be detected continuously with FTIR by following the water consumption in the region of the OH-stretching band during the reaction. This method is suitable for determining the extent of hydrolysis and fatty acid specificity of lipase within the class of triglycerides.

Nuclear Magnetic Resonance (NMR) Assay Nethod

O'Connor *et al.* (79) have developed a novel NMR assay to monitor lipase-catalyzed reactions without the need to extract and purify the individual components. This technique does not measure the amount of free fatty acid released but rather the ratio of ester: alcohol in the reactant mixture. Therefore, it may be used with relatively crude substrates and for high concentrations that are needed for biocatalytic ester synthesis for industrial purposes. This method has proven useful as a quick screen for the effect of varying conditions on the extent of esterification in various biphasic solvent systems and can be applied to both saturated and unsaturated long chain fatty acid and alcohol substrate. However, one disadvantage of the NMR ratio technique is that a NMR spectrometer may not be available for industry use. Nevertheless, this new technique could well be used in research and development of specific aspects of enzyme-catalyzed reaction involving long chain fatty acids and alcohols in nonaqueous solvents.

Circular Dichroism (CD) Assay Method

Uzawa *et al.* (111) proposed a highly sensitive circular dichroism (CD) method for determining the optical purity and absolute configurations of 1,2-di-O-benzoyl-*sn*-glycerol, which was used to determine the stereoselective hydrolysis of tri-O-benzoylglycerol by lipases from various origins. In this method, the deacylation was conducted without racemization using the Grignard degradation and this method was proposed as a general method to determine the optical purity of 1,2-di-O-acyl-*sn*-glycerol independent of the structures of acyl groups. This method was successfully applied to determine the stereoselectivity of lipases such as *Pseudomonas* (AP, 89% optical purity, *sn*-1 preference), porcine pancreatin (PPL, 9.3% optical purity, *sn*-3 preference) and *Candida* (CC, *sn*-2 preference) using tripalmitin. The advantage of this method is that it can be used to determine the optical purities and the absolute configuration by CD without authentic samples of known configurations.

CONCLUSIONS

Currently, there is a large and increasing interest in the development of application of lipases. For these

applications, a reliable method of determining lipase activity is essential. Although numerous methods for detection and determination of lipase activity have been developed, many research groups are still looking for more sensitive, less time consuming and continuous assay systems. This may serve as an indication that many of the methods are not entirely satisfactory and they are valid for the specific purposes only. In order to select the most suitable method for determining lipase activity, a variety of parameters should be considered, such as the number of samples to be analyzed, the desired sensitivity, the availability of equipment, enzymes and etc., and the advantages or disadvantages of each method must be compared at the experimental design stage.

REFERENCE

1. Abergel, C., J. Fontecilla, and C. Cambillau. 1991. Crystallization of gastric lipases. p. 28-30. In L. Alberghina, R.D. Schmid and R. Verger (ed.), *Lipases: Structure, Mechanism and Genetic Engineering*. GBF Monographs, Vol. **16**. Braunschweig, Germany.
2. Alford, J.A. and E.E. Steintle. 1967. A double layered plate method for the detection of microbial lipolysis. *J. Appl. Bacteriol.* **30**: 488-494.
3. Allenmark, S. and A. Ohlsson. 1992. Enantioselectivity of lipase-catalyzed hydrolysis of some 2-chlorethyl 2-arylpropanoates studied by chiral reversed-phase liquid chromatography. *Chirality* **4**: 98-102.
4. Anderson, M. 1979. Enzyme Immunoassay for measuring lipoprotein lipase activator in milk. *J. Dairy Sci.* **62**: 1380-1383.
5. Antonian, E. 1988. Recent advances in the purification characterization and structure determination of lipases. *Lipids* **23**: 1101-1106.
6. Baginsky, M.L. 1981. Measurement of lipoprotein lipase and hepatic triglyceride lipase in human postheparin plasma. *Methods in Enzymol.* **72**: 325-346.
7. Bjokling, F., S.E. Godfredsen, and O. Kirk. 1991. The future impact of industrial lipases. *TIBTECH.* **9**: 360-363.
8. Borgstrom, B. and R.L. Ory. 1970. Castor bean lipase: specificity of action. *Biochim. Biophys. Acta.* **212**: 521-526.
9. Bozoglu, F., H.E. Swaisgood, and D.M. Jr. Adams. 1984. Isolation and characterization of an extracellular heat-stable lipase produced by *Pseudomonas fluorescens* MC50. *J. Agric. Food Chem.* **32**: 2-6.
10. Brandle E., and T. Zizer. 1973. On the hydrolysis of aromatic esters by microbial preparations with lipolytic and proteolytic activity. *Osterreich Milchwirtsch.* **28**: 15-24.
11. Breckenridge, W.C. and F.B.St.C. Palmer. 1982. Composition of human plasma lipoprotein phosphatidylinositols. *Biochim. Biophys. Acta.* **72**: 707-711.
12. Breuil, C. and D.J. Kushmer. 1975. Lipase and esterase formation by psychrophilic and mesophilic *Acinobacto* species. *Can. J. Microbiol.* **21**: 423-433.
13. Brockman, H.L. 1984. General features of lipolysis: reaction scheme, interfacial structure and experimental approaches. p. 3-47. In B. Borgstrom B. and H.L. Brockman (ed.), *Lipases*, Elsevier.
14. Brunk, S.D. and J.R. Swanson. 1981. Colorimetric method for free fatty acids in serum validated by comparison with gas chromatography. *Clin. Chem.* **27**: 924-926.
15. Cambillau, C. 1991. Crystallization and X-ray studies of lipases. p. 17-26. In L. Alberghina, R.D. Schmid and R. Verger (ed.), *Lipases: Structure, Mechanism and Genetic Engineering*. GBF Monographs, Vol. **16**. Braunschweig, Germany.
16. Castberg, H.B., P. Solberg, and T. Egelrud. 1975. Tributyrate as a substrate for the determination of lipase activity in milk. *J. Dairy Res.* **42**: 247-253.
17. Chapman, G.W. Jr. 1979. Gas chromatographic determination of free fatty acids in vegetable oils by a modified esterification procedure. *J. Am. Oil Chem.* **56**: 77-79.
18. Chapus, C., K. Abousalham, B. Kerfelec, M. Moulard, E. Foglizzo, and D. Lombardo. 1991. Pancreatic lipase: crystallization, domain investigation and cloning. p. 35-38. In L. Alberghina, R.D. Schmid and R. Verger (ed.), *Lipases: Structure, Mechanism and Genetic Engineering*. GBF Monographs, Vol. **16**. Braunschweig, Germany.
19. Chapus, C., M. Semeriva, C. Bovier-Lapierre, and P. Desnuelle. 1976. Mechanism of pancreatic lipase action. 1. Interfacial activation of pancreatic lipase. *Biochem.* **15**: 4980-4987.
20. Chemnitius, G.C., H. Erdmann, and R.D. Schmid. 1992. Solubilized substrates for the on-line measurement of lipases by flow injection analysis during chromatographic enzyme purification. *Anal. Biochem.* **202**: 16-24.
21. Chemnitius, G.C., H. Erdmann, and R.D. Schmid. 1990. Proceeding 5th european congress on biotechnology (Copenhagen) pp. 544-547.
22. Cheung, A.H., A. Bensadoun, and C-F. Chen. 1979. Direct solid phase radioimmunoassay for chicken lipoprotein lipase. *Anal. Biochem.* **94**: 346-357.
23. Chopra, A.K., H. Chander, and J. Singh. 1982. Lipolytic activity of *Syncephalastrum racemosum*. *J. Dairy Sci.* **65**: 1890-1894.
24. Christie, W.W. 1985. The analysis of fatty acid. p. 133-166. In *H.P.L.C. and lipids*. Pergamon press, Oxford.
25. Chung, K.H., Y.P. Lee, K.H. Jeohn, O.J. Yoo, and J.S. Rhee. 1991. Cloning and nucleotide sequence of thermostable lipase gene from *Pseudomonas fluorescens* SIK W1. *Agric. Biol. Chem.* **55**: 2359-2365.
26. Cousin, M.A. 1982. Presence and activity of psychrotrophic microorganism in milk and dairy product: A review. *J. Food Prot.* **45**: 172-207.
27. Deepth, H.C., C.H. Fitz-Gerald, and A.Z. Snow. 1983. A gas chromatographic method for the quantitative determination of free fatty acids in milk and milk products. *N.Z. J. Dairy Sci. Technol.* **18**: 13-20.
28. Desnuelle. P. 1972. Aldol cleavage and condensation, Other C-C cleavage, phosphorolysis, Hydrolysis (Fats, Glycosides). p. 575-615. In P.D. Boyer(ed.), *The Enzymes*, Vol. **7**. Elimination and addition. Academic Press, New York.
29. Duncombe, W.G. 1963. The colorimetric micro-determinations of long-chain fatty acids. *Biochem. J.* **88**: 7-10.
30. Dundas, D.G.A., H.M. Henderson, and N.A.M. Eskin. 1978. Lipase from *Vicia faba* minor. *Food Chem.* **3**: 171-176.

31. Ergan, F. and G. Andre. 1989. Simple high performance liquid chromatography methods for monitoring lipase reaction. *Lipids* **24**: 76-78.
32. Farooqui, A.A., W.A. Taylor, C.E. Pendly II, J.W. Cox, and L.A. Horrocks. 1984. Spectrophotometric determination of lipase, lysophospholipases and phospholipases. *J. Lipid Res.* **25**: 1555-1562.
33. Fitz-Gerald, C.H. and H.C. Deeth. 1983. Factors influencing lipolysis by skim milk cultures of some psychrotrophic microorganism. *Aus. J. Dairy Technol.* **38**: 97-103.
34. Fujiki, Y., Y. Aizono, and M. Funatsu. 1978. Chemical properties of major subunit of rice bran lipase. *Agric. Biol. Chem.* **42**: 599-654.
35. Gargouri, Y., H. Moreau, and R. Verger. 1989. Gastric lipase: biochemical and physiological studies. *Biochim. Biophys. Acta.* **1006**: 255-271.
36. Gatt, S., Y. Barenholz, R. Goldberg, T. Dinur, G. Besley, J. Rosenthal, R.G. Desnick, E.A. Devine, and F. Tsuruki. 1981. Assay of enzymes of lipid metabolism with colored and fluorescent derivatives of natural lipids. *Methods in Enzymol.* **72**: 351-375.
37. Gills, A. 1988. Research discover new roles for lipase. *J. Am. Oil Chem. Soc.* **65**: 846-852.
38. Grenner, G., G. Deutsch, R. Schmidtberger, and F. Kati. 1982. A high sensitive enzyme immunoassay for the determination of human pancreatic lipase. *J. Clin. Chem. Clin. Biochem.* **20**: 515-519.
39. Gubemator, K., K. Muller, and F. Winkler. 1991. The structure of human pancreatic lipase suggests a locally inverted, trypsin-like mechanism. p. 9-16. In L. Alberghina, R.D. Schmid and R. Verger (ed.), *Lipases: Structure, Mechanism and Genetic Engineering*. GBF Monographs, Vol. **16**. Braunschweig, Germany.
40. Halling, P.J. 1990. Lipase-catalyzed modification of oils and fats in organic two phase system. *Fat Sci. Technol.* **6**: 15-19.
41. Hassing, G.S. 1971. Partial purification and some properties of a lipase of *Corynebacterium acnes*. *Biochim. Biophys. Acta.* **242**: 381-394.
42. Hills, M.J. and K.D. Mukherjee. 1988. Assay for triacylglycerol lipase by a rapid thin-layer chromatographic techniques. *J. Lipid Res.* **29**: 1397-1399.
43. Hochkoeppler, A. and S. Palmieri. 1990. A spectrophotometric lipase activity assay by coupled lipase-lipoxygenase in reverse micelles. *Biocatalysis* **3**: 357-365.
44. Hofelmann, M., R. Kittsteiner-Eberle, and P. Schreier. 1983. Ultra thin-layer agar gel: a novel print technique for ultra thin layer isoelectric focusing of enzymes. *Anal. Biochem.* **128**: 217-222.
45. Horowitz, W. 1980. Association of Official Analytical Chemists in official method (13ed.) *Assoc. Off. Anal. Chemists*. Washington D.C.
46. Iwai, M. and Y. Tsujisaka. 1984. Fungal lipase. p. 443-470. In *lipases*. B. Borgstrom and H.L. Brockman(ed.), Elsevier Sci. Publisher. Amsterdam.
47. Jacobson, T., J. Olson, and K. Allenmam. 1989. Production, partial purification and immunochemical characterization of multiple forms of lipase from *Geotrichum candidum*. *Enzyme Microb. Technol.* **11**: 90-95.
48. Jensen, R.G., D.R. Galluzzo, and V.J. Bush. 1990. Selectivity is an important characteristics of lipases. *Biocatalysis* **3**: 307-316.
49. Jensen, R.G. 1983. Detection and determination of lipase (Acylglycerol hydrolase) activity from various sources. *Lipids* **18**: 650-657.
50. Jonsson, U. and B.G. Snygg. 1974. Lipase production and activity as a function of incubation time, pH and temperature of four lipolytic microorganisms. *J. Appl. Bacteriol.* **37**: 571-581.
51. Karnetova, J., J. Mateju, T. Rezanka, P. Prochazka, M. Nohyenk, and T. Rokos. 1984. Estimation of lipase activity by the diffusion plate method. *Folia Microbiol.* **29**: 346-347.
52. Kaur, J., V. Ramamurthy, and R.M. Kothari. 1993. Characterization of oat lipase for lipolysis of rice bran oil. *Biotechnol. Lett.* **15**: 257-262.
53. Kawase, M., K. Sonomoto, and A. Tanaka. 1992. Inspection of an acyl-enzyme intermediate in a lipase reaction by gas chromatography-mass spectrometry and modelling of the reaction mechanism. *Biocatalysis* **6**: 43-50.
54. Kawase, M., H. Takahashi, K. Sonomoto, K. Nakamura, A. Tanaka. 1991. Effect of chemical modification of tyrosine residues on activities of bacterial lipase. *J. Ferment. Biotechnol.* **72**: 317-319.
55. Kermasha, S., F.R. Van de Voort, M. Metche. 1986. Characterization of french bean seed lipase. *Can. Inst. Food Sci. Technol. J.* **19**: 23-27.
56. Kierkels, J.G.T., I.F.W., Vieugels, J.H.A. Kem, E.M. Meijer, and M. Kloosterman. 1990. Lipase kinetics: on-line measurement of the interfacial area of emulsions. *Enzyme Microb. Technol.* **12**: 760-763.
57. Kilara, A. 1985. Enzyme-modified lipid food ingredients. *Process Biochem.* **20**: 36-45.
58. Klibanov, A.M. 1989. Enzymatic catalysis in anhydrous organic solvents. *Trends Biochem. Sci.* **14**: 141-144.
59. Koops, J. and H. Klomp. 1977. Rapid colorimetric determination of free fatty acids in milk by the copper soap method. *Netherlands Milk Dairy J.* **31**: 56-59.
60. Kouker, G. and K.E. Jaeger. 1987. Specific and sensitive plate assay for bacterial lipase. *Appl. Environ. Microbiol.* **53**: 211-213.
61. Kugimiya, W., Y. Otami, Y. Hashimoto, and Y. Takagi. 1986. Molecular cloning and nucleotide sequence of the lipase gene from *Pseudomonas fragi*. *Biochim. Biophys. Res. Commun.* **141**: 185-190.
62. Kurooka, S., S. Okamoto, and M. Hashimoto. 1977. A novel and simple colorimetric assay for human serum lipase. *J. Biochem (Tokyo)* **81**: 361-369.
63. Kwon, D.Y. and J.S. Rhee. 1986. A simple and rapid colorimetric method for determination of free fatty acid for lipase assay. *J. Am. Oil. Chem. Soc* **63**: 89-92.
64. Kwon, S.J., S.Y. Lee, S.W. Cho, and J.S. Rhee. 1993. A rapid gas chromatography method for quantitation of free fatty acids, mono-, diacyl-, and triacylglycerols without derivatization. *Biotechnol. Technique* **7**: 727-732.
65. Lawrence, R.C., T.F. Fryer, and B. Reiter. 1967. The production and characterization of lipase from a Micrococcus and a *Pseudomonas*. *J. Gen. Microbiol.* **48**: 401-418.
66. Lee, S.Y. and J.S. Rhee. 1993. Production and partial purification of a lipase from *Pseudomonas putida* SK. *Enzyme Microb. Technol.* **15**: 617-623.

67. Lee, S.Y. and B.H. Lee. 1989. Production and characterization of esterase-lipase of *Lactobacillus casei* subsp. *pseudoplantarum* LE2. *Biotechnol. Appl. Biochem.* **11**: 552-563.
68. Lowery, R.R. and I.I. Tinsley. 1976. Rapid colorimetric determination of free fatty acids. *J. Am. Oil Chem. Soc.* **53**: 470-472.
69. Macrae, A.R. 1983. Extracellular microbial lipases. p. 225-250. In *Microbial enzymes in Biotechnology*. W.H. Forgarty (ed.), *Appl. Sci.* Publishers, New York.
70. Malcata, F.X., H.R. Reyes, H.S. Garcia, C.G. Hill. Jr., and C.H. Amundson. 1992. Kinetics and mechanisms of reactions catalysed by immobilized lipases. *Enzyme Microb. Technol.* **14**: 426-446.
71. McKellar, R.C. 1986. A rapid colorimetric assay for the extracellular lipase of *Pseudomonas fluorescens* B52 using β-napthyl caprylate. *J. Dairy Res.*, **53**: 117-127.
72. Miles, J., R. Glasscock, J. Aikens, J. Gerich, and M. Haymond. 1983. A microfluorometric method for the determination of free fatty acids in plasma. *J. Lipid Res.* **27**: 163-165.
73. Mizuno, K., M. Toyosato, S. Yabumoto, I. Tauimizu, and H. Hirakaua. 1980. A new enzymatic method for colorimetric determination of free fatty acids. *Anal. Biochem.* **108**: 6-10.
74. Mosmuller, E.W.J., J.D.H. Van Heemst, C.J. Van Delden, M.C.R. Franssen, and J.F.J. Engbersen. 1992. A new spectrophotometric method for the detection of lipase activity using 2,4-dinitrophenyl butyrate as a substrate. *Biocatalysis* **5**: 279-287.
75. Mukherjee, K.D. 1990. Lipase catalyzed reactions for modification of fats and other lipids. *Biocatalysis* **3**: 277-293.
76. Nachlas, M.M. and R. Blackburn. 1958. The colorimetric determination of urinary lipase. *J. Biol. Chem.* **230**: 1051-1061.
77. Nadkarni, S.R. 1971. Studies on bacterial lipase. Part 2. Study of the characteristics of partially purified lipase from *Pseudomonas aeruginosa*. *Enzymologica.* **40**: 302-313.
78. Nelson, J.H., R.G. Jensen, and R.E. Pitas. 1977. Pregastric esterase and other oral lipases. A review. *J. Dairy Sci.* **60**: 327-362.
79. O'Connor, C.J., S.F. Petricevic, J.M. Coddington, and R.A. Stanley. 1992. An NMR assay for quantitating lipase activity in biphasic macroemulsions. *J. Am. Oil Chem. Soc.* **69**: 295-300.
80. Perrella, F.W., R. Jankewicz, and E.A. Dandrow. 1991. Phospholipase C from human melanoma: purification and characterization of a phosphatidylinositol-selective enzyme. *Biochim. Biophys. Acta.* **1076**: 209-214.
81. Piazza, G., A. Bilyk, D. Schwartz, and M. Haas. 1989. Lipolysis of olive oil and tallow in an emulsifier-free two-phase system by the lipase from oat seeds (*Avena sativa l.*). *Biotechnol. Technique* **11**: 487-492.
82. Poch, M.V.F., C. Sola, L.R.A. Santos, and L.J.L.F. Costa. 1991. On-line monitoring of lipase production in fermentation process. *Biotechnol. Lett.* **5**: 251-254.
83. Posorske, L.H. 1984. Industrial-scale application of enzymes to the fats and oil industry. *J. Am. Oil Chem. Soc.* **61**: 1758-1760.
84. Privett, O.S., M.L. Blank, and W.O. Lundberg. 1961. Determination of mono-, di-, and triglycerides by molecular distillation and thin layer chromatography. *J. Am. Oil Chem. Soc.* **38**: 312-316.
85. Pronk, W. and K. Riet. 1991. The interfacial behavior of lipase in free form and immobilized in a hydrophilic membrane reactor. *Biotechnol. Appl. Biochem.* **14**: 146-154.
86. Ransac, S., E. Rogalska, Y. Gargouri, A.M.T.J. Deveer, F. Platauf, G.H. De Hass, and R. Verger. 1990. Stereoselectivity of lipases. I. hydrolysis of enantiomeric glyceride analogues by gastric and pancreatic lipases, a kinetic study using the monomolecular film technique. *J. Biol. Chem.* **265**: 20263-20270.
87. Ratledge, C. 1987. Lipid biotechnology: a wonderland for the microbial physiologist. *J. Am. Oil Chem. Soc.* **64**: 1647-1656.
88. Rattray, J.B.M. 1984. Biotechnology and the fats and oils industry-an overview. *J. Am. Oil Chem. Soc.* **61**: 1701-1712.
89. Redding, W., G.G. Mayer, and J.W. Lornell. 1983. A semiautomated assay foı nonesterified fatty acids in the 0.02 to 0.04 µed/ml range. *J. Lipid Res.* **24**: 100-104.
90. Renard. G., J. Grimaud, A. El Zant, M. Pina, and J. Graille. 1987. An improved method for the colorimetric assay of lipase activity using an optically clear medium. *Lipids* **22**: 539-541.
91. Riisom, T. and L. Hoffmeyer. 1992. High performance liquid chromatography analysis of emulsifiers: 1. Quantitative determinations of mono and diacylglycerols of saturated fatty acids. *J. Am. Oil Chem. Soc.* **55**: 649-652.
92. Sahasrabudhe, M.R. 1982. Measurement of lipase activity in single grains of oat. (*Avena sativa*). *J. Am. Oil Chem. Soc.* **59**: 354-355.
93. San Clemente, C.L. and D.V. Vadehra. 1967. Instrumental assay of microbial lipase at constant pH. *Appl. Environ. Microbiol.* **15**: 110-113.
94. Schmid, R.D. 1987. Biotechnology; application to oleochemistry. *J. Am. Oil Chem. Soc.* **65**: 252-254.
95. Schuch, R. and K.D. Mukherjee. 1989. Fast radio thin layer chromatography for assay of lipase-catalyzed esterification and interesterification reactions. *J. Chromatogr.* **450**: 448-451.
96. Schuch, R. and K.D. Mukherjee. 1988. Radiochemical techniques for the assay of lipase catalyzed reactions. p. 330-331. In *Proceedings of the World Conference on Biotechnology for the Fats and Oils Industry*. T.H. Applewhite (ed.), Am. Oil Chem. Soc., Glenview, IL.
97. Shipe, W.F., G.F. Senyk, and K.B. Fountain. 1980. Modified copper soap solvent extraction method for measuring free fatty acids in milk. *J. Dairy Sci.* **63**: 193-198.
98. Smeltzer, M, M.E. Hart, and J.J. Iandolo. 1992. Quantitative spectrophotometric assay for Staphylococcal lipase. *Appl. Environ. Microbiol.* **58**: 2815-2819.
99. Sonnet, P.E. and J.A. Gazzillo. 1991. Evaluation of lipase selectivity for hydrolysis. *J. Am. Oil Chem. Soc.* **68**: 11-15.
100. Sonnet, P.E. 1988. Lipase selectivities. *J. Am. Oil Chem. Soc.* **65**: 900-904.
101. Stead, D. 1986. Microbial lipases: their characteristics; role in food spoilage and industrial uses. *J. Dairy Res.* **53**: 481-505.
102. Stead, D. 1984. Evaluation of a fluorimetric assay on the

lipases from strains of milk psychrotrophic bacteria. *J. Dairy Res.* **51**: 123-130.

103. Stead, D. 1983. A fluorimetric method for the determination of *Pseudomonas fluorescence* AR 11 lipase in milk. *J. Dairy Res.* **50**: 491-502.
104. Stralfors, P., N.O. Nilsson, G. Fredrikson, and P. Belfrage. 1981. Hormone-sensitive from adipose tissue of rat. *Methods in Enzymol.* **71**: 319-325.
105. Sztajar, H. and I. Maliszewska. 1988. The effect of culture conditions on lipolytic productivity of microorganisms. *Biotechnol. Lett.* **10**: 199-204.
106. Taipa, M.A., M.R. Aires-Barros, and J.M.S. Cabral. 1992. Purification of lipases. *J. Biotechnol.* **26**: 111-142.
107. Taylor, F. 1985. Flow through pH-stat method for lipase activity. *Anal. Biochem.* **148**: 149-153.
108. Tsai, S.W., G.H. Wu, and C.L. Chiang. 1991. Kinetics of enzymatic hydrolysis of olive oil in biphasic organic-aqueous system. *Biotechnol. Bioeng.* **38**: 761-766.
109. Tsemg, I-Y., R.M. Kliegman, E-I. Miettinen, and S.E. Kalhan. 1981. A rapid, simple, and sensitive procedure for the determination of free fatty acids in plasma using glass capillary columns gas-liquid chromatography. *J. Lipid Res.* **22**: 852-858.
110. Tyski, S., W. Hryniewiez, and J. Jeljaszewicz. 1983. Purification and some properties of the *staphylococcal* extracellular lipase. *Biochim. Biophys. Acta.* **749**: 312-317.
111. Uzawa, H., T. Noguchi, Y. Nishida, H. Ohrui, and H. Meguro. 1993. Determination of the lipase stereoselectivities using circular dichroism (CD); lipases produces chiral di-O-acylglycrols from achiral tri-O-acylglycerols. *Biochim. Biophys. Acta.* **1168**: 253-260.
112. Verger, R. 1984. Pancreatic lipase. p.83-150. In *Lipases*, B. Borgstrom and H.L. Brockman (ed.), Lipases, Elsevier Science Publishers, Amsterdam.
113. Vorderwulbecke, T., K. Kieslich, and H. Erdmann. 1992. Comparison of lipases by different assays. *Enzyme Microb. Technol.* **14**: 631-639.
114. Walde, P. 1990. A colorimetric determination of fatty acids as a new assay of lipases in reverse micelles. *J. Am. Oil Chem. Soc.* **67**: 110-115.
115. Walde, P. and P.L. Luisi. 1990. Detection of water in lipase catalyzed reactions in reverse micelles as studied by infrared spectroscopy. *Biocatalysis* **3**: 381-383.
116. Walde, P. and P.L. Luisi. 1989. A continuous assay for lipases in reverse micelles based on fourier transform infrared spectroscopy. *Biochem.* **28**: 3353-3360.
117. Winkler, U.K. and M. Stuckmam. 1979. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens. J. Bacteriol.* **138**: 663-670.
118. Woodley, J.M., A.J. Brazier, and M.D. Lilly. 1991. Lewis cell studies to determine reactor design data for two liquid phase bacterial and enzymic reactions. *Biotechnol. Bioeng.* **37**: 133-140.
119. Yamane, T. 1987. A comparison of assays on hydrolytic activity of lipase with and without surfactant. *J. Jpn. Oil Chem. Soc.* **36**: 402-408.
120. Yamane, T., M.M. Hoq, S. Itoh, and S. Shimizu. 1986. Glycerolysis of fat by lipase. *J. Jpn. Oil Chem. Soc.* **35**: 45-51.

(Received December 13, 1993)