Eduard W.J. Mosmuller

Studies on the incorporation of lipase in synthetic polymerisable vesicles

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Studies on the incorporation of lipase in synthetic polymerisable vesicles

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op donderdag 13 mei 1993
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

voor Jos

Jij hebt me α geleerd,
jammer dat je dit Ω niet
meer mag meemaken.

CIP-gegevens Koninklijke Bibliotheek, Den Haag

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STELLINGEN

1. Bij dehydratatie (vriesdrogen) van een vesikeloplossing blijft de structuur van het membraan grotendeels intact.
   Dit proefschrift

2. Toyota en Asakawa concluderen ten onrechte dat het bij twee van de door hen geïsoleerde sesquiterpenen gaat om bicyclogermacranen.
   M. Toyota en Y. Asakawa (1993) Sesqui- and triterpenoids of the liverwort *Conocephalum japonicum*. Phytochemistry, 32, 1235-1237

3. Bij zijn representatie van de interactie van triglyceriden met een geactiveerd lipase houdt Blow, bij de oriëntatie van deze triglyceriden, te weinig rekening met de verschillen in polariteit in het substraat.
   D. Blow (1991) Lipases reach the surface. Nature, 351, 444-445

4. Sánchez-Ferrer en García-Carmona voeren onvoldoende experimenteel bewijs aan voor het bestaan van *reverse vesicles* in het door hen gebruikte surfactantsysteem.
   A. Sánchez-Ferrer en F. García-Carmona (1992) Reverse vesicles as a new system for studying enzymes in organic solvents. Biochem. J., 285, 373-376

5. Het is onduidelijk waarom Formelová et al. bij de scheiding van vrij trypsine en trypsine dat in eiwit-lecithine liposomen is ingesloten, geen gebruik hebben gemaakt van een gelpermeatie-chromatografiekolom met een groter scheidend vermogen.
   J. Formelová, A. Breier, P. Gemeiner en L. Kurillová (1991) Trypsin entrapped within liposomes. Partition of a low-molecular-mass substrate as the main factor in kinetic control of hydrolysis. Collect. Czech. Chem. Commun., 56, 712-717

6. Het door Tolbert et al. voorgestelde mechanisme voor de vorming van cyclische allenen uit chlorocarbanionen is discutabel.
   L.M. Tolbert, M.N. Islam, R.P. Johnson, P.M. Loiselle en W.C. Shakespeare (1990) Carbanion photochemistry: a new photochemical route to strained cyclic allenes. J. Am. Chem. Soc., 112, 6416-6417

7. Het vermoeden van Walter en Ballschmiter dat de bromering van anisool door chloroperoxidase van *Caldariomyces fumago* een regioselectieve reactie is, wordt onvoldoende door experimenten onderbouwd.
   B. Walter en K. Ballschmiter (1991) Biohalogenation as a source of halogenated anisoles in air. Chemosphere, 22, 557-567

8. Bij interdisciplinair onderzoek is niet alleen het hebben van de juiste kennis van belang, maar ook het hebben van de juiste kennissen.

9. De kieskeurige consument is mede schuldig aan het overmatig gebruik van gewasbeschermingsmiddelen.

10. De hoge snelheden, die recreatieve wielrenners vaak ontwikkelen, en het daarmee gepaard gaande, veelal onverantwoordelijke weggedrag rechtvaardigen de verplichting tot het dragen van een goedgekeurde valhelm.

11. Bij de gang naar een fietswinkel voor de aanschaf van een aërodynamisch hulpmiddel moet de matig getrainde triathleet zich afvragen of hij niet meer gebaat is bij een extra training.

12. Een schoner milieu is een mentaliteitskwestie.

Eduard W.J. Mosmuller

Studies on the incorporation of lipase in synthetic polymerisable vesicles

13 mei 1993, Wageningen

CONTENTS

SCOPE AND CONTENTS OF THIS THESIS

The general aim of this thesis was to investigate the possibilities of incorporation of enzymes in synthetic polymerisable vesicles and to study the effects of incorporation on activity and stability of the enzyme. *Candida cylindracea* lipase was taken as the model enzyme. The choice for a lipolytic enzyme was prompted by the ability of this class of enzymes to perform their catalytic action preferably at interfaces. It was anticipated that the properties of vesicular systems could positively influence the (mode of) action of lipases.

Vesicles, spherical bilayer structures build up from amphiphilic molecules, have well-defined surface properties. Due to the great flexibility in the design of vesicular systems, there is an interesting development and diversification in the application of vesicles as membrane model systems. Enzyme-vesicle systems can be used for therapeutic purposes or in biotechnological applications and may lead to the development of multi-enzyme systems that can be used as micro-bioreactors. For practical applications of these systems, e.g. for the immobilisation and stabilisation of enzymes, it is desirable to have samples of high stability. Therefore, in this thesis special attention has been paid to enzyme-vesicle systems with polymerised bilayer structures.

Chapter 1 describes some general characteristics of lipases. In chapter 2 the properties of surfactant molecules and vesicles are reviewed. These chapters are introductions into the field of lipases and surfactant vesicles.

Despite extensive investigations on lipases and on vesicular systems, surprisingly little is known about the incorporation of enzymes, and in particular lipases, in synthetic vesicles. Therefore, in first instance the incorporation of lipases in vesicles was investigated, and the effect of this incorporation on enzymatic activity was shown. In chapter 3 the activity of free lipase from *Candida cylindracea* (CCL) is compared with CCL incorporated in polymerisable positively charged quaternary dialkyl ammonium surfactant vesicles for a variety of glyceryl and 2,4-dinitrophenyl ester assays. The effects of several surfactant addenda on lipase activity are studied.

Lipases have not been incorporated earlier into polymerisable vesicles, in spite of the improved stability of polymeric vesicle systems. Chapter 4 focuses on the stability of CCL incorporated in polymerisable vesicles composed of zwitterionic vesicles. The stability of the incorporated lipase is investigated for a number of protein denaturing conditions.

Important aspects in studies concerning the incorporation of proteins in vesicles are the characterisation of the interaction between enzyme and vesicle during the incorporation process and the determination of the location of the incorporated enzyme. Fluorescence techniques are very suitable for elucidating protein-vesicle interactions. In chapter 5, the interaction of lipase with the vesicle membrane is emphasised, based on fluorescence energy transfer and fluorescence anisotropy studies.

For the application of lipases, a reliable method for the determination of enzymatic activity is required. Chapter 6 describes a new lipase assay system based on the spectrophotometric detection of a variety of newly synthesised 2,4-dinitrophenyl esters.

In chapter 7 the results of the preceding chapters are reviewed in the summarising discussion.

The list of abbreviations and symbols, the list of references and the abstract are appended to the thesis.

1 TRIACYLGLYCEROL LIPASES

Keyword phrases:

MODE OF ACTION OF LIPASES, INTERFACIAL ACTIVITY AND STRUCTURAL ASPECTS

REACTION SYSTEMS FOR LIPASES

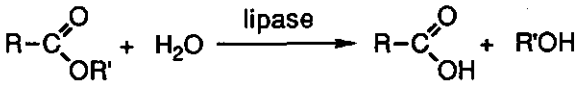
LIPASES AND AMPHIPHILES

APPLICATIONS OF LIPASES

1.1 INTRODUCTION

Enzymes that catalyse the hydrolytic cleavage of organic compounds are called hydrolases. According to the rules of IUPAC-IUB Commission on Nomenclature [106], hydrolases are listed under the code EC 3. Further subdivision of hydrolases is first based on the nature of the bond hydrolysed and then on the nature of the substrate.

The enzyme used in this thesis is a triacylglycerol lipase (EC 3.1.1.3), which belongs to the class of carboxylic ester hydrolases. The (bio)chemical reaction in which carboxylic ester hydrolases are involved, is the hydrolysis of an ester into a free fatty acid and an alcohol:



In accordance with the IUPAC-IUB rules, triacylglycerol lipases, mostly referred to as lipases, are listed as enzymes that catalyse the reaction:

$$\text{triacylglycerol} + H_2O \longrightarrow \text{diacylglycerol} + \text{fatty acid}$$

Lipases are found in all organisms: they are identified in mammals, plants and micro-organisms. Lipases play a key role in the lipid metabolism of the organism. In general terms, they are necessary for the lipid transfer from one organism to the other. Within organisms, lipases play an important role in the deposition and mobilisation of fat, an important energy source. In addition, they are involved in the metabolism of intracellular lipids and therefore, in the functioning of biological membranes [16,25,47].

Although the natural substrates for lipases are triacylglycerols, their catalytic action is not restricted to these compounds: also diacylglycerols, monoacylglycerols and a broad variety of other esters [14,16,25,39,47,64] ranging from simple carboxylic acid esters [31,32] or carbohydrates [132,221] to tricyclic acetates [55,181] can be hydrolysed.

Apart from a broad substrate specificity, lipases show regio- and stereoselectivity for different alcohol or (fatty) acid moieties or combinations of these selectivities. Jensen et al. [111,112], Bariszlovich et al. [6] and Sonnet [227] have used these parameters in order to define a practical approach for the determination of lipase selectivity for acylglycerols. The selectivity of lipase from *Candida cylindracea*, the enzyme that is used in this thesis, is illustrated on the basis of some examples in table 1.1.

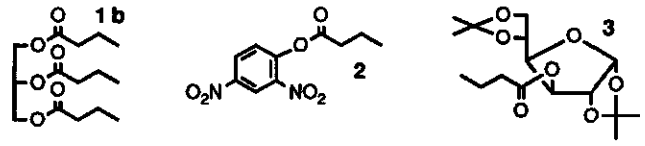
Lipases are generally water soluble. Most natural substrates of lipases on the other hand, are not soluble in aqueous environment and therefore, the reaction system is heterogeneous. Consequently, lipases have evolved to enzymes that perform their catalytic action at the substrate-water interface. The physical state of lipase substrates in aqueous environment - soluble or insoluble - is of great importance in lipase reactions (see next paragraph for a detailed discussion). In order to indicate the difference in the physical state of

substrates, the terms esterolytic and lipolytic activity are sometimes used to refer to the hydrolysis of soluble respectively insoluble substrates.

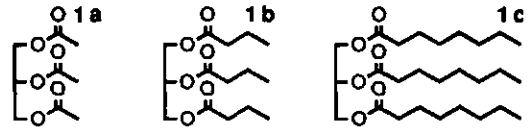
Table 1.1 Selectivity of lipase from *Candida cylindracea*, in hydrolytic reactions.

Substrate selectivity (hydrolysis of different types of substrates)

Same carboxylic acid, different alcohol moiety (**1b**, [168]; **2**, [169]; **3**, [132])

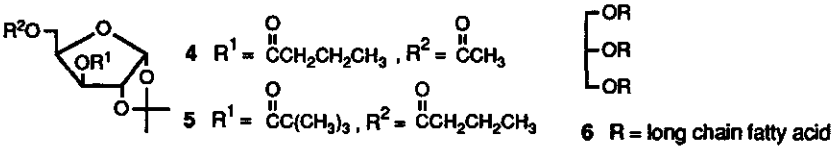


Same alcohol, different carboxylic acid moiety (**1 a-c** [168])



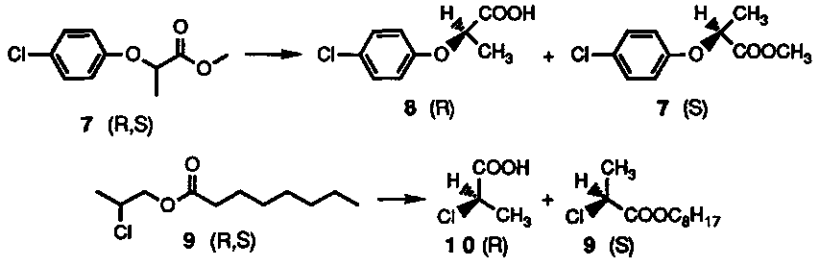
Regioselectivity

Hydrolysis of primary esters (**4**, [132]),
secondary esters (**5**, [132]) or
all esters (nonselective: **6**, [10])



Stereoselectivity

Stereoselective hydrolysis (**7**, [31]; **9**, [32])



1.2 INTERFACIAL ACTIVITY OF LIPASES

Since lipases hydrolyse carboxylic ester bonds, they can in this respect be considered as carboxylesterases (EC 3.1.1.1). However, the fundamental difference between carboxylesterases and lipases lies in the physical state of the substrate. Whereas carboxylesterases only hydrolyse solubilised substrates, i.e. esterolytic activity, lipases perform their catalytic action preferably at a substrate-water interface, i.e. lipolytic activity.

Already in 1936 the importance of the substrate interface for lipase action was noted by Holwerda et al. [97]. In their work concerning the hydrolysis of triglycerides by pancreas extract they stated: "... *Wir haben in exakten Versuchen zeigen können, dass der Verteilungszustand von flüssigen Triglyceriden im allgemeinen von grossem Einfluss auf ihre Verseifungsgeschwindigheid unter Einfluss von Pankreaslipase ist. (...) Es ist erforderlich dafür zu sorgen, dass die Grenzfläche Lipid-wässerige Phase in bezug auf die benutzte Menge Pankreasextrakt relativ hinreichend gross ist und bleibt. ...*". They found, among other things, that by simply increasing the shaking speed for a mixture of 0.4 mM tricaprylin and pancreas lipase, the amount of fatty acid hydrolysed in 20 minutes was raised more then twenty-fold.

However, it lasted until the fifties and sixties before this phenomenon was studied in more detail. The first attempts to characterise lipase activity were made by the group of Desnuelle [8,58,213]. They investigated the hydrolytic activities of horse liver esterase and porcine pancreas lipase at various triacetin concentrations, below and beyond the solubility limit.

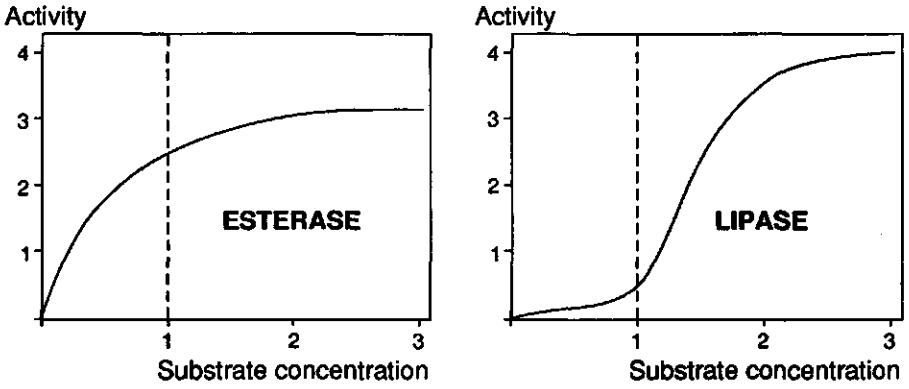


Figure 1.1 Hydrolysis of triacetin by horse liver esterase and porcine pancreatic lipase. Enzymatic activities are given as a function of substrate concentration, which is expressed in multiples of the saturation concentration (dashed vertical line) [213].

The esterase displayed normal Michaelis-Menten kinetics in the concentration range where the substrate is completely soluble. On the other hand, lipase showed almost no activity as long as triacetin was in its dissolved state. However, when the substrate concentration was increased beyond the solubility limit, a sharp increase in enzyme activity was observed (figure 1.1).

Apparently, the esterase is active on monomolecularly dispersed substrate molecules, whereas lipase clearly needs a substrate interface for attaining maximal activity. Thereupon, Benzonana and Desnuelle [8] defined lipase reaction kinetics. By using two emulsions with substrate droplets of different size, they showed that the reaction rate does not depend so much on the concentration of the substrate as on the area of its interface. Lipase kinetics could now be characterised by replacing normal units of concentration (g/l or mole/l) by 'interfacial concentration', i.e. the interfacial area of the substrate per volume unit (m^2/l).

A few years later, it was recognised that the catalytic action of lipases is basically a two-step process, quite distinct from esterases [22,24,27,250]. First the water soluble enzyme E is activated by binding to the (substrate) interface ($E \leftrightarrows E^*$). Through binding, the specific orientation of the activated enzyme E* at the interface makes the active site accessible for the substrate. The second step is the actual binding of a substrate molecule to the active site of the enzyme ($E^* + S \leftrightarrows E^*S$). Once the enzyme-substrate complex E*S is formed, the actual catalysis takes place: product P is formed and the enzyme is regenerated in its activated form ($E^*S \rightarrow E^* + P$). Figure 1.2 illustrates this model for the action of a soluble enzyme at an interface, which was proposed for the first time by Verger et al. [250]. Although in first instance, this model was defined for phospholipase A_2 (EC 3.1.1.4), it also appeared applicable for triacylglycerol lipases [248,249].

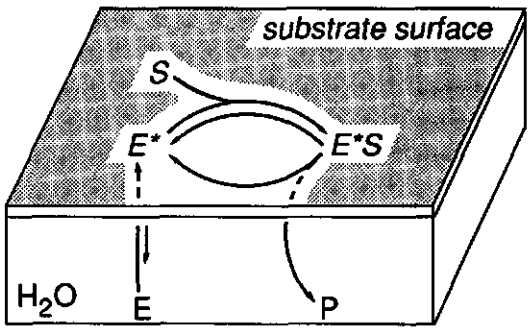


Figure 1.2. Model for the interaction of lipase at a substrate interface (adapted from Verger et al. [250]).

The importance of the first step in lipase action - the adsorption of enzyme to the substrate interface - is nicely demonstrated by pancreatic lipases under physiological conditions. Pancreatic lipases are inhibited by amphiphilic substances like bile salts. This inhibition is caused by the desorption of the enzyme from the substrate interface into the aqueous bulk phase. However, colipase can prevent or overcome inhibition by bile salts. Colipase, a polypeptide cofactor, facilitates the (re-)adsorption of the enzyme to the surface of an emulsified substrate [15,17,18,23]. In this way, the pancreatic lipase is anchored to the substrate surface and hydrolysis of the substrate can proceed.

1.3 STRUCTURAL ASPECTS OF LIPASE ACTION

Knowing the general mode of action of lipases, it is interesting to focus on a description of the action of lipases on the molecular level. In the early seventies, Sémériva et al. [219] and Brockerhoff [24] suggested that lipase was a serine hydrolase with an active site similar to that of chymotrypsin. However, complete insight in the three-dimensional structure and the anatomy of the active site could not be obtained until sequence and X-ray crystallographic data of lipases became available. Up to now, the complete X-ray structure of the lipases of human pancreas [257], *Rhizomucor miehei* [19] and *Geotrichum candidum* [216] have been published. The amino acid residues in the active site responsible for the catalytic action are Ser-His-Asp or, in the case of *Geotrichum candidum* lipase, Ser-His-Glu. These residues form a special spatial arrangement, the so called catalytic triad, which enables the serine residue - the actual chemical operator - to attack the carbonyl group of the substrate (figure 1.3). In this way, an acyl-enzyme intermediate is formed and the alcohol moiety (R"-OH) of the substrate is liberated (step 1). Subsequently, the acyl-enzyme intermediate can be (hydro-)lysed by the attack of a nucleophile (Nu). This regenerates the enzyme and, in the case of aqueous solutions where water is the nucleophile, releases a carboxylic acid (R'-C(O)-OH).

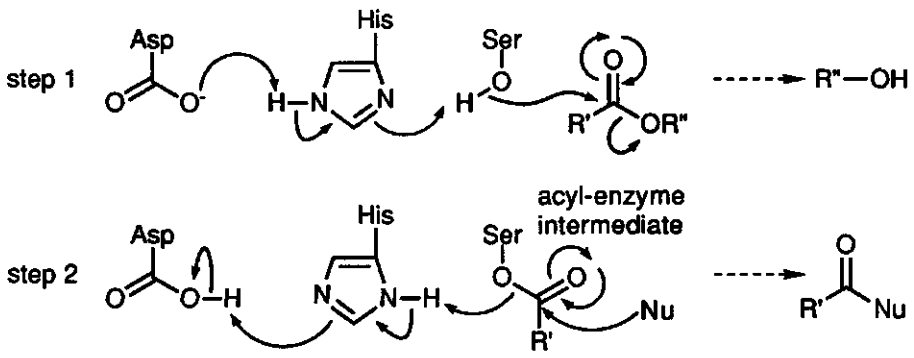


Figure 1.3 The molecular mechanism of lipases.

A comparative analysis of some twenty lipases from different sources showed that these lipases have structural similarities [134]. In all cases the active site is not exposed to the solvent but it is buried in the core of the enzyme and covered with a peptide loop, often referred to as 'flap' or 'lid'. When the lipase adsorbes to a (substrate) surface, a rearrangement process takes place in which the flap is displaced. In this process, the hydrophilic part of the flap becomes partly buried and is anchored to the main domain of the protein. The hydrophobic part of the flap then becomes completely exposed, thereby increasing the apolar surface around the active site [29,45,90]. Thus, interfacial activation occurs by displacement of the peptide loop; this movement exposes the catalytic groups and creates an apolar surface around the active site which stabilises the contact between enzyme and substrate interface. A schematic representation of this process is depicted in figure 1.4.

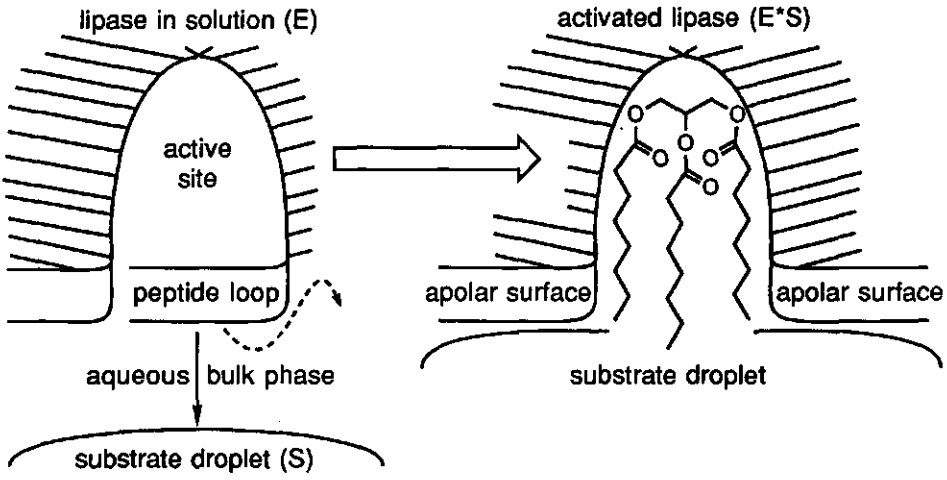


Figure 1.4 Diagram of the interfacial activation of lipase. This diagram shows only the active site of enzyme.

1.4 REACTION SYSTEMS FOR LIPASES

As described in the previous paragraphs, lipases perform their catalytic action preferably at a substrate-water interface. Two major factors that influence lipase activity are (i) the amount of substrate molecules at the interface (surface quantity) and (ii) the nature or structural properties of the interface (surface quality). Many different types of reaction systems have been developed for lipases (figure 1.5), in order to control the surface quantity and/or surface quality.

In order to improve enzyme performance, several possibilities for the formulation of the substrate are advanced. The least complicated system is a substrate that is completely dissolved in water. However, in these type of homogeneous systems lipase activity is low due to the lack of an interface (see section 1.2). The activity of lipase on soluble substrates can be enhanced by the addition of small amounts of water miscible organic solvents, but the effect is relatively small. Entressangles and Desnuelle have reported an increase in activity of maximal 6% in case of the addition of 2 mole % dioxane in the hydrolysis of tripropionin [59]. Moreover, the reaction rates for water soluble substrates can be enhanced by the addition of a (non-substrate) interface, e.g. siliconised glass beads [27], nonpolar organic solvents [244] or amphiphiles [174].

In the case of a reaction system consisting of an emulsified substrate, the interfacial area of the emulsion is of great importance. This interfacial area, or interfacial concentration, can be quantified by determination of the droplet size distribution of the heterogeneous reaction system. Droplet size distributions can be measured with microscopic techniques [207], or by the use of a coulter counter [8,180] or an optical particle sizer [123]. However, for emulsions that merely consist of substrate, the stability and consequently the maximal surface is limited. Emulsifiers like polyvinyl alcohol [236,262] or gum arabic [167] are

therefore often used to stabilise substrate emulsions for the only purpose of obtaining maximal activity.

Several other useful strategies have been developed for studying lipase reactions, making use of their interfacial activity. These strategies include enzymatic reactions in microemulsions (or reversed micelles) [96,122,157,158], and in two phase systems [56,244]. Basically, these media are composed of a mixture of an aqueous phase, containing the enzyme, and an organic phase in which the substrate is solubilised. The enzymatic reaction takes place at the interface of the phases. In the case of microemulsions, the aqueous phase consists of small discrete water droplets that are stabilised by amphiphiles. Two-phase systems lack amphiphiles and the maximum contact surface of lipase solution and substrate solution is only preserved by constant agitation.

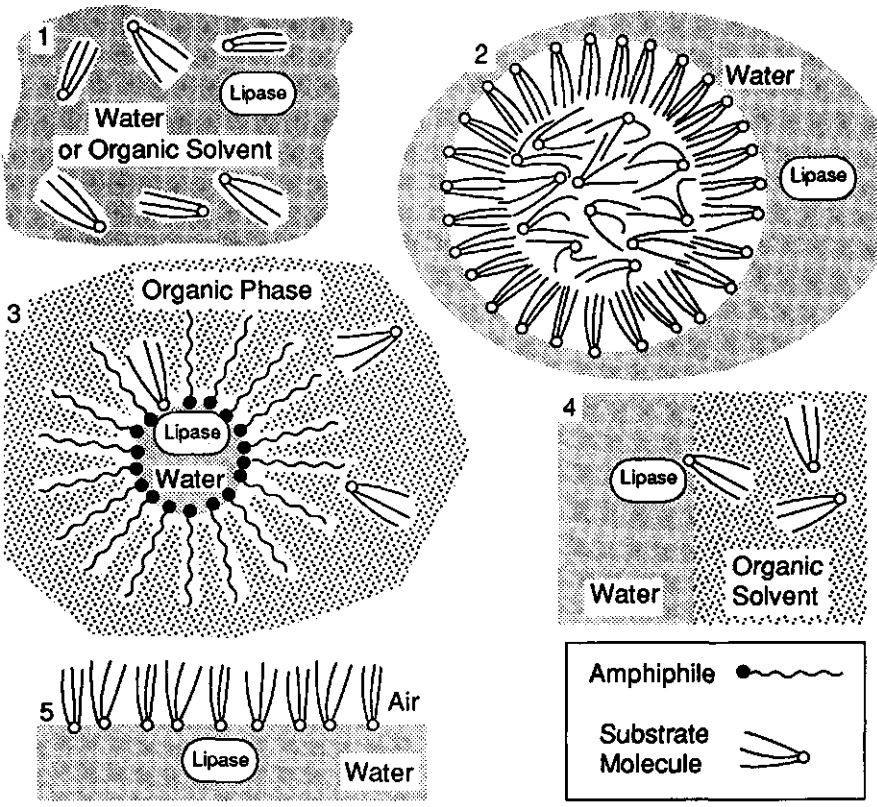


Figure 1.5 Different reaction systems for lipases: 1. solubilised substrate, 2. emulsified substrate, 3. microemulsion/reversed micelle, 4. two-phase system, 5. monomolecular film.

Since it has been demonstrated that enzymes are able to work in organic solvents with low water content [33], lipases are successfully employed as biocatalysts in organic media [39,51,129,266]. The substrates are fully dissolved, whereas lipases are insoluble in most organic solvents. Thus, the enzyme is suspended in the organic medium and therefore, the reaction system is heterogeneous. The solid-liquid interface of enzyme and substrate is

responsible for the activity of the enzyme. In many cases, lipases are found to have an altered selectivity and specificity compared to aqueous systems and they display an exceptional stability in anhydrous media [127,264,265].

The monomolecular film technique [28,193] and membrane reactors [92,198] are very useful for studying lipase kinetics. In the monomolecular film technique a monolayer of substrate molecules is created at a gas-liquid interface, which allows a very accurate monitoring and controlling of the interfacial quality. Membrane reactors have also well defined interfacial characteristics. The principle of the reactor is based on the separation of two different phases, e.g. an organic phase containing the substrate and an aqueous phase for the removal of products, by the use of a porous membrane. Membrane reactors have the additional advantage that the enzyme is immobilised on the reactor-membrane, which allows the application for a long period in consecutive reaction cycles. In general, immobilisation is a useful tool in enhancing the operational stability and the lifetime of lipases [155].

Other methods that improve the performance of the enzyme are based on chemical modification of the enzyme, e.g. by polyethylene glycol [7], or by modification of amino acid residues with chemical reagents like tetranitromethane [89] or *N*-(4-azidobenzoyl)oxysuccinimide [118]. In some cases the simple addition of a chemical compound to the reaction mixture is already sufficient to influence the enzyme performance. The rate of hydrolysis can be improved by the addition of carbohydrates [211] or cyclodextrins [40]; chiral amines are reported to improve the enantioselectivity of lipase systems [93]. The role of amphiphiles is discussed in the next paragraph.

1.5 LIPASES AND AMPHIPHILES

In lipase reaction systems, the effects of many additives have been investigated, including amphiphiles. The effects of these surface active agents (surfactants) however, are not always unequivocal and sometimes difficult to interpret, because they can interact with many parts of the system. Surfactants interfere with the substrate surface and can affect both surface quantity and quality. Furthermore, they can interact with the product(s), thereby affecting the surface quality. Finally, surfactants can directly bind to the enzyme. In this case, the influence of the amphiphile depends on its concentration (relative to the critical micelle concentration), on structural properties (e.g. enzyme structure, chain length of the amphiphile) and on environmental factors (ionic strength, temperature, pH and, consequently, charge of the enzyme). An overview of the effects of amphiphiles is given in table 1.2.

When amphiphiles, like bile salts or polyoxyethylenes (nonionic), ammonium salts (cationic) or sulphates (anionic), are added to the reaction medium prior to the administration of the enzyme, they inhibit in most cases the activity. This is the result of accumulation of these surfactants at the substrate surface (pollution of the interface) [15,34,119,174,240]. This prevents the enzyme from reaching the substrate molecules and/or causes desorption of the enzyme from the interface.

Table 1.2 Effects of amphiphiles on lipases and lipase reaction systems.

target	effect	result
substrates	- accumulation at the interface	- (mostly) decrease in activity
products	- desorption products from interface, and/or solubilisation	- increase in activity
enzyme	- binding	- increase in activity and/or in enantioselectivity
	- binding and unfolding of the native conformation	- deactivation

However, surface active additives can also prevent inhibition that is caused by formation of some fatty acids during hydrolysis. These fatty acids can be solubilised by amphiphiles [9], or can be accommodated in the surfactant core of liposomes [208] or micelles [161] and thus prevent their accumulation at the substrate surface.

Contact between surfactant and lipase before addition to the substrate, inactivates the enzyme in most cases. For lipases, the effect is reported to be strongest when cationic amphiphiles are applied [74,174,215,232,240,251]. In general, ionic amphiphiles are more effective denaturants than nonionic amphiphiles as a result of both electrostatic and hydrophobic interactions. The denaturation process is initiated, preferentially by electrostatic interactions, leading to (partly) unfolded protein conformations. This induces previously buried hydrophobic protein residues to become exposed and thus available to further amphiphile binding, which finally results in denaturation of the enzyme [214,233,252]. However, the application of amphiphiles to enzyme preparations does not necessarily lead to denaturation. On the contrary, treatment of lipase with phospholipids or deoxycholate is reported to yield stable lipid-lipase complexes which display enhanced activity [107,108,261] or enhanced enantioselectivity [5,182,261].

Despite the variable effects that surface active additives can have on lipases and on their reaction systems, in many applications amphiphiles are successfully employed, e.g. as emulsifiers or in reversed micellar systems.

1.6 APPLICATIONS OF LIPASES

The structure of a chemical compound and, especially in the case of lipases, its formulation determine whether a compound is accepted as a substrate. Furthermore, the specificity of an enzyme depends on the stereochemical architecture of its active site. For the application of lipases, the choice of the enzyme source is important, since lipases from different sources have different steric characteristics. The cavity of the active site in lipases from *Aspergillus* species for example, is larger than that of *Pseudomonas* and *Mucor* species, whereas the size of the active site cavity in *Candida* lipase is in between. As a consequence, *Aspergillus* lipases are better suited for hydrolysing bulky esters

[64]. Insight in the steric requirements of the lipase allows the prediction of its reactivity (figure 1.6).

For predetermining the selectivity of lipases, several models have been developed. Kloosterman et al. [131] and Hultin and Jones [102] have developed active site models for *Pseudomonas fluorescens* and porcine pancreatic lipase. Their models describe the steric constraints in the active site, with the aim of anticipating whether a compound is accepted as a substrate. Kazlauskas et al. have analysed the conversion of a range of different substrates and they were able to define rules to predict the enantiomeric preference of lipases from *Pseudomonas cepacia* and from *Candida cylindracea* for esters of secondary alcohols [120]. These lipase models may be helpful in designing strategies for the application of lipases in organic synthesis.

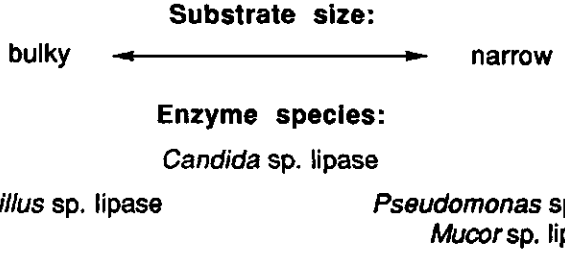


Figure 1.6 Steric requirements of lipases [64].

The broad substrate specificity makes lipases usable in a wide field of applications and their market is still growing [13,113,153,170,218]. Lipases are used in the dairy and food industries for cheese ripening [46], the production of flavours [53,167] and the hydrolysis [150,199] or interesterification [149,173,194] of oils and fats [228]. In addition, they are used in detergents and laundry cleaning systems [50,57,78,235], in the production of (biodegradable) surfactants [12,128] and for analytical purposes [136,245]. One of the most promising fields of application is in the production of optically active agrochemicals and pharmaceuticals [117,130,131]. Lipases act, most often, in a highly selective manner. It is therefore possible to catalyse a large number of useful reactions in preparative organic chemistry by using different lipases, each with its own specific selectivity [14,39,64].

2 VESICLES

Keyword phrases:

SURFACTANTS AND SURFACTANT ASSEMBLIES

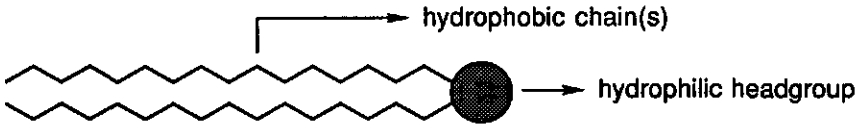
VESICLES: MORPHOLOGY AND PREPARATION

STABILISATION OF VESICLES BY THE USE OF POLYMERISABLE SURFACTANTS

USE AND APPLICATION OF VESICLE SYSTEMS

2.1 SURFACTANTS

Surfactants owe their name to their characteristic behaviour at interfaces: they strongly adhere to them and reduce surface tension. The word surfactant is a contraction of the term surface active agents. These substances possess a characteristic chemical structure with two distinct regions, *viz.* a hydrophilic and a hydrophobic part. The hydrophilic part comprises ionic or highly polar groups, whereas the hydrophobic moiety generally consists of one or more apolar aliphatic chains [165,172]. The dual polarity combined in one molecule is expressed with the term amphiphilic. The general structure of amphiphiles is often depicted as a globule attached to one or two chains:



The most useful classification of surfactants is based on the nature of the headgroup moiety. The four basic classes are: anionic, cationic, zwitterionic and nonionic surfactants. Some examples of surfactants are depicted in figure 2.1.

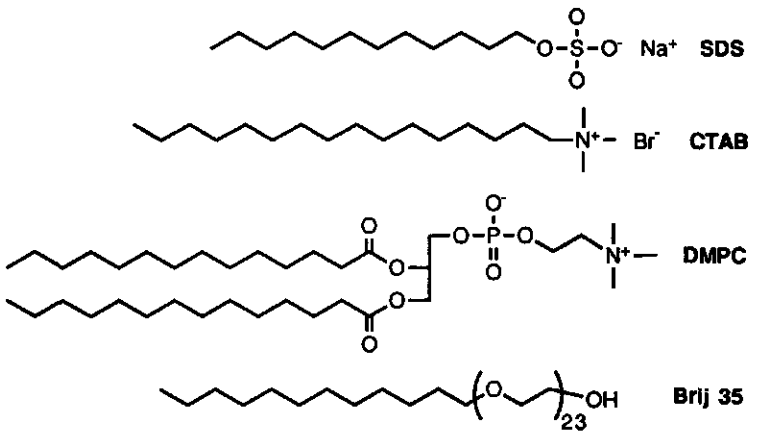


Figure 2.1 Examples of an anionic, cationic, zwitterionic and nonionic surfactant: SDS, sodium dodecyl sulphate; CTAB, cetyltrimethylammonium bromide; DMPC, dimyristoyl phosphatidylcholine; Brij 35, polyoxyethylene(23) lauryl ether.

2.2 SURFACTANT ASSEMBLIES

In aqueous environment, the amphiphilic nature of the surfactant is responsible for the association of the molecules into a structured orientation. The hydrophilic groups interact with water, and the hydrophobic regions are

directed away from the bulk aqueous phase. Hydrophobic interactions between the apolar chains are the major driving force in the phenomenon of (self-)association of amphiphiles. Furthermore, association is attended with a large increase in entropy as the result of the release of water molecules from the hydrophobic chains. In addition to these effects, Van der Waals forces between adjacent surfactant tails, and electrostatic and hydrogen bonds between surfactant headgroups and the surrounding aqueous environment stabilise the derived amphiphile assemblies [11,62,105]. This leads to several distinct geometrical surfactant aggregations (figure 2.2). The form of the aggregate strongly depends on the molecular shape and concentration of the surfactant and on environmental parameters, like electrolyte concentration, pH, and temperature [67,154,191].

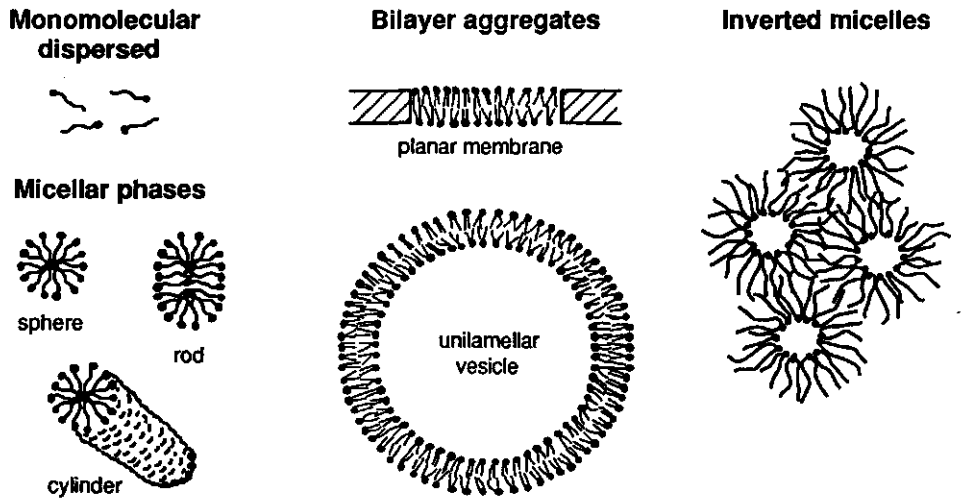


Figure 2.2 Amphiphiles can associate into a variety of structures in aqueous solutions, which can be transformed in one to another by changing the solution conditions.

Israelachvili has defined geometrical factors that control the packing of surfactants into aggregates [103,104]. The volume of the hydrophobic part v, the optimal headgroup area a_o and the critical length l_c of the hydrophobic chain[1] are formulated in the critical packing parameter:

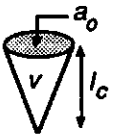
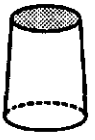
$$\text{critical packing parameter} = \frac{v}{a_o * l_c}$$

A summary of the structures, which are expected from molecules with increasing values of the critical packing parameters, is presented in table 2.1. Amphiphiles with relatively large headgroup areas and small hydrocarbon volumes, as in molecules with a single hydrocarbon chain, assemble into micellar aggregates. Amphiphiles with two (or three or four) hydrocarbon chains are too bulky to pack into such small micellar aggregates and they will

[1]The maximal extent to which the chain can be stretched out.

predominantly form bilayers. The shape of bilayers may be planar or spherical, in which latter case the bilayer structure is referred to as vesicle.

Table 2.1 Effect of the critical packing parameter on the association behaviour of surfactant structures in aqueous solutions. Adapted from references [104] and [172].

critical packing parameter $v/a_o l_c$	surfactant type	critical packing shape	expected aggregate shape
< 0.33	single chained surfactants with large head groups		cone: spherical micelles or ellipsoidal micelles
0.33 - 0.5	single chained surfactants with relatively small head groups		truncated cone: large cylindrical or rod-shaped micelles
0.5 - 1.0	double chained surfactants with relatively large head-groups and flexible chains		truncated cone: vesicles or flexible bilayer structures
~ 1.0	double chained surfactants with small head groups or rigid chains		cylinder: planar extended bilayers
> 1.0	double chained surfactants with small head groups and very bulky chains		inverted truncated cone: reversed micelles or inverted micelles

2.3 BILAYER FORMING LIPIDS AND SYNTHETIC SURFACTANTS

Bilayer membranes can be constituted from a broad variety of surfactants, or lipids in the case of naturally occurring amphiphiles. Nature distinguishes four major classes of lipids [81] (figure 2.3). Phosphoglycerides (i) are the most commonly found biomembrane lipids. They consist of a glycerol moiety linked to a polar phosphate-containing group and two hydrophobic chains. A representative example in this class is dimyristoylphosphatidylcholine, a phosphatidylcholine. The major backbone of phosphosphingolipids (ii), the second class, consists of a sphingosine moiety. One of the hydroxyl groups in sphingosine is derivatised with a phosphate containing group and the amino

group of sphingosine is coupled with a carboxylic acid residue. Sphingomyelin is an example in this lipid class. In the case of glycolipids (iii), the polar phosphate group in the phosphoglyceride or phosphosphingolipid is replaced with a sugar residue as is shown in glucocerebroside, a glucose-derivatised sphingolipid. The fourth major group of natural surfactants is formed by the sterols (iv), of which cholesterol is the most common.

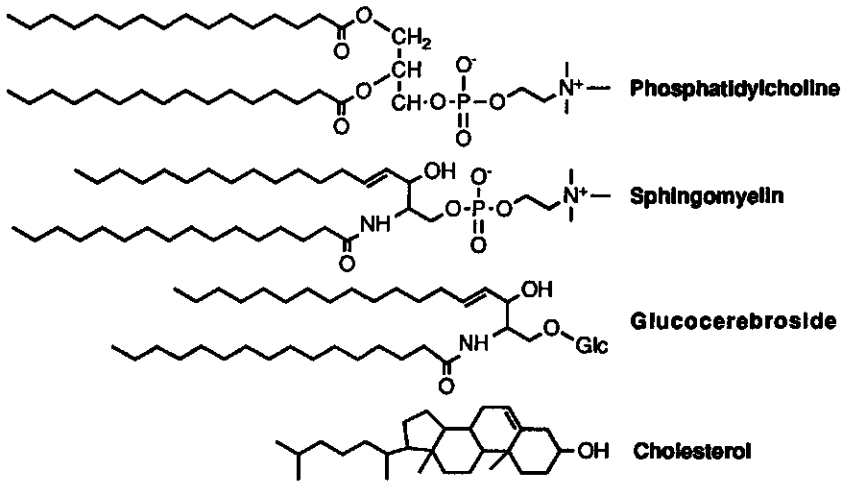


Figure 2.3 Four examples of lipids, one from each major lipid class.

In the late seventies it was reported that membrane structures could also be formed from compounds other than lipids. Kunitake and Okahata [141,142] showed the first example of a totally synthetic bilayer membrane. The surfactant they used was didodecyldimethylammonium bromide (DDDAB; figure 2.4, **1**).

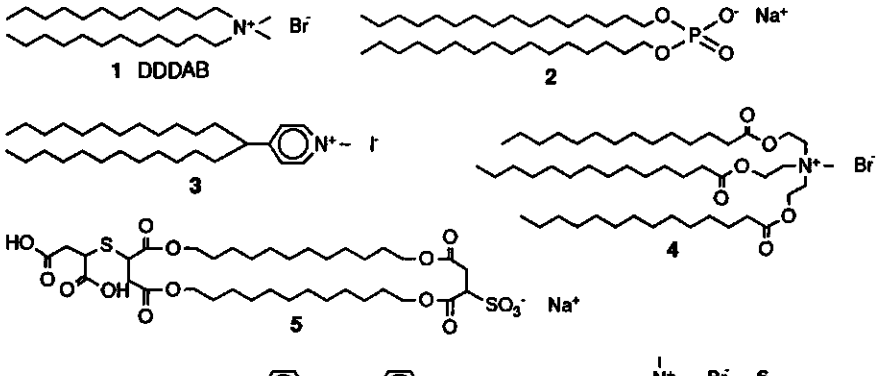


Figure 2.4 Some examples of synthetic amphiphiles that can form vesicles in aqueous solution (**1**, [142]; **2**, [166]; **3**, [230]; **4**, [138]; **5**, [76]; **6**, [139]).

The authors recognised the importance of this finding by their statement that this system could be used "... apart from its relevance to the physical chemistry of biomembranes, for preparing well-defined molecular organisations which possess various functions. ..."Ever since, many different types of amphiphiles have shown to yield vesicles. A few examples of vesicle-forming molecules are listed in figure 2.4: surfactants with two alkyl chains containing positively charged ammonium (1) or pyridinium (3) headgroups, negatively charged phosphate groups (2), surfactants with three alkyl chains (4), bola-formed or membrane-spanning surfactants (5) with two hydrophilic groups attached to a double-chained hydrophobic moiety, and amphiphilic structures with one alkyl chain in which a rigid chromophore is inserted (6).

2.4 VESICLES: MORPHOLOGY AND PREPARATION

The term vesicle is derived from the Latin word *vesicula*, which means hollow globule. Indeed, vesicles are spherical bilayer surfactant aggregates that enclose an amount of the aqueous phase in which they were formed. In the case of naturally occurring surfactants, lipids, spherical assemblies are mostly referred to as liposomes. The size of the vesicles can vary from 20 nm to several μm. The thickness of its bilayer ranges from 4 to 6 nm, depending on the length and bulkiness of the hydrophobic part of the surfactant.

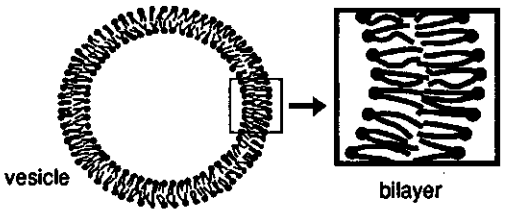


Figure 2.5 Representation of a unilamellar vesicle.

Mainly three types of vesicles are distinguished, as is depicted in figure 2.6. Structures with multiple bimolecular lamellae are called multilamellar vesicles (MLV) and their size varies from 100 nm to several μm. Unilamellar vesicles, which consist of only one bilayer, are subdivided into small unilamellar vesicles (SUV) with sizes ranging from 20-100 nm and large unilamellar vesicles (LUV) with sizes from 100 nm to several μm. Size determination of vesicles can be performed by electron microscopy, gel filtration, light scattering techniques, and density centrifugation [91,217,260].

Vesicles can be prepared by different procedures [66,84,163,231]. The method of preparation determines for a great deal the size, size distribution and morphology of vesicles [66,81,231,260].

The type of vesicles which can be obtained by the different methods of preparation are listed in table 2.2. The most straightforward method is sonication of a surfactant dispersion, either by a probe or a bath-type sonifier, or extrusion of aqueous surfactant dispersions through porous membranes or a small orifice.

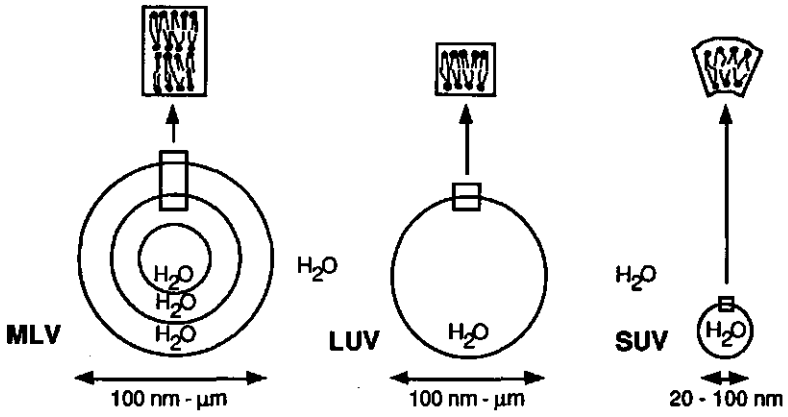


Figure 2.6 Schematic drawing of the three distinct vesicle types (from [191]).

The clusters of surfactant molecules are disrupted, the water layer around the hydrophobic tails is removed and the surfactants can aggregate into organised structures. The stability of these structures is the result of the increase in entropy due to the release of water molecules during aggregation and of hydrophobic interactions between the amphiphile chains [61,62]. These favourable effects nullify the unfavourable head group repulsion.

Table 2.2 Methods of preparation of surfactant vesicles and some characteristics.

Method of preparation	Type of vesicle[1]	Remarks
Sonication	SUV/LUV	uniform size distribution, fast
Extrusion		defined size, uniform size distribution, fast
Porous membranes	MLV	
French press (small orifice)	SUV / LUV	
Detergent dialysis or dilution	LUV (SUV)	elaborate
Hydration of dried lipid film	MLV / LUV	easy, non-uniform size distribution
Solvent vaporation and REV[2]	LUV	non-uniform size distribution
Injection method		fast, non-uniform size distribution
aqueous in organic	MLV	
organic in aqueous	SUV / LUV	
Fusion methods	SUV	elaborate

[1] LUV = Large unilamellar vesicles; MLV = multilamellar vesicle; SUV = Small unilamellar vesicle
[2] REV = reverse phase evaporation vesicles

Other methods involve the use of detergents or organic solvents. Vesicle forming surfactants, which are poorly soluble in water, can be co-solubilised by detergents. Removal of the detergent by dialysis or gel filtration yields vesicles. The surfactants can also be solubilised in an organic solvent. Removal of the solvent by a rotary evaporator yields a film of amphiphiles on the wall of the

flask and subsequent hydration of this film results in the formation of vesicles. In addition, a surfactant solution in organic solvent can be injected into (heated) water or vice versa. In any of these cases, the aggregation into vesicles is initiated by the organisation of the amphiphilic molecules on the water/organic phase interface. Preparation of reversed phase evaporation vesicles (REV) is based on the same principle. In this case, the organic phase, containing the surfactant, is removed under reduced pressure from a water-organic phase emulsion.

2.5 POLYMERISABLE VESICLES

Vesicles tend to aggregate spontaneously and they fuse under the influence of fusogenic agents such as bivalent ions. This causes changes in size and size distribution and can ultimately lead to flocculation [210]. In addition, the administration of several addenda, like salts or proteins, may destabilise the membrane structure. However, studies in which vesicles are applied, often require systems with a well-defined morphology and a long-term stability [187]. Therefore, strategies for membrane stabilisation had to be developed. Polymer science offers elegant means to tackle the problem of a limited stability [206].

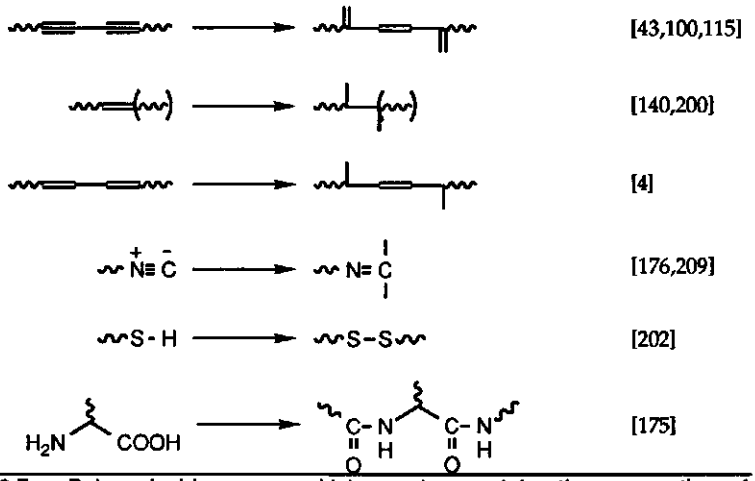


Figure 2.7 Polymerisable groups which can be used for the preparation of polymerisable amphiphiles.

The introduction of a polymerisable group into the amphiphilic molecule allows the linkage of the membrane components. Consequently, the vesicle structure is 'zipped up' and therefore becomes preserved. Moreover, the enhanced stability is accompanied by controllable particle sizes and enhanced membrane rigidity [68,114,186,205]. The first examples of these type of amphiphiles were introduced in the early eighties by several groups [98,115,143,179,200,242]. The tools that polymer science offer are versatile, as illustrated in figure 2.7.

Two approaches have been used to construct polymeric vesicles. On the one hand, amphiphiles can be aggregated and subsequently, the polymerisable groups in the organised vesicle structures can be polymerised (figure 2.8, **1, 2**). On the other hand the concept is reversed; first the surfactants are polymerised and then these (pre)polymers are vesiculated (**3**). In the former case the polymerisable group may be either fixed to the headgroup (**1**) or can be inside or at the end of the aliphatic chains of the surfactant (**2**). In the latter case, the polymerisable group should be attached to the headgroup area, mostly by the use of spacers or co-monomers (**3**). Apart from these approaches however, there is an alternative method in which the polymerisable group is not part of the surfactant itself. These monomeric vesicles can be stabilised by applying a polymer coating or polymer framework. This can either be achieved by the use of polymeric counter ions (**4**) or polymer chains that contain hydrophobic anchor groups (**5**) which penetrate into the vesicle bilayer. The concept of inserting stabilising groups into the membrane can be extended by combining monomeric surfactants with bipolar membrane-spanning surfactants (**6**). The different strategies for stabilising vesicles are depicted in figure 2.8.

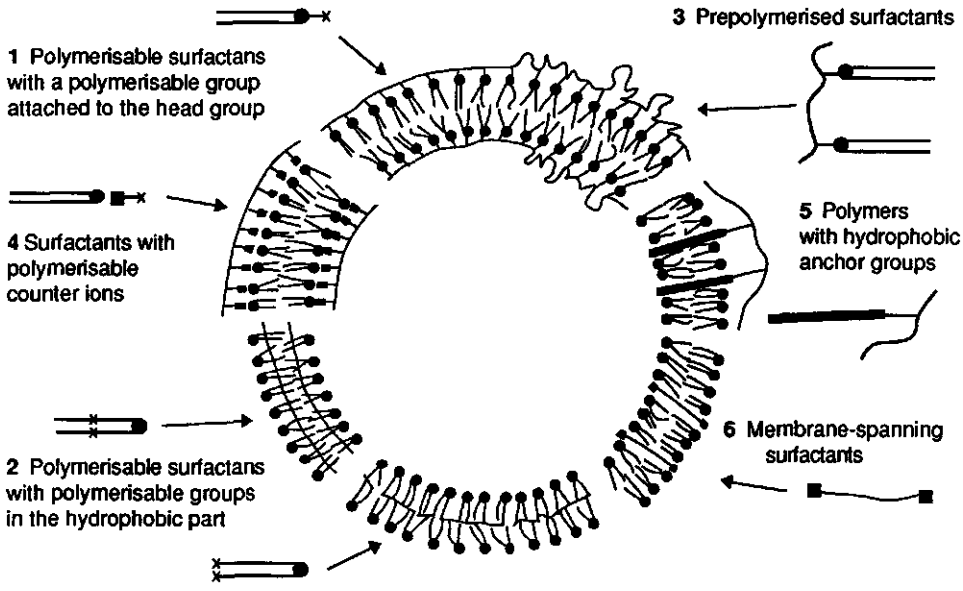


Figure 2.8 Different strategies for the stabilisation of vesicles. The polymerisable group is indicated as 'x'. Adjusted from [205].

Covalent linking of the constituents of the vesicle membrane indeed markedly improves its stability. The shelf-life of polymeric vesicles can be extended to several months compared to several hours or days for their nonpolymerised analogues. Although polymerisation of vesicles mostly takes place without morphological changes [72], the state of the membrane is affected. The packing density of the vesiculated surfactant molecules changes and herewith the properties of the membrane bilayer. Linkage of polymerisable groups attached to the headgroup exhibits a decrease in the

surface area per molecule compared to monomeric amphiphiles [52], and clefts are formed between the polymeric surfactant strands [177]. However, the fluidity or phase transition[2] of the vesicle membrane is not drastically altered by surface polymerisation, since the hydrophobic interior of the bilayer remains unchanged. In contrast, vesicles in which the hydrophobic core is polymerised lack phase transition due to the formation of a rigid polymer backbone [115,206]. As a consequence, polymeric vesicles have a greater resistance to osmotic shock [98] and the bilayer structure is retained in the presence of alcohols [200] or detergents [52]. In addition, the leakage rate of entrapped substances can be reduced due to decreased permeability of polymeric vesicles [88,201,242].

Finally, the development of polymerisable surfactants offers the possibility for the preparation of extremely stable vesicle systems. By adjusting the chemistry of the surface active compounds, it should be possible to find a suitable system for many applications.

2.6 Use and application of vesicle systems

Although membrane mimetic chemistry is a rather young research area, it is rapidly expanding and researchers of many disciplines participate in this area. Covering the whole field of uses and application is almost an endless task. The following examples will therefore mainly be focussed on biological studies.

Liposomes and vesicles are very useful as model systems for biological membranes. Biomembranes are complex systems with a variety of membrane lipids and proteins as the major components [81]. The use of simple model systems can yield valuable information on the physicochemical properties of membranes [44,66]. The packing, mobility and orientation of the amphiphile in the membrane and the phase transition of the vesicle membrane can much easier be determined in membrane systems composed of one single type of amphiphile [37,210,222]. The interaction of proteins with vesicles has received much attention [48,63,124]. Proteins can bind to liposomes by adsorption, by incorporation into the membrane during its formation or during incubation of the protein with preformed liposomes. Furthermore, proteins can be immobilised by covalent binding, either by modification of the proteins or by modification of the vesicle surfaces [82,238,239]. The latter method was successfully applied by Powers et al. [195,196] for the purification of specific enzymes from a protein mixture. They modified the vesicle surface with ligands. The proteins specific to the affinity ligand bind to these vesicles and were separated from the mixture by ultrafiltration.

Many membrane bound enzymes are inactivated upon purification due to the changed microenvironment of the protein. However, in many cases the activity can be reconstituted by the use of liposomes, as was shown e.g. for D-fructose dehydrogenase [121] and protein kinase C [183]. The group of Ringsdorf showed that ATP synthethase [254] and bacteriorhodopsin [185] could be incorporated in synthetic vesicles with retention of the enzymatic activity. By

[2]The phase transition indicates the change from a gel-like to a fluid state of the aliphatic chains of vesiculated surfactants upon rising the temperature.

using polymerisable surfactants, they were able to design enzyme-vesicle systems with long term stability. Furthermore, Adrian et al. [2] and Miranda et al. [164] showed that entrapment of proteins in liposomes increases their resistance to proteolysis.

Liposomes are further successfully employed in the elucidation of the mode of action of phospholipases, which are capable of hydrolysing phospholipids in the associated form [41,109]. In the case of lipases, which generally cannot hydrolyse phospholipids, vesicular systems have merely been used on a limited scale. Walde et al. [255] investigated the effect of liposomes on milk lipase and found that the hydrolysis of solubilised substrates was enhanced in the presence of liposomes. Hormone sensitive lipase could successfully be reconstituted by Holm et al. in phosphatidylcholine vesicles [95]. In a comparison of phospholipid coated triolein emulsion particles and liposomes containing this triglyceride, Rojas et al. [208] reported that the emulsified triolein was hydrolysed at a much higher rate than liposomal substrate due to a continued supply of substrate in the former case. Chang et al. [35] used liposomes for the immobilisation of lipase in continuous olive oil glycerolysis.

Besides the use as model membranes in biologically oriented studies, liposomes have received growing attention for incorporation and encapsulation[3] of all sorts of solutes. In this respect, vesicular systems have proven to be useful for the delivery of drugs and genetic materials [87,148,187]. The aim of these studies is twofold. Firstly, the targeting of the biologically active compound can be improved and secondly, the biological activity may be better preserved by encapsulation. In biomedical applications, the effectiveness of a drug can be improved, which leads to the administration of lower dosages. More recently, liposomes are reported to be utilised in the food industry for inclusion of food ingredients with the aim of improved performance and stability [253].

In physical chemistry, (polymerisable) vesicles are attractive for their controllable size and defined surface and due to compartmentalisation properties. Accordingly, vesicular systems have been applied in the formation of efficient colloidal catalysts, semiconductors, magnetic particles and in photochemical solar energy conversion [69,70].

The chemical diversity of the surfactants which lead to various surface functionalised vesicle systems surely will lead to the development of additional applications [71,77,137,205].

[3]Incorporation is defined as entrapment in the membrane bilayer, whereas encapsulation describes the entrapment of a compound in the aqueous interior of a vesicle.

3 LIPASE ACTIVITY IN VESICULAR SYSTEMS*

Keyword Phrases:

PURIFICATION AND CHARACTERISATION OF LIPASE FROM *CANDIDA CYLINDRACEA*

PREPARATION OF POLYMERISABLE SYNTHETIC SURFACTANT VESICLES

INCORPORATION OF CCL** IN SYNTHETIC SURFACTANT VESICLES

EFFECTS OF INCORPORATION ON THE ENZYMATIC ACTIVITY

EFFECTS OF SURFACTANT ADDENDA ON LIPASE ACTIVITY

* This chapter is published in a condensed form as: Mosmuller, E.W.J., Franssen, M.C.R., Engbersen, J.F.J. (1993) Lipase activity in vesicular systems: Characterisation of *Candida cylindracea* lipase and its activity in polymerisable dialkylammonium surfactant vesicles. *Biotechnol. Bioeng.*, accepted

** *Candida cylindracea* lipase

3.1 INTRODUCTION

For practical applications of enzyme-vesicle systems, e.g. for the stabilisation of enzymes under denaturing conditions, the development of drug targeting systems or in order to create micro-bioreactors, it is desirable to study enzyme vesicle interactions in more detail. Therefore, we were interested in the behaviour of enzymes incorporated in polymerisable surfactant vesicles.

In this chapter, a study is presented on lipase from *Candida cylindracea* (CCL) entrapped in vesicles composed of polymerisable surfactants. The lipase-vesicle system has been characterised in terms of activity and stability. The activities of free (non-incorporated) and incorporated lipase were compared using three different triglycerides and 2,4-dinitrophenyl butyrate as substrates. Furthermore, the effects are described that vesicles and some other surface active additives have on the (re)activity of free CCL towards the triglycerides. Finally, the protective influence of vesicles is illustrated by comparing the resistance for free and incorporated enzyme against proteolysis by trypsin.

3.2 MATERIALS AND METHODS

Materials

Crude lipase from *Candida cylindracea* was supplied by Meito Sangyo (lipase OF). According to the supplier the specific activity was 360 U per mg dry material (based on triolein hydrolysis). The protein content was 15%; one third of this is lipase. Trypsin (Bovine type XI) was obtained from Sigma.

Allyl bromide (99%), azo-bisisobutyronitrile (AIBN; 98%), *N,N*-dimethylformamide (DMF; 99%, dried on molsieves), dodecanoyl chloride (98%), *N*-methyldiethanolamine (97%, distilled), poly(ethylenimine) (PEI; 50 Wt.% aqueous solution, average Mw. 50-60,000), triacetin (99%, distilled) and tris-(hydroxymethyl)aminomethane (Tris; 99+%) were obtained from Janssen Chimica. 2,4-Dinitrophenyl butyrate was synthesised as described in chapter 6.2. Hexadecanoyl chloride (98%), methyl bromide (99.5+%) and polyoxyethylene(23) lauryl ether (Brij 35) were purchased from Aldrich. Tributyrin was obtained from Aldrich and Janssen Chimica (Aldrich: 98%, distilled. Janssen Chimica: 98%), 10-undecenoyl chloride (pract., >97%, distilled) from Fluka A.G., calcium chloride (p.a.), *N*-cetyl-*N,N,N*-trimethylammonium bromide (CTAB; p.a.) and uranyl acetate (p.a., dihydrate) from Merck, Sephacryl S-300 and Sephadex G-100 Superfine from Pharmacia and tricaprylin (97-98%) from Sigma.

All organic solvents were distilled before use. All aqueous solutions were prepared in particle free, demineralised and deionised water, purified by a Seralpur Pro 90 C/Seradest LFM water purification system.

Purification and characterisation of the enzyme

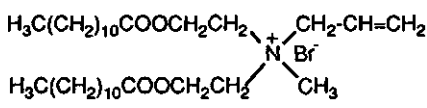
Prior to use, the crude lipase preparation was purified. Dry enzyme powder was suspended in 10 mM Tris-HCl buffer (pH 7) and centrifuged 30 minutes at

15,000 g in a Sigma 202 M centrifuge. The supernatant was centrifuged again at 48,500 g at 10°C for one hour in a Beckman 2-21 centrifuge using a JA-21 rotor in order to remove all traces of solid impurities. Subsequently, the clear enzyme solution was applied to a gel permeation column (26/60 Sephadex G-100 or Hiload 26/60 Sephacryl S-300 HR) coupled to an automated FPLC system controlled by a personal computer with FPLC-manager software (Pharmacia). The columns were equilibrated and run with a 20 mM Tris-HCl buffer pH 7.1 containing 150 mM NaCl. Finally, the enzyme solution was desalted on a Sephadex G-25 column and freeze-dried before storage.

The protein content was determined using the bicinchoninic acid protein assay reagent kit (Pierce) according to Smith et al. [224], using bovine serum albumin (Pierce) as the standard protein. Polyacrylamide gel electrophoresis (PAGE) was performed on a Midget system (Pharmacia) holding 8x5x0.75 mm gels. SDS-polyacrylamide gels according to Laemmli [145] had a 4% T, 2.5% C stacking gel and a 15% T, 0.6% C separating gel. Proteins with molecular weights ranging from 14.4 to 94 kD were used as markers (Pharmacia, Electrophoresis calibration kit). Isoelectric focussing was carried out according to the procedure of Wolbert et al. [259] with Servalyte ultrathin precoated gels with a pH gradient of 3-10 (Serva), in combination with a Pharmacia-LKB Ultraphor electrophoresis unit at 4°C and a Pharmacia-LKB 2297 Macrodrive 5 power supply. Cytochrome c (pI 9.9), ribonuclease A (8.0), alcohol dehydrogenase (6.8), carbonic anhydrase (5.8) trypsin inhibitor (4.6) and flavodoxin (3.8) were used as marker proteins.

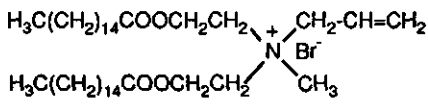
Surfactant synthesis, vesicle formation and polymerisation

The quaternary dialkylammonium surfactants (see scheme) were synthesised according to the methods described by Kippenberger et al. [125] and Tundo et al. [242] with some modifications.



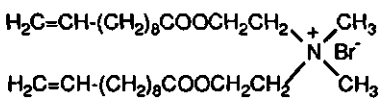
ABDEMA Br

N-Allylbis[2-(dodecanoyloxy)ethyl]methylammonium Bromide



ABHEMA Br

N-Allylbis[2-(hexadecanoyloxy)ethyl]methylammonium Bromide



BUEDMA Br

Bis[2-(10-undecenoyloxy)ethyl]dimethylammonium Bromide

N-Allylbis[2-(dodecanoyloxy)ethyl]methylammonium bromide

To a solution of 11.92 g (100 mmol) of *N*-methyldiethanolamine in 80 ml of dry DMF, 51 ml (220 mmol) of dodecanoyl chloride was added dropwise. Within a few minutes the product started to crystallise. After one hour, the

reaction mixture was diluted with 150 ml of ethyl acetate and filtered. Subsequent recrystallisation from ethyl acetate yielded 43.5 g (84%) of bis[2-(dodecanoyloxy)ethyl]methylammonium chloride as white crystals.

Mp 105-107°C; ^{1}H-NMR (200 MHz, $CDCl_3$), δ 8.0 (br s, 1 H, N-H), 4.55 (t, 4 H, O-CH_2), 3.42 (br t, 4 H, N-CH_2), 2.88 (s, 3 H, N-CH_3), 2.33 (t, 4 H, C(O)-CH_2), 1.57 (t, 4 H, C(O)-C-CH_2), 1.24 (br s, 32 H, CH_2), 0.83 (t, 6 H, alkyl-CH_3).

A solution of 5,20 g bis[2-(dodecanoyloxy)ethyl]methylammonium chloride (10 mmol) in 60 ml of dichloromethane was treated with 50 ml of 1.0 M aqueous sodium hydroxide solution. The organic layer was separated and dried on magnesium sulphate. After solvent removal the liquid bis[2-(dodecanoyloxy)ethyl]methylamine was obtained in a 95% yield (4.6 g).

^{1}H-NMR (200 MHz, $CDCl_3$), δ 4.12 (t, 4 H, O-CH_2), 2.66 (t, 4 H, N-CH_2), 2.31 (s, 3 H, N-CH_3), 2.26 (t, 4 H, C(O)-CH_2), 1.60 (t, 4 H, C(O)-C-CH_2), 1.26 (br s, 32 H, CH_2), 0.83 (t, 6 H, alkyl-CH_3).

The liquid amine was then refluxed with an excess of allyl bromide (5 ml, 58 mmol) for 7 hours. After the removal of the unreacted allyl bromide by evaporation, the product was recrystallised twice from ethyl acetate, yielding 5.15 g (85%) of *N*-allylbis[2-(dodecanoyloxy)ethyl]methylammonium bromide.

Mp 74-76°C; ^{1}H-NMR (200 MHz, $CDCl_3$), δ 6.15-5.70 (m, 3 H, vinyl-H), 4.58 (br t, 4 H, O-CH_2), 4.46 (d, 2 H, N-CH_2-C=C), 4.00 (br t, 4 H, N-CH_2), 3.40 (s, 3 H, N-CH_3), 2.29 (t, 4 H, C(O)-CH_2), 1.53 (t, 4 H, C(O)-C-CH_2), 1.22 (t, 32 H, CH_2), 0.81 (t, 6 H, alkyl-CH_3).

_N_-Allylbis[2-(hexadecanoyloxy)ethyl]methylammonium bromide

Hexadecanoyl chloride (15.17 g, 55 mmol) was added dropwise to a solution of 2.99 g (25 mmol) of *N*-methyldiethanolamine in 50 ml of dry DMF; the product immediately started to crystallise. The reaction mixture was stirred for one hour; then the mixture was diluted with 75 ml of methanol and filtered. The product was recrystallised twice in methanol, yielding 12.38 g (78%) of white crystals of bis[2-(hexadecanoyloxy)ethyl]methylammonium chloride.

Mp 117-118°C; ^{1}H-NMR (200 MHz, $CDCl_3$), δ 8.0 (br s, 1 H, N-H), 4.57 (t, 4 H, O-CH_2), 3.37 (m, 4 H, N-CH_2), 2.87 (d, 3 H, N-CH_3), 2.34 (t, 4 H, C(O)-CH_2), 1.58 (m, 4 H, C(O)-C-CH_2), 1.22 (br s, 48 H, CH_2), 0.85 (t, 6 H, alkyl-CH_3).

The ammonium salt (12.02 g, 19 mmol) was suspended in 150 ml of dichloromethane. The suspension was washed with 50 ml of a 1.0 M aqueous sodium hydroxide solution. The clear organic layer was washed with 50 ml of water and subsequently dried over magnesium sulphate. After removal of the solvent and recrystallisation in methanol-ethyl acetate 5:1 (v/v), 9.7 g (16.3 mmol, 86%) of the solid bis[2-(hexadecanoyloxy)ethyl]methylamine was obtained.

Mp 40°C; ^{1}H-NMR (200 MHz, $CDCl_3$), δ 4.14 (t, 4 H, O-CH_2), 2.67 (t, 4 H, N-CH_2), 2.32 (s, 3 H, N-CH_3), 2.28 (t, 4 H, C(O)-CH_2), 1.58 (t, 4 H, C(O)-C-CH_2), 1.26 (br s, 48 H, CH_2), 0.85 (t, 6 H, alkyl-CH_3).

A solution of bis[2-(hexadecanoyloxy)ethyl]methylamine (8.94 g, 15 mmol) in petroleum ether 60-80 was refluxed for 5 hours with an excess of allyl bromide (6.05 g, 50 mmol). After removal of the solvent and the unreacted allyl bromide by evaporation, the product was recrystallised twice from ethyl acetate yielding 8.93 g (83%) of *N*-allylbis[2-(hexadecanoyloxy)ethyl]methyl-ammonium bromide.

Mp 82-84°C; ^{1}H-NMR (200 MHz, $CDCl_3$), δ 6.1-5.8 (m, 3 H, vinyl-H), 4.58 (t, 4 H, O-CH_2), 4.51 (d, 2 H, N-CH_2-C=C), 4.06 (t, 4 H, N-CH_2), 3.44 (s, 3 H, N-CH_3), 2.33 (t, 4 H, C(O)-CH_2), 1.57 (t, 4 H, C(O)-C-CH_2), 1.26 (br s, 48 H, CH_2), 0.85 (t, 6 H, alkyl-CH_3).

Bis[2-(10-undecenoyloxy)ethyl]dimethylammonium bromide

To a solution of 2.98 g (25 mmol) of *N*-methyldiethanolamine in 20 ml of dry DMF, 12.38 g (61 mmol) of 10-undecenoyl chloride was added. The product started to crystallise within a few minutes. After one hour, the mixture was diluted with 75 ml of ether, cooled to -15°C and filtered. The crystals were washed twice with small portions of cold ether. Recrystallisation from acetonitrile yielded 10.94 g (86%) of bis[2-(10-undecenoyloxy)ethyl]methylammonium chloride as white crystals.

Mp 91-93°C; ^{1}H-NMR (200 MHz, $CDCl_3$), δ 8.1 (br s, 1 H, N-H), 5.87-5.67 (m, 2 H, C=CH-), 5.01-4.85 (m, 4 H, C=CH_2), 4.56 (t, 4 H, O-CH_2), 3.37 (br d, 4 H, N-CH_2), 2.87 (s, 3 H, N-CH_3), 2.34 (t, 4 H, C(O)-CH_2), 2.00 (q, 4 H, C=C-CH_2) 1.45 (qui, 4 H, C(O)-C-CH_2), 1.17 (br s, 20 H, alkyl-CH_2).

After treatment of a solution of 4.98 g (10.2 mmol) of the ammonium chloride in chloroform with an aqueous solution of 1.0 M sodium hydroxide, drying on magnesium sulphate and subsequent solvent removal, 4.29 g (91%) of bis[2-(10-undecenoyloxy)ethyl]methylamine was obtained as a liquid.

^{1}H-NMR (200 MHz, $CDCl_3$), δ 5.91-5.68 (m, 2 H, C=CH-), 5.05-4.88 (m, 4 H, C=CH_2), 4.14 (t, 4 H, O-CH_2), 2.68 (t, 4 H, N-CH_2), 2.32 (s, 3 H, N-CH_3), 2.28 (t, 4 H, C(O)-CH_2), 2.01 (q, 4 H, C=C-CH_2) 1.61 (qui, 4 H, C(O)-C-CH_2), 1.27 (br s, 20 H, alkyl-CH_2).

The liquid amine (2.3 g, 5 mmol) was treated with a saturated ethanolic methyl bromide solution. This mixture was heated for 5 hours on a 60°C water-bath. After removal of the solvent and the excess of methyl bromide, the crude product was crystallised twice from petroleum ether-chloroform 25:2 (v/v), to yield 2.6 g (95%) of bis[2-(10-undecenoyloxy)ethyl]dimethylammonium bromide.

Mp 134-137 °C; ^{1}H-NMR (200 MHz, $CDCl_3$), δ 5.90-5.70 (m, 2 H, C=CH-), 5.15-4.90 (m, 4 H, C=CH_2), 4.62 (br s, 4 H, O-CH_2), 4.20 (br s, 4 H, N-CH_2), 3.58 (s, 6 H, N-CH_3), 2.39 (t, 4 H, C(O)-CH_2), 2.05 (m, 4 H, C=C-CH_2), 1.62 (m, 4 H, C(O)-C-CH_2), 1.30 (br s, 20 H, CH_2).

Vesicle preparation and polymerisation

Nonpolymerised vesicles were prepared by sonication of 5 mM surfactant suspensions that initially were thermostated to 60°C, using a standard 13 mm tip of a VC-300 Sonics & Materials sonifier, output control set at level 8 (corresponding to 60 W output power). Slightly opaque solutions were obtained in 10-20 minutes.

Polymeric vesicles were prepared by two distinct procedures. In one procedure the free radical initiator azo-bisisobutyronitrile (AIBN, 1-5% w/w with regard to the surfactant) was co-sonicated with a 5 mM aqueous surfactant suspension. The solution was then placed in an oil bath thermostated to 70°C for 8-12 hours. The resulting solution was in most cases more clear than the solution at the start. Alternatively, in the second procedure ABHEMA Br was polymerised before vesiculation. Therefore, AIBN (1-5% w/w with regard to the surfactant) was added to 5 mM solutions of surfactant in tetrahydrofuran. This solution was placed in an oil bath thermostated to 70°C for 8-12 hours.

Subsequently, the solvent was removed and a proper amount of water was added. The prepolymerised surfactants were vesiculated by sonication (13 mm standard tip, 60 W) at 60°C; opaque solutions were obtained in 20-60 minutes.

The polymerisation process was followed by means of NMR. Nonpolymerised or polymeric vesicle solutions were freeze-dried and were then solubilised in deuterated pyridine or trifluoroacetic acid. The disappearance of the vinyl signal was taken as a measure for the degree of polymerisation. NMR spectra were recorded on a Bruker 200 E at 200 MHz at room temperature. All vesicle solutions were centrifuged before further use for 30 minutes at 13,500 rpm (15,500 g) in a Sigma 202 M centrifuge (10x10 ml rotor) to remove any titanium particles from the sonifier tip. The stability of the vesicle solutions was examined by mixing equal amounts of vesicle solution with various buffers or salt solutions. The time in which the solutions remained opaque (hence, did not flocculate) was taken as a measure of stability.

Sample preparation for electron microscopic investigation

Samples for the transmission electron microscope (JEOL 1200EX) were prepared by two distinct methods. In one procedure samples of polymerised vesicle solutions were prepared by mixing equal amounts of 5 mM vesicle solutions and 2% (w/v) uranyl acetate to realise a negative staining. Drops of these mixtures were placed on a 200 mesh copper grid coated with a Formvar film. The excess of fluid was absorbed into a piece of filter paper and then the grids were dried before examination. Uranyl staining of nonpolymerised vesicles gave no satisfactory pictures. This is due to the limited stability of these systems. Mixtures of nonpolymerised vesicles and uranyl acetate started to precipitate within a few minutes from mixing. Alternatively, samples were rapidly frozen in liquid propane. After this, the samples were partly freeze-dried in a BAF 400 freeze-fracturing apparatus (Balzers Union, Liechtenstein), platinum shadowed (2 nm) at an angle of 40° and coated with carbon. The replicas were floated off in distilled water, washed overnight in 20% sodium hypochlorite, followed by rinsing in distilled water. The replicas were then transferred onto 200 mesh copper grids.

Enzyme incorporation in vesicles

Lipase entrapment in vesicles is accomplished by the dehydration-rehydration method or by the incubation method.

The dehydration-rehydration method as described by Kirby and Gregoriadis [126] was slightly modified. A solution of lipase (278 μg protein/ml) was added to a freeze-dried 5 mM vesicle solution resulting in a final protein-to-surfactant ratio of 56 mg protein/mol surfactant. This mixture was homogenised by short sonication (microtip, output control set at level 3, pulser set at 50%). The enzyme-vesicle mixture was then freeze-dried and rehydrated by the addition of a proper amount of water. The slightly opaque solution was sonicated at 0°C (microtip, level 3, pulser at 50%) in order to prepare unilamellar vesicles, since trapping efficiencies are reported to be higher in unilamellar vesicles than in multilamellar vesicles [124,126].

Alternatively, equal amounts of lipase solutions (56 μg protein/ml) and 5 mM vesicle solutions (protein-to-surfactant ratio 11 mg/mol) were mixed by the use of a vortex at room temperature; the mixtures became slightly turbid.

The preparations were incubated for 1 hour at room temperature and stored at 4°C.

The entrapped enzyme was isolated by centrifugation for 2 to 4 hours at 48,500 g at 10°C. The pellets, containing the vesicle entrapped enzyme, were resuspended and centrifuged again in order to remove all nonentrapped enzyme. Enzymatic activity in both supernatants and pellets was measured for the determination of the trapping efficiency.

Assay for testing the protection against proteolysis with trypsin

A sample of CCL, incorporated in polymerised ABHEMA Br vesicles, (113 µg protein/ml, 2.5 mM surfactant) was prepared as described above.

Equal amounts of free or incorporated enzyme were mixed with a 1 mg/ml solution of trypsin in 10 mM Tris-HCl pH 8.5 and 0.2 mM calcium chloride, and these mixtures were incubated at 30°C. After certain time intervals, samples were drawn from the mixture and lipase activity was determined using the standard assay method.

Determination of lipase activity

In the standard assay method, lipase activity was determined by a titrimetric method using tributyrin as the substrate. Tributyrin is slightly soluble in water, the solubility limit is 0.4 mM [225]. Thus, 0.2 g tributyrin was emulsified in 40 ml of water by 2 minutes of sonication (13 mm standard tip; output control set at level 8, corresponding to 60 W). The enzymatic hydrolysis was started by addition of a proper amount of enzyme. The assay mixtures were titrated with 2 to 10 mM KOH solutions. Base consumption due to hydrolysis of the triglycerides was monitored using a Schott Geräte T100 titrating and dosing system equipped with an AUTOLAB/ISEC personal computer controlled automatic titrator (Eco Chemie, Utrecht, NL). The reactions were carried out at pH 7, 37°C and in a nitrogen atmosphere.

For the determination of the enzymatic activity at variable triglyceride concentrations, the assay method was slightly modified. The term concentration refers to the overall amount of substrate in the assay. Three substrates were tested: triacetin, tributyrin and tricaprylin. Triacetin is soluble in water up to 6% v/v (440 mM); at higher concentrations, this substrate forms emulsions. Tributyrin is slightly soluble in water (up to 0.4 mM), whereas tricaprylin is insoluble in aqueous solutions. Several triglyceride stock solutions were prepared in acetonitrile. Assay mixtures were prepared by addition of 1% v/v of these substrate solutions to 30-50 ml of water. In the case of tributyrin and tricaprylin the mixtures were sonified (13 mm tip, level 8) for 2, respectively 5 minutes to effect maximal emulsification. In the case of triacetin, the addition of base was monitored for at least 5 minutes before the enzyme was added, in order to determine base consumption due to spontaneous hydrolysis. At low triacetin concentration, the blank reaction was not negligible and the final values for enzyme activity were corrected for this spontaneous hydrolysis. The enzymatic reactions were started by addition of free or incorporated CCL with final concentrations of 95-158 ng protein /ml of assay mixture. Furthermore, lipase activity was assayed with 2,4-dinitrophenyl butyrate (DNPB) at a concentration of 1.0 mM, which is beyond the solubility limit. The hydrolysis of the dinitrophenyl ester can be spectrophotometrically monitored due to the increase in absorbance at 360 nm as the result of the formation of 2,4-

dinitrophenol during the enzymatic reaction. The assay mixture consisted of 2.5 ml 5 mM Tris-HCl pH 7, 2% (v/v) acetonitrile and 1.0 mM DNPB. The enzymatic activity was started by addition of 20 µl of a 2.8 µg protein/ml CCL solution (free or incorporated) in Tris-HCl buffer, 1 mM and pH 7; the dehydration-rehydration ratio was 22 mg/mol. Further details of the spectrophotometrical detection of lipase activity with 2,4-dinitrophenyl esters is outlined in chapter 6.

The effect of additives on the enzymatic activity of CCL was tested by the addition of ABHEMA Br (as a monomolecular dispersed solution or as nonpolymerised and polymerised vesicles), cetyltrimethylammonium bromide (CTAB), polyoxyethylene(23)lauryl ether (Brij 35) and poly(ethylenimine) (PEI) to the assay solution. The concentration of the additives in the triglyceride assays amounted to 5 µM and to 1 µM in the DNPB assays on monomer basis. The enzymatic hydrolysis was started by the addition of a proper amount of CCL (112 ng protein/ml) to the triglyceride assay solutions and was monitored for 5 minutes. Then, the additive was administered and the activity was again monitored for 5 minutes. In the case of the DNPB assay, the activity of free CCL was measured for 2.5 minutes, then the additive was administered and the reaction was followed for another 2.5 minutes. Monomolecular dispersed ABHEMA Br was obtained by adding 0.5 ml of a stock solution of the surfactant in acetonitrile to the sample solution. Poly(ethylenimine) was dissolved in water and adjusted to pH 7 before use. In these experiments the assay mixtures consisted of 50 ml of water, 1% (v/v) acetonitrile, triacetin (15 mM, homogeneous solution), tributyrin (0.32, homogeneous solution, or 5 mM, emulsion) or tricaprylin (0.32, emulsion, or 5 mM, emulsion), or 2.5 ml of 5 mM Tris-HCl pH 7 containing 2% v/v acetonitrile and 1.0 mM of emulsified DNPB.

All measurements were taken in triplicate or, when the standard deviation was lower than 2%, in duplicate. The reaction rates for triacetin, tributyrin and DNPB were linear versus time. However, the *initial* reaction rate for CCL in the case of tricaprylin was not linear in time. Initial reaction rates were extrapolated and, in addition, were determined at a point where the hydrolysis was linear (at 5 minutes reaction time). Lipase activity (U) is defined as µmol fatty acid released per min, conform the rules of the IUPAC-IUB. It was confirmed by separate measurements that the ester bonds in the surfactant were not hydrolysed by the enzyme.

3.3 RESULTS

Purification and characterisation of *Candida cylindracea* lipase

The crude enzyme mixture was centrifuged twice at 15,500 and 48,500 g to remove insoluble material; 96% of the lipase activity is recovered in the supernatant. The lipase could be purified by applying the crude enzyme preparation on a 26/60 Sephacryl S-300 column (figure 3.1). On this column, with a bed volume of 320 ml and an elution volume of 120 ml, 5 ml aliquots of a 100 mg dry material/ml CCL solution could be fractioned. Fraction 2 was the most active fraction.

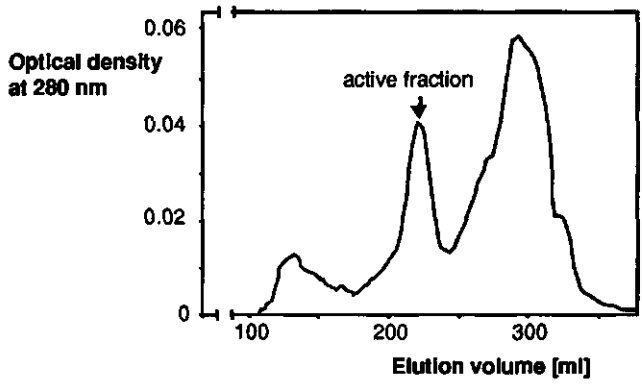


Figure 3.1 Elution profile of CCL (crude) on a Sephacryl S-300 HR column.

After freeze-drying and desalting on a Sephadex G-25 column, 24% of the activity can be recovered, corresponding to 998 U/mg protein (table 3.1).

Table 3.1 Purification table of lipase from *Candida cylindracea*.

purification steps	protein content [%]	specific activity[1] [U/mg protein]	recovery [%]
crude enzyme	14	372	100
centrifugation	20	341	96
gel permeation[2]	56	998	24

[1] Specific activities are measured with the standard assay method.
[2] The values in this row are taken after application of a Sephacryl S-300 or Sephadex G-100 column and after desalting on a Sephadex G-25 column.

Some characteristics of the enzyme were determined, using tributyrin as the substrate (standard assay method, see Materials and Methods section). The pH optimum is broad, ranging from 6 to 9 (figure 3.2), and the optimum temperature for CCL is 45°C (figure 3.3).

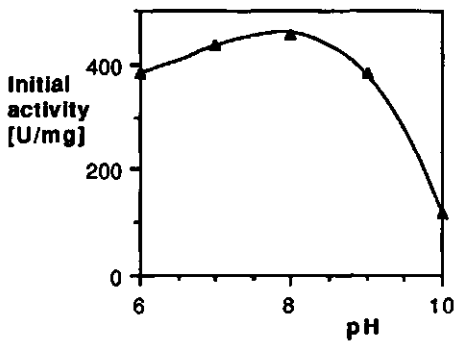


Figure 3.2 pH profile of CCL.

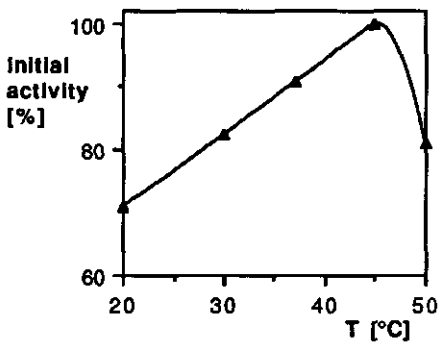


Figure 3.3 Temperature profile of CCL.

The protein migrates to a molecular weight of 58.5 kD on SDS-PAGE (figure 3.4, left gel). Isoelectric focussing shows that the purified lipase (lane 2) has an isoelectric point of 4.1 (figure 3.4, right gel).

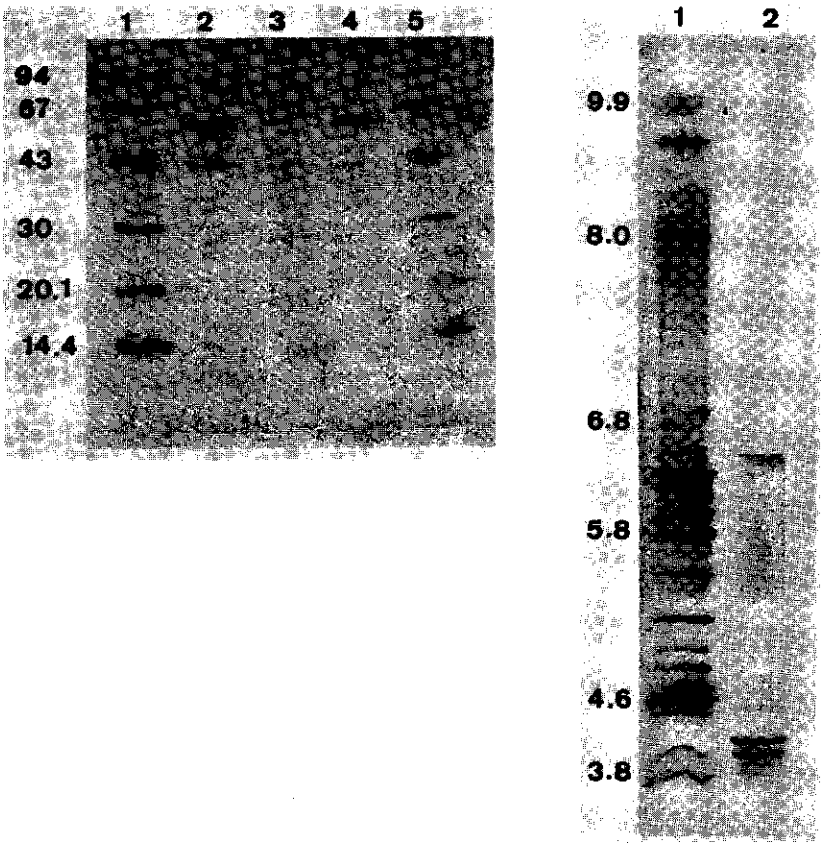


Figure 3.4 SDS-PAGE (left gel) of crude (lane 2) and purified CCL (lane 3, 4); marker proteins are shown in lane 1 and 5. Isoelectric focussing (right gel) of purified CCL (lane 2); marker proteins are depicted in lane 1.

Characterisation of the vesicle systems

Vesicles of the three different surfactants *N*-allylbis[2-(dodecanoyloxy)ethyl]-methylammonium bromide (ABDEMA Br), *N*-allylbis[2-(hexadecanoyloxy)-ethyl]methylammonium bromide (ABHEMA Br) and bis[2-(10-undecenoyl-oxy)ethyl]dimethylammonium bromide (BUEDMA Br) can be prepared by sonication of a surfactant suspension. Upon sonication, the mixtures become opaque. Examination of these solutions by transmission electron microscopy demonstrate that sonication produces uniformly sized, unilamellar vesicles.

Figure 3.5 shows a typical example of a polymerised ABHEMA Br vesicle that is partly freeze-dried and subsequently platinum shadowed (left photograph). An example of a sample of an enzyme-vesicle mixture, stained with uranyl acetate, is depicted in the right photograph of figure 3.5. The size of the vesicles is 200-400 nm. According to the classification made by Vuillemard [253] vesicles of this size are referred to as large unilamellar vesicles (LUV).

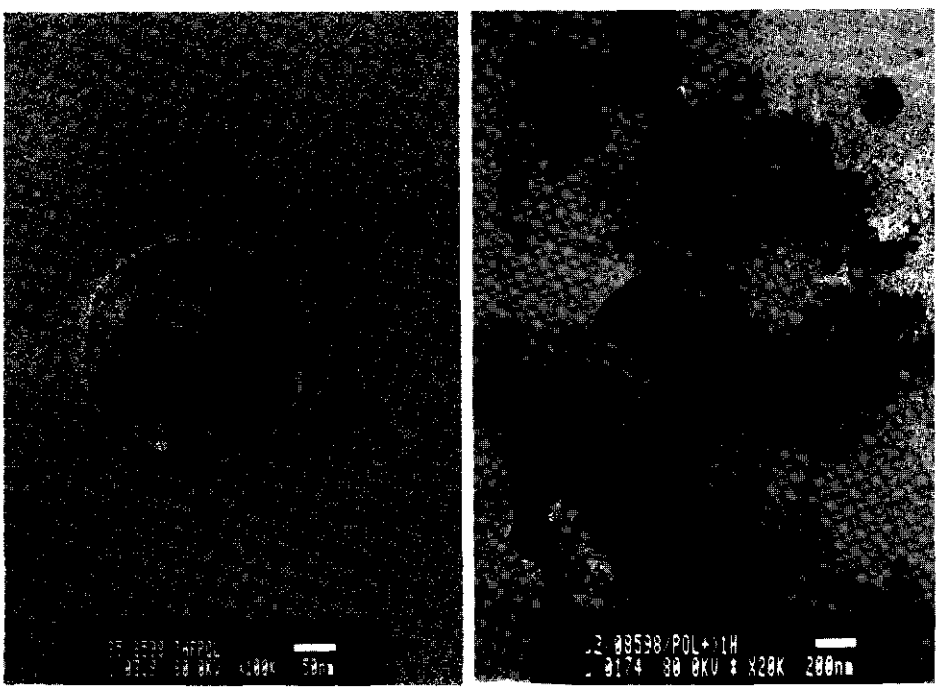


Figure 3.5 TEM photographs of polymeric ABHEMA Br vesicles, stained by platinum shadowing (left photograph) or by mixing with uranyl acetate (right photograph).

The degree of polymerisation is estimated by NMR through comparison of the integration of the vinyl signals of the nonpolymerised and the polymeric surfactants. This method revealed that the vesicles can be polymerised in water to a maximal extent of 32%. In case of prepolymerisation of surfactants in THF and subsequent vesiculation nearly no vinyl signal can be detected.

The stability of the vesicles is controlled by the medium that is used. In pure water all nonpolymerised and polymeric vesicles are stable for several months. In 10 mM potassium phosphate buffer (pH 6 or 8) or in 10 mM Tris-HCl buffer (pH 7 or 8.5), the polymeric vesicles are stable for at least one month. Upon prolonged standing of polymeric ABHEMA Br vesicles in

phosphate buffer an increase in viscosity can be observed. This is most probably due to the fact that phosphate can act as a crosslinking agent between the positively charged headgroups of the surfactant polymers. In contrast, the stability of nonpolymerised ABDEMA Br vesicles was restricted to several hours at pH 6-7 and even decreased to a few minutes when buffers of pH 8 or 8.5 were applied. In 100 mM phosphate buffer the nonpolymerised vesicles precipitated almost instantly, whereas the polymerised analogues flocculated after several minutes (ABDEMA Br) or after 12-15 hours (BUEDMA Br and ABHEMA Br).

Incorporation of CCL in vesicles, effects on enzymatic activity

Incorporation of *Candida cylindracea* lipase in synthetic surfactant vesicles

The incorporation of CCL in vesicles, using the dehydration-rehydration method, was investigated for two different surfactants: ABDEMA Br and BUEDMA Br. The extent of incorporation, based on activity measurements, is expressed as the trapping efficiency (table 3.2). Recovery is defined as the sum of total activities of entrapped and non-entrapped lipase divided by the total activity of initially added enzyme. Enzyme activity is determined with the standard assay method.

In the case of the use of ABDEMA Br vesicles, there is a considerable difference in activity between enzyme entrapped in polymerised or in non-polymerised vesicles, as is shown in table 3.2. In the case of BUEDMA Br vesicles, there is neither any difference in trapping efficiency for polymerised and nonpolymerised vesicle solutions. However, the recovery for the polymerised analogue is lower.

Table 3.2 Trapping efficiency and recovery for CCL incorporated into ABDEMA Br and BUEDMA Br vesicles by means of the dehydration-rehydration method.

CCL incorporated into vesicles of:		trapping efficiency[1] [%]	recovery[1] [%]
ABDEMA Br:	nonpolymerised	43	83
	polymerised[2]	19	55
BUEDMA Br:	nonpolymerised	21	77
	polymerised[2]	21	50

[1] Trapping efficiency and recovery are normalised to the activity of the free enzyme (=100%). The activities are determined with the standard assay method.

[2] The extent of polymerisation is 28% and 32% respectively for ABDEMA Br and BUEDMA Br vesicles.

When CCL is incubated with vesicle solutions, trapping efficiencies of up to 100% can be obtained. This indicates that all of the initially added enzyme is incorporated into the vesicles. The concentrations used in the experiments are 56 μg CCL/ml (1 μM) and 5 mM ABHEMA Br. This corresponds to a molar protein to surfactant ratio of 1 : 5000. From appendix B it can be seen that a protein to surfactant ratio of 1 : 5000 is equal to a protein to vesicle ratio of $1 : 5000 * 10^{-4}$ or 2 : 1. The surface ratio, which indicates the amount of vesicle surface that is occupied with enzyme, amounts to 1 : 125 (2*50 : 1*125,000). As will be shown in chapter 5 (see section Results and Discussion, subsection

Energy transfer studies) the enzyme-vesicle ratio in these experiments is much lower than the saturation ratio (1 : 4).

The trapping efficiencies and recoveries vary with incubation time. It appears that directly after mixing of enzyme and vesicle, the activity of this mixture is very low. After an incubation time of one hour the activity of the lipase-vesicle mixture is maximal (figure 3.6).

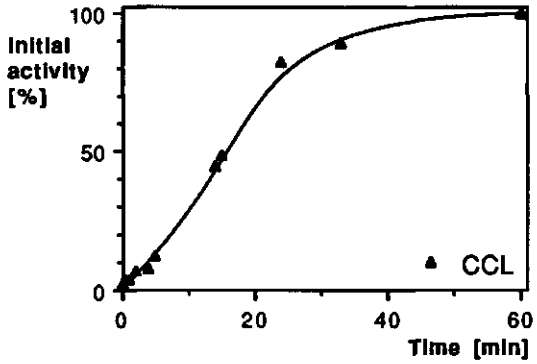


Figure 3.6 Time dependence of enzymatic activity during the incorporation of CCL in polymerised ABHEMA Br vesicles. Equal amounts of lipase and vesicle solution were incubated at 20°C and at various time intervals lipase activity was measured.

It was not possible to prepare vesicles by sonication in the presence of lipase with full retention of its activity. Sonication of surfactant-enzyme mixtures at 60°C resulted in a complete loss of enzymatic activity. The loss of activity is the result of thermal inactivation and is not due to shear inactivation caused by sonication, as was demonstrated in separate experiments. Sonication of a lipase solution in the absence of surfactant according to the standard procedure (initial temperature set to 60°C, followed by 10 minutes of sonication), resulted in complete inactivation. Sonication of a lipase solution for 10 minutes on an ice-bath merely resulted in a 10% decrease in activity. However, under these conditions it was not possible to obtain vesicles.

Activity of free and incorporated CCL

Changes in activity by incorporating the enzyme in vesicles were investigated by comparing the activities of free versus incorporated lipase for triacetin, tributyrin, tricaprylin and 2,4-dinitrophenyl butyrate.

The activity of free and vesicle incorporated CCL is compared for the three triglycerides by assaying lipase activity in a range of substrate concentrations. For triacetin the lipase activity was assayed at concentrations of 0.4-30 mM, below the solubility limit. The activity profile of free and incorporated *Candida cylindracea* lipase for triacetin is plotted in figure 3.7. It is clear that the activity for the incorporated lipase is higher than for the free lipase.

A similar behaviour can be observed in the case of tributyrin (figure 3.8). Especially at lower concentrations, slightly under the solubility limit, the incorporated enzyme is up to 4 times more active than the free enzyme.

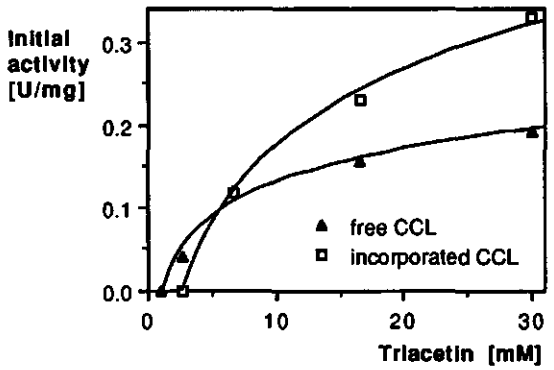


Figure 3.7 Activity profiles of free CCL and CCL incorporated in polymerised ABHEMA Br vesicles with varying triacetin concentrations.

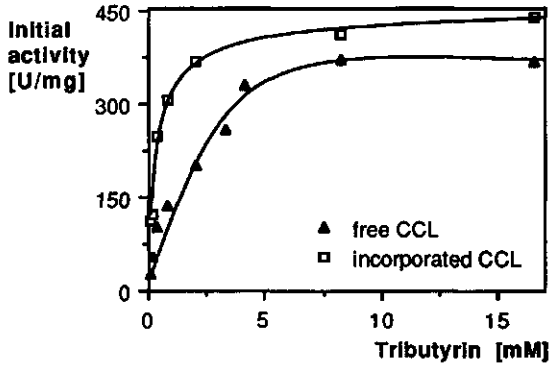


Figure 3.8 Activity profiles for free CCL and CCL incorporated in polymerised ABHEMA Br vesicles with varying tributyrin concentrations.

Table 3.3 Activity of vesiculated versus free *Candida cylindracea* lipase in dinitrophenyl butyrate assays.

	relative initial activity[1]	
sample	0.1 mM DNPB (homogeneous)	1 mM DNPB (heterogeneous)
CCL: free	100	100
in polymeric vesicles	109	137

[1] The activities are normalised to the activity of the free enzyme (=100%). The activities are 69.6 and 265.5 U/mg in case of 0.1, respectively 1.0 mM DNPB.

Merely in tricaprylin assays the free enzyme is more active than the incorporated enzyme when looking at the initial rate of hydrolysis (figure 3.9, left side). However, the reaction rates are not constant in time for this

substrate; the activities after 5 minutes for free and entrapped enzyme are almost equal (figure 3.9, right side).

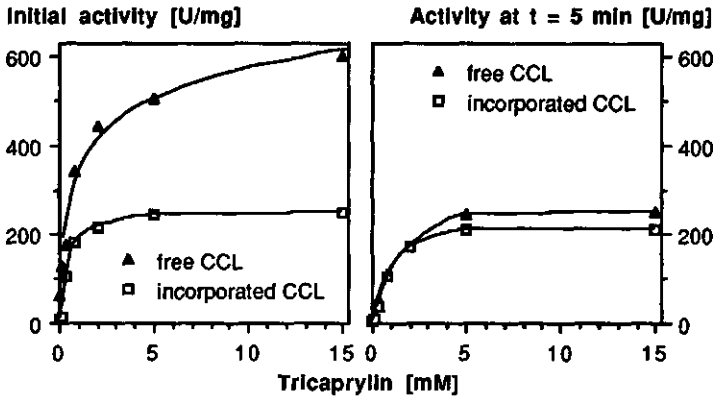


Figure 3.9 Activity profiles for free CCL and CCL incorporated in polymerised ABHEMA Br vesicles with varying tricaprylin concentrations. The left graph represents initial activities and the right graph represents the activities after 5 minutes.

In the case of DNPB, the activity for the homogeneous system (0.1 mM DNPB) and the heterogeneous reaction system (1.0 mM DNPB) is enhanced upon incorporation (table 3.3).

Effect of incorporation on protection against trypsin digestion

The stability of free and incorporated enzyme was tested by treatment of enzyme samples with the proteolytic enzyme trypsin. The effect of trypsin digestion on lipase activity is depicted in figure 3.10. Hydrolytic activity of free lipase is reduced to 50% within 2 hours of incubation, whereas incorporated enzyme is almost not attacked by the proteolytic enzyme.

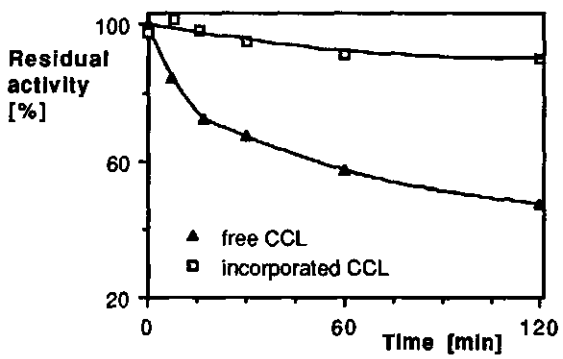


Figure 3.10 Proteolytic stability of free CCL and CCL incorporated in polymerised ABHEMA Br vesicles.

Effects of surfactant addenda on the activity of free lipase

The influence of several surface active additives on the hydrolytic activity of CCL was studied by monitoring the immediate change in activity upon their addition. The surfactant ABHEMA Br was added in three forms, which are monomolecular dispersed, as nonpolymerised vesicles or as polymerised vesicles. The commercially available surfactants, cetyltrimethylammonium bromide (CTAB) and polyoxyethylene(23)lauryl ether (Brij 35), were added in concentrations (5 μM) well below the critical micelle concentration. Polyethylenimine (PEI), a positively charged poly-electrolyte at pH 7, was added in the same concentration as the other compounds (based on ethylenimine monomer). The results are summarised in table 3.4.

Table 3.4 Influence of additives on the activity of *Candida cylindracea* lipase in assays with triacetin (15 mM), tributyrin (5 mM), tricaprylin (5 mM), or DNPB (1.0 mM).

	relative initial activity			
additive in assay[1]	triacetin	tributyrin	tricaprylin	DNPB
free CCL, no additive[2]	100	100	100	100
ABHEMA Br,				
monomolecular dispersed	17	6	311	69
vesiculated, not polymerised	3	55	309	92
vesiculated, polymerised	140	85	345	106
CTAB	50	3	331	-
Brij 35	100	100	100	-
PEI	-	70	312	-

[1] The additive concentrations amounted to 5 μM in the case of the triglyceride assays and 1 μM in the case of the DNPB assay.

[2] The activities are normalised to the activity of the free enzyme. In the case of tricaprylin, the activity after 5 minutes (244 U/mg) is taken as 100%. The initial activity is 521 U/mg, corresponding to 214 % of the activity after 5 minutes.

In the case of tricaprylin, lipase activity is improved more than threefold by most additives. In the tributyrin assay the enzymatic activity is slightly reduced by polymerised vesicles and PEI, whereas CTAB and nonvesiculated ABHEMA Br vesicles reduce the activity nearly to zero. For nonpolymerised ABHEMA Br the result was not very consistent; activities dropped to 46-63% with a mean value of 55% (n = 9). In the homogeneous triacetin assay system, addition of polymerised vesicles causes enhancement of the hydrolytic activity, whereas addition of monomolecular dispersed ABHEMA Br or nonpolymerised vesicles considerably reduce the activity. In the case of DNPB, addition of vesicles hardly changes the activity. Administration of monomolecular dispersed ABHEMA Br causes a reduction of 31% in the activity. Addition of Brij 35, a nonionic surfactant, has no influence on the enzymatic activity.

For tributyrin and tricaprylin the effect of polymerised vesicles on enzyme activity was also investigated at a substrate concentration of 0.32 mM. The results are shown in table 3.5. In both cases there is a considerable rate enhancement, for tributyrin 10 times and for tricaprylin 9 times.

Table 3.5 Influence of additives in 0.32 mM triglyceride assays on the activity of *Candida cylindracea* lipase.

	relative initial activity	
additive in assay	tributyrin	tricaprylin
free CCL, no additive	100	100 [1]
+ ABHEMA Br, vesiculated, polymerised	973	907

[1] The activity in the tricaprylin assay after 5 minutes (59 U/mg) is taken as 100%, because the rate of tricaprylin hydrolysis is not constant in time. The initial activity is 177 U/mg, corresponding to 300% compared to the activity after 5 minutes.

3.4 DISCUSSION

Characterisation of purified *Candida cylindracea* lipase

The crude enzyme mixture has a protein content of 15%, of which one third is the active lipase, according to the supplier. *Candida cylindracea* lipase was purified in three steps resulting in a sample with a protein content of 56% (table 3.1). As indicated by the elution profile of the Sephacryl S-300 column in figure 3.1, lipase OF (Meito Sangyo) is a mixture of proteins, which all have some lipase activity. However, peak 2 is the most active fraction and is used further in this work; this enzyme fraction is referred to as CCL. This protein has nearly the same elution volume as the marker protein BSA (66 kD) on the gel permeation column. The molecular weight of the protein is 58.5 kD on SDS-PAGE and is therefore considered to be monomeric. Although CCL is a widely used enzyme, only few studies have been devoted towards its purification and its characterisation. Furthermore, these studies do not reveal consistent data. Tomizuka et al. [236,237] have reported the purification and characterisation of a lipase from *Candida cylindracea* (Meito Sangyo) by means of precipitation and chromatographic techniques. They describe the purified enzyme as a high molecular weight glycoprotein with a high content of hydrophobic residues. Sigma lipase L.1754 was purified on a Mono Q anion exchange column by Veeraragavan and Gibbs [247]. Two lipases were isolated with molecular weights of 58 kD (SDS-PAGE) and pI's of 5.6 and 5.8. Shaw et al. [220] report the isolation of three distinct forms of lipase from a *Candida* preparation (Sigma) which consists of a hexamer (362 kD), a trimer (200 kD) and a dimer (143 kD), all with a 62 kD subunit. The three forms differ in optimum temperature and optimum pH. In a study of the esterase profile of the Sigma *Candida cylindracea* lipase type VII, Brahimi-Horn et al. [20] infer that the preparation consists of several enzymes with different substrate specificities and slightly different ionic states and hydrophobicities.

Isoelectric focussing revealed one major and one minor band with pI's of 4.1 and 4.0. Brahimi-Horn et al. [20] have reported also two bands for the crude CCL (pI's 4.3 and 4.7), whereas Veeraragavan and Gibbs [247] found much higher isoelectric points for his isolated enzymes. The effects of pH and temperature on the enzymatic activity are very similar to the pH and temperature profile of the lipase isolated by Tomizuka et al. [236]. However,

they report a much higher molecular weight of 120 kD, estimated on the basis of sedimentation techniques.

It is clear that it is possible to purify CCL to a considerable extent by simple techniques. Comparing our data and the data of Brahimi-Horn, Shaw, Tomizuka and Veeraragavan, it seems very likely that different *Candida* preparations or batches contain different enzymes. Therefore, it is advisable, depending on the application, to characterise the sample that is used.

Vesicle polymerisation and its effect on the morphology

Tundo et al. [242] and Kippenberger et al. [125] claim that it is possible to completely polymerise the dialkylammonium bromide surfactant vesicles of ABDEMA Br, ABHEMA Br and BUEDMA Br by ultraviolet radiation or selectively polymerise the outer surface of the membrane by thermal radical polymerisation using an initiator. However, our experiments showed that it was not possible to polymerise the vesicles by UV-radiation. Thermal radical polymerisation proceeded to a maximal extent of 32%, whereas Kippenberger et al. report 56%, corresponding to a complete polymerisation of the outer vesicle surface. Although polymerisation in aqueous solution takes place in a highly ordered system, which should favour the polymerisation process, the low extent of polymerisation can be explained by the isolated nature of the double bond in the surfactant molecule. In order to obtain higher degrees of surfactant crosslinking, the surfactants were prepolymerised in THF using AIBN as an initiator. In this way it was possible to completely polymerise the surfactants, although vesiculation of the oligomeric surfactants was more difficult than in the case of monomeric (nonpolymerised) surfactants, as is reflected by the increased sonication times. Due to polymerisation of the double bonds, the vesicles were appreciably more stable than their nonpolymerised analogues. This clearly shows the advantage of the use of polymerisable surfactants.

Morphological investigation of the vesicles indicates that freeze-drying and platinum shadowing give micrographs which have better quality and are more true to nature than vesicles stained with uranyl acetate, since no additional chemicals are needed in the former case. The freeze-drying method further confirms that the vesicle membrane structure is conserved during the dehydration process, as was claimed by Kirby and Gregoriadis [126] and Gregoriadis [85,86] without experimental evidence. It was difficult to find isolated vesicle structures in the case of polymerised vesicles stained with uranyl acetate, due to clotting of the material (figure 3.5, right photograph). As can be seen from the same micrograph, addition of lipase to a vesicle solution does not alter the size of the vesicles.

Incorporation of *Candida cylindracea* lipase in vesicles

Several techniques for the entrapment of biologically active substances have been proposed. For an overview the reader is referred to Fendler [66] Gregoriadis [83,84], Mayer et al. [163] and Szoka and Papahadjopoulos [231].

Dehydration-rehydration method

In first instance the dehydration-rehydration method was used for the incorporation of CCL in the positively charged dialkylammonium bromide surfactant vesicles. This method is attractive for incorporating enzymes

because very mild conditions can be used. No organic solvents and no or very short sonication are required, which may inactivate the enzyme. For this method trapping efficiencies for proteins are reported to vary from 1 to 40% [86,126]. Even with the same proteins, large variations are possible when, for instance, the protein-to-surfactant ratio is changed. In our approach the trapping efficiency varied from 19-43%. These results are in the same order of magnitude as reported, although we have not tried to optimise the entrapment.

Incubation method

Since the dehydration-rehydration method is rather laborious, we have investigated the possibility of direct incorporation of the enzyme into vesicles in solution. Therefore, lipase was added to a polymerised vesicle solution, incubated for one hour and the activity of the mixture, assayed at various (incubation) time intervals, was measured. The results, which are represented in figure 3.6, show that lipase activity directly after mixing is rather low but that it is restored to 100% within one hour. The trapping efficiency after one hour of incubation, reflected by the activity in the pellet after centrifugation, was 100%. This indicates that the enzyme can be incorporated in preformed vesicles without additional treatment.

The initial stage in the interaction between *Candida cylindracea* lipase and the vesicles is most probably caused by electrostatic attraction, since vesicle surface and enzyme have opposite charges at pH 7 (see also chapter 5). The importance of charge and charge distribution in the spontaneous transfer of enzymes from aqueous solution into a more hydrophobic environment has already been outlined by Wolbert et al. [259]. These authors found that the uptake of proteins into reversed micelles is controlled by the pH of the aqueous solution, and the isoelectric point and size of the enzyme. Subsequently, the hydrophobic part of the vesicle membrane will then induce further incorporation of the enzyme into the vesicle membrane [124,188,238,263]. Insertion of the protein into the bilayer will be accompanied by rearrangement of surfactant molecules around the enzyme and by changes in its state of hydration, the distribution of charged groups and other rearrangements in the protein structure (for recent reviews on thermodynamical aspects of protein-interface interactions, see Norde and Lyklema [178] and Rialdi, Battistel and Barisas [204]). These processes may, especially in the case of application of polymerisable vesicles, be responsible for the fact that enzymatic activity lowered upon mixing of enzyme and vesicle solutions. For several other lipases, similar behaviour is found. Iwai et al. [108] reported that the increase in lipase activity due to binding of the enzyme to phospholipid preparations showed a lag period compared to the rate of binding of the lipase to the lipids. Ekiz et al. [56] reported that, in the adsorption of CCL to a *n*-heptane/water interface, activity was fully restored after periods as long as 150 minutes.

Effects of incorporation on enzymatic activity

The effect of vesiculation upon enzyme activity is studied with a series of triglycerides with increasing hydrophobicity, viz. triacetin, tributyrin and tricaprylin and for the spectrophotometrically detectable 2,4-dinitrophenyl butyrate.

Triacetin is used in a concentration range well below its solubility limit. Both free and incorporated enzyme have low hydrolysis rates over the whole

concentration range, when compared to emulsified substrates (figure 3.7). However, incorporated enzyme is more active than free enzyme. This may be caused by the catalytic mechanism of lipolytic enzymes. Recently the structure of three lipases has been elucidated, *viz.* human pancreatic lipase [257], *Rhizomucor miehei* lipase [19] and *Geotrichum candidum* lipase [216]. The active site in these enzymes is buried and covered by a hydrophobic flap. The lipases can be activated by moving this flap away from the active centre [29]. This is accomplished by (exclusively hydrophobic) contacts between a hydrophobic surface and the protein [90]. In a comparative analysis of lipases Kordel et al. [134] found that many lipases have structural similarities. In *Candida cylindracea* lipase (ATCC14830) the essential amino acids in the active site form a helix which is also buried. Solubilised substrates, like triacetin, have no interface that can remove the flap, i.e. activate the enzyme, and, in general, lead to much lower enzyme activities than emulsified substrates. Recent X-ray crystallographic studies on lipase from *Rhizomucor miehei*, inhibited by diethyl 4-nitrophenyl phosphate, confirm that binding of lipase is accompanied by structural changes, which are essential for the activation of the enzyme [45]. In case of entrapment in vesicles, the lipase is activated by the membrane bilayer which induces the conformational changes in the enzyme. Hereby the lid is removed and the active site becomes accessible. This explains why the incorporated enzyme has a higher activity for triacetin than the free enzyme. The same behaviour is observed by Kordel and Schmid [135] in a similar system. They have investigated the inhibition of a *Pseudomonas* lipase by diethyl 4-nitrophenyl phosphate and found that the inhibition was accelerated by the addition of phosphatidylcholine micelles. According to the authors, this is caused by conformational changes in the enzyme, making the active site more accessible for the inhibitor. Uçar et al. [243] report that the hydrolysis of triacetin is enhanced when synthetic (nonsubstrate) surfaces are included in the reaction medium. In this case, the interface is provided by the addition of nonpolar *n*-hydrocarbons. At low tributyrin concentrations (the solubility limit of tributyrin is 0.44 mM) the activation of the enzyme by incorporation is even more pronounced (figure 3.8). The incorporated enzyme is up to 4 times more active than free CCL.

In tricaprylin emulsions the free enzyme is more active than the entrapped enzyme, when comparing initial activities (figure 3.9, left side). However, the reaction rate of the free enzyme gradually decreases during the initial stage of the reaction until a constant rate of 47% of the initial value within 4 minutes. In the case of incorporated lipase on the other hand, the reaction rate hardly changes with time (figure 3.9, right graph). The decreasing rate in the reaction of the free enzyme is most probably caused by the formation of caproate, one of the products during tricaprylin hydrolysis. This compound is, in contrast to butyrate and acetate, practically insoluble in water and acts as a (co-)surfactant. It will accumulate at the substrate-water interface, thereby decreasing the accessibility of the enzyme to the substrate [9,73,162]. A second contribution to the decrease in activity of the free enzyme might be the interaction of the carboxylate anion with the enzyme. It has been found that fatty acids can act as competitive inhibitors [241]. In the case of incorporated lipase, the vesicle bilayer can accommodate the caproate, thereby preventing the pollution of the substrate interface [15,161,208].

Trypsin digestion of free and incorporated lipase

Free lipase is inactivated by trypsin as the result of hydrolysis of the peptide bonds. For the incorporated CCL the major backbone of the enzyme is buried in the vesicle bilayer. The lipase is therefore not accessible for trypsin, resulting in a higher residual activity than in the case of free lipase (figure 3.10). This clearly demonstrates the protective influence of the vesicle bilayer. Similar results were obtained by Miranda et al. [164] in their studies on mushroom tyrosinase, incorporated in lecithin-cholesterol vesicles.

Effect of surfactant additives on the activity of free lipase

In the triacetin assay, only the addition of polymerised vesicles yields an increased activity (table 3.4). The introduction of a hydrophobic interface stimulates lipase activity. The conclusion that is underlined further by the tenfold enhancement of the activity in the assay with solubilised tributyrin (see table 3.5).

The decrease in activity by addition of nonpolymerised vesicles to the enzyme-substrate system may be due to the presence of the ester bond in the surfactant. Although these ester groups are not hydrolysed by the enzyme, they can act as inhibitors. As vesiculated ABHEMA Br provides an interface for the enzyme, the ester groups in this system can be more effective in the inhibition process than those in monomolecular dispersed ABHEMA Br. In the tributyrin and DNPB assays, where the free enzyme is already adsorbed to the substrate droplets before the addition of vesicle solution, this effect seems to play a minor role [79]. However, due to activation of the lipase at the substrate interface, monomolecular dispersed ABHEMA Br and CTAB can interfere more effectively with the enzyme than in the case of the homogeneous ('non-activating') triacetin system. The structural displacements within the enzyme due to adsorption to an interface, make the enzyme more susceptible to the operation of inhibitors [134]. CTAB is reported earlier to be an effective inhibitor for lipases [215,240,251].

In the case of tricaprylin as the substrate all positively charged additives increase the activity. Clearly, the inhibitory effect of the negatively charged caproate is prevented by association with these additives.

The addition of the non-ionic surfactant Brij 35 had no effect, although in literature it is reported that several polyoxyethylene ethers can enhance the activity to some extent [34,119,174].

3.5 Conclusions

This study shows that crude *Candida cylindracea* lipase can be purified by simple means. Comparison with data from literature indicate that different *Candida* preparations contain different proteins with lipolytic activity. Therefore, especially in kinetic studies, it is important that lipase preparations are purified before use.

Lipase from *Candida cylindracea* can be effectively entrapped in vesicles by simple incubation. In most cases, incorporated lipase has a higher activity than free lipase due to interfacial activation induced by the vesicle bilayer. This study further indicates that through addition of an interface to a homogeneous

solution, solubilised substrates are hydrolysed at a higher rate and that the enzyme becomes more sensitive to inhibitors.

Incorporation of enzymes in vesicles increases their stability towards surfactant additives and proteolytic attack, which can make these systems useful for drug targeting [148] and in detergent formulations, where surfactants are already used for enzyme stabilisation [1].

Vesicle-enzyme mixtures are optically clear, so they can be used in combination with spectroscopic techniques.

Understanding of enzyme vesicle interactions may lead to the development of multi-enzyme systems in vesicles, which can be used as micro-bioreactors.

4 LIPASE STABILITY IN VESICULAR SYSTEMS*

Keyword Phrases:

PREPARATION AND MORPHOLOGICAL CHARACTERISATION OF VESICLES COMPOSED OF BPAS**, A POLYMERISABLE ZWITTERIONIC SYNTHETIC SURFACTANT

INCORPORATION OF LIPASE FROM *CANDIDA CYLINDRACEA* IN BPAS VESICLES

STABILITY OF THE ENZYME-VESICLE SYSTEM IN THE PRESENCE OF WATER-MISCIBLE ORGANIC SOLVENTS

THERMAL STABILITY OF INCORPORATED LIPASE

PROTEOLYTIC STABILITY OF INCORPORATED LIPASE

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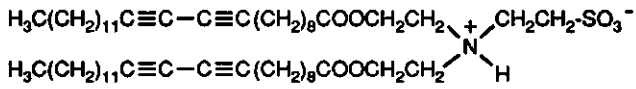
** BPAS is bis[2-(pentacosa-10,12-diynoyloxy)ethyl]-2-aminoethanesulfonic acid.

4.1 INTRODUCTION

For practical applications of vesicles as well as enzymes it is desirable to have samples of high stability. In medicine the life time of vesicle systems for targeting purposes should be controlled, whereas enzymatic activity should be preserved in an environment with proteases and antibodies, to ensure low doses and low dosing frequencies. In the detergent industries vesicles and enzymes have to maintain their stability in the presence of surface active compounds and proteolytic enzymes, at elevated temperatures and in alkaline environment. In other industrial applications reasons for destabilisation or inactivation are the presence of organic solvents or the necessity of elevated temperatures.

In this chapter, the incorporation of *Candida cylindracea* lipase (CCL) in vesicles from the synthetic polymerisable zwitterionic surfactant bis[2-(pentacosa-10,12-diynoyloxy)ethyl]-2-aminoethanesulphonic acid (BPAS) is described.



BPAS

Bis[2-(pentacosa-10,12-diynoyloxy)ethyl]-2-aminoethanesulfonic acid

The stability of the vesicle system is guaranteed by polymerising the diyn-containing surfactant by photopolymerisation, in contrast to ABDEMA Br, ABHEMA Br and BUEDMA Br (chapter 3), which are polymerised by radical polymerisation. In addition, BPAS is distinct in the site where the surfactants are linked together. Whereas BPAS surfactants are polymerised across the hydrophobic part of the apolar region, the headgroups are linked in ABDEMA Br and ABHEMA Br vesicles and in BUEDMA Br vesicles, the end of the amphiphilic chains are linked together.

The stability of free and incorporated CCL is monitored by comparing the activities of lipase preparations when stored under several denaturating conditions. These conditions are storage in buffer/ethanol or buffer/2-(*n*-butoxy)ethanol mixtures, at 50°C and 60°C, and in the presence of the proteolytic enzyme trypsin.

4.2 MATERIALS AND METHODS

Materials

Crude lipase from *Candida cylindracea* (CCL, lipase OF) was supplied by Meito Sangyo. The specific activity of the lipase preparation after purification (chapter 3) was 998 U tributyrin per mg protein; the protein content was 56%. Trypsin (bovine, type XI) was purchased from Sigma.

Pentacosa-10,12-diynoic acid was obtained from ABCR, 4-dimethylamino-pyridine (99%) from Aldrich, and 4-(2-hydroxyethyl)-1-piperazineethane-

sulphonic acid (HEPES, >99%) from BDH Chemicals. *N,N*-Bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (99+%), 2-(*n*-butoxy)ethanol (99%) and tributyrin (98%, distilled) were purchased from Janssen Chimica, oxalyl chloride (p.a.) and potassium hydroxide (p.a.) from Merck.

All organic solvents were distilled before use. The aqueous solutions were prepared with particle free, demineralised and deionised water purified by a Seralpur Pro 90C/Seradest LFM water purification system. Vesicle and enzyme were dissolved in 5 mM HEPES-KOH buffer pH 8.

Surfactant synthesis, vesicle formation and polymerisation

Bis[2-(pentacosa-10,12-diynoyloxy)ethyl]-2-aminoethanesulphonic acid (BPAS)

Pentacosa-10,12-diynoyl chloride was prepared from pentacosa-10,12-diynoic acid and oxalyl chloride in a 79% yield according to the procedure of Mattson and Volpenhein [160].

The surfactant bis[2-(pentacosa-10,12-diynoyloxy)ethyl]-2-aminoethanesulphonic acid (BPAS) was synthesised according to the method described by Hub, Hupfer and Ringsdorf [98-100] with some modifications. To a solution of 1.97 g (5.0 mmol) of pentacosa-10,12-diynoyl chloride in 10 ml of chloroform 0.4 ml (5 mmol) of pyridine, 0.1 g (0.8 mmol) of 4-dimethylaminopyridine and 0.53 g (2.5 mmol) of *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid were added. The mixture was refluxed for 20 hours. After cooling, 150 ml chloroform was added. The mixture was then washed with water, dried over magnesium sulphate, filtered and evaporated. The remaining solid was subsequently recrystallised in a chloroform-methanol mixture, yielding 1.70 g (1.8 mmol, 73%) of BPAS

Mp 102-103°C; ^{1}H-NMR (200 MHz, $CDCl_3$), δ 4.50 (t, 4 H, O-CH_2), 3.68 (br signal, 2 H, N-CH_2), 3.59 (br signal, 4 H, CH_2-N), 3.21 (br signal, 2 H, CH_2-SO_3H), 2.38 (t, 4 H, CH_2-C(O)), 2.22 (t, 8 H, CH_2-C≡C), 1.53 (m, 12 H, C*H*$_2$-CH_2-C≡C-C≡C-CH_2-C*H*$_2$-$(CH_2)_4$-C*H*$_2$), 1.23 (br signal, 52 H, CH_2), 0.86 (t, 6 H, CH_3).

Vesicle preparation and polymerisation

BPAS vesicles were prepared by sonicating 10 ml of 5 mM surfactant suspensions in HEPES-KOH buffer (5 mM, pH 8.0) for 30 minutes at 60°C using a 13 mm tip of a VC-300 Sonics & Materials sonifier, output control set at level 8 (corresponding to 60 W output power) and pulser set at a 50% pulse rate. After 10 minutes the suspension becomes opaque, which is an indication for vesicle formation. Before further use, all vesicle solutions were centrifuged for 30 minutes at 15,500 g in a Sigma 202M centrifuge in order to remove any possible titanium particles from the sonifier tip.

Polymerisation was accomplished by irradiation of the vesicle solutions in a quartz cuvette at room temperature with a Hanau TQ150 high-pressure mercury arc connected to a Hanau Q150 power supply. The starting vesicle solutions were opaque colourless and turned from purple and carmine for partly polymerised vesicle solutions, to bright red for completely polymerised vesicle solutions. Vesicle solutions were considered to be completely polymerised when the absorbance at 538 nm reached a plateau value (molar absorption 1700 $M^{-1}cm^{-1}$).

Preparation of vesicle samples for electron microscopic investigation

Samples for the Transmission Electron Microscope (JEOL 1200EX) were prepared by placing drops of the vesicle preparations on 250 mesh copper grids coated with a Formvar film. The excess of fluid was absorbed into a piece of filter paper and the grids were immediately frozen in liquid propane. Subsequently, the samples were freeze-dried in a BAF 400 freeze-fracturing apparatus (Balzers Union, Liechtenstein) and rotary shadowed with platinum (2 nm) at an angle of 40°.

Enzyme assays

Lipase activity was determined by a titrimetric method using tributyrin as the substrate. Tributyrin is slightly soluble in water; the solubility limit is 0.4 mM [225]. The activity of lipase was studied at two substrate concentrations[1], at 0.32 mM, where tributyrin is soluble, and at 16.5 mM, where tributyrin is present as an emulsion. In the former case 500 µl of a 32 mM tributyrin solution in acetonitrile was added to 50 ml of water and was stirred until all tributyrin was solubilised. In the latter case 0.2 g of tributyrin was emulsified in 40 ml of water by 2 minutes of sonication (13 mm tip, output control set at level 8). The enzyme reactions were started by the addition of free or in vesicle incorporated CCL to a final concentration of 141-197 ng protein/ml. The hydrolysis was monitored by titration of the produced butyric acid with 2 to 10 mM KOH solutions; the reaction was followed for at least 5 minutes. The addition of base was administered by a Schott Geräte T100 titrating and dosing system equipped with an AUTOLAB/ISEC personal computer controlled automatic titrator (Eco Chemie, Utrecht, NL). The reactions were carried out at pH 7, 37°C and under a nitrogen atmosphere. It was confirmed by separate measurements that the ester groups in the surfactant were not hydrolysed by CCL.

Incorporation of lipase in BPAS vesicles

The incorporation of *Candida cylindracea* lipase into nonpolymerised and polymerised vesicles was carried out according to the incubation method described in chapter 3. In a typical example, CCL and BPAS vesicles were mixed in HEPES buffer (5 mM, pH 8) and incubated. Final concentrations of enzyme and vesicle were 71 µg protein/ml and 1.25 mM (protein-to-surfactant ratio 56 ng/mol). The incorporated enzyme could be isolated by centrifugation of the enzyme-vesicle mixture for 4 hours at 48,500 g at 20°C in a Beckman 2-21 centrifuge using a JA-21 rotor. By measuring the enzymatic activity in both the supernatant (non-incorporated lipase) and the resuspended pellet (lipase incorporated in vesicle) it was determined that all of the initially added enzyme was incorporated.

For the preparation of an enzyme-vesicle sample in which the vesicles were polymerised in the presence of CCL, a slightly different method was applied, since CCL was deactivated by the high intensity of the light of the mercury arc. First, partly polymerised vesicles were prepared by irradiating a monomeric nonpolymerised vesicle solution through a glass filter (wavelength cut-off 300 nm) for one hour, giving a molar absorption of 45 $M^{-1}cm^{-1}$ at 538 nm. By

[1]The term 'concentration' refers to the overall amount of substrate in the assay.

using a filter, the degree of polymerisation is better controllable. Then, the enzyme was added and this mixture was irradiated through the glass filter for another two hours (molar absorption: 95 $M^{-1}cm^{-1}$). Hereby, the loss in activity is reduced to 14 %. In a control experiment, free enzyme is deactivated to the same extent.

Sample preparation for stability studies

The stability of free and incorporated CCL was compared by incubating the enzyme preparations under several denaturating conditions.

The lipase samples were mixed with ethanol or an aqueous solution of 2-(*n*-butoxy)ethanol (75% v/v, adjusted to pH 7) to final concentrations of 71 µg protein/ml, 1.25 mM BPAS vesicles, 50% v/v ethanol or 37.5% v/v 2-(*n*-butoxy)ethanol.

For studying the thermal stability, the enzyme solutions were incubated at 50°C or 60°C.

Proteolytic stability was tested by mixing equal amounts of lipase solution (free or incorporated) with a trypsin solution. The final concentrations of the reactants were 71 µg protein/ml, 1.25 mM BPAS vesicles, 500 µg trypsin/ml in 10 mM Tris-HCl buffer pH 8.5 and 0.2 mM calcium chloride. The mixtures were incubated at 30°C. Due to the relatively low rate of the proteolytic reaction it was not necessary to inhibit trypsin activity during the lipase assay. Separate measurements showed that trypsin did not alter lipase reaction kinetics.

From the above mentioned preparations, samples were taken and the lipase activity was determined using the 16.5 mM heterogeneous tributyrin assay.

4.3 Results and Discussion

Characterisation of the vesicle system

Nonpolymerised BPAS vesicles are stable (i.e. do not flocculate) for one month in HEPES-KOH buffer (5 mM, pH 8). The polymerised analogues have shelf-lives of at least 6 months in this medium. Polymerisation of BPAS vesicles was carried out at room temperature, since polymerisation is inhibited at temperatures above the phase transition temperature of the nonpolymerised diacetylenic surfactant vesicles [151,152]. As can be seen in figure 4.1, the size of nonpolymerised vesicles is 60 (± 10) nm (left photograph). The size is not altered upon polymerisation (figure 4.1, right photograph). When the pH of nonpolymerised and polymeric vesicle solutions is lowered to 4, all solutions immediately start to flocculate. The dissociation constant for the amine moiety in BPAS is 7.06 [42]. Consequently, at a pH lower than 5 the surfactant is almost completely in its zwitterionic form and the surfactant is net uncharged. Due to changes in the charge of the headgroups of the surfactants, the packing density of the membrane is altered. Apparently, under these conditions, the vesicles are destabilised and the vesicle solutions start to flocculate. In alkaline conditions, the amine moiety is completely deprotonated and the surfactant is converted to the anionic form. In this case, nonpolymerised vesicles can be destabilised due to electrostatic repulsion between adjacent headgroups. In polymeric vesicles however, destabilisation by electrostatic repulsion between the headgroups is compensated by the

covalent crosslinking of the alkyl chains. Indeed, whereas the stability of nonpolymerised vesicles at a pH higher than 10 is restricted to several minutes, polymerised vesicles do not flocculate, even at a pH of 12. Koch and Ringsdorf [133] observed similar behaviour for bis[2-(hexacosa-10,12-diynoyloxy)ethyl]-2-aminoethanesulphonic acid in monolayers and vesicles.

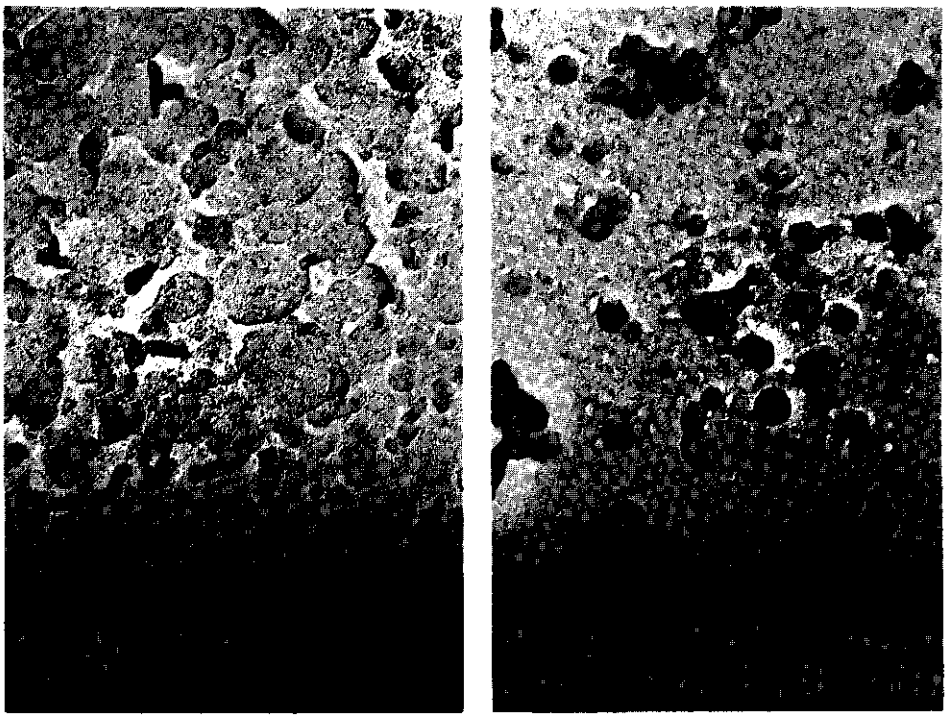


Figure 4.1 TEM photographs of platinum shadowed vesicle solutions. The left photograph depicts nonpolymerised BPAS vesicles. Polymerised BPAS vesicles are presented in the right photograph.

When ethanol or 2-(*n*-butoxy)ethanol is added to vesicle solutions the enhanced stability of polymeric vesicles is even demonstrated more pronounced. Whereas nonpolymerised vesicles start to flocculate upon addition of more than 20% (v/v) ethanol or small amounts of 2-(*n*-butoxy)-ethanol (less than 10%), the polymerised vesicles are even stable in the presence of 50% ethanol or 37.5% 2-(*n*-butoxy)ethanol. The morphology of polymeric vesicles is not affected in the presence of ethanol (figure 4.2); the size of the vesicles in the presence of 2-(*n*-butoxy)ethanol is decreased from 60 nm to 20 nm (not shown).

The morphology of enzyme loaded vesicles (protein-to-surfactant ratio 56 ng/mol) is shown in figure 4.3. A shift towards larger sizes of vesicles (aging), as reported by Dini et al. [49] is prevented by polymerisation.

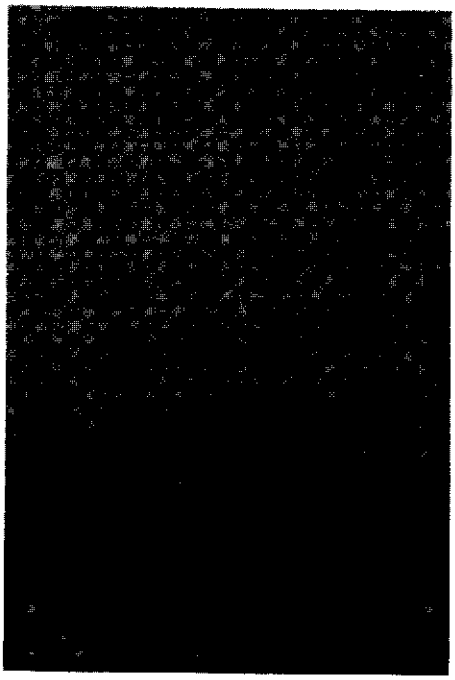


Figure 4.2 TEM photograph of polymeric vesicles in the presence of 50% ethanol. The vesicles were incubated for 2 months at 4°C.

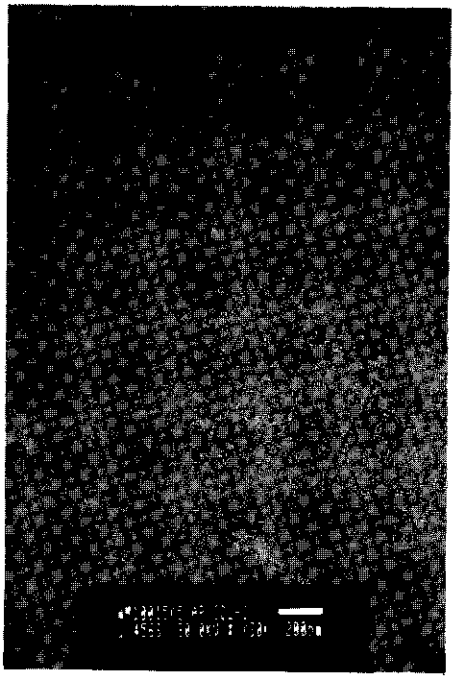


Figure 4.3 TEM photograph of enzyme loaded vesicles. The mixture was incubated for 2 months at 4°C.

Incorporation of *Candida cylindracea* lipase in BPAS vesicles

It is known that surfactants can inactivate proteins by interaction of surfactant monomers with the protein, followed by the exposure of the buried hydrophobic protein residues to the solvent. Unfolding of the protein results in denaturation, which is followed by a decrease in activity [252]. However, application of vesicles composed of quaternary dialkyl ammonium surfactants has shown that lipase from *Candida cylindracea* can be incorporated into vesicles from these surfactants with full retention of the enzymatic activity (chapter 3). The most effective method for incorporation is shown to be incubation of lipase and vesicles. This method is found to be also applicable for incorporating CCL in BPAS vesicles. When enzyme and vesicles are mixed and allowed to incubate for 15 minutes, the vesicle incorporated lipase can be isolated by centrifugation. A control experiment, in which the same procedure was followed using enzyme, but without addition of vesicles, showed that free enzyme remained in the supernatant after centrifugation. In contrast, after incubating lipase with vesicles, all activity is found in the pellet, which indicates that all of the initially added lipase is incorporated into the vesicles at

these conditions. Therefore, in the subsequent experiments lipase-vesicle mixtures were used without prior isolation.

Table 4.1 Activity of free and incorporated CCL at tributyrin concentrations of 0.32 mM (homogeneous) and 16.5 mM (heterogeneous).

	Specific activity [U/mg]	
sample	0.32 mM	16.5 mM
CCL: free[1]	100	100
in nonpolymerised vesicles	831	127
in polymeric vesicles[2]	640	103
in partly polymerised vesicles	-	86

[1] The initial activities at 0.32 and 16.5 mM were 42, respectively 488 U/mg.

[2] In this case, a nonpolymerised vesicle solution was exposed for one day to the mercury arc. Mixing of enzyme and vesicles with exposure times of 5, 15 or 60 minutes (and thus with different degrees of polymerisation) yielded similar results.

As can be seen from the table 4.1, CCL incorporated in BPAS vesicles is 3 to 27% more active in the heterogeneous assay at high tributyrin concentration. The activity remains constant for a period of minimal 10 days. The higher activity is most probably caused by an increased accessibility of substrate to the active site of the enzyme. Recently, X-ray structure analysis of several lipases [19,216,257] revealed that the active site in these enzymes is buried and covered with a peptide loop, often referred to as flap or lid. Adsorption of lipase to a surface activates the enzyme by displacement of the flap, which makes the active site accessible for substrate molecules [29,90]. It was found that many lipases have the same structural features, including lipase from *Candida cylindracea* [134]. The adsorption and subsequent activation of the enzyme at the substrate-water interface is considered to be the rate limiting step in lipolysis [248]. However, in a vesicle system the enzyme is already present at an interface. The interaction between lipase and the vesicle membrane induces the structural changes in the enzyme necessary for activation. Therefore, substrate molecules can be more readily attacked by the enzyme. In chapter 5 the interaction between lipase and the vesicle membrane is elucidated using fluorescence techniques. The incorporation process of the lipase into the vesicle bilayer is caused by electrostatic and hydrophobic interactions. After the initial interaction, which is of an electrostatic nature, the hydrophobic part of the membrane will induce further incorporation of the enzyme into the vesicle membrane [124,188,238,259,263]. Insertion of the protein into the vesicle bilayer will be accompanied by rearrangement of surfactant molecules around the enzyme and by changes in its state of hydration, charge distribution and other rearrangements in the protein structure [178,204]. The structural changes in the enzyme and the change in the medium around the enzyme may explain the larger activity of lipase when incorporated in nonpolymerised vesicles. In polymerised vesicles this effect is less pronounced, since the enzyme is located between polymeric surfactants [177], which restricts the free movement of the lipase molecule. In the partly polymerised vesicle system the vesiculated BPAS monomers have been polymerised in the presence of lipase. This results in a polymer network in which the enzyme is completely

embedded and leads to a larger restriction in enzyme movement. It was anticipated that this should lead to higher enzyme activities. However, data indicate that the positive effect is undone, probably due to a decreased accessibility of the active site for the substrate caused by the polymer network.

Substrates that are completely soluble in water, have no interface that can trigger the removal of the flap, i.e. activate the enzyme, and lead in general to much lower enzyme activities than emulsified substrates. Recent X-ray crystallographic studies confirm that binding of lipase is accompanied by structural changes, which are essential for the activation of the enzyme [45]. Therefore, the homogeneous reaction mixture with 0.32 mM tributyrin shows an even more pronounced increase in activity through incorporation of the enzyme in vesicles, than in case of the heterogeneous assay system. The activities in the former case are 6 to 8 times higher than for free lipase. Furthermore, the lag-phase that is observed when free enzyme is added to the assay, is not present when incorporated lipase is added to the tributyrin solution. This confirms the assumption that the lipase is already activated when it is incorporated into a vesicle membrane.

It is possible to polymerise the vesiculated BPAS surfactant molecules in the presence of lipase with retention of the enzymatic activity. Due to the high intensity of the light of a mercury arc, lipase is deactivated completely within 15 minutes, if quartz glassware is used. However, when normal glassware is used, the denaturation is controllable, and the loss in activity can be reduced to 14%.

Stability of free and incorporated *Candida cylindracea* lipase

The stability of CCL, free or incorporated in polymerised vesicles, was tested by incubation of the enzyme under several denaturating conditions. The stability was expressed as residual activity versus incubation time. The denaturating conditions are: addition of water-miscible organic solvents (ethanol and 2-(*n*-butoxy)ethanol), storage of the enzyme system at 50°C and at 60°C and addition of the proteolytic enzyme trypsin to the enzyme-vesicle system.

Influence of organic solvent

Addition of ethanol (50%, v/v) results in an immediate decrease in activity of 37% and 34% respectively, for free and in polymerised vesicles[2] incorporated CCL (not shown in the figure). When the activity of the enzyme-ethanol mixtures is monitored after this immediate decrease, the free lipase is deactivated completely within 300 hours, whereas the incorporated enzyme has a residual activity of 10% (figure 4.4[3]). Addition of 2-(*n*-butoxy)ethanol (37.5% v/v) results in a similar effect as in the case of ethanol (figure 4.5[2]). Again, there is an immediate decrease in activity of 21 or 40% for free, respectively incorporated lipase, when the solvent is added (not shown in the figure).

[2]The results for CCL incorporated in nonpolymerised vesicles were similar to the results with polymerised vesicles. (not shown in graph 4.4 and 4.5).

[3]In figure 4.4 and 4.5 the activity of the enzyme solution directly after addition of the organic solvent is taken as 100%.

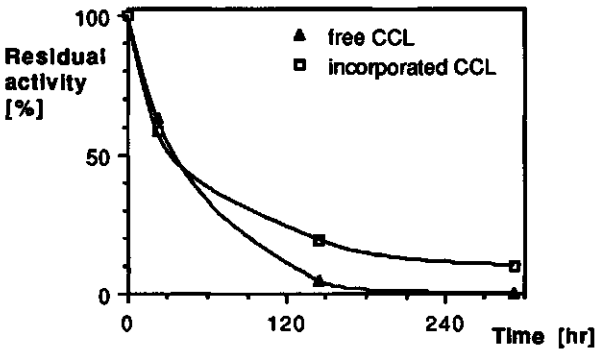


Figure 4.4 Storage stability of free CCL and CCL incorporated in polymerised vesicles in the presence of 50% ethanol.

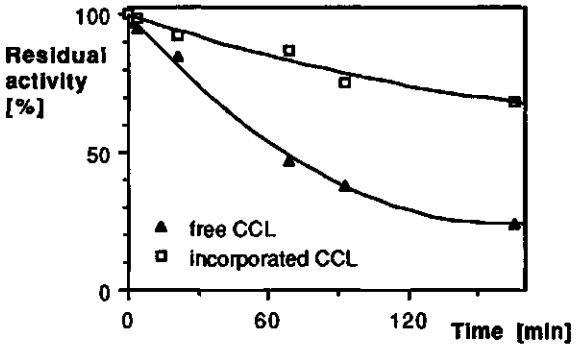


Figure 4.5 Storage stability of free CCL and CCL incorporated in polymerised vesicles in the presence of 37.5% 2-(*n*-butoxy)ethanol.

In case of 2-(*n*-butoxy)ethanol the deactivation of the enzyme is considerably limited in surfactant environment. The residual activity of free CCL is 24% after 165 minutes, whereas the enzyme in vesicle preparation has a residual activity of 68%.

There are two main factors that may influence the inactivation of enzymes upon the addition of water miscible organic solvents. Firstly, the binding of solvent molecules to the protein interferes with binding of substrate to the enzyme. Secondly, organic solvents alter the dielectric constant of the solution and affect the balance of noncovalent forces that maintain the protein in its native conformation. The stabilising effect of vesicles is most probably caused by the altered medium surrounding the enzyme. In contrast to an enzyme in solution, the free movement of the incorporated protein is decreased and the number of drastic conformational changes, which lead to denaturation, are minimised [75,233,234,252].

Thermal stability

The effect of temperature on the stability of CCL upon incorporation in polymerised vesicles[4] is depicted in figure 4.6 (50°C) and 4.7 (60°C). It is clear from these results that the thermal stability of CCL is enhanced by incorporation. Due to thermal movement and decreased solvent structure, the native structure of a protein in solution is distorted at temperatures above the physiological range [21,214], and subsequently (irreversibly) inactivated [3,267]. Due to the fact that incorporated CCL has less freedom of motion and is probably less exposed to solvent molecules, the decrease in activity is reduced. Semi-logarithmic plots of residual activity of free CCL at 50°C versus time (not shown) match with first order inactivation kinetics. Curves for the incorporated enzyme preparations point to a biphasic inactivation model. The kinetics of inactivation seem to be somewhat different at a higher temperature (60°C), a phenomenon that is reported more often in the literature (see e.g. [155,184,189, 226]).

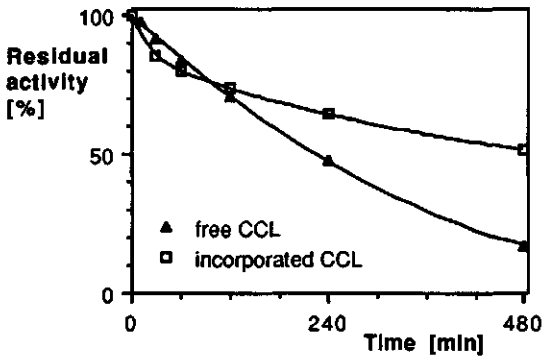


Figure 4.6 Thermal inactivation at 50°C.

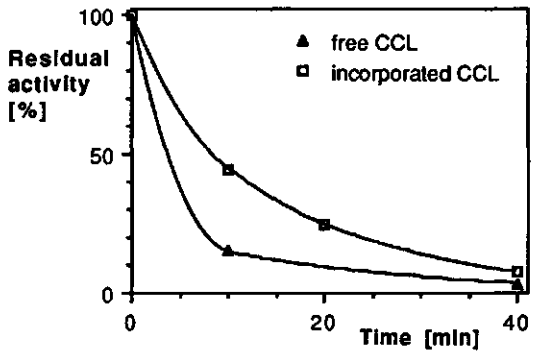


Figure 4.7 Thermal inactivation at 60°C.

[4]The results for CCL incorporated in nonpolymerised vesicles were similar to the results obtained for polymerised vesicles (not shown in graph 4.6 and 4.7)

Proteolytic stability

The protective influence of the vesicle bilayer is most clearly shown when the resistance against proteolytic degradation of different CCL preparations is tested. Hydrolytic activity of free lipase is reduced to 50% within 2 hours of incubation with trypsin, whereas the incorporated enzyme preparations are hardly attacked by the proteolytic enzyme (figure 4.8).The inactivation by trypsin is the result of hydrolysis of peptide bonds in the lipase molecule. Partial unfolding of a protein molecule makes it more susceptible to proteolytic degradation. For vesicle incorporated lipase, the major backbone of the enzyme is buried into the vesicle bilayer membrane. The freedom of movement - and with that, undesired unfolding - of the lipase molecule is therefore restricted. Thus, the enzyme is less exposed to the bulk solvent and therefore not accessible for the attack of trypsin that, according to Vaz et al. [246], interacts only with the outer surface of a vesicle membrane, but does not penetrate into the bilayer. Protection from proteases is only guaranteed when an enzyme is completely incorporated in the vesicle membrane, as was shown by Hebdon et al. [94]. Adenylate cyclase that was incorporated in egg yolk phosphatidylcholine vesicles, was still inactivated by trypsin, because part of the enzyme was exposed on the outer surface of the vesicle. Incorporation of lipase however, shields the enzyme and results in a higher residual activity. The best protection against the hydrolytic action of trypsin is offered by lipase-vesicle preparations in which the vesicles are (partly) polymerised in the presence of the protein. Since the surfactant molecules have been crosslinked in the presence of enzyme, the lipase molecules are embedded in the polymer network. This offers better protection from proteolytic enzymes than incorporation in nonpolymerised or polymerised vesicles. Similar results were obtained when quaternary dialkylammonium surfactants were used (chapter 3). Mushroom tyrosinase is also reported to be protected from proteolytic attack when incorporated in lecithin-cholesterol vesicles [164].

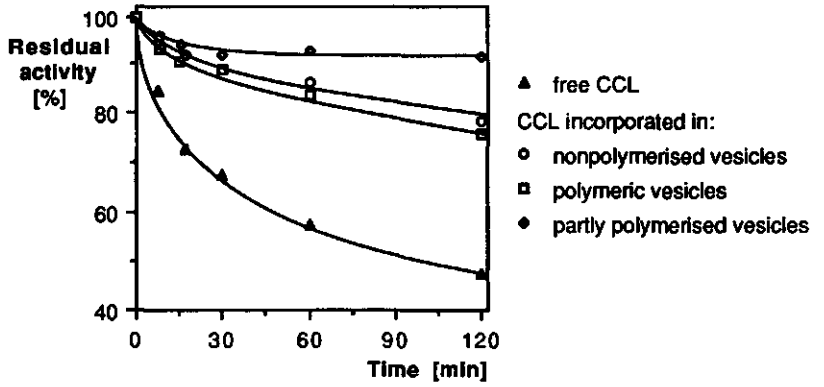


Figure 4.8 The stability of free and vesicle incorporated *Candida cylindracea* lipase in the presence of trypsin.

4.4 CONCLUSIONS

In this chapter it is shown that it is possible to incorporate lipase in synthetic vesicles with full retention of activity. By the use of polymerisable surfactants, enzyme-vesicle systems are created with high stability. In addition, the (storage) stability of the enzyme is enhanced by incorporation, since the vesicle bilayer protects the enzyme from inactivation. Therefore, this combination of enzymology and membrane mimetic chemistry will especially be useful and advantageous in applications of enzymes as well as vesicles in which stability and enzymatic activity are of great importance.

5 FLUORESCENCE STUDIES ON LIPASE-VESICLE INTERACTION*

Keyword Phrases:

DETERMINATION OF THE PHASE TRANSITION OF NONPOLYMERISED AND POLYMERIC ABHEMA Br** VESICLE BILAYERS BY THE USE OF STEADY STATE FLUORESCENCE ANISOTROPY

CHARACTERISATION OF THE INCORPORATION OF *CANDIDA CYLINDRACEA* LIPASE IN ABHEMA Br VESICLES BY ENERGY TRANSFER STUDIES

CHARACTERISATION OF THE INTERACTION BETWEEN RITC**-MODIFIED *CANDIDA CYLINDRACEA* LIPASE AND ABHEMA Br VESICLES BY THE USE OF STEADY STATE FLUORESCENCE ANISOTROPY

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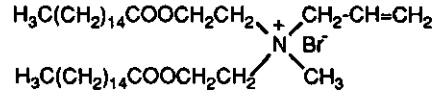
** ABHEMA Br: *N*-allylbis[2-(hexadecanoyloxy)ethyl]methylammonium bromide. RITC: rhodamine isothiocyanate

5.1 INTRODUCTION

This chapter describes studies on the interaction of *Candida cylindracea* lipase (CCL) with positively charged, polymerisable surfactant vesicles. By the use of fluorescence anisotropy, the microviscosity of the bilayer of nonpolymerised and polymeric *N*-allylbis[2-(hexadecanoyloxy)ethyl]methylammonium bromide (ABHEMA Br) vesicles and their phase transition temperatures could be determined.



ABHEMA Br

***N*-Allylbis[2-(hexadecanoyloxy)ethyl]methylammonium Bromide**

By the use of fluorescence energy transfer, the incorporation of native CCL from the bulk aqueous phase into the vesicle bilayer was monitored. In addition, anisotropy measurements were used for monitoring the interaction between vesicles and lipase modified with a rhodamine label.

5.2 INTRODUCTION INTO FLUORESCENCE

Fluorescence occurs when molecules are irradiated with ultraviolet or visible light. Upon irradiation, electrons are excited. They are transferred from the ground state (S_0) to energetically higher excited states (S_1, S_2). Light absorption, which results in excitation, will only take place when the energy difference between the ground state and the excited state is equal to the energy of the light (hν). The absorbance process is extremely fast in the range of 10^{-15} sec. Following light absorption, several deactivation processes can occur. The absorbance and emission of light is illustrated by the energy level diagram suggested by Jablonski (figure 5.1).

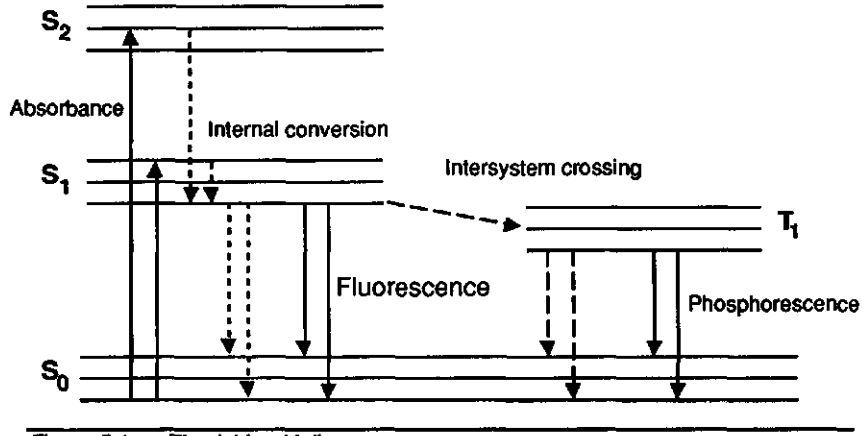


Figure 5.1 The Jablonski diagram.

Usually, molecules are excited to some higher vibrational level of the excited states, and then rapidly (10^{-12} sec) relax to the lowest vibrational level of S_1 (internal conversion). From this lowest excited state S_1, the molecule can return to the ground state by emitting light. This process is called fluorescence. Thus, fluorescence is the emission of light, which results from the return of an electron from the lowest excited state to the ground state. Fluorescence lifetimes are typically near 10^{-8} sec. The molecule can also return to the ground state by way of radiationless internal conversion or through conversion to the first triplet state T_1 (intersystem crossing). Emission from T_1 is termed phosphorescence (lifetime 10^{-3} - 1 sec) [147].

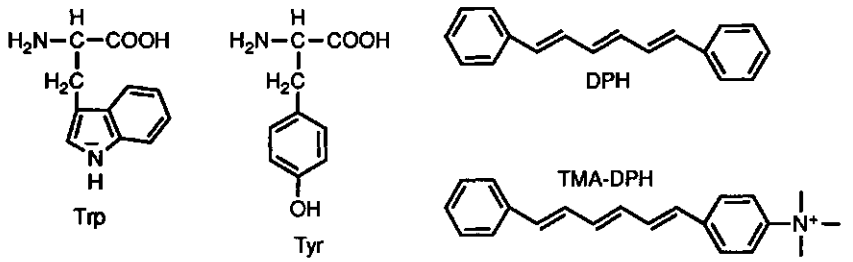


Figure 5.2 Some examples of fluorescent molecules. Trp: tryptophan, Tyr: tyrosine, DPH: 1,6-diphenyl-1,3,5-hexatriene, TMA-DPH: 1-[4-(trimethylammonium)-phenyl]-6-phenyl-1,3,5-hexatriene.

Molecules that display fluorescence generally possess delocalised electrons. Some typical fluorophores are depicted in figure 5.2.

Proteins show fluorescent behaviour due to the fluorescent amino acid residues tryptophan (Trp) and tyrosine (Tyr). The tyrosine fluorescence is often masked by energy transfer to the energetically lower emitting tryptophan residues. Especially the Trp residue is very sensitive to the polarity of the surrounding medium [30]. Conformational changes or binding of ligands is sometimes manifested in spectral shifts. The emission spectrum of proteins therefore reflects the average exposure of the tryptophan residues to the aqueous phase.

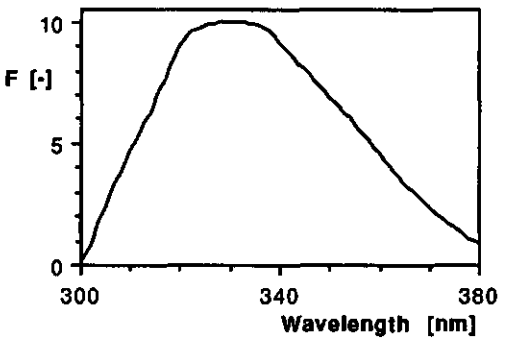


Figure 5.3 Emission spectrum of CCL. Excitation wavelength is 280 nm, emission wavelength maximum lies at 330 nm.

Proteins are excited at wavelengths around 280 nm and have emission maxima that can range from 320 to 350 nm. The emission spectrum (fluorescence intensity versus wavelength) of lipase from *Candida cylindracea* (CCL) is plotted in figure 5.3.

1,6-Diphenyl-1,3,5-hexatriene (DPH, figure 5.2) is a so-called hydrophobic fluorescent membrane probe. This substance is insoluble in water and partitions readily into the apolar region of bilayer membranes. By derivatisation of DPH, e.g. with a trimethylammonium group (TMA-DPH, figure 5.2), probes can be localised in selected areas of the membrane, as shown in figure 5.4 for both DPH and TMA-DPH partitioned into a bilayer membrane [171,197].

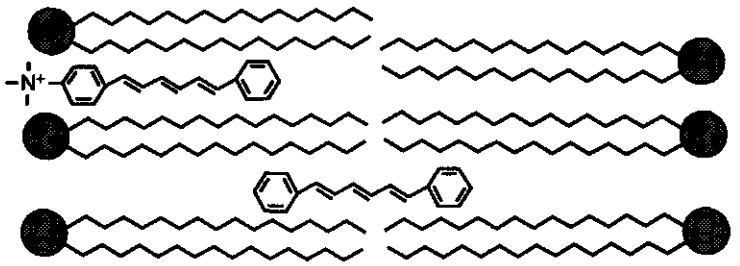


Figure 5.4 Schematic representation of the localisation of DPH and TMA-DPH in a bilayer membrane.

Whereas DPH is localised completely in the hydrophobic core of the membrane, TMA-DPH is thought to reside at the membrane-water interface. Thus DPH is an internal probe and TMA-DPH is an interfacial membrane probe. Emission spectra of DPH and TMA-DPH are shown in figure 5.5.

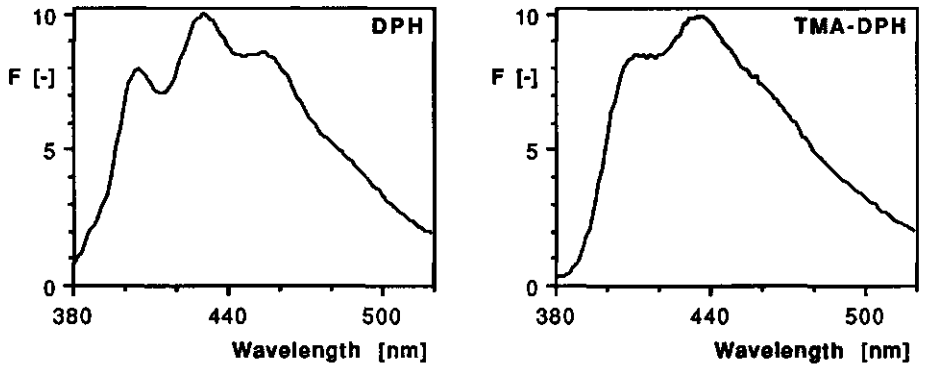


Figure 5.5 Emission spectra of the membrane probes DPH and TMA-DPH solubilised in chloroform; excitation wavelength is 360 nm.

Fluorescence anisotropy

In isotropic solutions, molecules are oriented randomly. Upon excitation with polarised light the emission from the fluorescent samples is also polarised. Fluorophores with transition moments aligned (nearly) parallel to the polarisation vector of the excitation will predominantly be excited. Thus,

photoselection, the selective excitation of an isotropic solution, results in an anisotropic distribution. The anisotropy can be measured by analysing the fluorescence as a function of the polarisation vector of the emission, as indicated in figure 5.6.

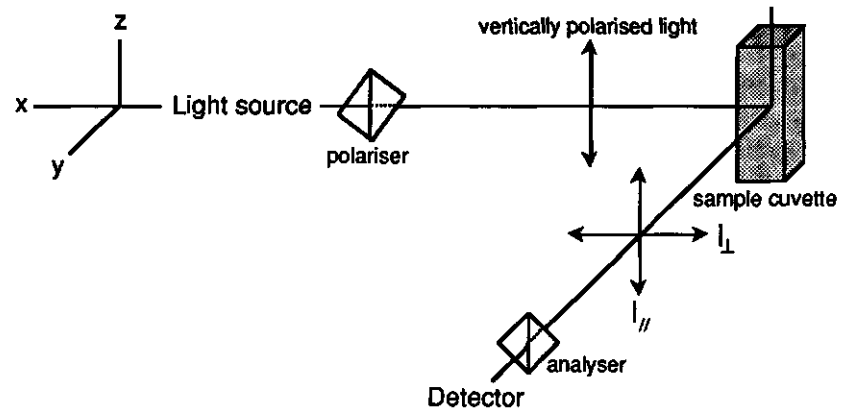


Figure 5.6 Schematic diagram for measurement of fluorescence anisotropy.

By irradiation of a sample with vertically polarised light (parallel to the z-axis), two different fluorescent intensities can be analysed. When the emission polariser or analyser is oriented parallel to the excitation, $I_{//}$ can be observed and likewise, when the analyser is oriented perpendicular, $I_\perp$ can be observed. From these observations, the anisotropy r is defined as:

$$r = \frac{I_{//} - I_\perp}{I_{//} + 2*I_\perp} \quad (5.1).$$

Measurement of anisotropy is widely used in biochemical and biophysical research, since changes in size, shape or (rotational) freedom of movement of (bio-)molecules will affect the anisotropy [48,246]. The latter effect is used for instance for the determination of the microviscosity, the motion of the surfactants chains, in bilayer membranes [222,229]. The internal structure of the membrane is in a gel-like state at temperatures under the phase transition temperature. Consequently, the freedom of motion of an inserted membrane probe like DPH is restricted. Upon exceeding the phase transition temperature, the membrane core goes from a gel-like to a fluid state. Thus, the rotational freedom of the label will also increase and a polarised excited DPH molecule will be depolarised more rapidly. By the use of anisotropy, the phase transition in membrane bilayers can therefore be measured.

In addition, fluorescence anisotropy can be used to indicate the interaction of proteins with each other or e.g. with vesicles [48]. Upon interaction of a relatively small fluorescent protein and a relatively large (non-fluorescent) entity, the rotational freedom of the protein is restricted and an increase in the anisotropy of the protein will occur (see figure 5.7). This results in an increased polarised emission.

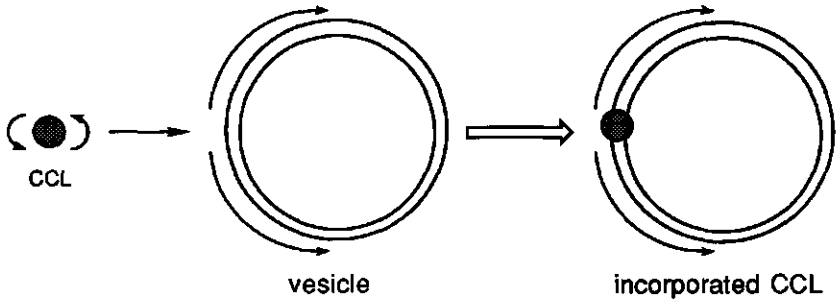


Figure 5.7 Schematic representation of the interaction and extent of freedom of a protein (small) and a vesicle (large).

Fluorescence energy transfer

In addition to fluorescence anisotropy, resonance energy transfer (ET) provides information on the interaction between proteins and vesicles [48,246]. The method utilises the nonradiative energy transfer of the excited state energy of a donor, e.g. the fluorescent groups in proteins, to an acceptor, e.g. a fluorophore present in the bilayer of vesicles. A weak dipolar coupling between donor and acceptor molecules is a requirement for energy transfer. The rate constant of transfer depends (i) on the overlap of the emission spectrum of the donor molecule and the excitation spectrum of the acceptor molecule, (ii) on the mutual orientation of donor and acceptor dipole moments and (iii) on the distance between these molecules. This overlap integral, the average orientation factors and some other quantities can be collected in the so-called critical distance between the fluorophores, which ranges from 2 to 5 nm. This range of values is comparable to the diameter of most proteins and the thickness of vesicle membranes. It can therefore be concluded that energy transfer will be manifested only if the protein and bilayer membrane are in very close range. The principle of energy transfer is depicted in figure 5.8.

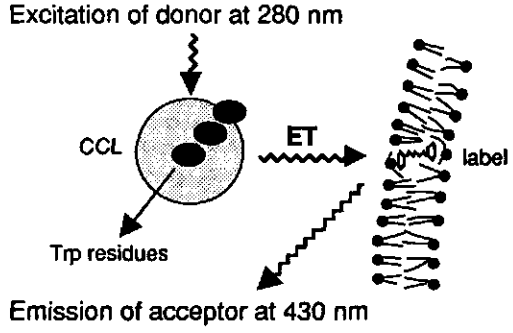


Figure 5.8 Schematic representation of energy transfer using CCL as a donor molecule and DPH as an acceptor molecule.

The interaction of CCL with vesicles can be monitored by energy transfer when DPH or TMA-DPH are used as membrane probes. The emission spectrum of the enzyme and the absorption spectrum of these probes (figure 5.9) have a sufficient overlap to let energy transfer take place.

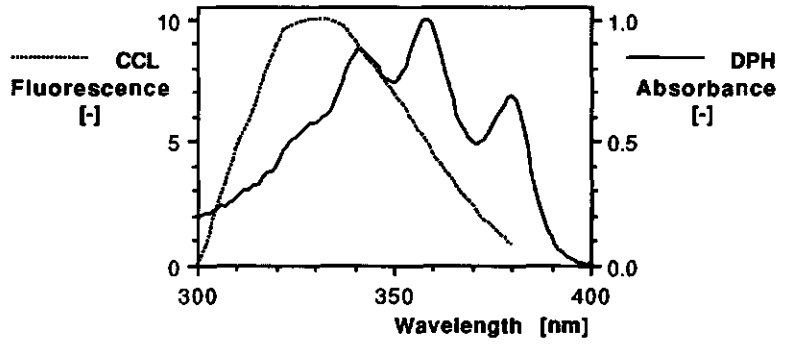


Figure 5.9 Emission spectrum of CCL and absorption spectrum of DPH.

5.3 MATERIALS AND METHODS

Enzymes/Reagents.

Purification of *Candida cylindracea* lipase (CCL; lipase OF, Meito Sangyo) and synthesis of the polymerisable surfactant *N*-allylbis[2-(hexadecanoyloxy)-ethyl]methylammonium bromide (ABHEMA Br) are described in chapter 3.

Tris(hydroxymethyl)aminomethane (Tris; 99+%) and 1,6-diphenyl-1,3,5-hexatriene (DPH; 98%) were obtained from Janssen Chimica; Sephadex G-25 (superfine) was purchased from Pharmacia. Rhodamine X isothiocyanate (RITC) and 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene *p*-toluene sulphonate (TMA-DPH) were supplied by Molecular Probes. All aqueous solutions were prepared in particle free, demineralised and deionised water, purified by a Seralpur Pro 90C/Seradest LFM water purification system.

Preparation of rhodamine labelled CCL

Candida cylindracea lipase was provided with a rhodamine label by mixing 3.46 mg of lipase (59 nmol) in 2 ml of 10 mM Tris-HCl buffer pH 9 and 0.5 mg (14.5 eq) rhodamine isothiocyanate (RITC) in 0.5 ml buffer. After 2 hours reaction time, the labelled protein was separated from the unreacted label by passing the reaction mixture through a sephadex G-25 column, using 1 mM Tris-HCl pH 7 as an eluens. The label to protein ratio was determined by means of UV/Vis spectrophotometry. In separate measurements, the extinction coefficients ε for RITC at 278 and 578 nm and for CCL at 278 nm[1] were determined. The label concentration in the modified enzyme was determined from the absorbance at 578 nm. The protein concentration was calculated from the absorbance at 278 nm, which had to be corrected for the

[1]The extinction of lipase at 578 nm was negligible.

contribution of the absorbance originating from the label at that wavelength. Thus, the label to protein ratio was calculated from:

$$\text{label to protein ratio} = \frac{\left(\frac{A_{578}}{\varepsilon_{578}^{RITC}}\right)}{\left(\frac{A_{278} - \left\{\frac{A_{578}}{\varepsilon_{578}^{RITC}} \times \varepsilon_{278}^{RITC}\right\}}{\varepsilon_{278}^{CCL}}\right)} \quad (5.2)$$

A = absorbance of the labelled protein at 278 nm (A_{278}) and at 578 nm (A_{578}), ε = molar extinction coefficients for CCL at 278 nm and RITC at 278 nm and 578 nm ($\varepsilon_{278}^{CCL} = 36{,}700\ M^{-1}$, $\varepsilon_{278}^{RITC} = 11{,}700\ M^{-1}$, $\varepsilon_{578}^{RITC} = 25{,}500\ M^{-1}$).

Preparation of vesicle solutions

Unlabelled vesicles were prepared by sonication of dispersions of nonpolymerised or polymeric *N*-allylbis[2-(hexadecanoyloxy)ethyl]methylammonium bromide. Polymeric ABHEMA Br was prepared by prepolymerisation of the surfactant in tetrahydrofuran via radical polymerisation as described earlier (chapter 3). Nonpolymerised vesicles were provided with membrane probe molecules by mixing stock solutions of nonpolymerised or polymeric ABHEMA Br in chloroform/ethanol (5/1, v/v) with DPH or TMA-DPH stock solutions in chloroform. The solvent was evaporated under a stream of nitrogen and the samples were kept under high vacuum for at least 15 minutes in order to remove any traces of organic solvent. Next, the samples were rehydrated with a proper amount of Tris-HCl buffer (1 mM, pH 7) and subsequently vesiculated by sonication for 10 minutes at 60°C using a VC-300 Sonics & Materials probe sonifier equipped with a microtip set at output level 3. Before use, all solutions were centrifuged for 30 minutes at 15,500 g in a Sigma 202M centrifuge, and were passed through a Millipore filter (pore size 0.45 μm) in order to remove any possible titanium particles from the sonifier tip.

Energy transfer measurements

Fluorescence energy transfer studies were carried out on an Aminco fluorimeter DMX-1000 from SLM Instruments with excitation and emission monochromator slits set at 5 nm. The various vesicles stock solutions (20 mM ABHEMA Br: nonpolymerised or polymeric) were titrated to a 50 μg/ml CCL (850 nM) solution (1 mM Tris-HCl, pH 7). The surfactant to label molar ratios were 40 : 1 (DPH) or 50 : 1 (TMA-DPH). The titrations were carried out at 20°C. The excitation wavelength was set to 280 nm; the experiments were carried out at room temperature with continuous stirring. The titrations with nonpolymerised vesicles were carried out to a maximum of 300 μM (in the case of DPH as the membrane probe) or 158 μM (in the case of TMA-DPH). At a concentration of 158 μM, the enzyme-vesicle solutions started to flocculate. In the case of titration with polymerised vesicles, this problem does not occur. All measurements were carried out in duplicate, the standard deviation in all cases was less than 3.1%.

Fluorescence anisotropy

Steady state fluorescence anisotropy measurements were carried out with a home build T-format fluorescence polarisation apparatus with single photon counting detection. All solutions were stirred continuously during the measurements. The titrations were carried out at 20°C. The fluorescence anisotropy r was calculated from equation 5.1. All measurements were carried out in duplicate, standard deviation never exceeded 1.9%.

Phase transition determination of ABHEMA Br vesicles

The phase transition of nonpolymerised and polymeric ABHEMA Br vesicle membranes was examined by monitoring the anisotropy of the membrane probe DPH (surfactant to label ratio 500 : 1). The temperature of the vesicle solutions was increased from 20°C to 60°C with 2°C increments. The temperature in the cuvettes was controlled by the use of a jacketed cuvette holder that was connected to a thermostated water bath (Lauda RMG). The cuvettes were allowed to equilibrate for 15 minutes before each measurement; the real temperature was determined by sampling with a thermometer. DPH was excited at 360 nm and fluorescence was measured at a maximum of 450 nm (Balzers K45 interference filter) in combination with a cutoff filter (Schott KV399, 3 mm).

Anisotropy of RITC-CCL

The incorporation of rhodamine-modified lipase in ABHEMA Br vesicles was determined by steady state fluorescence anisotropy measurements. Aliquots of 5 mM nonpolymerised or polymeric ABHEMA Br vesicles were titrated to solutions of 240 μg/ml (4.10 μM) RITC-CCL in 1 mM Tris-HCl buffer pH 7. The excitation wavelength was set to 580 nm and the emission was measured at 613 nm (Balzers 613 nm interference filter) in combination with a cutoff filter (Schott RG610, 3 mm).

5.4 Results and Discussion

Phase transition of ABHEMA Br bilayers

The gel to liquid crystalline phase transition in nonpolymerised and polymeric ABHEMA Br vesicles was determined via fluorescence anisotropy by the use of DPH as a membrane probe. The DPH molecule is oriented in the hydrophobic core of the bilayer and by this, serves as an excellent probe for studying the order in membrane bilayers [146,197]. The gel to liquid transition of the alkyl chains in the vesicle bilayer is characterised by a drastic change in anisotropy. The fluorescence anisotropy versus temperature profile, as depicted in figure 5.10, indicates that the trajectory of the phase transition of ABHEMA Br bilayer is broadened upon polymerisation. The phase transition temperature T_c[2] for nonpolymerised and polymeric vesicles are valued at 49°C and 45°C. Dorn and Ringsdorf [52] obtained similar results, lowering of T_c upon surface polymerisation, with differential scanning calorimetry studies using vesicles from quaternary dialkyl ammonium surfactants with a surface-polymerisable moiety attached to the headgroup.

[2]The phase transition temperature is defined as the centre of the transition trajectory.

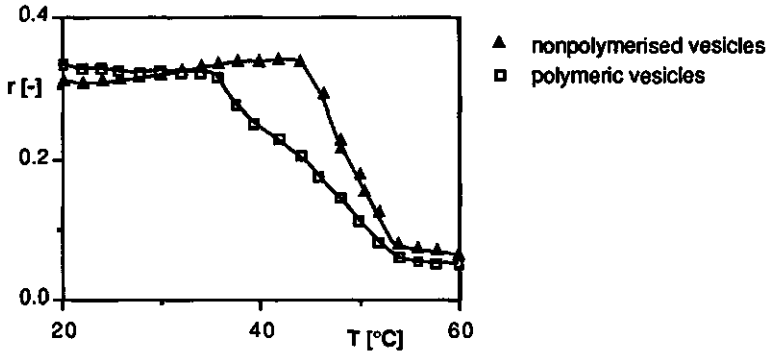


Figure 5.10 Phase transition trajectory for nonpolymerised and polymerised ABHEMA Br vesicles when heated from 20°C to 60°C.

It is a known phenomenon that alteration in the surfactant headgroup size results in an altered packing density of the headgroups and consequently in the phase transition behaviour of the membrane [54,156,212]. Headgroups are pulled together upon polymerisation, which causes clefts in the bilayer structure [177]. The surfactant chains near these clefts will have a higher degree of freedom, which results in a decrease in T_c. DPH molecules, which will be redistributed in the bilayer via translational diffusion, will have an increased rotational motion when they are located in these clefts. As a result of these inhomogeneous structures (clefts in bilayer), the phase transition trajectory will be broadened and shifted to lower values.

Energy transfer studies

Interaction of proteins with membranes can be monitored by fluorescence energy transfer [48,147]. This method is based on the nonradiative transfer of the excited state energy from a donor to an acceptor molecule. The extent of energy transfer depends mainly on the extent of overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, and on the orientation and distance between them. The critical distance for energy transfer ranges from 2 to 5 nm, which is comparable to the radius of *Candida* lipase (3.5 nm, derived from references [207] and [223], see appendix B) and the thickness of synthetic or biological membranes (4 - 6 nm) [81]. The transfer from protein molecules that remain free in solution (non-incorporated) to the membrane can be considered negligible. The protein that contains the fluorescent amino acid residues tyrosine (Tyr) and tryptophan (Trp), acts as the donor molecule for electronic excitation energy. The available data on the amino acid composition of lipase from *Candida cylindracea*, [237] suggest that CCL contains 4 or 5 Tyr residues and 16 Trp residues, which makes the enzyme very suitable for ET studies.

The membrane probes, DPH and TMA-DPH are used as acceptor molecules for the protein excitation energy. The exact location of the two labels in a bilayer membrane is different (see figure 5.4). Whereas DPH is located in the hydrophobic core of the membrane, TMA-DPH is considered to reside at the interface of the bilayer [171,197]. By the use of an internal and an interfacial label

molecule, it was anticipated that information could be obtained of any preferential orientation about the incorporated protein.

A typical example of the change in the fluorescence spectrum of *Candida* lipase upon titration with DPH-labelled vesicles is depicted in figure 5.11. The decline of the fluorescence peak at 330 nm, which originates from the protein, coincides with an increase of the signal emanating from the label. This clearly indicates that lipase is in close proximity of the probe molecules. Considering the dimensions of the enzyme and the width of the bilayer, the major part of the protein is most likely located in the vesicle bilayer.

The emission maximum of the tryptophan residues is a good probe for the exposure of these groups to the environment. In protein molecules, three different locations for tryptophan residues are distinguished, as is schematically indicated in figure 5.8. Peripheral tryptophans, which are fully exposed to the solvent, have an emission maximum from 340 to 350 nm. The maximum for semi-exposed tryptophans lies from 330-340 nm, and buried residues show maximal emission from 310-330 nm [30]. The shift of the spectral maximum position from 330 nm for the free lipase (dashed line) to 322 nm for the incorporated enzyme (solid lines), indicates that mainly peripheral and semi-peripheral Trp residues (emitting at longer wavelength) are involved in the energy transfer.

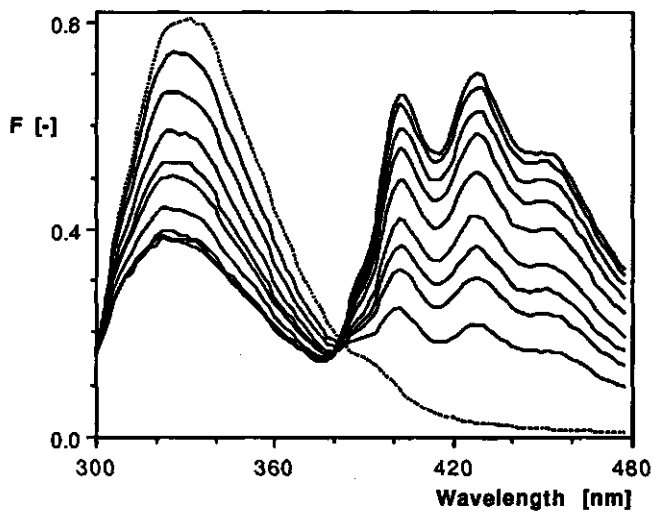


Figure 5.11 Changes in the fluorescence spectrum of *Candida cylindracea* lipase (50 µg/ml Tris, dashed line) upon addition of 2 µl aliquots of 20 mM nonpolymerised vesicles containing 0.4 mM DPH.

In order to compare the (extent of) incorporation in nonpolymerised and polymerised vesicles, the decrease in fluorescence at 330 nm was taken as a measure for binding (figure 5.12). The fluorescence increase at 430 nm, as the result of the energy transfer from the protein to the membrane probe, yielded similar results (not shown). All curves were corrected for the fluorescence of the label in the absence of protein. The steep slopes for titration of enzyme with nonpolymerised vesicles containing DPH or TMA-DPH suggest that

lipase is readily incorporated in the vesicle bilayer at temperatures below the phase transition temperature. Since the slopes for the titration curves for both DPH (figure 5.12, left graph) or TMA-DPH-containing (right graph) nonpolymerised vesicles are identical, it is assumed that the protein is fully dispersed/partitioned in the hydrophobic core of the vesicle membrane.

The span of time between successive measurements in the titration of CCL with vesicles, as illustrated in figure 5.11, was 3 minutes. The span of time, or incubation time, between two measurements, however, had no influence on the fluorescence. In separate experiments, it was shown that energy transfer was completed immediately[3] after addition of vesicles to the lipase solution - and that the fluorescence signal did not alter any more with time (for the duration of maximal one hour).

In the titration curve of lipase with nonpolymerised vesicles containing DPH, an estimation was made for the stoichiometry of the system (for further details see Appendix B). At a concentration of 158 μM vesicles the curve shows a minimum value in fluorescence intensity F, which suggest saturation of vesicles with enzyme. The enzyme-surfactant ratio is 50 μg/ml : 160 μM, corresponding to molar ratios of 1 : 160 protein : surfactant, and 1 : 0.016 protein : vesicle. Thus one vesicle contains (1/0.016 =) about 60 lipase molecules. As can be deduced from appendix B, 60 CCL molecules per vesicle means that 1/4 of the available vesicles surface is occupied by lipase.

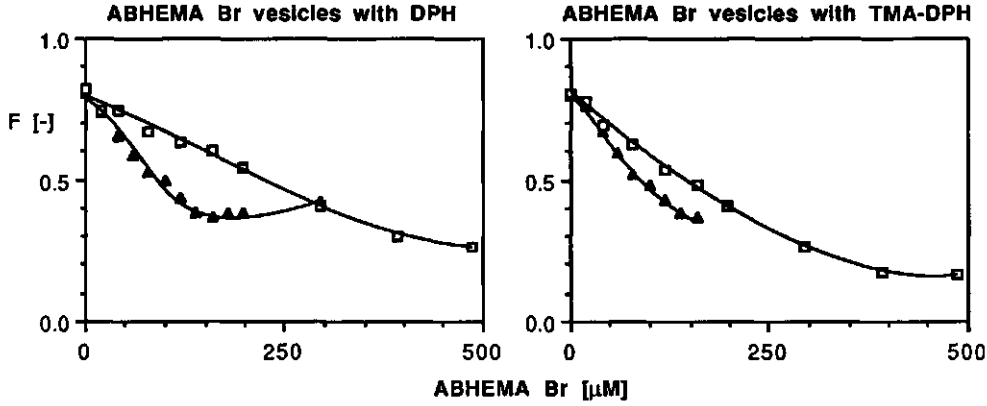


Figure 5.12 The fluorescence at 330 nm (vertical axis) monitored during the titration of a 50 μg/ml CCL solution with nonpolymerised (▲) or polymeric(□) vesicles containing the internal label DPH (left graph) and interfacial label TMA-DPH (right graph), the ABHEMA Br concentration is plotted on the horizontal axis.

In the case of polymerised vesicles (figure 5.12, open squares) the slope of the decrease in fluorescence at 330 nm is less steep than in the case of nonpolymerised vesicles (closed triangles). This is evidently due to the polymeric nature of the membrane. Surface polymerisation changes the distance between neighbouring surfactant headgroups as well as the packing density and order of the alkyl chains in the hydrophobic core of the bilayer.

[3]The term 'immediately' refers to the minimal time required for recording a fluorescence spectrum from 300 to 480 nm, which is amounted to 1.5 minutes.

This makes polymerised ABHEMA Br vesicles less accessible for protein than the nonpolymerised counterparts. The difference in slopes of the titration curves with polymeric vesicles containing DPH (left graph), an internal membrane probe, or TMA-DPH (right graph), a surface probe, suggests that CCL penetrates the vesicle bilayer less deeply than in the case of nonpolymerised vesicles.

The initial stage in the incorporation process will mainly be of an electrostatic nature [238,259]. However, lipases undergo structural changes upon contact with surfaces [19,216,257]. These structural changes generate the formation of an apolar surface around the active site [29], which might lead to complete insertion of the enzyme into the vesicle bilayer [178,263]. However, polymerisation decreases the accessibility of the bilayer for the enzyme. Incorporation of the protein will take place, but is reduced, as is illustrated by higher saturation concentrations in the titration curves of polymeric vesicles (400 - 500 μM, compared to 160 μM for nonpolymerised vesicles). In addition, a decreased accessibility of the polymeric bilayer is accompanied by a phenomenon shown in chapter 3, figure 3.6. Directly after mixing of enzyme with polymeric vesicles the activity of the enzyme is very low. After an incubation time of one hour the activity of the lipase-vesicle mixture is maximal. Although fluorescence studies show that incorporation is a rather fast process, the activity of the incorporated enzyme is not immediately maximal. This may be the result of reorientation of the protein in the membrane, or reorientation of the surfactant molecules around the enzyme, which imparts a decreased accessibility of the active site upon incorporation in polymerised vesicles. Thus, incorporation in polymeric vesicles proceeds less facile than in the case of nonpolymerised vesicles and the polymeric nature of the vesiculated surfactant molecules initially lowers the enzymatic activity.

Fluorescence anisotropy of RITC-labelled CCL

Steady state fluorescence anisotropy can be used for the characterisation of the incorporation process of a protein into a vesicle bilayer. The rotational freedom of the protein will be restricted upon incorporation. Consequently, the anisotropy of the enzyme will rise upon addition of vesicles [147]. This phenomenon was used to monitor the lipase-vesicle interaction.

It was not possible to use native enzyme for this purpose. Due to the high amount of tryptophans in *Candida cylindracea* lipase [237], a relatively small protein (58,5 kD, chapter 3), fluorescence depolarisation might be completely due to internal tryptophan-tryptophan energy transfer. This possibility prevents detection of protein-vesicle interaction by anisotropy measurements. Therefore, it was decided to use CCL surface-labeled with the red dye RITC. The rhodamine moiety is covalently coupled to lysine residues of the enzyme. From Tomizuka's data on the amino acid composition of *Candida cylindracea* it can be calculated that CCL contains 15 lysine residues [237]. Under the conditions used, four to five residues were derivatised (equation 5.2: A_{278} = 0.371 and A_{578} = 0.480, see Materials and Methods section). The derivatisation considerably affected the properties of the enzyme. The specific activity of the labelled enzyme was reduced to 10% of the activity of the native enzyme. The modified enzyme is less stable than the native enzyme and might be denatured upon derivatisation, which was denoted by precipitation of the

protein upon prolonged standing. The isoelectric point of the enzyme is also altered, since the isothiocyanate reacts preferentially with the (uncharged) amino groups of the lysine residues under basic conditions. Roberts and Tombs [207] have reported a decrease of 1.2 and 1.0 pI units for two different lipases that were modified with fluorescein isothiocyanate. In our case however, this effect could not be confirmed by isoelectric focussing. The enzyme did not move from the spot where it was administered. This is most likely due to the reduced stability of the modified lipase which causes precipitation of the protein in the gel.

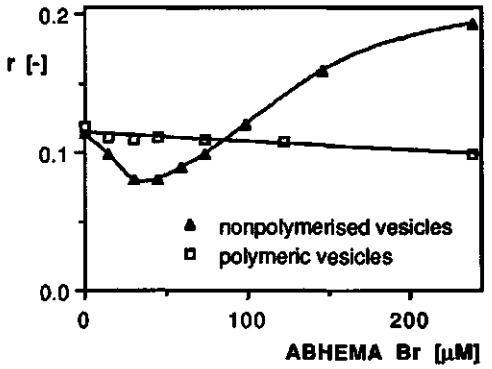


Figure 5.13 Anisotropy of rhodamine modified CCL when titrated with nonpolymerised or polymerised ABHEMA Br vesicles.

Incorporation of the protein in vesicles should yield a gradual increase in fluorescence anisotropy. However, the anisotropy of the rhodamine modified CCL first shows a decrease in case of titration with nonpolymerised vesicles (figure 5.13). This may be explained by the existence of oligomeric aggregates of the enzyme. Due to derivatisation of the lysine residues, the enzyme might tend to aggregate into oligomers because repulsive interactions, which stabilise the monomeric protein, have been diminished. Due to their amphiphilic nature, initial addition of vesicles may induce deaggregation of the oligomeric protein, which results in a decrease of the anisotropy. Further administering of vesicles will lead to an increase in anisotropy due to incorporation of the enzyme in the vesicles. Addition of polymerised vesicles to the modified enzyme solution exhibits almost no change in anisotropy. Obviously, the change in protein structure due to derivatisation prevents incorporation of the modified enzyme into polymeric vesicles. It should be noted that covalent labeling can considerably alter the characteristics of the enzyme, which also might affect the incorporation behaviour of the modified protein.

5.5 CONCLUSIONS

Fluorescence anisotropy and radiationless energy transfer are suitable techniques to monitor the interaction between lipase and synthetic vesicles. By the use of anisotropy, the phase transition temperature of nonpolymerised

and polymeric vesicles could be determined. The results indicate that the packing density in both types of vesicles is distinct. In addition, fluorescence anisotropy and energy transfer studies show that the enzyme is readily incorporated into the membrane. However, the incorporation proceeds more efficient in nonpolymerised vesicles. The lipase cannot penetrate the polymerised bilayer in the same extent as in the case of the nonpolymerised analogues, due to the altered membrane characteristics.

6 SPECTROPHOTOMETRIC DETECTION OF LIPASE ACTIVITY*

Keyword Phrases:

SYNTHESIS OF 2,4-DINITROPHENYL ESTERS

DESCRIPTION OF A LIPASE ASSAY METHOD FOR LIPASES USING 2,4-DINITROPHENYL ESTERS

SURVEY OF THE APPLICABILITY OF THE ASSAY METHOD FOR DIFFERENT LIPASE SPECIES

* This chapter is published in a condensed form as: Mosmuller, E.W.J., Van Heemst, J.D.H., Van Delden, C.J., Franssen, M.C.R., Engbersen, J.F.J. (1992) A new spectrophotometric method for the detection of lipase activity using 2,4-dinitrophenyl butyrate as a substrate. *Biocatalysis*, **5**, 279-287

6.1 INTRODUCTION

Lipases perform their biological action preferably at the interface of an aqueous and a hydrophobic phase. Maximal activity is observed when the substrates are emulsified or associated with micelles or vesicles [16,47]. The major factor that controls lipase activity is the amount of substrate molecules at the interface, which is proportional to the surface area of the emulsion [248,249]. This characteristic makes it difficult to develop a reliable assay method for lipase activity. However, for the application of lipases, a reliable method for determination of enzymatic activity is required. A surprisingly large number of different procedures for assaying lipase activity has been developed over the past years. This may serve as an indication that many of the methods are not entirely satisfactory. Methods for the quantification of lipase activity can be divided into four groups: (i) assays based on the physical changes in the reaction system during ester hydrolysis, (ii) assays monitoring the disappearance of the substrate ester, (iii) assays determining the amount of liberated alcohol during ester hydrolysis, and (iv) assays based on the quantification of the carboxylic acid formed. Several authors have reviewed the field of assay systems for lipases [6,16,25,47,60,110].

Nowadays, most assay systems for lipase activity are based on fatty acid titration during hydrolysis of tributyrin [26], triolein [167] or olive oil [256]. However, the presence of multiple susceptible bonds in triglycerides may complicate the kinetics of the reaction. Therefore, simple esters containing a single ester bond are in principle more appropriate for assaying lipase activity than are triglycerides. In addition, high sensitivity can be obtained when spectrophotometric methods are used in which single ester substrates are applied with large absorbance differences between substrate and product. For spectrophotometric detection of lipase activity various substrates have been used: 4-nitrophenyl esters [38,101,192], ω-trinitrophenylaminolauryl esters of glycerol [65,80] and long chain fatty thioesters of glycerol or glycerol analogues in combination with thiol trapping agents [65,203]. Most spectrophotometric methods are restricted to water soluble substrates. These homogeneous reaction systems however, lack the substrate interface that is required for optimal lipase activity. Furthermore, some assay systems contain additional amounts of solubiliser [174,262] or organic solvent [101]. Moreover, some of these methods have the disadvantage of discontinuous measurement, which makes the assay laborious [80].

In this chapter, a simple, rapid and reproducible method for the determination of lipase activity is described, using 2,4-dinitrophenyl esters as the substrates. The method is based on continuous spectrophotometric monitoring of the increase in absorbance at 360 nm due to the formation of 2,4-dinitrophenolate, which is produced during hydrolysis. The assay system is optimised for 2,4-dinitrophenyl butyrate (DNPB), which can be applied as a homogeneous system at a low concentration (0.1 mM), or as a heterogeneous system at a higher concentration (1.0 mM). At higher DNPB concentrations, this substrate is used in an emulsified form, which is obtained by short

sonication after injection of a concentrated solution of the substrate in acetonitrile into a cuvette (total amount of acetonitrile: 2% v/v). During hydrolysis there is also a small contribution to the absorbance change at 360 nm due to clearance of the emulsion. However, this can easily be corrected by simultaneous monitoring of the absorbance change at a reference wavelength (525 nm).

6.2 Materials and Methods

Materials

Reagents/enzymes

Acetyl chloride (98%, distilled), butyryl chloride (98%, distilled), 2,2-dimethylpropanoyl chloride (99%, distilled), octanoyl chloride (99%) and polyoxyethylene(23)lauryl ether (Brij 35) were obtained from Aldrich. 2,4-Dinitrophenol (DNP) was purchased from Merck and was recrystallised from a water-methanol mixture before use. For the molar extinction coefficient at 360 nm (pH 7.2) a value of 14800 ($M^{-1}cm^{-1}$) was recorded (literature [190]: 14700 $M^{-1}cm^{-1}$). Dodecanoyl chloride (98%), monobasic and dibasic potassium phosphate (p.a.) and tris(hydroxymethyl)aminomethane (Tris; 99+%) were purchased from Janssen Chimica and 10-undecenoyl chloride (pract., >97%, distilled) from Fluka A.G. Triethylamine (Merck) was dried for one day over potassium hydroxide and then distilled from calcium hydride. All solvents were distilled before use.

Porcine pancreatic lipase was purchased from Sigma (PPL, type II); lipase from *Candida cylindracea* was obtained from Sigma (CCL, type IV) and from Meito Sangyo (CCL, Lipase OF); CCL from Meito Sangyo is further referred to as CCL unless otherwise stated. Lipases from *Aspergillus niger* (LAP6) and from *Pseudomonas* sp. (LPS) were generous gifts from Amano. Enzyme stock solutions were prepared in 0.1 M potassium phosphate buffer pH 6.0. Porcine pancreatic lipase (19.7 mg/ml) and lipases from *Candida cylindracea* (0.1 mg/ml) and *Pseudomonas* sp. (2.1 mg/ml) were dissolved at room temperature in potassium phosphate buffer (0.1 M, pH 6.0) and centrifuged to remove insoluble material. The supernatant was used as the enzyme stock solution. In the case of porcine pancreatic lipase and lipases from *Candida cylindracea* and *Pseudomonas* sp. respectively 1, 10 and 10 μM Brij 35 was added in order to get satisfactorily reproducible results (less than 2% standard deviation). For lipase from *Aspergillus niger* addition of a solubiliser was not necessary.

Synthesis of 2,4-dinitrophenyl esters

2,4-Dinitrophenyl butyrate (DNPB) was synthesised by slowly adding 5.74 g (6.64 ml, 53.9 mmol, 1.4 eq) butyryl chloride to a stirred mixture of 7.10 g (38.6 mmol, 1 eq) 2,4-dinitrophenol and 4.68 g (46.3 mmol, 1.2 eq) triethylamine in 100 ml dichloromethane. The reaction mixture was stirred at room temperature for one hour, washed several times with a sodium bicarbonate solution and dried on sodium sulphate. After solvent removal, 6.96 g (27.3 mmol, 78%) of 2,4-dinitrophenyl butyrate was obtained as a yellow liquid. In

order to obtain a product that was completely free of dinitrophenol it was essential to use an excess of butyryl chloride.

Bp 162°C (0.2 mm Hg); ^{1}H-NMR (200 MHz, $CDCl_3$), δ 8.91 (d, 1 H, ArH), 8.49 (dd, 1 H, ArH), 7.45 (d, 1 H, ArH), 2.65 (t, 2 H, CH_2), 1.80 (m, 2 H, CH_2), 0.99 (t, 3 H, CH_3).

In addition to DNPB, the 2,4-dinitrophenyl esters of acetic, octanoic, dodecanoic, 10-undecenoic and 2,2-dimethylpropanoic acid were synthesised (table 6.1). The esters are synthesised in an analogous way; the NMR data of the esters are in full accordance with their structure. The esters can be stored at room temperature without any detectable decomposition.

Table 6.1 List of synthesised 2,4-dinitrophenyl esters.

2,4-dinitrophenyl ester	yield [%]	physical characteristics
acetate	76	solid, mp 54-60°C
butyrate	78	liquid
octanoate	60	liquid
dodecanoate	97	solid, mp 30-34°C
10-undecenoate	90	liquid
2,2-dimethylpropanoate	77	solid, mp 70°C

Enzyme assay

Assay mixtures of 2,4-dinitrophenyl butyrate were prepared by injecting 50 µl of a 50 mM stock solution of DNPB in acetonitrile into 2.45 ml of 5 mM Tris-HCl buffer pH 7.2 kept at 21°C. To ensure complete emulsification of the substrate, the mixture was sonicated for 30 seconds with a Sonics VC-300 sonifier (set at level 3, pulser set to 50%) using a microprobe. The temperature of the sample solution rose to 25°C upon sonication; subsequently, the assay was carried out at this temperature of 25°C. Enzymatic hydrolysis of the substrate was started by addition of a proper amount (5-100 µl) of the enzyme. The reaction mixture was kept at 25°C during the assay in a thermostated cuvette holder. All measurements were carried out in triplicate. Three samples could be measured at the same time by the use of a HP 89075C multicell transport. Reactions were followed for 6 to 8 minutes or to 10% completion and showed reproducibility within 1.3%.

Data analysis

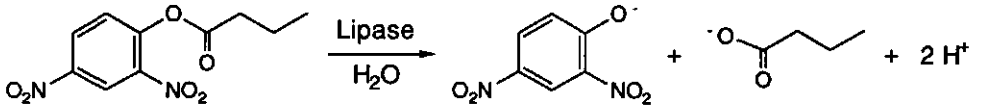
Spectrophotometric measurements were carried out on a HP 8452A diode-array spectrophotometer equipped with a HP 89500 ChemStation using the UV/VIS kinetics and general UV/VIS software. Changes in absorbance (ΔA) were obtained using wavelength averaging at λ_{max} (360 nm) of the 2,4-dinitrophenolate anion and internal referencing in a region where this compound has no absorption (525 nm):

$$\Delta A = (\text{Average from 356 to 364 nm}) - (\text{Average from 520 to 530 nm})$$

Linear increase of absorption (ΔA) was observed throughout hydrolysis. Enzyme rates were obtained by means of a linear fit program using highest linear slopes.

6.3 Results and Discussion

The standard assay method employs emulsified 2,4-dinitrophenyl butyrate (DNPB) as the substrate. Upon hydrolysis this substrate liberates the strongly absorbing 2,4-dinitrophenolate anion, making spectroscopic detection easily accessible. 2,4-Dinitrophenol (DNP) is a highly acidic phenol with a pK_a of 3.96. Consequently, DNP is completely in its anionic form at the operational pH of 7.2. This has the important advantage that the assay method is not sensitive to modest changes of pH during the reaction.



In general , the molar extinction of a phenolic solution is very dependent on the pH near its pK_a. Chapus et al. describe an assay method in which 4-nitrophenyl esters are used as the substrate [38]. Since the pK_a for 4-nitrophenol is 7.15, it is necessary that the pH is carefully kept constant during the assay of lipase activity. However, in the case of DNPB, the pH of the sample solution remains well above the pK_a of DNP during hydrolysis, which makes 2,4-dinitrophenyl esters more convenient chromogenic substrates than 4-nitrophenyl esters.

Figure 6.1 shows the change in the absorbance spectrum during enzymatic hydrolysis of DNPB. The absorbance in the 330 - 400 nm region is increasing due to the formation of 2,4-dinitrophenolate.

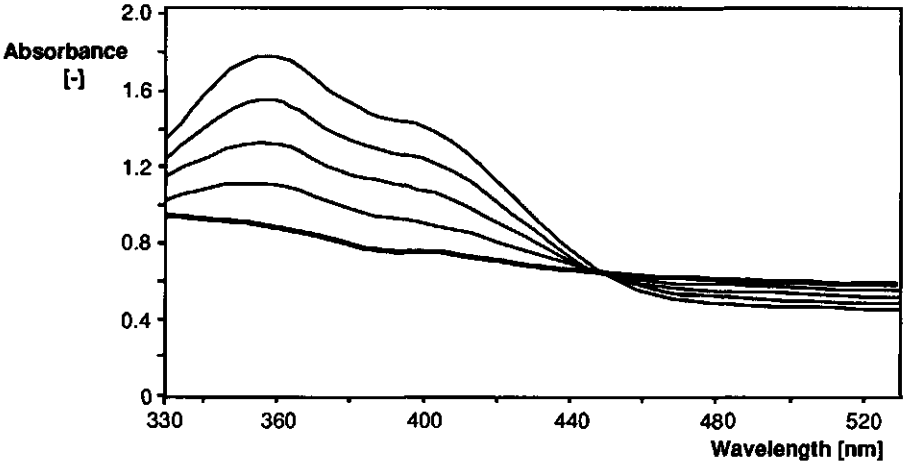


Figure 6.1 Changes in absorbance spectrum during the conversion of 2,4-dinitrophenyl butyrate (bold line) to 2,4-dinitrophenol (ionic form) by lipase from *Candida cylindracea*. The time span between two consecutive spectra is 20 sec.

Since the substrate is offered in an emulsified form, the assay solution is slightly turbid, which results in background absorption in the entire wavelength area. During enzymatic hydrolysis, the assay solution clarifies. For this change in background absorption can be corrected by internal referencing in a region where no absorption of substrate or products occurs (480 - 530 nm). Figure 6.2 gives the corrected spectra from figure 6.1; the absorbance is plotted relative to the absorbance at 525 nm. Absorbance changes in the region 480 - 800 nm indicated that the optical clearance is wavelength-independent.

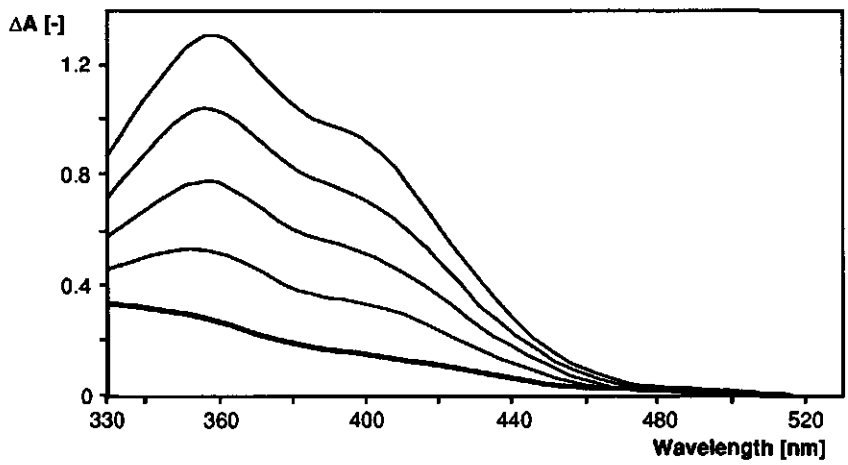


Figure 6.2 Absorbance spectra from figure 6.1 (conversion of 2,4-dinitrophenyl butyrate, bold line, to the 2,4-dinitrophenolate anion) that are corrected for interference of absorbance changes due to clearance of the emulsion during hydrolysis by subtraction of the absorbance at 525 nm.

The enzymatic activity is proportional to ΔA, which is the absorbance increase at 360 nm and corrected for absorbance changes due to clarification at 525 nm: $\Delta A = [\bar{A}(360\ nm) - \bar{A}(525\ nm)]$.

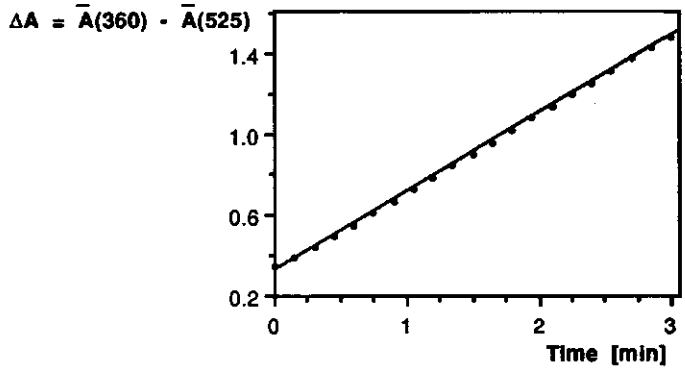


Figure 6.3 Linear increase of absorption $\Delta A = [\bar{A}(360) - \bar{A}(525)]$ by enzymatic hydrolysis of 2,4-dinitrophenyl butyrate by the use of wavelength averaging and internal referencing.

As a result, a linear increase in ΔA is obtained, as is depicted in figure 6.3. In order to obtain maximal reproducibility, the absorbance at 360 nm $\overline{A}(360)$ and at 525 nm $\overline{A}(525)$ are obtained by averaging the absorbance over a small wavelength area around 360 nm, *viz* $\overline{A}(360)$ = A(from 356 to 364 nm) and around 525 nm, *viz* $\overline{A}(525)$ = A(from 520 to 530).

In our approach we have tested four enzymes of various sources: mammalian lipase from porcine pancreas, lipase from the yeast *Candida cylindracea*, fungal lipase from *Aspergillus niger*, and microbial lipase from *Pseudomonas* sp. The results of the activity measurements are summarised in the table 6.2.

Table 6.2 Specific activity of four types of lipases for 2.4-dinitrophenyl butyrate and olive oil.

		specific activity[1] for	
Enzyme	Source	DNPB	olive oil
PPL	porcine pancreas	0.01 (± 0.5%)	1.17
CCL	*Candida cylindracea*	145.36 (± 0.9%)	50
LPS	*Pseudomonas* sp.	4.82 (± 1.2%)	30
LAP6	*Aspergillus niger*	25.00 (± 1.3%)	6

[1] Specific activity is defined as μmol substrate hydrolysed per minute per mg solid material (U/mg). The reaction conditions for the olive oil assay, as indicated by the supplier, are: PPL: pH 7.7, 37°C, 30 minutes incubation; CCL: pH 6.0, 30°C; LPS: pH 7.0 30-37°C; LAP6: pH 6.0, 37°C, 30 minutes incubation, 2%polyvinyl alcohol.

As can be seen from the table, porcine pancreatic lipase and lipase from *Pseudomonas* sp. show significantly lower specific activities for DNPB than for olive oil. However, it should be taken into account that the conditions for these substrates are not identical; the hydrolysis of olive oil is performed at higher temperatures and different pH.

Figure 6.4 Relationship between total amount of lipase (CCL) and enzymatic activity.

For purified lipase (Lipase OF, Meito Sangyo, see chapter 3), two different DNPB concentrations were applied: 0.1 mM, where DNPB is soluble, and 1.0

mM, which is beyond its solubility limit. The activity in the case of the solubilised DNPB system was 69.6 U/mg, and in the case of the emulsified substrate 265.5 U/mg. The higher activity in the latter heterogeneous assay system is the result of the interfacial activation of the enzyme at the substrate water interface [248,249]. The influence of variation of lipase concentration in the assay mixture on the enzymatic activity yields an almost linear relationship, as is depicted in figure 6.4 for CCL.

Since the assay system does not contain any solubiliser (e.g. for dissolving an insoluble substrate), inhibition of lipase due to the presence of a surfactant, as is found by Yamane [262], will not occur. However, for the preparation of three enzyme stock solutions it proved to be necessary to add small amounts of Brij 35 (polyoxyethylene(23)lauryl ether) in order to enhance complete solubilisation of the enzyme. In all cases the final Brij 35 concentration in the assay mixture did not exceed 0.04 mM, which is lower than the critical micelle concentration (0.5 mM) for this compound. Brij 35 is used as a solubiliser for the lipases in order to retain high reproducibility at low enzyme concentrations. Without the addition of Brij 35, the enzyme activity in the case of CCL showed poor reproducibility with a standard deviation of 7%. This could be markedly improved to 0.9% upon addition of 10.7 w/w% Brij 35 to the lipase stock solution. Moreover, the activity of the enzyme increased with 18% in the presence of Brij 35. For LPS, activity decreased with 28% after Brij 35 (0.6 w/w%) was added. The standard deviation, however, was improved from 4.5% to 1.6%. PPL did not yield a linear reaction progress without Brij 35. Upon addition of Brij 35 (0.006 w/w%) however, a linear relationship could be obtained. For LAP6, addition of solubiliser was superflous for getting satisfactory results.

2,4-Dinitrophenyl butyrate is scarcely soluble in water and therefore, stock solutions of DNPB were made up in acetonitrile. Assay mixtures were prepared by injecting 50 µl of 50 mM stock solution of DNPB into 2.45 ml of Tris-HCl buffer, giving a final concentration of 2% acetonitrile v/v. Addition of an organic cosolvent can affect the enzymatic activity [59]. Therefore, the influence of the acetonitrile concentration in the assay mixture was determined in more detail for the lipase from *Candida cylindracea*. Maximal activity was obtained at 2% v/v acetonitrile. Relative to the assay that contained 2% acetonitrile, addition of 1 or 4% acetonitrile lowered enzyme activity with 22% and 18% respectively. This is in good agreement with the results of Entressangles and Desnuelle [59], who found that the activity of pancreatic lipase for monomeric tripropionin was increased upon addition of small quantities of water-miscible organic solvents. Also in the case of pancreatic lipase, maximal activity was obtained at acetonitrile concentrations of 2% v/v.

In addition to 2,4-dinitrophenyl butyrate, some other esters with different chainlengths and bulkiness were tested as substrates for two *Candida cylindracea* preparations from different suppliers; the results are listed in table 6.3. 2,4-Dinitrophenyl acetate is a poor substrate for both *Candida* lipases, most probably because the system is homogeneous [249]. The 2,2-dimethylpropanoate ester was not hydrolysed by both enzymes. The 2,2-dimethylpropanoate residue is too bulky to be hydrolysed, as is also reported for other 2,2-dimethylpropanoate esters [132]. The Sigma lipase clearly prefers the short

chain acyl group (C_4), whereas the Meito Sangyo lipase has highest activity for the octanoate ester. The most striking result from this comparative analysis is the considerable difference in reactivity of lipases originating from the same source. The commercial preparations were used without further purification, and it is therefore very well possible that these samples contain multiple hydrolytic enzymes in different ratios, which consequently results in distinct selectivity patterns. This again indicates that data derived with enzyme samples from different suppliers should be used or compared with great care, as was already stated in chapter 3. There, the physical characteristics of several purified CCL preparations were compared.

Table 6.3 Reactivity of *Candida cylindracea* lipase from Sigma and Meito Sangyo for several 2,4-dinitrophenyl esters.

	Relative activity [%]	
2,4-dinitrophenyl ester	CCL Sigma	CCL Meito Sangyo
acetate	2	4
butyrate[1]	100	100
octanoate	56	191
dodecanoate	54	76
10-undecenoate	77	111
2,2-dimethylpropanoate	0	0

[1] The specific activities for the Sigma lipase and the Meito Sangyo lipase are 11.6, respectively 145.4 U/mg dry material.

6.4 CONCLUSIONS

The present method is a simple, rapid and sensitive assay for the detection of lipase activity. The standard substrate, 2,4-dinitrophenyl butyrate, can be applied in solubilised and emulsified form. The latter case is similar to natural occurring reaction systems for lipases By simultaneously monitoring the production of 2,4-dinitrophenolate (360 nm) and the clearance of the substrate emulsion (525 nm), reproducible enzyme activities are easily obtained.

All four types of tested lipases are readily able to hydrolyse the 2,4-dinitrophenyl butyrate substrate. This allows the assumption that this substrate can generally be used in lipase activity determination. As was illustrated with two *Candida cylindracea* lipases from different suppliers, several other 2,4-dinitrophenyl esters are also hydrolysed. However, the reactivities of these preparations towards the various esters were not alike. This demonstrates that data obtained from lipases derived from different sources should be cautiously used.

The small reaction volume and the high extinction coefficient of the product makes this method several orders of magnitude more sensitive than lipase assays based on fatty acid titration. It has the additional advantage that the assay mixture is not diluted by addition of a titrant, as is the case in titrimetric methods.

7 SUMMARISING DISCUSSION

In enzymologic applications, complex enzyme systems are increasingly used, mostly with the aim of increasing (re-)use and stability, or because the enzyme system obtains new or altered catalytic properties [36,144,258]. A new development in this area is the application of enzymes incorporated in polymerisable synthetic vesicles.

The incorporation of lipase from *Candida cylindracea* (CCL), the enzyme used throughout this thesis, in polymerisable dialkyl ammonium surfactant vesicles is described. CCL could be purified with simple techniques. The enzyme has a molecular weight of 58.5 kD and an isoelectric point of 4.1. *Candida cylindracea* lipase has a broad optimal pH (6 - 9), which makes the enzyme versatile for application; the optimal temperature for CCL is 45°C.

The protein could be readily incorporated in vesicles from three different polymerisable surfactants: *N*-Allylbis[2-(dodecanoyloxy)ethyl]methylammonium bromide (ABDEMA Br), *N*-Allylbis[2-hexadecanoyloxy)ethyl]methylammonium bromide (ABHEMA Br) and Bis[2-(10-undecenoyloxy)ethyl]-dimethylammonium bromide (BUEDMA Br). The vesicle systems were characterised in terms of morphology and stability. The polymerised vesicles were found to be considerably more stable than the nonpolymerised analogues.

We demonstrated that CCL can readily be incorporated in these vesicles by incubation without additional treatment, in contrast with many active incorporation procedures for soluble non-membrane associated enzymes [116,159,239]. The activities of free and entrapped CCL in homogeneous substrate systems (triacetin and low concentrations of tributyrin) clearly indicates the relevance of the presence of an interface for optimal activity of the lipase. The introduction of a (nonsubstrate) interface - in this case provided by the vesicle bilayer in which the enzyme is incorporated - causes activation of the lipase and leads to higher activities. In heterogeneous reaction systems, incorporation is especially advantageous in case of production of insoluble fatty acids (e.g. caproate), because this inhibitor can be accommodated in the bilayer, preventing it from interacting with the lipase.

CCL could also readily be incorporated into vesicles of the polymerisable zwitterionic surfactant bis[2-(pentacosa-10,12-diynoyloxy)ethyl]-2-amino-ethanesulphonic acid (BPAS). These vesicle systems have the advantage of easy and controllable UV polymerisation. BPAS vesicle systems were characterised in terms of morphology (Electron Microscopy) and stability.

Polymerisation of BPAS vesicles proceeds by linkage between the diacetylenic groups of the surfactant in the core of the bilayer membrane. This polymerisation did not alter the morphology, and polymeric vesicles were considerably more stable than the monomeric analogues. The enzyme remained fully active upon incorporation into the vesicle bilayer. Incorporated lipase was more stable than the free enzyme under denaturing conditions, as was established in CCL samples containing ethanol (50% v/v) or 2-(n-butoxy)ethanol (37.5% v/v). In addition, an increased stability was observed against temperatures above the physiological range (50°C, 60°C). The most striking stabilisation effect for the incorporated lipase was obtained in the presence of trypsin. Whereas the free enzyme was readily lysed by the proteolytic enzyme, the incorporated CCL was almost not attacked.

The polymeric network of the polymerised vesicles was shown to provide a suitable protective environment, especially when the enzyme was incorporated before (partial) polymerisation of the membrane layer. The protective effect proceeds most probably from the decreased freedom of movement and the reduced exposure of the protein to the aqueous phase. This makes the enzyme less accessible to unfolding, which generally leads to inactivation and denaturation [214,233,252].

Thus, incorporation of enzymes in polymeric vesicles can considerably enhance their stability. This may make these type of systems useful and advantageous in applications of enzymes and vesicles in which stability and enzymatic activity are of great importance. Advantages may be expected in therapeutical or laundering applications.

Fluorescence has shown to be a suitable technique to illustrate the interaction of lipase from *Candida cylindracea* with ABHEMA Br vesicles.

The phase transition of vesicles composed of nonpolymerised and polymerised *N*-allylbis[2-(hexadecanoyloxy)ethyl]methylammonium bromide could be determined in the absence of lipase, by measuring the change in fluorescence anisotropy of the membrane probe diphenylhexatriene. The phase transition temperature for nonpolymerised vesicles was 49°C and for the polymerised analogues 45°C.

Fluorescence energy transfer studies with native lipase as the donor molecule and an interfacial membrane probe (trimethylammonium diphenylhexatriene) or an internal membrane probe (diphenylhexatriene) as acceptor molecules demonstrated that *Candida cylindracea* lipase is readily incorporated into the hydrophobic bilayer of the vesicle. Furthermore, it could be determined that the lipase is incorporated more efficiently into nonpolymerised vesicles, and that the penetration of the enzyme into the bilayer is most probably less deeply in the case of the polymerised vesicles. Fluorescence anisotropy studies with a lipase preparation modified with a rhodamine label underlined these observations.

For practical application of lipases, a reliable method for the determination of enzyme activity is required. A rapid and sensitive assay for the detection of lipase activity was developed. The method is based on the increase in absorbance at 360 nm due to the formation of the 2,4-dinitrophenolate anion during enzymatic hydrolysis of 2,4-dinitrophenyl esters. The standard substrate

2,4-dinitrophenyl butyrate can be used in homogeneous or emulsified form. Using a diode array spectrophotometer with internal referencing, a correction can be made for absorbance changes due to clearance of the emulsion during hydrolysis. Small reaction volume and high extinction coefficient of the product makes the method applicable for the detection of low enzyme concentration.

The assay method has a broad applicability, as was tested for lipases from different sources: porcine pancreas, *Candida cylindracea, Pseudomonas* sp. and *Aspergillus niger*. All enzymes readily hydrolysed the standard substrate. In addition to 2,4-dinitrophenyl butyrate, the octanoate, dodecanoate and 10-undecenoate esters were also hydrolysed by lipase from *Candida cylindracea*.

APPENDIX A: LIST OF ABBREVIATIONS AND SYMBOLS

A	absorbance [-]
$\overline{A}$	absorbance obtained by wavelength averaging
ΔA	change in absorbance
ABDEMA Br	*N*-allylbis[2-(dodecanoyloxy)ethyl]methylammonium bromide
ABHEMA Br	*N*-allylbis[2-(hexadecanoyloxy)ethyl]methylammonium bromide
AIBN	azo-bisisobutyronitrile (or: 2,2'-azobis(2-methylpropionitrile))
BPAS	bis[2-(pentacosa-10,12-diynoyloxy)ethyl]-2-aminoethanesulfonic acid
Brij 35	polyoxyethylene(23) lauryl ether
BUEDMA Br	bis[2-(10-undecenoyloxy)ethyl]dimethylammonium bromide
CCL	*Candida cylindracea* lipase
CTAB	*N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide
DMF	*N*,*N*-dimethylformamide
DNP	2,4-dinitrophenol
DNPB	2,4-dinitrophenyl butyrate
DPH	1,6-diphenyl-1,3,5-hexatriene
ET	energy transfer
F	fluorescence (intensity) [-]
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
$I_{\perp}$	fluorescence intensity perpendicular to the polarised excitation
$I_{//}$	fluorescence intensity parallel to the polarised excitation
LAP 6	lipase from *Aspergillus niger*
LPS	lipase from *Pseudomonas* sp.
LUV	large unilamellar vesicles
PEI	poly(ethylenimine)
PPL	porcine pancreas lipase
r	anisotropy [-]
RITC	rhodamine X isothiocyanate
RITC-CCL	rhodamine labelled *Candida cylindracea* lipase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide-gel electrophoresis
T_c	phase transition temperature [°C]
TEM	transmission electron microscope
THF	tetrahydrofuran
TMA-DPH	1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate
Tris	tris(hydroxymethyl)aminomethane
Trp	tryptophan
Tyr	tyrosine
U	enzyme unit [μmole/min]
ε	molar extinction coefficient [$M^{-1}cm^{-1}$]
λ	wavelength [nm]
[-]	arbitrary or dimensionless unit

APPENDIX B: VESICLE-ENZYME STOICHIOMETRY

Surface area of surfactant headgroups a_o

- Egg lecithin: a_o = 0.72 nm^2 (depends on several parameters, like salt concentration) [81]
- DMPC: $a_o \approx 5$ nm^2 (depends on several parameters; varies with temperature from 4.6 to 7.4 nm^2, T = 10 -30°C) [66]

Estimated a_o for a quaternary dialkyl ammonium group:

$a_o = 2.5\ nm^2$

Surface area of vesicles

- Surface area of a sphere $A = 4\pi r^2$
- Outer surface area of a vesicle = $A_v = 12.6 * r^2$
- Total surface area of a vesicle $A_{v,t} \approx 2A$ (inside and outside) = $25r^2$
- Radius of ABHEMA Br vesicles r ~ 100 nm (derived from EM, see chapter 3)

Calculated surface areas of a vesicle

$A_v = 125{,}000\ nm^2$

$A_{v,t} = 250{,}000\ nm^2$

Number of surfactants per vesicle N_v, vesicle concentration C_v

Number of surfactants per vesicle $Nv = a_o / A_{v,t} = 2.5/250{,}000$

$N_v = 100{,}000$ (compare: reported N_v = 80,000 - 100,000 [67])

Vesicle concentration $C_v = C_s / N_v$ (C_s = surfactant concentration)

$C_v = 10^{-5} C_s$

Surface area of CCL

The surface area of globular proteins with a molecular weight M_w of 58.5 kD can be derived from relations between M_w and protein radius r:

- r = 3.45 nm (based on gel permeation chromatography [223])
- r = 3.62 nm (based on binding/adsorption measurements [207])

The estimated r for CCL is:

$r_{CCL} = 3.5\ nm$

The surface area A of CCL is put at the surface area of a square $(2 * r_{CCL})^2$:

$A_{CCL} = \sim 50\ nm^2$

Protein-vesicle ratio for CCL and ABHEMA Br vesicles

- Concentration of a 50 - 60 μg/ml CCL solution $C_{CCL} \approx 1$ μM
- 10μM (vesiculated) ABHEMA Br corresponds with a ABHEMA Br vesicle concentration C_V of 10^{-4} μM

At these concentrations, the following ratios can be derived:

Mole ratio's:

CCL : ABHEMA Br = 1 : 10

CCL : ABHEMA Br vesicles = 1 : 10^{-4}

Surface ratio's (C_{CCL}*A_{CCL} : C_V*A_V = 1*50 : 10-4*125,000):

CCL : ABHEMA Br vesicles = 4 : 1

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ABSTRACT

This thesis describes studies on the suitability of synthetic polymerisable vesicles for the incorporation and stabilisation of lipase for the bioconversion of organic chemical compounds.

In **chapter 1,** some characteristics are reviewed of hydrolytic enzymes, and more specific those of lipases. In **chapter 2** an overview is presented of the features and properties of surfactants and vesicles.

In **chapter 3,** the incorporation is described of lipase from *Candida cylindracea* (CCL) into polymerisable positively charged dialkylammonium bromide surfactant vesicles.

Before incorporation the lipase has been purified and characterised. The enzyme has a molecular weight of 58.5 kD and an isoelectric point of 4.1; the pH optimum is broad, ranging from pH 4 to 6 and the optimal temperature is 45°C.

The synthesis of several polymerisable surfactants and the preparation of nonpolymerised and polymerised vesicles from these surfactants are described. The vesicle systems were characterised in terms of morphology (electron microscopy) and stability. It appeared that polymerised vesicles are considerably more stable than their nonpolymerised analogues.

The enzyme was incorporated in the vesicle by the use of the dehydration-rehydration method or by incubation. In the latter case, trapping efficiencies are obtained of up to 100%. Activities of free and vesicle incorporated CCL are tested for three triglycerides: triacetin, tributyrin and tricaprylin and for 2,4-dinitrophenyl butyrate. Enzyme activity is lowest in homogeneous mixtures (triacetin and relatively low concentrations of tributyrin) and highest in heterogeneous mixtures (tricaprylin and relatively high concentrations of tributyrin and 2,4-dinitrophenyl butyrate). Incorporation of the enzyme in vesicular systems is advantageous for the activity, especially in homogeneous reaction mixtures, due to the presence of hydrophobic sites of the vesicles. Moreover, in the case of the production of insoluble fatty acid (caproate), inhibition by the acid is suppressed.

The influence of several surface active additives is tested on the activity of lipase. Vesicles have a positive influence on the activity, whereas positively charged surfactant addenda act as inhibitors. In the case of tricaprylin assays, the positively charged surfactant addenda increase enzymatic activity.

In addition, the sensitivity for tryptic digestion of free and incorporated CCL is compared. Free CCL is readily inactivated, whereas incorporated enzyme is protected from proteolytic degradation.

In **chapter 4** the stability of vesicle incorporated *Candida cylindracea* lipase is described.

For this purpose, the enzyme was incorporated into vesicles of the polymerisable zwitterionic surfactant bis[2-(pentacosa-10,12-diynoyloxy)ethyl]-2-aminoethanesulfonic acid (BPAS). Vesicle systems of BPAS were characterised

in terms of morphology (electron microscopy) and stability. Polymerisation of vesiculated BPAS surfactants does not alter the vesicle morphology. Polymeric vesicles are considerably more stable than the monomeric analogues. CCL incorporated into the vesicle membrane by the incubation method remains fully active; especially in homogeneous assay mixtures the vesicle incorporated enzyme shows an increased activity when compared to the free lipase. The stability of free and incorporated lipase was determined by measuring the residual activity of the various systems when mixed with ethanol (50% v/v) or 2-(n-butoxy)ethanol (37.5% v/v), at 50°C and 60°C and in the presence of the proteolytic enzyme trypsin. In all cases the vesicle incorporated enzyme displays an increased stability against denaturating conditions.

The interaction of lipase from *Candida cylindracea* with positively charged polymerisable surfactant vesicles was studied by the use of steady state fluorescence techniques. The results of these studies are described in **chapter 5.**

The phase transition of vesicles composed of nonpolymerised and polymerised *N*-allylbis[2-(hexadecanoyloxy)ethyl]methylammonium bromide was determined by measuring the change in fluorescence anisotropy of the membrane probe diphenylhexatriene. The phase transition temperature for nonpolymerised vesicles is 49°C and for the polymerised analogues 45°C. Fluorescence anisotropy and energy transfer measurements were used to demonstrate that *Candida cylindracea* lipase is readily incorporated into the hydrophobic bilayer of the vesicle. By using an interfacial membrane probe (trimethylammonium diphenylhexatriene) and an internal membrane probe (diphenylhexatriene), it could be determined that the lipase is incorporated more efficiently into the nonpolymerised vesicles, and that the penetration of the enzyme into the bilayer is less deeply in the case of polymerised vesicles.

In **chapter 6,** a rapid and sensitive assay for the detection of lipase activity is described. The method is based upon the increase in absorbance at 360 nm due to the formation of the 2,4-dinitrophenolate anion during the enzymatic hydrolysis of 2,4-dinitrophenyl esters. Several esters with different acyl chain length have been tested. 2,4-Dinitrophenyl butyrate proved to be a suitable standard substrate. This substrate can be used in homogeneous reaction systems and in emulsified form. In the latter case, a correction can be made for absorbance changes due to clearance of the emulsion during hydrolysis by using a diode array spectrophotometer with internal referencing. The small reaction volume and the high extinction coefficient of the product makes this method suitable for assay mixtures of low substrate and low enzyme concentration.

In **chapter 7** the results from the preceding chapters are reviewed in a general discussion.

NAWOORD

40.000 km... ik fiets onder het *panneau rouge* door. De hele *Tour de Dissertation* flitst door mijn hoofd. Prof.dr. H.C. van der Plas 'promote' de wedstrijd, maar dr. J.F.J. Engbersen werd uiteindelijk de ploegleider van de eenmansformatie *Enzymen in vesikels*. Eenmansformatie?, niet helemaal. Dr. Maurice Franssen bleek een uitstekend wegkapitein en Hugo Jongejan deed met name in etappe 4 het nodige kopwerk voor me. De neo-profs Jasper van Heemst, Freek van Keulen en Coen van Delden hebben tijdens hun trainingsstages prima sleurwerk verricht. Daarnaast hebben de 'buitenlandse' ploegen *Biochemie* (dr. Ton Visser, ir. Eward Pap, Ron Wolbert) en *Plantencytologie en -morfologie* (Adriaan van Aelst) me geregeld uit de wind gehouden. Ik had de bergen *hors catégorie* ook nooit kunnen nemen zonder de goede zorgen van soigneurs en materiaalverzorgers en de mensen die dit routeboek hebben doorgekamd (Door, Hannie en alle - deels buitenlandse - leden van het Cellar Cake Committee; Beb, Sies, Bart, Willem, Gert, Jurrie, Pleun en Ronald; Hugo, Monique en Ruud). Iedereen die me onderweg heeft aangemoedigd met goede adviezen ben ik bijzonder erkentelijk.

Vlak voor de meet kijk ik nog even om. Ik heb al die tijd op een tandem gefietst. Syl, altijd in dat ondankbare tweede zadel, maar wij weten wel beter: geen tandem zonder machinist, geen machinist zonder stoker!

Tzeoiegtw: IhCCC!

CURRICULUM VITAE

E.W.J. Mosmuller (Ward) werd op 26 maart 1961 in Heerlen geboren. De lagere school volgde hij in Nieuwenhagen. In 1981 behaalde hij het diploma Gymnasium B aan het Coriovallum College te Heerlen. In datzelfde jaar begon hij aan de toenmalige Landbouwhogeschool te Wageningen aan zijn studie Moleculaire Wetenschappen; hij volgde een biotechnologisch studieprofiel. Zijn eerste doctoraalvak deed hij bij prof.dr. H.C. van der Plas aan de vakgroep Organische Chemie van de Landbouwuniversiteit Wageningen onder leiding van dr. M.C.R. Franssen. Zijn onderzoeksopdracht lag op het gebied van haloperoxidasen (immobilisatie van chloroperoxidase; karakterisatie van bromoperoxidase). Voor zijn doctoraalvak Bio-organische Chemie was hij te gast bij prof. dr. E.M. Meijer, sectie Bio-Organische Chemie, DSM-Research in Geleen. Hij deed aldaar onder leiding van dr. M. Kloosterman onderzoek aan reacties met lipasen (hydrolyse van suikeresters; acylering van dihydroxyverbindingen). In 1987 werd zijn student-onderzoeksvoorstel gehonoreerd op het gebied van enzymen in synthetische vesikels. In 1988 behaalde hij zijn ingenieurstitel aan de Landbouwuniversiteit Wageningen. Zijn wetenschappelijke opleiding kreeg een vervolg met het in dit proefschrift beschreven promotie onderzoek, dat hij onder leiding van prof.dr. H.C. van der Plas, dr. J.F.J. Engbersen en dr. M.C.R. Franssen uitvoerde aan de vakgroep Organische Chemie van de Landbouwuniversiteit in Wageningen

Sinds 1986 is hij actief als correspondent voor het Chemisch Weekblad, Chemisch Magazine en Biotechnologie in Nederland/BioNieuws. Van 1988 tot en met 1992 is hij bestuurslid geweest van de Afdeling Jonge Leden, Werkgroep Midden, van het Koninklijk Instituut van Ingenieurs; van november 1989 tot februari 1991 was hij voorzitter.

Momenteel is hij werkzaam als projectadviseur Biotechnologie bij Senter in Den Haag. Hij is ondermeer belast met de coördinatie van het Innovatiegerichte Onderzoek Programma Katalyse.