TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 977

MEDICA - ODONTOLOGICA

IMMOBILIZATION OF *BURKHOLDERIA CEPACIA* LIPASE: KINETIC RESOLUTION IN ORGANIC SOLVENTS, IONIC LIQUIDS AND IN THEIR MIXTURES

by

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UNIVERSITY OF TURKU
Turku 2011

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ISBN 978-951-29-4720-1 (PRINT)
ISBN 978-951-29-4721-8 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2011

ABSTRACT

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Biocatalysis opens the door to green and sustainable processes in synthetic chemistry allowing the preparation of single enantiomers, since the enzymes are chiral and accordingly able to catalyze chemical reactions under mild conditions. Immobilization of enzymes enhances process robustness, often stabilizes and activates the enzyme, and enables reuse of the same enzyme preparation in multiple cycles. Although hundreds of variations of immobilization methods exist, there is no universal method to yield the highly active, selective and stable enzyme catalysts. Therefore, new methods need to be developed to obtain suitable catalysts for different substrates and reaction environments.

Lipases are the most widely used enzymes in synthetic organic chemistry. The literature part together with the experimental part of this thesis discusses of the effects of immobilization methods mostly used to enhance lipase activity, stability and enantioselectivity. Moreover, the use of lipases in the kinetic resolution of secondary alcohols in organic solvents and in ionic liquids is discussed.

The experimental work consists of the studies of immobilization of *Burkholderia cepacia* lipase (lipase PS) using three different methods: encapsulation in sol-gels, cross-linked enzyme aggregates (CLEAs) and supported ionic liquids enzyme catalysts (SILEs). In addition, adsorption of lipase PS on celite was studied to compare the results obtained with sol-gels, CLEAs and SILEs. The effects of immobilization on enzyme activity, enantioselectivity and hydrolysis side reactions were studied in kinetic resolution of three secondary alcohols in organic solvents, in ionic liquids (ILs), and in their mixtures. Lipase PS sol-gels were shown to be active and stable catalysts in organic solvents and solvent:IL mixtures. CLEAs and SILEs were highly active and enantioselective in organic solvents. Sol-gels and SILEs were reusable in several cycles. Hydrolysis side reaction was suppressed in the presence of sol-gels and CLEAs.

Keywords: CLEA, immobilization, ionic liquid, kinetic resolution, lipase, organic solvent, secondary alcohol, SILE, sol-gel.

TIIVISTELMÄ

Piia Hara

***Burkholderia cepacia*n lipaasin immobilisointi ja kineettinen resoluutio orgaanisissa liuottimissa, ionisissa nesteissä ja näiden seoksissa**

Farmakologia, lääkekehitys ja lääkehoito/ Synteettisen lääkekemian laboratorio, Turun Yliopisto

Annales Universitatis Turkuensis, Painosalama Oy, Turku, Finland, 2011.

Biokatalyysin käyttö synteettisessä kemiassa on ympäristöystävällinen vaihtoehto puhtaiden enantiomeerien valmistamiseksi, sillä entsyymit eivät ole toksisia ja ne katalysoivat monivaiheisia kemiallisia reaktioita suoraviivaisesti, usein miedoissa olosuhteissa. Katalyytteinä käytettävien entsyymien immobilisointi lisää usein prosessien vakautta, stabiloi entsyymiä eri reaktio-olosuhteissa, aktivoi entsyymiä sekä mahdollistaa saman entsyymin uudelleenkäytön useissa reaktioissa. Vaikka lukemattomia sovelluksia erilaisista immobilisointimenetelmistä on kehitetty, ei ole yhtä yleispätevää menetelmää erittäin aktiivisten, selektiivisten ja stabiilien entsyymikatalyyttien valmistamiseksi. Uusia menetelmiä entsyymien immobilisointiin tarvitaan, jotta saadaan erilaisille yhdisteille ja reaktioympäristöille sopivia tehokkaita katalyyttejä.

Lipaasit ovat yleisimmin käytettyjä entsyymejä synteettisessä orgaanisessa kemiassa. Väitöskirjassa tarkastellaan yleisimmin lipaasien immobilisoinnissa käytettyjä menetelmiä, sekä niiden vaikutusta lipaasien aktiivisuuteen, stabiilisuuteen ja enantioselektiivisyyteen. Lisäksi tarkastellaan lipaasin katalysoimaa kineettistä resoluutiota orgaanisissa liuottimissa ja ionisissa nesteissä.

Väitöskirjan kokeellisessa osassa *Burkholderia cepacia*n lipaasi (lipaasi PS) immobilisoitiin käyttäen sooli-geeli-, ristiinsidottu entsyymiaggregaatti- (CLEA) ja kiinteän kantajan päälle immobilisoitua nestekatalyytti- (SILE) menetelmiä. Lisäksi lipaasi PS adsorboitiin celiteen tulosten vertailemiseksi. Immobilisoinnin vaikutusta entsyymin aktiivisuuteen, enantioselektiivisyyteen ja hydrolyysisivureaktioon tutkittiin kolmen sekundäärisen alkoholin kineettisellä resoluutiolla orgaanisissa liuottimissa, ionisissa nesteissä (IL) ja näiden seoksissa. Lipaasi PS sooli-geelit olivat aktiivisia ja enantioselektiivisiä katalyyttejä orgaanisissa liuottimissa sekä liuottimen ja ILn seoksissa. CLEAt ja SILEt olivat aktiivisia ja enantioselektiivisiä orgaanisissa liuottimissa. Sooli-geelit ja SILEt olivat uudelleenkäytettäviä useissa reaktioissa. Sooli-geelien ja CLEAn käyttö vähensi hydrolyysisivureaktiota.

Avainsanat: CLEA, immobilisointi, ioninen neste, kineettinen resoluutio, lipaasi, orgaaninen liuotin, sekundäärinen alkoholi, SILE, sooli-geeli.

TABLE OF CONTENTS

ABBREVIATIONS

AC	active carbon
AnL	*Aspergillus niger* lipase
Asp	aspartic acid
a_w	water activity
[BMIM][BF_4]	1-butyl-3-methylimidazolium tetrafluoroborate
[BMIM][NTf_2]	1-butyl-3-methyl bis(trifluoromethylsulfonyl)imide
[BMIM][PF_6]	1-butyl-3-methyl hexafluorophosphate
BTMS	butyl trimethoxysilane
c	conversion
CAL A	*Candida antarctica* lipase A
CAL B	*Candida antarctica* lipase B
CLE	cross-linked dissolved enzyme
CLEA	cross-linked enzyme aggregate
CLEC	cross-linked enzyme crystal
CrL	*Candida rugosa* lipase
CSDE	cross-linked spray-dried enzyme
DES	deep euthetic solvent
DIPE	diisopropyl ether
DKR	dynamic kinetic resolution
DMAP	4,4-dimethylaminopyridine
DME	dimethoxyethane
E	enantiomeric ratio
ee	enantiomeric excess
E^N_T	Reichardt´s normalized polarity scale
[EMIM][BF_4]	1-ethyl-3-methylimidazolium tetrafluoroborate
[EMIM][NTf_2]	1-ethyl-3-methyl bis(trifluoromethylsulfonyl)imide
EC	Enzyme Commission
FDA	U.S. Food & Drug Administration
FFA	free fatty acid
GC	gas chromatography

Gln	glutamine
His	histidine
IL	ionic liquid
IPA	isopropylalcohol
iBTMS	isobutyl trimethoxysilane
k	rate constant
K_m	Michaelis constant
KR	kinetic resolution
Leu	leucine
Lipase PS	*Burkholderia cepacia* lipase
log P	logarithm for the partition coefficient of a solvent between 1-octanol and water
MmL	*Mucor miehei* lipase
MTMS	methyl trimethoxysilane
PEI	polyethyleneimine
PfL	*Pseudomonas fluorescens* lipase
PpL	pig pancreatic lipase
PrL	*Penicillium roqueforti* lipase
PTMS	propyl trimethoxysilane
PVA	polyvinylalcohol
RmL	*Rhizomucor miehei* lipase
Ser	serine
SDS	sodium dodecyl sulfate
SILE	supported ionic liquids enzyme
SILP	supported ionic liquid phases
TBME	*tert*-butyl methylether
TEOS	tetraethoxysilane
TlL	*Thermomyces lanuginosus* lipase
TMOS	tetramethoxysilane
UV	ultraviolet
V_{max}	maximum reaction rate

LIST OF ORIGINAL PAPERS

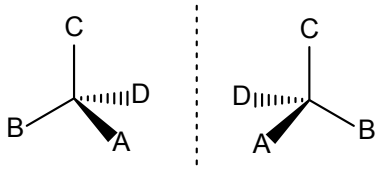
This thesis is based on the following papers referred by Roman numerals (I - III) in the text.

I. Hara, P.; Hanefeld, U; Kanerva, L.T. Sol-gels and cross-linked aggregates of lipase PS from *Burkholderia cepacia* and their application in dry organic solvents. *J. Mol. Catal. B: Enzym*., **2008**, *50*, 80-86.

II. Hara, P; Hanefeld, U.; Kanerva, L.T. Immobilized *Burkholderia cepacia* lipase in dry organic solvents and ionic liquids: A comparison. *Green Chem*., **2009**, *11*, 250-256.

III. Hara, P.; Mikkola, J.-P.; Murzin, D. Yu.; Kanerva, L.T. Supported ionic liquids in *Burkholderia cepacia* lipase-catalyzed asymmetric acylation. *J. Mol. Catal. B: Enzym*., **2010**, *67*, 129-134.

DEFINITIONS



Enantiomers

Biocatalysis	Chemical conversion of a substance into desired product with the aid of a free or immobilized enzyme or enzymes inside whole cells.[1]
Chiral	A molecule having the property of chirality.[2]
Chirality	Geometric property of a rigid object (or spatial arrangement of points or atoms) of being nonsuperimposable on its mirror image; such an object has no symmetry elements of the second kind (a mirror plane, centre of inversion, a rotation-reflection axis). If the object is superimposable on its mirror image the object is described as being *achiral*.[2]
Conformation	Spatial arrangement of the atoms affording distinction between stereoisomers which can be interconverted by rotations about formally single bonds.[2]
Enantiomer	One pair of molecular entities which are mirror images of each other and non-superimposable.[2]
Enantiopure	A sample all of whose molecules having (within limits of detection) the same chirality sense.[2]
Enantioselectivity	Preferential formation in a chemical reaction of one enantiomer over another.[2]
Racemate	An equimolar mixture of a pair of enantiomers.[2]
Stereochemistry	The area of chemistry that deals with spatial arrangements of atoms in molecules and the effects of these arrangements on the chemical and physical properties of substances.
Transesterification	A reaction where the alcohol part of an ester is replaced by another alcohol, leading to a formation of the new ester.[3]

1. INTRODUCTION

Chirality is one of the most important factors in drug discovery and development. During the last decade there has been a growing trend to focus on single enantiomers of chiral drugs. For the first time in 2004, all the approved chiral synthetic drugs went to market as single enantiomers,[4] and in 2006, 80 % of small-molecule drugs approved by FDA, U.S. Food & Drug Administration, were chiral and 75 % were single enantiomers.[5]

The synthesis of enantiopure compounds is a challenging task. Single enantiomers can be produced by chemical, chemo-enzymatic or purely biocatalytic synthesis. Biocatalysis is one of oldest chemical transformation methods known to humans. Biocatalysis offers advantages over chemical synthesis as the enzymes display high enantio-, chemo- and regioselectivity under mild conditions. Because of their excellent functional properties (activity, specificity and selectivity) enzymes are able to catalyze fast modifications of an individual functional group with a high degree of substrate specificity. Moreover, biocatalysis is a clean and ecological way to perform chemical processes.

In synthetic chemistry, enzymes are used in different reaction media like aqueous buffers, organic solvents, ionic liquids, supercritical fluids, and even in solvent free systems. Also enzymes need to have large substrate tolerance for natural and unnatural substrates. For synthetic purposes, enzymes are usually stabilized. The stabilization of enzymes towards reaction conditions and substrates is often achieved by immobilizing enzymes on different solid supports, encapsulating or cross-linking. Immobilization often improves activity, specificity and selectivity, and reduces the inhibition caused by substrates, products or a reaction medium. The immobilization of enzymes also makes the handling of the catalyst more convenient, since it enables filtration of the catalysts from reaction mixtures and makes the use of reactors easier. In industrial scale of enzyme catalysis, the immobilization of enzymes is desirable since, in addition to increased stability for reactions, immobilization allows the reuse of the catalyst and the use of continuous processes, which is beneficial from economical and environmental aspects.

Literature review in this thesis is focused on lipase catalysis in organic media and in ionic liquids, and on the methods and benefits of lipase immobilization. In the experimental section, the immobilization of *Burkholderia cepacia* lipase (lipase PS) as sol-gels, CLEAs and supported ionic liquid catalysts, together with the application of the obtained immobilized lipase preparations in enzymatic kinetic resolution of secondary alcohols in organic solvents, ionic liquids and in their mixture is discussed. The results are compared to those obtained with lipase PS on celite, method widely used for lipase immobilization in the Laboratory of Synthetic Drug Chemistry since 1993.[6] Moreover, lipase PS on celite is a commercial product as lipase PS-D from Amano Enzyme Inc. Lipase PS sol-gels, CLEAs and supported ionic liquids catalysts are shown to be stable and highly active and selective catalyst for preparing enantiopure secondary alcohols.

2. REVIEW OF LITERATURE

This Review of literature focuses on two main subjects. Firstly, lipases and lipase-catalyzed kinetic resolution and the effects of organic solvents and ionic liquids on enzymatic kinetic resolution are discussed. Secondly, the modification of crude lipase preparations by immobilization is discussed. The examples given primarily employ *Burkholderia cepacia* lipase, lipase PS, which is the catalyst used also in experimental part of the thesis. In addition, some other generally used lipases are discussed.

2.1. Lipases

2.1.1. Classification and general properties of lipases

Enzymes are proteins which have a specific 3D structure for catalysis, and they catalyze most of the biological reactions in nature. In official enzyme nomenclature enzymes are numerically classified (EC numbers) to six classes according to the reactions they catalyze (Table 1).[7] The naming system is recommended by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology.[7] The main industrial applications for enzymes such as proteases and lipases are detergent and food industries. Enzymes, especially lipases, can be produced in high yields from microbial organisms like fungi, yeast and bacteria. Enzymes can also be isolated from slaughter waste or cheap mammalian organs such as kidney or liver.

Table 1. Classification of enzymes.[7]

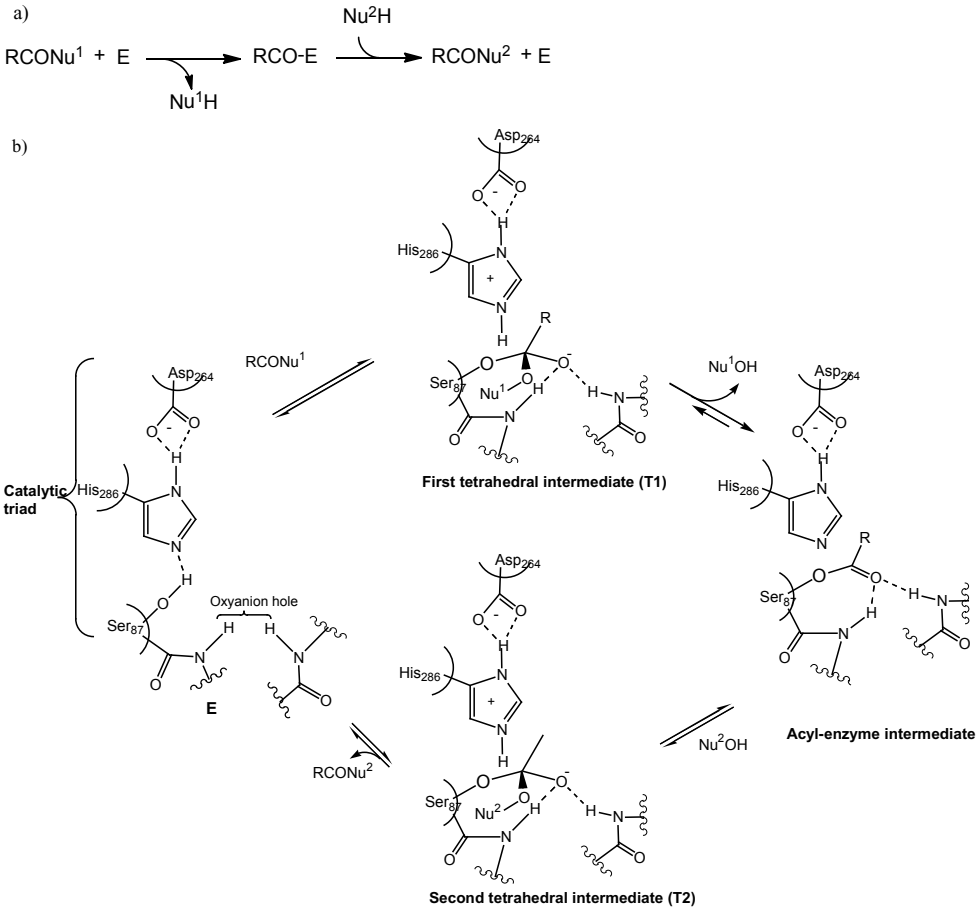
Enzyme class	Reaction type	Example of the enzyme
1. Oxidoreductases	Oxidation/reduction of C-H, C-C or C=C bonds.	Dehydrogenase, oxidase
2. Transferases	Tranfers methyl, aldehyde, ketone, acyl, sugar, alkyl, aryl, phosphoryl, nitrogenous, sulphur or selenium-containing groups.	Transaminase, kinase
3. Hydrolases	Hydrolysis/formation of esters, amides, lactones, lactams, epoxides, nitriles, anhydrides and glycosides.	Lipase, amylase, protease
4. Lyases	Addition/elimination of small molecules on C=C, C=N and C=O bonds.	Decarboxylase
5. Isomerases	Isomerizations including racemization and epimerization.	Glucose-isomerase, mutase
6. Ligases	Formation/cleavage of C-O, C-S, C-N, C-C bonds with concomitant triphosphate cleavage.	Synthetase

Lipases are most widely used enzymes in organic chemistry. There are hundreds of references of the use of lipases in organic solvents, and they have been reviewed in many books and articles.[3,9-11] Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are hydrolytic enzymes that catalyze hydrolysis of triglyserides into fatty acids and glycerol. In non-aqueous solvents

reactions may be shifted towards synthetic direction enabling the formation of esters from acyl donors and alcohols. Lipases exhibit wide substrate specificity, being therefore usable catalysts in organic synthesis. Various lipases are nowadays commercially available as free and immobilized forms. Lipases are used in various industrial applications in preparation of detergents, food, flavours, pharmaceuticals, esters and amino acid derivatives, fine chemicals, agrochemicals, cosmetics and perfumery as well as biosensors.[12]

2.1.2. Mechanism of the lipase catalysis

Based on the structure of the active site, lipases belong to serine hydrolases and they catalyze reactions by the same mechanism as serine proteases do (Scheme 1).[13] The active site consists of the catalytic triad and oxyanion hole. The catalytic triad is formed by the nucleophilic serine and an aspartate or glutamate that is hydrogen bonded to the histidine residue acting as a general acid-base catalyst. The oxyanion hole is located next to the catalytic triad, so that one of the backbone NHs of the oxyanion hole is that of the residue next to the catalytic Ser. Oxyanion hole stabilizes the negative charge of the tetrahedral intermediates by hydrogen bonding. Reactions follow the ping-pong bi-bi mechanism (Scheme 1).

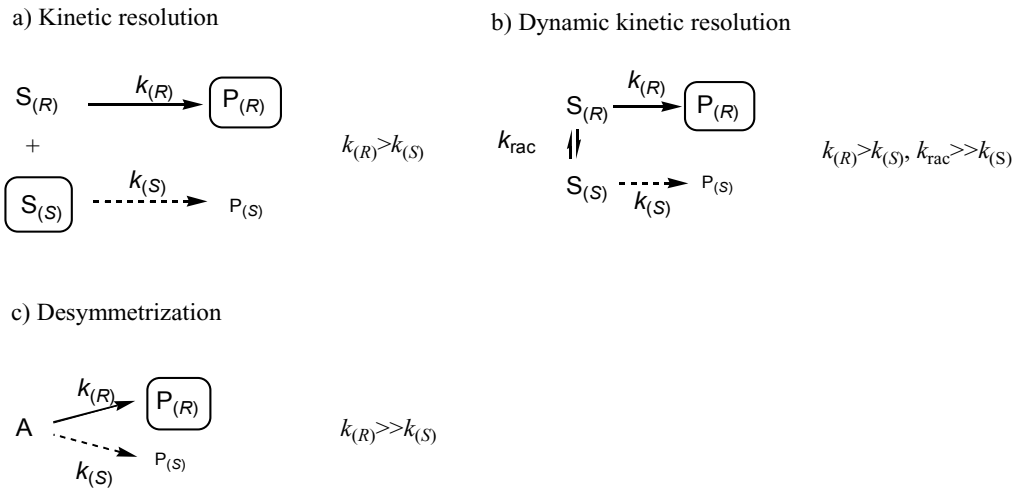


Scheme 1. Ping-pong bi-bi mechanism of lipases. a) General equation of lipase-catalyzed reaction; b) Catalytic cycle for lipase PS-catalyzed transesterification reaction. E=enzyme

Burkholderia cepacia lipase (lipase PS, previously *Pseudomonas cepacia*) is one of the most popular lipases used in organic synthesis. Lipase PS has a broad substrate tolerance and has widely been used for regio- and enantioselective hydrolysis and transesterification reactions. Lipase PS is a protein of 320 amino acids and 33 kDa molecular weight. The crystal structure of lipase PS has been determined as an open conformation.[14,15] The catalytic triad of lipase PS is formed by the residues Ser87, His286 and Asp264 (Scheme 1 b), and the oxyanion hole by the residues Gln88 and Leu17. Lipase PS contains an essential Ca^{2+} -site which stabilizes the β-hairpin in residues 214-228.[16] The active site is covered by a lid which undergoes conformational rearrangements and switches the enzyme between inactive (closed lid) and active (open lid) states. Lipase PS has high activity over a wide range of pH (3.5-11). The optimum pH is 7.0-8.0. Lipase PS is commercially available as its free and immobilized forms from e.g. Amano Enzyme Inc. (lipase PS "Amano" SD powder and lipase PS "Amano" IM on diatomaceus earth), Sigma-Aldrich (Amano products lipase PS "Amano" SD, lipase PS "Amano" C-I and lipase PS "Amano" C-II, on ceramic, lipase PS "Amano" IM, and lipase PS powder (Sigma)), Sprin Technologies (lipase PS covalently on epoxy acrylic resin and adsorbed on polystyrene resin), and Iris Biotech (adsorbed on DVB cross-linked polystyrene).

2.1.3. Enantioselective applications of lipases

Three main lipase-catalyzed methods to get enantiopure compounds are kinetic resolution (KR), dynamic kinetic resolution (DKR) and desymmetrization (Scheme 2).

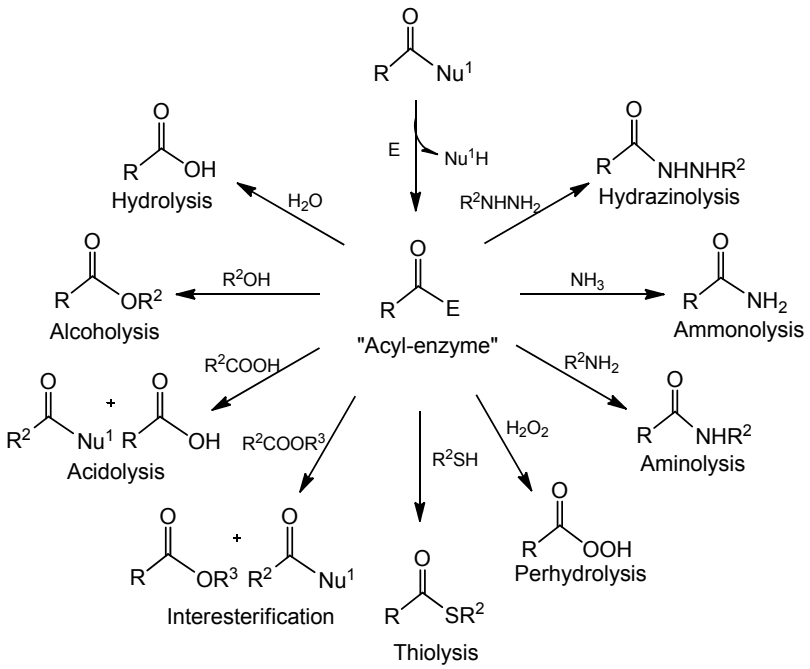


Scheme 2. Lipase-catalyzed methods to yield enantiopure compounds. $S_{(S)}$, $S_{(R)}$ substrate enantiomers, $P_{(S)}$, $P_{(R)}$ product enantiomers, A prochiral substrate.

In kinetic resolution, the substrate enantiomers ($S_{(R)}$ and $S_{(S)}$) react with different rates to product enantiomers ($P_{(R)}$ and $P_{(S)}$) (Scheme 2a). When the rate difference between the enantiomers is high enough, both the unreacted substrate enantiomer $S_{(S)}$ and the product enantiomer $P_{(R)}$ are obtained in enantiopure forms at 50 % conversion, expecting that the reaction to product proceeds irreversibly.[8] Kinetic resolution provides only 50% theoretical

yield of the enantiopure compounds, which might be a limitation if the other enantiomer is unwanted. In lipase-catalyzed reactions, ester $RCOONu^1$ (acyl donor) reacts with the enzyme to form an acyl-enzyme intermediate which is attacked by nucleophile (Nu^2H) to form the product (Scheme 1a). In kinetic resolution, either an acyl donor or a nucleophile can be chiral, and usually the other one is achiral. In lipase-catalyzed kinetic resolution focused on this thesis, acyl donor is achiral and substrate secondary alcohol, Nu^2H, chiral.

Lipases catalyze several types of reactions in organic media (Scheme 3), which makes them highly potential catalysts in the preparation of chiral compounds, such as pharmaceuticals and fine chemicals, in enantiopure forms. The reactions like hydrolysis, alcoholysis, interesterification, acidolysis, aminolysis, perhydrolysis, ammonolysis and thiolysis can take place. Alcoholysis, thiolysis and interesterification are in fact transesterification reactions where the alcohol part of the acyl donor is replaced by another nucleophile (Nu^2H) leading to the formation of another ester.



Scheme 3. Lipase-catalyzed reactions in organic media.[3,9,11]

Enantiomeric ratio E expresses the enantioselectivity of a kinetic resolution reaction where no side reactions occur. E is determined kinetically $E=(k_{cat}/K_m)_A/(k_{cat}/K_m)_B$ where k_{cat}/K_m is the apparent second-order rate constant for the reaction of the enzyme and the substrate at infinitely low substrate concentrations to give product(s).[17] k_{cat} and K_m denote catalytic and Michaelis constants, respectively, and A and B are substrate enantiomers. For synthetic purposes E value can be calculated more conveniently from equation $E=\ln[(1-c)(1-ee_S)]/\ln[(1-c)(1+ee_S)]$ based on substrate ee and $E=\ln[1-c(1+ee_p)]/\ln[1-c(1-$

ee_p)] based on product ee.[18,19,158] *E* can be regarded as moderate (15-30), good (30-100) or excellent (>200). The linkage of *E* to ee and conversion at *E*-values *E*=5, *E*=30 and *E*=200 is given in Fig. 1. It can be seen that when *E*=5, only the starting material can be isolated enantiopure at very high conversion (low yield). When *E*=30, the starting material can be separated enantiopure after 60% conversion and when *E*=200, both substrate and product enantiomers can be separated enantiopure at 50% conversion.

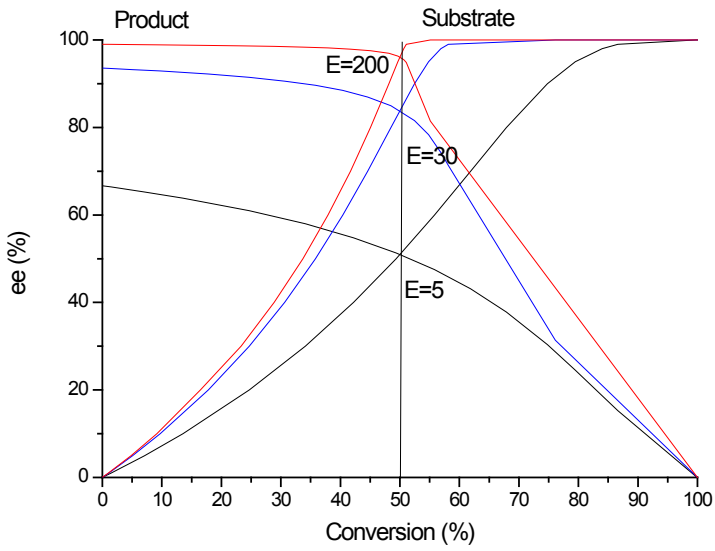


Figure 1. Dependence of ee on conversion. *E*=enantiomeric ratio.

Dynamic kinetic resolution (Scheme 2b) overcomes the problem of 50 % yield in kinetic resolution, but another enzyme is needed when both enantiomers are required. The unwanted $S_{(S)}$ enantiomer is racemized and recycled in situ giving a theoretical yield of 100 % for the product $P_{(R)}$. The rate constant of racemization (k_{rac}) should be much higher than $k_{(S)}$ for achieving very high selectivity. Racemization can be performed by a chemocatalyst, a biocatalyst or it can occur spontaneously. The method has been extensively reviewed.[20-23] As another effective method, desymmetrization (Scheme 2c) of prochiral or *meso*-componds offers a route to enantiopure compounds with 100% theoretical yield. The method has been widely used for the preparation of enantiopure cyanohydrins, diols and diesters.

Secondary alcohols as important organic building blocks are by far the most frequently used racemic targets in lipase-catalyzed transesterification reactions. Lipases usually show much higher enantioselectivity in the resolution of secondary than primary or tertiary alcohols. Vinyl acetate is the most common acyl donor for the acylation of secondary alcohols in organic solvents. The use of vinyl esters as acyl donors leads to irreversible reactions. In the case of primary alcohols, the enantioselectivity is frequently from low to moderate and can be increased by optimizing the reaction conditions. Tertiary alcohols are often unreactive towards lipases. There are only few examples of lipase-catalyzed resolution of

tertiary alcohols.[24-26] In the experimental part of this thesis, racemic secondary alcohols were used as nucleophiles and vinyl acetate as achiral acyl donor in transesterification.

2.2. Solvent effects in lipase catalysis

2.2.1. Lipase catalysis in organic solvents

The choice of reaction medium is an important issue for enzymatic reactions. Moreover, tightening environmental legislations and FDA guidelines set strict demands for the nature of the solvent. For instance, many chlorinated hydrocarbon solvents have already been or are likely to be banned in the near future. In the context of green chemistry, the solvent should be relatively non-toxic, safe and low volatile. The current trend leads away from hydrocarbons and chlorinated hydrocarbons towards lower alcohols, esters and, in some cases, also ethers. Inexpensive natural solvents, such as ethanol, have also the advantage of being biodegradable.[27]

The nature of the solvent may have clear effect on enzymatic activity, stability and selectivities, and finding the best solvent usually needs solvent screenings and optimizations. Ethers like *tert*-butyl methyl ether, diisopropyl ether and tetrahydrofuran, esters like ethyl acetate and vinyl acetate, and aliphatic and aromatic hydrocarbons like hexane and toluene have been widely used in lipase-catalyzed transesterifications.[3]

The catalytic activity of lipases is often lower in neat organic solvents than in aqueous solutions.[28] The main reason for this is the rigidity of enzymes in organic solvents. An enzyme needs an essential water layer which maintains the flexibility of the protein molecule necessary to catalysis, and can not be replaced without denaturation of the protein.[29] Generally, in hydrophobic solvents the conformation of the active site stays stable and, accordingly, the enzyme maintains its activity and thermostability, whereas in hydrophilic solvents the solvent molecules reach into the active site and strip the essential water from the enzyme.[30-32] Some lipases remain active with very small amount of residual water, whereas many others seem to require more bound water.[33] In organic solvents the enzymes have "pH memory" meaning that the catalytic activity and conformation of enzymes reflect to the last aqueous solution they have been dissolved.[30] Thus, the enzymatic activity can be enhanced by lyophilizing enzymes from aqueous solutions of optimal pH before use in organic solvents.

Generally, the partition coefficient of a solvent between water and *n*-octanol, log *P*, is used as an indicator of solvent polarity and suitability for enzyme catalysis, however, no clear correlation between log *P* and enantioselectivity has been observed.[34-36] Polar organic solvents having log $P<2$ are often thought to be unsuitable for biocatalysis due to inactivation or denaturation of enzymes by affecting the essential water layer. Midpolar solvents having log *P* between 2 and 4 weakly dissolve the water, and their effect on activity and selectivity is unpredictable. Nonpolar solvents having log $P>4$ do not dissolve the water coat thereby leaving the biocatalyst in an active state. Log *P* values of commonly used solvents in

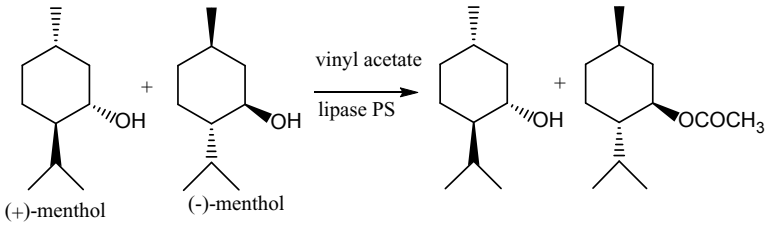
biocatalysis are presented in Table 2. Instability of an enzyme in polar solvents can, however, often be partially overcome e.g. by immobilizing the enzyme. Many reactions with free and immobilized lipases have been performed even in THF and acetonitrile.[37]

Table 2. Log *P* values of commonly used organic solvents.[34]

Solvent	log *P*
acetonitrile	-0.33
ethanol	-0.24
acetone	-0.23
tetrahydrofuran	0.49
ethyl acetate	0.68
isopropanol	0.8
tert-butyl methyl ether (TBME)	1.35
diisopropyl ether (DIPE)	1.9
toluene	2.5
cyclohexane	3.2
hexane	3.5

Table 3 presents the results of solvent screening for the acylation of (±)-menthol with lipase PS. The results indicate that highest *E* was obtained in CCl_4 (*E*=139, log *P* 3.0) and the lowest in acetone (*E*=33, log *P* -0.23). The results also show that other solvent properties than polarity, e.g. the chain length of alkanes (hexane *E*=73, decane *E*=63) and branching of the chain (hexane *E*=73, cyclohexane *E*=82), may affect enantioselectivity.

Table 3. Acylation of (±)-menthol with vinyl acetate in organic solvents in the presence of lipase PS.[38]

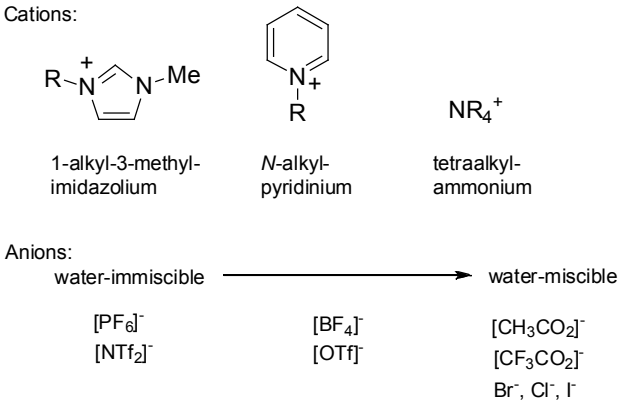


Solvent	log *P*[34]	*E*
hexane	3.5	73
cyclohexane	3.2	82
CCl_4	3.0	139
toluene	2.5	53
benzene	2.0	45
NEt_3	1.6	38
THF	0.49	40
acetone	-0.23	33
dodecane	6.6	65
decane	5.6	63

2.2.2. Lipase catalysis in ionic liquids

Room temperature ionic liquids (RT)ILs have recently received increasing attention as environmentally preferable alternative to organic solvents. They are considered green solvents since they are non-volatile (produce no atmospheric pollution), non-flammable and non-explosive. Additionally, they have high chemical and thermal stability and low melting points (<100°C). Although ionic liquids are considered as green solvents, mostly due to their negligible vapour pressure, the preparation and purification of 1-alkyl-3-methylimidazolium halide ionic liquids are not green according to the twelve principles of green chemistry.[39] Also ILs used in biocatalysis have not been designed biocompatible nor biodegradable, and many of them have been observed to be (eco)toxic.[40] They also have high solubility in water through which ILs might be run into the environment.[40]

ILs consist of an organic cation and an inorganic anion. In second generation ILs, mostly used in biocatalysis, hydrophobic anions such as trifluoromethanesulfonate $[CF_3SO_3]^-$ (triflate $[OTf]^-$), bis((trifluoromethyl)sulfonyl)imide $[N(CF_3)SO_2)_2]^-$ (bistriflamide, $[NTf_2]^-$) and tris((trifluoromethyl)sulfonyl)methanide $[C(CF_3SO_2)_3]^-$ ($[CTf_3]^-$) are popular due to their low reactivity with water and their large electrochemical windows.[41,42] Most common cations are dialkylimidazolium or pyridinium cations. (Scheme 4, Table 4). ILs are beneficial as designer solvents, meaning that their physical and chemical properties like viscosity, density, solubility, polarity and hydrophilicity/hydrophobicity can be tuned optimal for a certain reaction by changing the cation or anion.[27,41] ILs can be immiscible with many organic solvents such as hexane and ether, and their water-miscibility varies unpredictably depending more on the anion part.[43-46] As an attractive feature, ILs improve water solubility of polar substrates of low solubility like carbohydrates, making them more accessible for the reactions.



Scheme 4. Typically used cations and anions in ILs.[41,42]

Table 4. Properties of ionic liquids commonly used in biocatalysis.

Ionic liquid	Melting point[7] [°C]	Density[47] [g cm^{-3}]	Viscosity[48] [cP]	E^N_T [49,a]	Water miscibility[50]
[EMIM][BF_4]	15	1.34	43	0.710	yes
[EMIM][NTf_2]	-15	1.53	28	0.676	no
[BMIM][BF_4]	-71	1.20	219	0.673	yes
[BMIM][NTf_2]	2	1.44	69	0.642	no
[BMIM][PF_6]	12	1.38	450	0.667	no

[a] E^N_T : Reichardt´s normalized polarity scale.

In 2002, BASF published the first publicly-announced industrial use of ILs, the BASIL™ process for the production of the generic photoinitiator precursors alcoxyphenylphosphines.[41] Since then several industial methods utilizing ILs have been introduced by BASF, Eastman Chemical Company, IFP, Degussa, BP and other companies.

The first biocatalysis in ILs was reported in 2000, when *Z*-aspartame was synthesized in thermolysin-catalyzed reaction in [BMIM][PF_6].[51] The first lipase-catalyzed reaction in ILs was published in the same year.[52] Since then several applications of lipases in ILs have been performed, and the use of lipases have been extensively reviewed by several authors.[43-46,50,53-55] The use of ionic liquids or their mixtures with organic solvents as reaction media has improved stability, activity, regio- and enantioselectivity of various enzymes. Frequently used lipases CAL-B and lipase PS have been found to be catalytically active in 1-alkyl-3-methylimidazolium and 1-alkylpyridinium ILs in combination of anions [BF_4], [PF_6], [OTf] and [NTf_2] that are most widely used ILs for biocatalysis (Table 4).[46]

ILs seem to affect enzymes in much the same way as conventional organic solvents do.[44] The factors affecting the activity and stability of lipases in ILs are e.g. ion kosmotropicity and chaotropicity, polarity, ability to form hydrogen bonds, viscosity and hydrophobicity, but no clear correlation between IL property and enzyme activity/stability has been found.[43,54,56] Generally, lipase stability and activity has been higher in hydrophobic ILs.[54,57,58] Lipases also have the pH memory in ILs like in organic solvents. Thus, they maintain the pH of the last aqueous environment.[59]

ILs have high polarity, e.g. [BMIM][PF_6], often used in biocatalysis, has log *P* of -2.39 [60], that is much lower than that of suitable organic solvents for biocatalysis (Table 2 vs. Table 4). Although polar organic solvents have been found to denaturate enzymes, ILs with similar polarity do not.[42,43] Therefore, log *P* does not seem to be a useful parameter for indicating enzyme activity in ILs.[60] More useful magnitude of polarity is Reichardt´s normalized polarity scale, E^N_T, (Table 4). [EMIM] and [BMIM] ILs commonly used in lipase catalysis has E^N_T between 0.6 and 0.8 whereas water has E^N_T 1 and cyclohexane 0.006. This indicates high polarity of ILs which is not in line with polarity of organic solvents in determination of effects of ILs on enzymes. That is why enzyme activity may

be related more on viscosity and less to the polarity of ILs.[43] ILs have high viscosity (Table 4), which can thus cause mass-transfer limitations in enzymatic reactions.

Physical properties of ILs depend much on their purity, and users of ILs should be aware of impurities in ILs. Common impurities in ILs are water, halides and organic salts.[61-63] Water is a common contaminant in ILs either due to ineffective drying after preparation or due to absorption from atmosphere due to hygroscopicity of an IL. [BMIM][NTf_2] saturates with about 1.4% (w/w) of water, and for more hydrophilic ILs, water uptake from air can be even much greater.[64] Most hydrophobic ILs can dissolve up to 1% of water. The presence of water (or other cosolvents) reduces the viscosity[63], and it can also cause partial hydrolysis of [BF_4] and [PF_6] with formation of HF which denatures enzymes.[46] The common impurity in 3-alkyl-1-methylimidazolium ILs is 3-alkyl-1-methylimidazolium halide (generally chloride) from incomplete metathesis reaction.[62] Chloride contamination increases viscosity and inactivates lipases.[62,63] ILs should be carefully purified and dried before use in biocatalysis.

Third generation ionic liquids (advanced ILs) retain the moderate polarity, stability and distributed negative charge of second generation ILs.[42] These third generation ILs use biodegradable, readily available cations or anions of lower toxicity. Cation may be e.g. choline, amino acid or alkylimidazolium, and the anion sugars/sugar analog, amino acid, organic acid, alkyl phosphate or alkyl sulfate. One example of third generation ILs is choline citrate. Third generation ILs tend to be more hydrophilic than second generation ILs and they are often water-miscible. Advanced ionic liquids also contain deep euthetic solvents, DES´s, which are mixtures of salts, such as choline chloride, and uncharged hydrogen bond donors, such as urea, oxalic acid or glycerol.[42,65] Their properties are similar to ionic liquids. These advanced ionic liquids are new, and only few examples of biocatalysis have been published in those media. In transesterification of ethyl valerate with 1-butanol, lipase PS, CAL B and CAL A showed good activity in deep euthetic solvents, and in choline chloride:glycine the activity was similar to activity in toluene with all three lipases.[65] In the experimental part of this thesis, second generation ILs [EMIM][NTf_2], [EMIM][BF_4] and [BMIM][PF_6] have been prepared and used in kinetic resolution of secondary alcohols.

2.2.3. Effect of water in lipase-catalyzed transesterification reactions in non-aqueous media

Thermodynamic water activity (a_w) determines the mass action effects of water on hydrolytic equilibrium. It also describes the distribution of water between the various phases that can compete in binding of water.[66] It is commonly accepted that water requirements should be discussed in terms of a_w.

In lipase-catalyzed transesterification reactions, water may act as a competing nucleophile with an alcohol (substrate), causing hydrolytic side reactions of ester substrates and products to liberate free acids (Fig. 2). This water originates by the adsorption from the atmosphere, incomplete drying of the reagents, seemingly dry support materials

or side reactions producing water. This side reaction proceeds until a_w is reduced to the level where hydrolysis is no longer favourable. In this way water also influences the equilibrium concentrations. Water will tend to equilibrate between the immediate environment of the biocatalyst and the bulk phase, such that they reach the same a_w.[66] Also other type of side reactions like racemization, polymerization or decomposing of the reagents may take place due to water in the reaction system. Water can also change solvent properties, like viscosity of ILs. Elevated water activity increases the activity, V_{max} and K_m of lipase PS [67,68], and with many lipases, e.g. with CAL B, increasing a_w leads to decreased enantioselectivity.[69] The components of the system able to compete with the water are bulk phase, reactants and the support. Most support materials like celite does not have effect on activity-a_w profile with enzymes.[66]

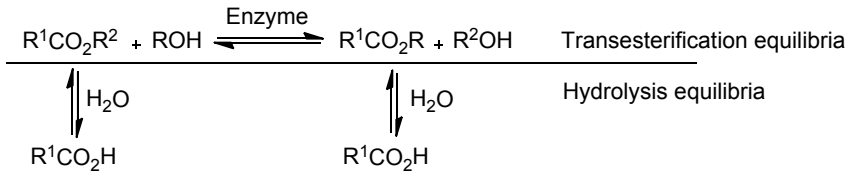


Figure 2. Equilibria caused by water in transesterification.

The enzyme activity can be greatly influenced by water activity. In lipase PS -catalyzed acylation of 1-phenylethanol with lauric acid in isooctane, enantioselectivity was not affected by initial water content ranging from 0 to 0.5% (v/v). However, the activity of lipase PS had decreasing trend when initial water content increased from 0.1 to 0.5% (v/v). As the water content increased, the amount of water in the bulk phase increased, and the reaction became favourable toward hydrolysis. Specific activity decreased from 4.17 to 0.65 mmol/min/g at 2% (v/v) initial water concentration.[70] It was explained that since the bulk phase is saturated with water, bound water is more difficult to expel from the enzyme to the bulk phase for the reaction to take place.[70] This decrease in activity may also be due to competing nucleophile of 1-phenylethanol with water.

Therefore, attention should be paid for proper drying of the components and choice of the reagents. While acylation with acids produces water, the acylation with vinyl esters produces acetaldehyde. Still, in lipase PS –catalyzed acylation of 1-phenylethanol with vinyl acetate in benzene-d_6 the main reaction was hydrolysis of vinyl acetate (lipase catalyzed), not the acylation of the substrate even in dry conditions. Product ester hydrolysis was not observed.[71]

2.3. Immobilization of lipases

2.3.1. General aspects

Immobilization of lipases has been thoroughly reviewed.[72-77] Immobilization often increases stability, activity, selectivity and offers possibilities to use higher substrate

concentrations and different solvent systems, e.g. organic solvents, ionic liquids and supercritical fluids. In hydrophobic media immobilization optimizes enzyme dispersion and helps to avoid aggregation. In addition, immobilization provides more convenient handling, facile separation and efficient re-use of catalyst. Moreover, protein contaminations in the product can be minimized or eliminated. For an industrial scale, immobilization is considered favourable in many applications. Many lipases also undergo interfacial activation, and they need to face a hydrophobic interface to adopt the open/active conformation. This can take place when using hydrophobic supports.

Immobilization protocols and supports are strongly dependent on the reaction medium, and several parameters must be taken into account to reach the highest possible stability and activity (Table 5).[8,72,73,75-77] Native enzymes that are commercially available as crude preparations contain various additives, such as polyols and sugars, which are added as stabilizers. As a result, the actual protein content may be very low (typically 1-30%). Also the contaminations from the fermentation broth like inactive proteins, medium components, nutrients, buffer salts or carbohydrates can be present. It should be kept in mind that crude preparations are often more stable than purified enzymes. Maximal specific activity is often obtained when enzyme forms a monolayer on the surface of the support.[78] Low enzyme loading can be harmful because of the strong interactions with matrix. On the contrary, high loading leads to multilayers of enzyme molecules which decreases the accessibility of the substrate to the active site of the enzyme.

Table 5. Enzyme and immobilization parameters affecting to efficient immobilization.[75,76]

Size of an enzyme
Stability of an enzyme under immobilization conditions
Conformational flexibility
Isoelectric point
Surface functional groups
Glycosylation
Additives in enzyme preparation
Immobilization time
Immobilization pH - optimal to enzyme
Immobilization temperature
Immobilization buffers
Nature and properties of carrier
Reaction medium

Immobilization techniques applied to lipases include 1) support binding (adsorption and deposition, ionic binding, covalent attachment), 2) entrapment in a polymeric gel, membrane or capsule and 3) cross-linking with polyfunctional agent. The comparison of those techniques is given in Table 6. The first two techniques involve the use of solid support or entrapment of the enzyme. The last method entails covalent linking of enzyme molecules with each other without additional support. There are hundreds of

variations based on combinations of these methods. Covalent coupling, entrapment and cross-linking are irreversible methods, and adsorption, ionic binding, affinity binding, chelation, metal binding and the formation of disulfide bonds are reversible methods where the support can be regenerated and re-loaded when enzyme activity decays. In organic media, non-covalent immobilization methods are often used because enzymes are generally insoluble to that media.

Table 6. Comparison of enzyme immobilization techniques.[61,73,75,79]

Method	Advantages	Disadvantages
Adsorption	Simple No chemical modification of an enzyme Reversible Possible to recycle supports Possible to use crude enzyme preparations Often inexpensive	Weak binding (leaking) Little or no stabilization Non-specific binding May limit mass-transfer
Covalent attachment	Tight binding, no leakage Wide variety of supports and linkers available Rational control of enzyme loading, distribution and microenvironment Can be used in any medium	Chemical modification of enzyme Support not recyclable Often expensive Activity diluted by carrier May limit mass-transfer Too high rigidity Irreversible
Sol-gel entrapment	No chemical modification of enzyme Can be simple Inexpensive Mild Usually very stable Open for chemical modification of matrix by changing components Retain high activity Easily recyclable High thermal and mechanical resistance	Little or no stabilization Environmental changes can disrupt network and cause leakage May limit mass-transfer Can be brittle Not all entrapped enzymes are catalytically active
Cross-linking	High volumetric activity Compatible with elevated temperature and organic solvents No carrier required Tight binding Simple Possible to use crude enzyme preparations No leaching Contains high proportion of active enzyme Improved storage and operational stability Easy to recover and recycle Possible to coimmobilize two or more enzymes	Chemical modification of enzyme Little control of particle properties CLEC requires crystallization of enzymes May limit mass-transfer Irreversible Weak resistance of mechanical stresses such as stirring

In recent years several immobilization methods have been utilized with lipases for different substrates, reaction conditions and reactors, but there is still no universal method for obtaining highly active and stable immobilized enzyme for each conditions. Below the most common lipase immobilization methods are presented. Attention is paid especially to supported ionic liquids, sol-gel entrapment and cross-linking, the methods which are used in the experimental work of this thesis. More detailed procedures of immobilization of lipase PS via sol-gel entrapment, CLEA and supported ionic liquids are presented in the experimental part of this thesis.

2.3.2. Support binding

Most commonly used support binding methods are adsorption and covalent attachment. Those and supported ionic liquids, a newer technique utilizing ionic liquids to protect or improve the properties of an enzyme, as well as support materials are discussed below.

In Figure 3 different immobilization methods on solid supports are presented. Ionic binding is a simple and reversible method for enzyme binding, however, it is difficult to find conditions under which the enzyme remains strongly bound and fully active.[72,75] Any ion exchange resin can act as a carrier, and the charge of an ion exchanger (positively/negatively charged) depends on the charge of the enzyme. The affinity of ionic binding depends on pH and salt concentrations during immobilization, but also during application. During ionic binding, enzyme properties like pH optimum or pH stability may change, and there may be problems with charged substrates and products.

The benefits in immobilization of lipases on porous supports encompass fully dispersion of enzyme molecules, which prevents aggregation, autolysis or proteolysis. Also the enzyme will not be in contact with external hydrophobic interface.[76] Immobilization of enzymes on solid supports can also increase the interfacial surface area between protein and solvent thereby increasing reaction rate.[61] In an ideal situation the immobilized enzyme molecules are uniformly spread over the available surface, and monolayer of enzyme can be formed on the wall of the pores.[73]

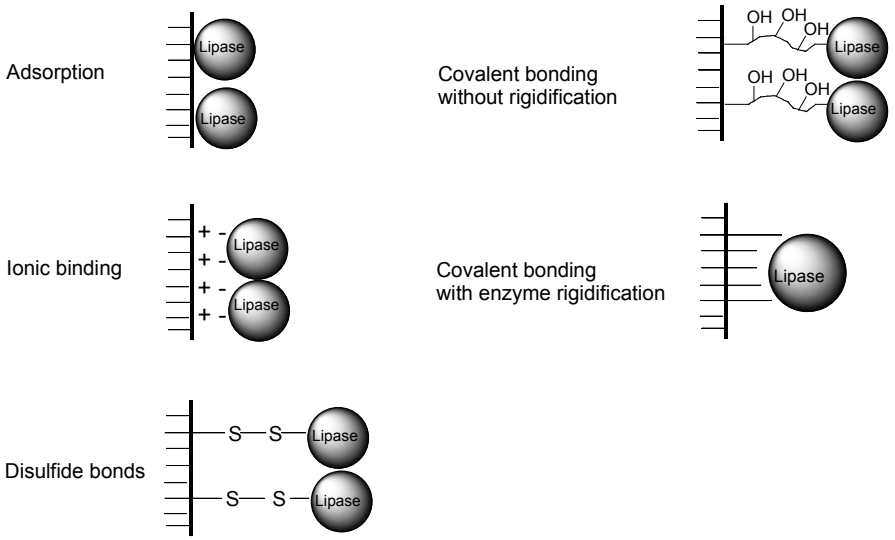


Figure 3. Approaches to immobilization of lipases on supports.

2.3.2.1 Support materials for adsorption and covalent attachment

Numerous support materials are available for immobilization of enzymes (Table 7.). Physical and chemical properties of the support material strongly affect an enzyme. The support needs to be chemically and mechanically stable. Hydrophilicity/hydrophobicity of the material is one of the most important characters of a support, in addition to mean particle diameter, swelling, mechanical strength, compression behaviour and surface properties.[72,73] It is essential to have large surface area, either by small particle size or highly porous materials. In particular pore parameters and particle size determine the total surface area and thus critically affect the capacity for binding enzymes. Porous supports are preferred, since high surface area allows higher enzyme loading and porous materials provides greater protection for the enzyme. The supports are classified as inorganic and organic supports (Table 7).

Table 7. Classification of support materials for immobilization of enzymes.[61,72]

Organic supports		**Inorganic supports**	
Natural polymers	Synthetic polymers	Natural minerals	Processed minerals
Polysaccharides e.g. cellulose, dextran, agar, agarose, alginate, starch Proteins e.g. collagen, albumin Carbon Carrageenan	Polystyrene Other polymers like polyacrylate polymethacrylates, polyacrylamide, polyamides, vinyl and allyl polymers Nylon	Bentonite Silica Mesoporous silicas Alumina Zeolites Diatomaceous earth Clays	Glass (non-porous and controlled pore) Metals Controlled pore metal Oxides Sol-gel

The activity of immobilized enzymes depends on the pore size of the support relative to enzyme and particle sizes. Microporous carriers have pores in the range of 0.1-10 μm. Mesoporous pore size is 3-10 nm which is of the same size with enzymes, and macroporous pores with specific areas of 25-100 m^2/g have diameter of 8-1000 nm.[73] In adsorption of lipases, retention of activity depends on the pore size in the range below 100 nm.[73] Above this value, activity is independent of the pore size. Activity of enzymes immobilized on carriers with pore size below 100 nm (e.g. Eupergit C, pore size 10-20 nm) is lower and strongly related to enzyme loading and pore size, whereas pore size >100 nm results increased accessibility of pores. Thus, when adsorbing lipase PS on mesoporous silicates SBA-15-55Å (55 Å pore diameter) and SBA-15-240 Å (pore diameter 240 Å), the SBA-15-55Å-lipase PS exhibited reduced activity due to limited accessibility of a substrate to enzyme active site.[80] Also loadings were lower, suggesting that significant portion of internal pore volume is not available for lipase adsorption. SBA-15-240 Å-lipase PS had sufficient space to allow lipase penetrate deeply into pores, and thus showed similar catalytic activity to free lipase PS.

Substrate sorption into support material can cause problems in kinetics. In the esterification of 4-methyloctanoic acid with Novozym 435, *Candida antarctica* lipase B adsorbed on hydrophobic macroporous polymer, sorption of substrate into pores of hydrophobic beads caused problems in determining *E* of the esterification.[81] Similar problems did not occur in hydrolysis or transesterification reactions. The volume of the beads increased 175-250% by sorption of substrate which correlated 7.2 mmol of substrate per gram of lipase. This amount is not taking part in esterification, and calculation of *E* must be corrected.

Lipase PS and CAL B have been successfully immobilized on several types of supports, such as porous ceramic support Toyonite 200-M[82] and Tungsten(VI)oxide coated metal oxides[83], supramagnetic nanoparticles based on magnetite Fe_3O_4[84,85] and zirconia nanoparticles[86], and macroporous polystryrene microspheres[87] and mesoporous silicates[80,88,89,90].

Typical hydrophobic and hydrophilic supports used in immobilization of lipases are presented in Table 8. Hydrophobic nature of the support facilitates the opening of the hydrophobic lid and thus activation of the enzyme.[77] Acrylic resins are widely used in immobilization of enzymes. Eupergit® C is macroporous copolymer of *N,N´*-methylene-bi-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide.[77] Reactive epoxy group has made Eupergit C and Eupergit C 250 L popular. Eupergit C binds to proteins via the reaction of its oxirane moieties with free amino groups of an enzyme to form covalent bonds at neutral or alkaline pH.[75] Due to high density of oxirane groups on the surface of the beads, enzymes are immobilized at various sites of their structure (=multipoint attachment). Sepabeads® are methacrylic carriers that can be fuctionalized either by epoxy or amino groups and covalently attached to enzymes.[75]

Table 8. Examples of hydrophilic and hydrophobic supports used in enzyme immobilization.

Hydrophilic supports	Hydrophobic supports
Cellulose	EP-100 polypropylene
Lignin	Accurel MP 100 polypropylene
Avicel (microcrystalline cellulose)	Octyl-silica
Celite	Octyl-agarose
Porous glass	XAD-7 (acrylic resin)
Silica gel	PVA (polyvinyl alcohol)
	Agarose

Celite (diatomaceous earth, diatomite) is one of the most popular carriers in immobilization of lipases, and it can be utilized both in adsorption and covalent attacment. Celite is hydrophilic, and its structure and properties can vary significantly as a function of production process. Several celite types are commercially available. The pore size of celite can vary from μm to several mm, and the shape of the particles can be rods or beads. The shape and porosity greatly affects the adsorption of enzymes as well as the ability to retain water inside the pores. Many commercial lipase preparations are based on celite powder, e.g. Amano lipase PS-D from *Burkholderia cepacia*.

The support material and immobilization method can also induce traces of different compounds to the reaction mixture. It was noticed that number of compounds migrated from Novozym 435 (adsorbed on polyacrylate beads Lewatit® VP OC 1600, poly(methyl methacrylate) cross-linked with divinylbenzene) to organic solvents and ILs.[91] Five major components were glycerol, benzoic acid, 2-hydroxyethyl benzoate, 2-hydroxyethyl sorbate and sorbic acid. The presence of these compounds may not have considerable impact on reactions with high substrate concentrations and when an enzymatic reaction is fast, but their presence is good to keep in mind especially in chromatographic analysis.

2.3.2.2. Adsorption

Adsorption onto a water-insoluble macroscopic carrier (Fig. 3) is the easiest and oldest method of immobilization.[8,72,73] In a most simple case, a lipase in a buffer solution is mixed with a support. The advantages and disadvantages of adsorption method are presented in Table 6.

Adsorption forces are relatively weak.[8,72,73,75] Basically any type of carrier in Tables 7 and 8 can be used to adsorb an enzyme via physical adsorption. Enzymes with large hydrophobic surface area will interact well with hydrophobic carriers, whereas enzymes with large hydrophilic areas interact with hydrophilic carriers. An enzyme is attached to the matrix through hydrogen bonding, van der Waals forces, or hydrophobic interactions. Although the adsorption of a lipase may be quite strong, it can be reversed by changing the conditions that influence the strength of interaction (e.g. pH and ionic strength in aqueous environment, temperature, polarity of solvent) to allow the recovery and reuse of the support. Deposition method can be used when enzymes are not adsorbed efficiently

enough.[72] The enzyme is dissolved in a minimal volume of an aqueous buffer, which is then mixed with the support followed by drying of the complete mixture. Everything present in the solution is deposited on support. Deposition is useful for wide range of enzymes and supports. Celite is typical support used in a deposition method.

Most lipases show interfacial activation (Fig. 4.), where the conformational change from lid closed to lid open form.[72,75] Adsorption of lipases on hydrophobic supports at low ionic strength is thought to mimic the interfacial activation. It has been suggested that lipases are immobilized in their active conformation (lid open). The final immobilized preparation may be even more active than the native enzyme.

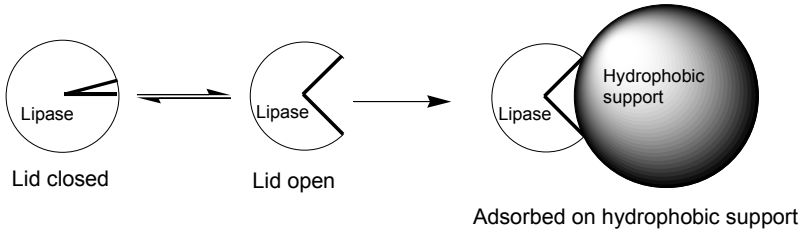


Figure 4. Interfacial activation of lipases on hydrophobic supports.

When a lipase is immobilized on a hydrophobic support the only interactions between the support and the enzyme are van der Waals forces.[75] For efficient immobilization both the support and the enzyme need to have large lipophilic surface. Too high hydrophobicity of the support prevents the access of the enzyme to the pores and thus leads to decreased enzyme loadings.[89] The presence of ethanol has been used to decrease the hydrophobicity enabling better accessibility of the enzyme to pore channels.

In many enzymes, hydrophilic amino acid residues, which can be also glycosylated, prevail on the surfaces. Therefore, they can easily form hydrogen bonds and be immobilized on hydrophilic supports like cellulose, lignine, celite, porous glass and silica gel. Particularly popular hydrophilic support in lipase adsorption is celite (diatomaceous earth). Lipases immobilized on celite have been extensively used in organic solvents without any significant leaching. Lipase PS has been adsorbed on celite to enhance the activity and stability of the enzyme directly [92,93] or in the presence of sucrose[6,94-97] in several applications. Also commercial preparations of lipase PS on celite has been available, e.g. Amano lipase PS-D and lipase PS "Amano" IM. However, adsorption forces are not strong enough to keep lipase PS adsorbed in polar ILs.[98] Celite supports can also be used to control the water activity in the reaction systems. Celite powder adsorbs only minimum amounts of water, but porous celite rods adsorb and release water such way that water content is maintained constant in the reaction system.[99] When celite rods R-640 were ground and reduced to fine powder, celite lost most of its ability of adsorbing water.

2.3.2.3. Covalent attachment

Covalent bonding with or without enzyme rigidification (Fig. 3) is widely used because of the stable nature of the bonds between an enzyme and a matrix. This method, usable in any medium, is employed when there are strict requirements for the absence of enzyme in the product.[72,75] The advantages and disadvantages of covalent bonding were presented in Table 6. Covalently immobilized enzyme preparation usually contains the support, spacer, linker and enzyme.

Covalent bonding of an enzyme to a carrier is based on a chemical reaction between the active amino acid residues located on the enzyme surface and active functionalities that are attached to the carrier surface.[73] The most commonly used reactive groups in covalent binding are presented in Fig. 5.[72,73] Attention must be paid to amino acid residues essential for catalytic activity since they can not be involved in the covalent linkage to the support. Coupling methods can be divided in 1) activation of the matrix by addition of a reactive functionality to a polymer and 2) modification of polymer backbone to produce activated group.[72]

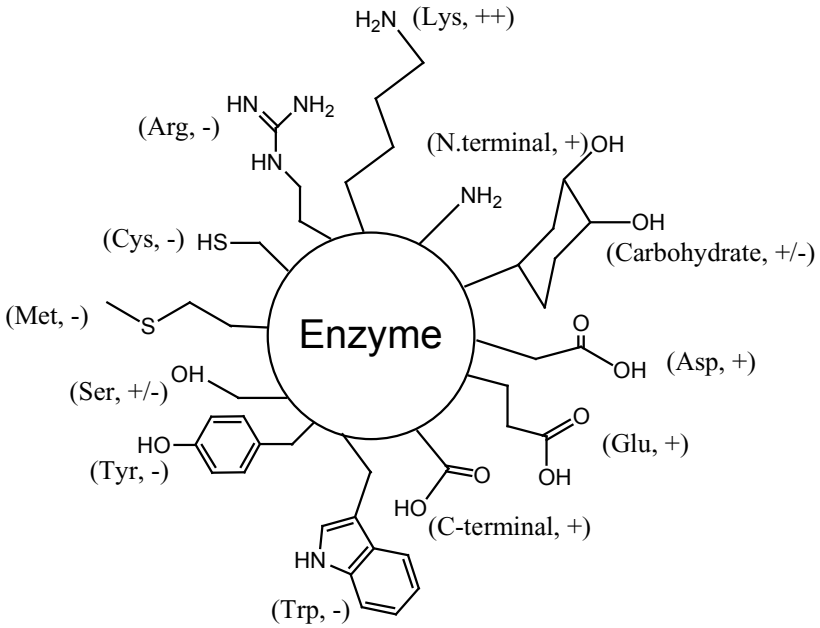
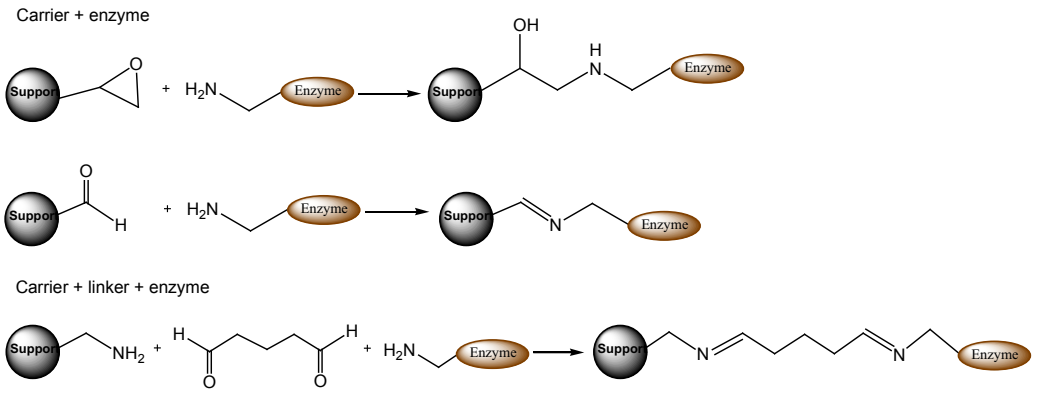


Figure 5. Reactive amino acid residues of an enzyme. ++: very frequently used; +: frequently used; +/-: not frequently used; -: not used in enzyme immobilization.[73]

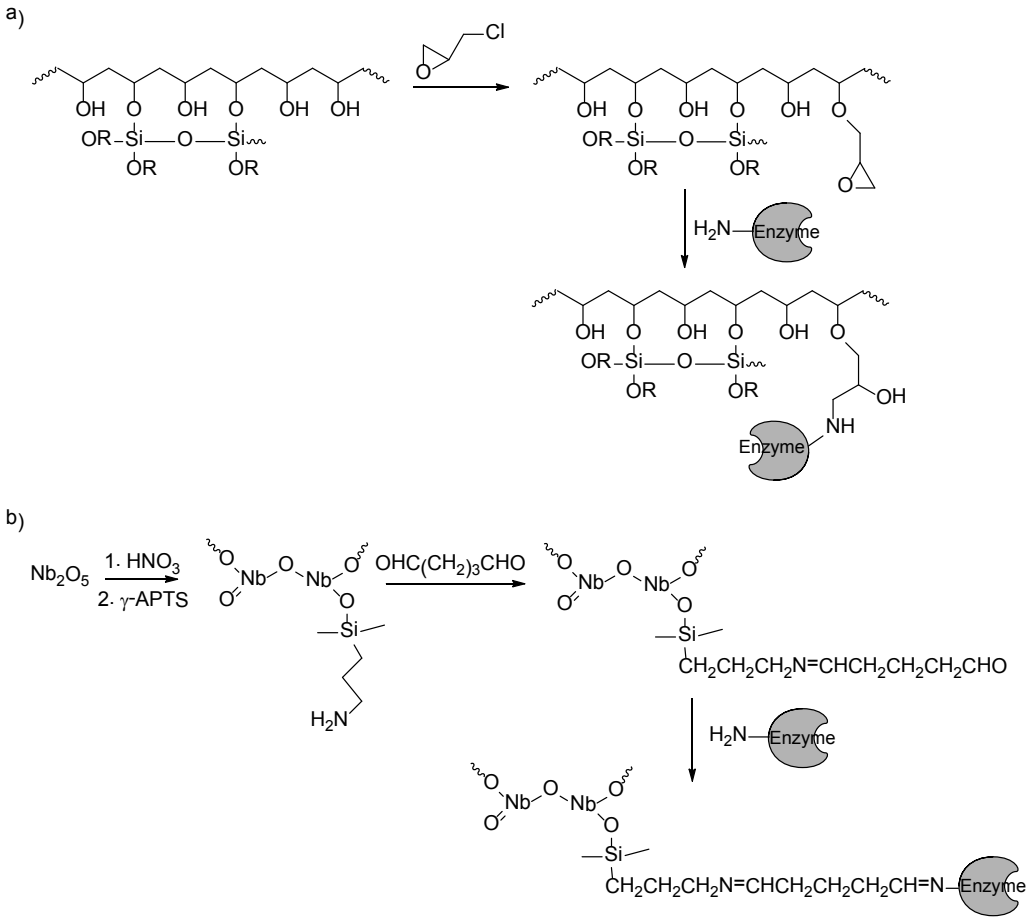
In covalent immobilization, both hydrophilic and hydrophobic supports can be used. The supports are often activated before use for binding enzymes. Reactive groups on the support can be attached via short or long spacers to the enzyme. Lipases are known to have remarkable conformational changes during catalysis, and longer spacers may be advantageous since they are expected to allow a wider conformational flexibility.[75] Multipoint covalent attachment of enzymes on supports via short spacers involve

many residues on the enzyme surface, which promotes rigidification of the enzyme.[72,76] There are numerous reactive groups and linkers used in covalent immobilization.[73] Supports functionalized with reactive epoxy, amino or glyoxyl groups are often used in covalent immobilization of lipases. Some examples of covalent attachment of enzymes on supports are presented in Scheme 5. Often used linkers are glutaraldehyde and carbodiimides.



Scheme 5. Examples of activation methods of the carriers in covalent immobilization.

Lipase PS was covalently immobilized on niobium oxide (Nb_2O_5) and polysiloxane-polyvinyl alcohol (SiO_2-PVA) (Scheme 6).[100] SiO_2-PVA was activated with epichlorohydrin (epoxy support) (Scheme 6a) and Nb_2O_5 was pretreated with nitric acid, silanized with γ-aminopropyltriethoxysilane (γ-APTS) and activated with glutaraldehyde (Scheme 6b). SiO_2-PVA has larger pore volume and specific surface area than Nb_2O_5, and lipase PS on SiO_2-PVA was 2 times more stable than lipase PS on Nb_2O_5 and 17 times more stable than free lipase. Transesterification yield was also higher with lipase PS on SiO_2-PVA (89% vs. 40%). When *Burkholderia* sp. C20 lipase was immobilized covalently on celite carriers by cross-linking with glutaraldehyde, the stability and thermal tolerance was increased.[101]



Scheme 6. Covalent immobilization of lipase PS on SiO_2-PVA (a) and Nb_2O_5 (b).[100,102,103]

As an example of environmentally friendly covalent immobilization of lipases, fully biodegradable catalyst was prepared by cross-linking lipase from *Aspergillus niger* by glutaraldehyde on silk fibers.[104] The silk-fiber lipase was successfully used for hydrolysis of sunflower oil for fatty acids (biodiesel production).

2.3.2.4. Supported ionic liquids

Supported IL enzyme (SILE) catalysis is a relatively new technique combining the advantages of ionic liquids with those of heterogenous support materials. Immobilization and supporting of ionic liquids and enzymes can be carried out in many ways, such as with simple impregnation, covalent linking, sol-gel method, or using ionic liquid as a vector between a support and an enzyme (Fig. 6).[105,106] In SILEs, the ionic liquids are used to protect or improve the properties of the catalyst.[107,108] Ionic liquids provide adequate microenvironment for an enzyme allowing high selectivities. The benefits of SILEs are the easier handling of heterogenous catalyst, and use of lower volumes of costly ILs compared to reactions in ILs without loosing the benefits of ILs.[106] The diffusion of

substrates through IL phase may cause mass-transfer limitations during catalysis. Since SILE method is new, only few examples of the method applied to enzymes are available. The work described in Paper III was the first example of lipase PS SILE.

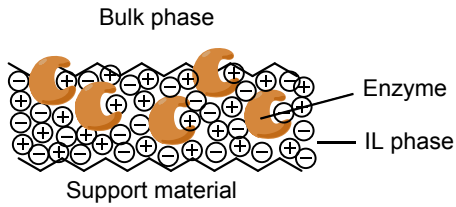
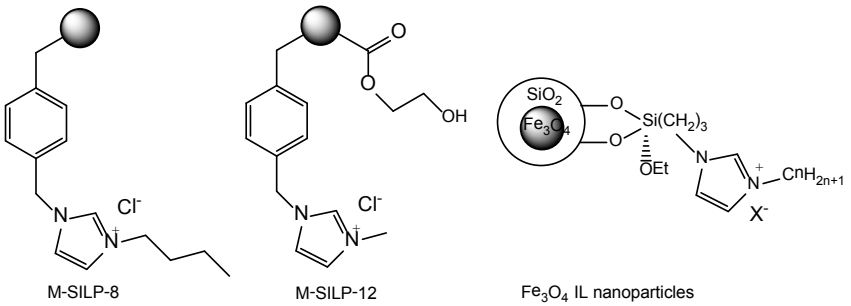


Figure 6. Supported ionic liquid enzymes where IL and enzyme are deposited on a support material.

On the other hand, CAL B SILEs were described already in 2005. ILs were used as a vector between ceramic supports and CAL B.[105] CAL B was supported to α-alumina macroporous tubular supports with [BMIM][PF_6], [BMIM][NTf_2] and [EMIM][NTf_2]. The activities of the SILEs were tested in the esterification of lauric acid with butyl acetate in hexane. The SILEs were active and stable. The activities were in the order no IL>[BMIM][NTf_2]>[EMIM][NTf_2]>[BMIM][PF_6]. CAL B was also immobilized on α-alumina macroporous tubular supports first by cross-linking CAL B with the support and then coating the active membranes with six different ILs.[109,110] The most suitable IL was [OMIM][PF_6]. The activities of SILEs were tested in the acylation of 1-butanol with vinyl propionate. Although the activity was lower when ILs were used (compared to absence of IL), the selectivity increased reaching >99 %.

In covalently supported ionic liquids phases (SILP), the SILP is first prepared and the enzyme is adsorbed. The technique has been applied to CAL B which was adsorbed on macroporous monolith-supported ionic liquid phase M-SILP-8 and M-SILP-12 (Scheme 7) with high IL/enzyme ratio to ensure full interaction between the protein and IL phases. The catalyst obtained was applied to the synthesis of citronellyl propionate from citronellol and vinyl propionate in $ScCO_2$.[111] The M-SILP-8-CAL B and M-SILP-12-CAL B were found to be very active and selective, the productivity of M-SILP-8-CAL B being 4.5 times higher (yield 93%) than that obtained for M-SILP-12-CAL B. The product hydrolysis was negligible. No enzyme leaching was observed.



Scheme 7. Covalently supported ionic liquids.[111,112]

Candida rugosa lipase (CrL) was adsorbed on magnetic nanoparticles supported ionic liquids (Fe_3O_4-IL) with cation length C_1, C_4 and C_8 and anions Cl^-, BF_4^- and PF_6^- (Scheme 7).[112] Magnetic nanoparticles supported ILs were obtained by covalent bonding of IL-silane on magnetic Fe_3O_4 nanoparticles before adsorbing the enzyme. The activities of the prepared SILP-CrL were analyzed in the esterification of oleic acid with 1-butanol without a solvent. All lipase preparations showed higher activity than the native enzyme (Table 9). Also the operational stability of $[C_1C(S)Im]Cl$-CrL was 92% after 5 cycles, whereas its native counterpart retained 35 % of the initial activity.

Table 9. Lipase activity with magnetic nanoparticles supported ionic liquids CrL.[112]

Catalyst	Activity [μmol min^{-1} g^{-1}]	[%]
Native CrL	106.5	100
$[C_1C(S)Im]Cl$-CrL	119.3	112
$[C_1C(S)Im]BF_4$-CrL	125.1	118
$[C_1C(S)Im]PF_6$-CrL	126.9	119
$[C_4C(S)Im]PF_6$-CrL	130.7	123
$[C_8C(S)Im]PF_6$-CrL	132.3	124

2.3.3. Entrapment

Enzymes can be entrapped in polymeric network that allows substrates and products to pass through but retains the enzyme. Entrapment matrix is generally formed during the immobilization process. Enzyme molecules can be physically embedded or covalently linked to the matrix. Several methods for entrapment have been developed, sol-gel method being the most prominent and widely used. Other methods for entrapment of enzymes comprise, for example, fiber entrapping, micro-encapsulation, double entrapment, covalent entrapment, adsorption entrapment, cross-linking entrapment, attachment entrapment and entrapment-coating.[73] Entrapment is a mild method and can be useful for enzymes which are easily deactivated e.g. in covalent immobilization. More detailed procedure of sol-gel immobilization of lipase PS is presented in the experimental section and the original paper I.

2.3.3.1. Sol-gel entrapment

Conventional sol-gel entrapment refers to a process where enzyme is mixed with sol-gel solution, followed by gelation process under the influence of pH and aging process. Sol-gel entrapment of enzymes is thoroughly reviewed.[72,113-116] In sol-gel method, an inert gel network is built by chemical condensation around each enzyme macromolecule. In nano-cages, the enzyme must have room enough to change its conformation required for catalytic cycles, and substrates and products must remain free to diffuse in and out. It is assumed that the enzyme is captured in the matrix in a "lid-opened" (active) conformation.[72,113] Lipases entrapped in sol-gel are usually more stable than native enzymes.[73]

Sol-gels are porous inorganic matrices that have controllable surface area. After aging, the pore sizes of the gel formed are usually in the range of 2-20 nm (mesoporous).[73] Materials concerned in sol-gel processes are mostly oxides: silica, alumina, aluminosilicates, titanium dioxide, zirconium dioxide.[113] Most favoured precursors are silicon alkoxides $Si(OR)_4$ or alkoxysilanes ($XSi(OR)_3$ or $XX´Si(OR)_2$ where X and X´are organic groups). In alkoxides, R is often methyl (tetramethoxysilane, TMOS) or ethyl (tetraethoxysilane, TEOS). TMOS and TEOS can liberate significant amount of alcohol during hydrolysis.[115] Since methanol is less harmful than ethanol, TMOS is often used instead of TEOS.[116] Previously the problem of liberating alcohols was solved by changing the precursor to sodium silicate and silicic acid or by using glycerated silanes.[115] However, these materials showed large degree of shrinkage. In this thesis, evaporation of alcohol before enzyme addition, the method used for hydroxynitrile lyase,[117] was used first time in immobilization of lipase PS. By adding trialkoxysilanes (e.g. methyltrimethoxysilane, MTMS), sol-gels with hydrophobic surface can be obtained. Hydrophobic surfaces can have positive influence on the reactivity of lipases, since they might induce interfacial activation, meaning that the lipase is in its active conformation. They also reduce the leaching of the enzyme (Fig. 7).

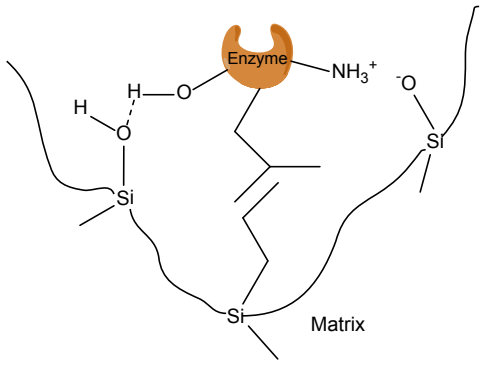
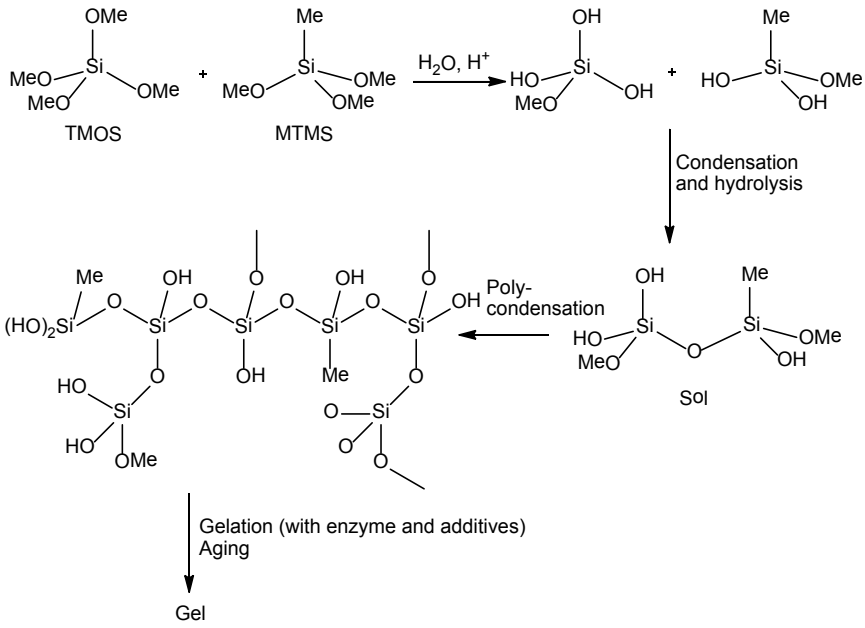


Figure 7. Non-covalent interactions between gel matrix and the enzyme.[114]

The synthesis of sol-gels includes 1) hydrolysis of precursors, 2) condensation, 3) gelation and aging and 4) drying (Scheme 8).[72,73,75] The enzyme and possible additives can be added prior or after hydrolysis and condensation. As the hydrolysis and condensation of

silicon alkoxides are slow, they need to be catalyzed either by acids (H^+), bases (O^-) or strong Lewis bases, such as F^- ions.[113] This sol mixture consists of partially hydrolyzed and partially condensed monomers. The change in pH along with the presence of a catalyst and additives promotes a large scale polymerization reaction over the period of minutes to hours, resulting in gelation of the sol and thus, entrapment of enzyme.[115] Aging promotes further condensation and strengthens the network. After aging, the gels are dried with an appropriate method (Table 10, Fig. 8) Most gels studied for encapsulation have been dried by evaporation of solvents to form xerogels (Fig. 8b).[113] Ambigels (Fig. 8c) are obtained when the proportion of alkoxysilane in a precursor mixture is sufficiently high, and the proportion of hydrophobic groups in the pore surface will be sufficient to give hydrophobic character to gels. Dry ambigels have the same size as the wet gels. The stability of the sol-gel entrapped enzyme was found to be highly dependent on enzyme concentration, pH, ionic strength and other immobilization conditions.[73]



Scheme 8. Synthesis of sol-gels.[75,115]

Table 10. Drying methods and gel types of sol-gels (Fig. 8.).[113]

Gel type	Drying method	Gel size
Aquagel (a)	Pores of the gel filled with water and alcohol	Actual size
Xerogel (b)	Evaporation	Shrinking of the gel due capillary forces
Ambigel (c)	Hydrophobization+evaporation	Same size than wet gels
Aerogel (d)	Supercritical CO_2 drying with or without acetone dialysis	Size and structure of gel remains

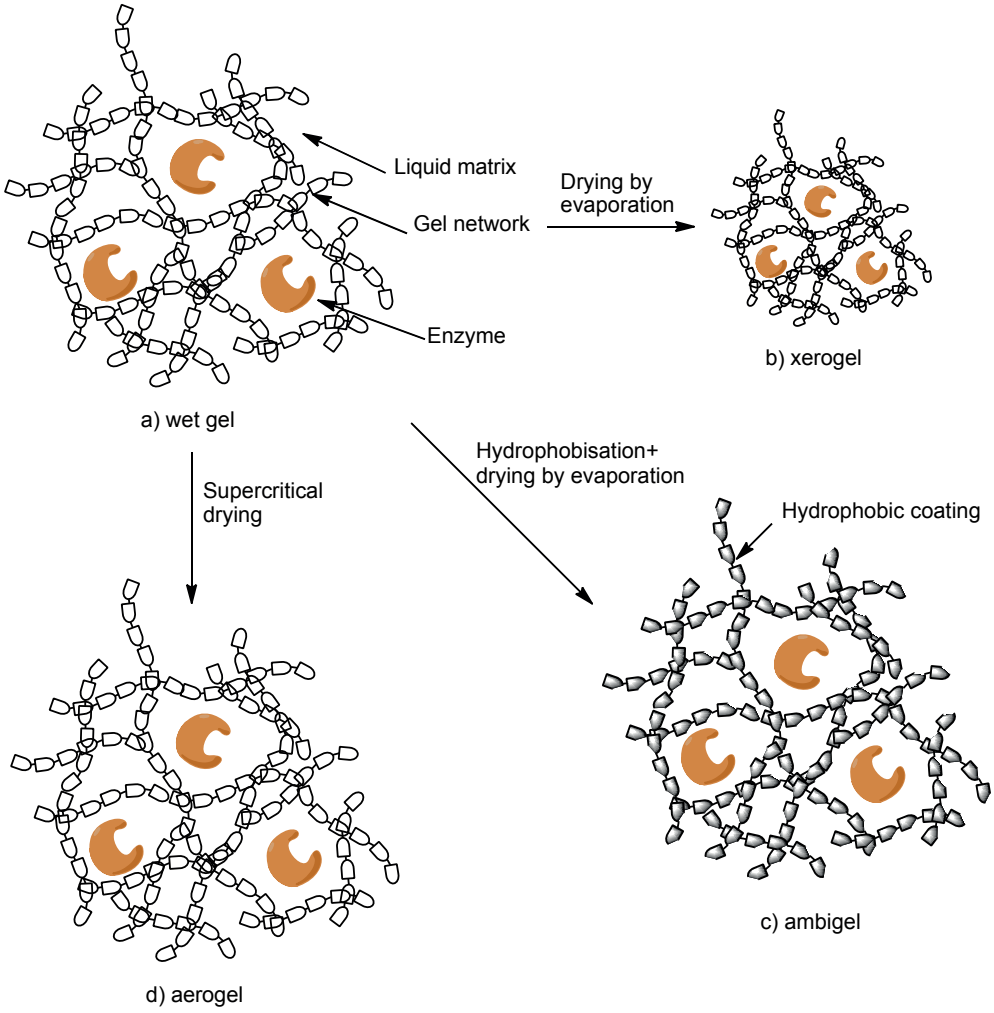


Figure 8. Drying of sol-gels.[113]

Reetz *et al*. showed that immobilization of lipase PS in TMOS gels has relative activities only less than 5%, whereas gels containing more hydrophobic silanes $RSi(OCH_3)_3$ (ambigels) has significantly higher activities.[118] The relative activity increased from 30% with 50% MTMS up to 1300% for pure MTMS. The relative activity increased with increasing chain length (CH_3<C_2H_5<C_3H_7<C_4H_9) and hydrophobicity. In all cases excellent activity was obtained, and up to 93% of the protein was immobilized. The best results were obtained with TMOS/PTMS (1/5), the activity being 14 times higher than the activity of free lipase PS.[118,119] The use of additives, such as PVA, albumin or gelatine, significantly enhanced activity.[119] The sol-gels were successfully reused, 30 runs resulted only 12-15% decrease in activity.[119]

Lipase PS sol-gels have also been prepared by hydrolysis of TMOS with MTMS, isobutyltrimethoxysilane (i-BTMS) and *n*-butyltrimethoxysilane.[120] TMOS/i-BTMS

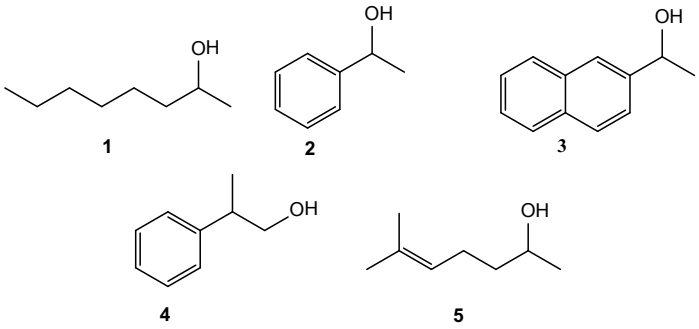
showed highest activity, with formation of 61.2% in the hydrolysis of soybean oil to FFA. The thermostability was improved, lipase PS sol-gel being stable up to 70 °C. The sol-gels retained more than 95% activity after 12 reactions. Pierre *et al*. describe the encapsulation of lipase PS in silica aerogels obtained by supercritical CO_2 drying.[121,122,123] These aerogels perform better than xerogels, since diffusion of substrates and products through the silica matrix is much easier.[121] No strong interactions between the enzyme and the gel network could be observed in aerogels, contrary to xerogels. Enzymes remained free to adjust their conformation to perform good catalytic activity. In xerogels, drying can at least partially crush the enzyme. Recently, *Rhizopus oryzae* lipase was immobilized using sol-gel coating using dimethyldimethoxysilane and TMOS in supercritical CO_2.[124] The sc-CO_2-sol-gels exhibited 5-7 times higher esterification activity in esterification of (*R*)-glycidol with *n*-butyrate in isooctane than sol-gels made in aqueous sol-gel route.

These first generation sol-gels has been markedly improved by higher enzyme loading, variation in alkyl group in silane precursor and the use of appropriate additives in the second generation sol-gels. *n*-Butyl- and isobutylsilanes (BTMS or iBTMS, respectively) were used with TMOS (5/1), and isopropyl alcohol and PVA (polyvinyl alcohol) was included in each gel due to their beneficial effects in preliminary studies.[125] Crown ethers, β-cyclodextrin derivatives, salts and surfactant Tween 80 were used as additives (Table 11). These second generation sol-gels proved to be highly active, robust and recyclable. The effects of additives on activity and selectivity of lipases is discussed below.

Additives encapsulated together with enzymes can be beneficial for the enzyme performance. Usually additives have following functions: mitigation of diffusion constraints, increase of the porosity, effect on enzyme activity, stability and selectivity, and modulated enzyme conformation.[73] Lipases need the presence of an interface between hydrophobic and hydrophilic medium which can be tailored by introducing hydrophobic sites in otherwise hydrophilic silica gel, or adding a partially stable organic material, such as PVA.[121] Additives such as triethylamine, crown ethers, surfactants e.g. Triton X-100, salt hydrates e.g. LiCl and KCl, carbamates, isopropanol, polyvinyl alcohol, cyclodextrins or surfactants enhance efficiency of lipase catalyzed reactions and immobilization. [72,126] The presence of crown ether or surfactant can alter not only the structure of sol-gel matrix but also the conformational flexibility of the enzyme.[73] The effects of additives are individual, and the effects depend also on the sol-gels precursors used, the enzyme and substrates (Table 11).

Second generation BTMS/TMOS and i-BTMS/TMOS sol-gels containing IPA+PVA were prepared with several lipases using Celite, 18-crown-6, Tween 80, methyl-β-cyclodextrin or KCl as additives (Table 11, entries 1-6).[125] These sol-gels were employed in the acylation of **1-5** (Scheme 9). The obtained sol-gels were highly active. The additives had individual effects on activity depending on enzyme and/or silane used. Lipase PS sol-gels had 2 to 6 fold activities compared to those without additives, depending on additive and/or silane precursor. PVA as an additive introduces hydrophobic sites to sol-gels. PVA increased the activity of MTMS and TMOS gels of lipase PS (Table 11, entry

13).[122,123] The most active preparation was hydrophobic aerogel made from MTMS and TMOS with PVA.



Scheme 9. Substrates used to test the sol-gels made with Celite, 18-crown-6, Tween 80, methyl-β-cyclodextrin or KCl

Nanostructured supramagnetic magnetite (Fe_3O_4) was entrapped simultaneously with lipase PS or CAL B in sol-gels with gelatine or PVA as an additive (Table 11, entries 7,8).[127] The catalyst was highly active and easily separated from the reaction mixture by using a magnet. Lipase PS co-lyophilized with peracetylated β-cyclodextrin was entrapped in MTMS sol-gels and employed in transesterification of 13 secondary alcohols with isopropenyl acetate in toluene (Table 11. entry 9).[128] The enhancement of reaction rate and enantioselectivity was observed, and it is suggested to be based on the beneficial effect of the additive on enzyme.

ILs in sol-gel can act as a template during gelation and behave as a stabilizer to protect the enzyme from inactivation by released alcohol or heat.[45] ILs have been used as additives in sol-gels of *Candida rugosa* lipase [108,129] and *Pseudomonas fluorescens* lipase [130] (Table 11, entries 10-12). The activities and stabilities were higher in each case.

Table 11. Additives used in sol-gels.

Entry	Lipase	Additive	Effect	Ref.
1	PfL, CrL, TlL, PpL, PrL, lipase PS	PVA+IPA+Celite	Increase in activity in acylation of **1**	125
2	CAL B, MmL, AnL, CrL L-3	PVA+IPA+Celite	Decrease in activity in acylation of **1**	125
3	TlL, PrL	PVA+IPA+18-crown-6	Decrease in activity in acylation of **1**	125
4	CAL B, PfL, lipase PS	PVA+IPA+18-crown-6	Increase in activity in acylation of **1-5**	125
5	CAL B	PVA+IPA+KCl	decrease in activity with BTMS, increase with i-BTMS	125
6	lipase PS	PVA+IPA+Tween 80	Increase in activity **1-5**	125
7	lipase PS	gelatin + Fe_3O_4, PVA+ Fe_3O_4	Higher activities, highly reusable	127
8	CAL B	PVA + Fe_3O_4	Higher activities, high ees	127
9	lipase PS	peracetylated β-cyclodextrin	Enhancement of reaction rate and E in kinetic resolution of 13 sec alcohols	128
10	CrL	[EMIM][BF_4], [OMIM][BF_4], [BMIM][PF_6], [OMIM][PF_6], [EMIM][NTf_2], [OMIM][NTf_2]	Hydrolytic activity 5-fold, esterification activity 16-fold greater than without IL	108
11	CrL	[EMIM][BF_4], [EMIM][PF_6], [EMIM][NTf_2], [OMIM][PF_6], [C_{16}MIM][BF_4], [C_{16}MIM][Cl], [C_{16}MIM][NTf_2]	Hydrolysis activity at 1:1 [EMIM][BF_4]:[C_{16}MIM][NTf_2] 10-fold and esterification activity 14-fold higher	129
12	PfL	[EMIM][BF_4], [PMIM][BF_4], [BMIM][BF_4], [HMIM][BF_4], [OMIM][BF_4], [EMIM][$COOCH_3$], [EMIM][$COOCF_3$]	Higher activities, similar E with OTMOS:TMOS 1:1 gels	130
13	lipase PS	PVA	Increase in activity when mixture of MTMS:TMOS, no affect in TMOS gel	121, 123

2.3.4. Cross-linking of enzymes

Cross-linked enzymes are formed in practice 100% of protein, and they are carrier-free. They can be made by cross-linking dissolved enzyme (CLEs), crystalline enzyme (CLEC), spray-dried enzyme (CSDEs) or physically aggregated enzyme (CLEAs). Although CLEs and CSDEs were not usable catalysts, CLECs and CLEAs have shown to be highly active and stable, being also commercially available.[74] In this thesis, CLEAs of lipase PS were prepared.

2.3.4.1. CLECs

CLECs are solid cross-linked enzyme crystals that are insoluble in both water and organic solvents.[131,132] In 1992 St. Clair and Navia demonstrated that CLEC of thermolysin retain catalytic activity even under harsh conditions.[133] Subsequently several CLECs of lipases, proteases and acylases have been commercialized as biocatalysts for asymmetric syntheses.[132] The advantages and disadvantages of cross-linking method of enzymes are presented in Table 6. CLECs have proven to be robust, highly active and stable catalysts with controllable particle size.[131,132]

CLEC method has two steps: 1) batch crystallization of an enzyme and 2) chemical cross-linking of the crystals. Important parameters for activity and stability of CLECs are crystallization conditions, size of crystals, size of substrates, conformation of enzymes and optimization of cross-linking to prevent excessive cross-linking.[131,132] Cross-linking is performed with bifunctional agent, glutaraldehyde being most popular.[131] CLECs have collaborate particle size (1-100 μm).[77] Minimizing crystal size is crucial for retention of high activity.[74] The size of particles depends on crystallization conditions. Many lipases, like lipase PS, can be crystallized with lid-open (active) conformation.[15] When CLEC of *Candida rugosa* lipase was prepared with open and closed forms, the activity of the open form was three times higher than that of the closed form.[132] Also enantioselectivity was higher. CLECs of lipase PS were considerably more active than crude enzyme powder in the acylation of 1-phenylethanol, sulcatol and 2-octanol.[134]

The limitation in using CLECs is the need of crystalline enzyme, since optimization of crystallization conditions is time-consuming and expensive procedure requiring an enzyme of high purity.

2.3.4.2. CLEAs

In year 2000, Sheldon´s group suggested the replacement of crystallization by precipitation of the enzyme from an aqueous buffer. This simpler and less expensive method led to the development of cross-linked enzyme aggregates, CLEAs.[74,79,135,136] The CLEA method is an extreme case of covalent binding, where enzyme acts as its own carrier, and virtually pure carrier-free enzyme is obtained eliminating the advantages and disadvantages connected to the use of a carrier. The advantages and disadvantages of the CLEA method are presented in Table 6. In this thesis, the CLEA method was used to prepare cross-linked enzyme aggregates of lipase PS.

CLEA methods contains 1) precipitation of enzyme from an aqueous buffer, 2) addition of selected additive, and 3) intermolecular cross-linking of the precipitated enzyme to insoluble high-molecular aggregates (Fig. 9).[74,79,136] Co-aggregation and cross-linking of two or more enzymes affords combi-CLEAs that are ideally suitable for catalyzing one-pot enzymatic cascade processes.[79] Cross-linking occurs via reaction of the free amino groups (mostly lysine residues) on the enzyme surface (Fig. 5), on the surface

of neighbouring enzyme molecules, with oligomers or polymers resulting from aldol condenzation of glutaraldehyde or other cross-linking agents.[79]

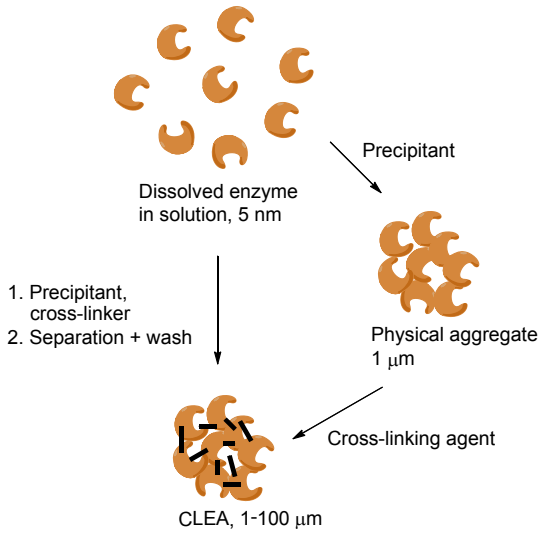


Figure 9. Preparation of CLEA.[77,137]

The CLEA method includes the choice of a suitable precipitant and a cross-linking agent. Choice of the best applicable precipitant is usually empirical and depends on the nature of enzyme.[138] Common precipitants used to aggregate the enzyme are salts e.g. saturated ammonium sulphate ($(NH_4)_2SO_4$), or water miscible organic solvents like alcohols, acetone, and 1,2-dimethoxyethane (DME).[136] Also PEG200 and PEG600 gave high activities with lipases.[139] Quenching the enzyme solution with high precipitant concentrations (even 90 vol-%) gave much better results than slow addition of the precipitant used in crystallization, since quenching results shockwise aggregation during which there is little chance for the protein denaturation.[136] Precipitation temperature (RT vs. 4°C) had little effect on activity recovery. Maximal activity of cross-linked enzyme was recovered at the point where the majority of the protein is precipitated out of the solution.[74] Cross-linker can be added after or prior to the precipitation step. Glutaraldehyde is a versatile cross-linker, although some enzymes can be inactivated by glutaraldehyde due to its reactivity and small size that allows it to penetrate the interior of the protein.[77,136] Bulky polyaldehydes e.g. dextran polyaldehyde or galactose dialdehyde, can be used as cross-linkers instead of glutaraldehyde in the case of enzyme deactivation.[77] Since glutaraldehyde may be harmful, optimization of cross-linking step often means minimizing the amount of cross-linker. Time needed for complete cross-linking depends on temperature.[136]

Since enzymes differ as to the number of lysine residues, they behave differently under cross-linking conditions. Optimization of the CLEA method includes the optimization of temperature, pH, concentration, stirring rate, precipitant, additives and cross-linking agent. If cross-linker concentration is too low, sufficient cross-linking does not occur,

and if the cross-linker concentration is too high, too much cross-linking occurs resulting complete loss of enzyme´s flexibility necessary for activity.[79]

Important property of CLEAs is the particle size (varying from 1 to 100 μm, mostly 1-50 μm, Fig. 9) which obviously has direct effect on mass transfer limitations and filterability.[77,79,138] CLEAs can form larger clusters which have mass-transport limitations, especially in fast reactions. Washing and redispersing easily increase clotting. Recovering the CLEA by centrifugation easily leads to increased cluster formation since it squeezes the CLEA particles close together. When clusters are put to an aqueous organic solvent they break up. Another recovery is filtration but CLEAs easily pass through even very fine filters.[136]

First lipase CLEAs were prepared 2002 by the Sheldon group using several lipases (Table 12).[137] Activities exceeding that of native enzyme were observed. This hyperactivation is thought to have its origin in conformational changes of the protein induced by aggregated state.

Table 12. Activity of CLEAs in the hydrolysis of ethyl octanoate in aqueous 1,2-dimethoxyethane.[137]

Precipitant	Additive[a]	Lipase, activity[b]				
		CAL A	CAL B	TlL	RmL	CrL
Ammonium sulfate	None	15	4400	18	21	0.4
	SDS	21	4200	87	35	1.5
	Triton	12	9400	22	22	2.3
1,2-dimethoxyethane	None	6	10300	186	20	0.3
	CR	9	8500	154	20	0.6
Acetone	None	6.7	8500	80	21	0
Native enzyme		2.3	14000	247	14	0.5

[a] SDS=sodium dodecyl sulfate, Triton=Triton X-100, CR=dibenzo-18-crown-6
[b] Activity in nmol of octanoic acid per h per μl in enzyme suspension.

BSA has been used as a proteic feeder to form CLEA from lipase PS using acetone as precipitant and glutaraldehyde as a cross-linker.[140] The excess cross-linker was noticed to lead to enzyme inactivation, and the highest activity without BSA was only 0.4% of activity of free enzyme. When BSA was used as proteic feeder, the hydrolytic activity was 100%. There was an optimum range for BSA concentration, below which there are not enough free amino groups to prevent excessive cross-linking. Beyond this optimum range, the free amino groups of BSA compete with free amino groups of lipase PS and prevent the necessary cross-linking. The use of BSA as proteic feeder has been noticed to be beneficial when the initiate protein concentration is low or the enzyme is vulnerable to loss on activity due to extensive cross-linking.[140] The lipase PS CLEA preparation, made with BSA, had also excellent activity and selectivity in [BMIM][PF_6] [141], where the activity of lipase PS had been poor [62]. The concentration of glutaraldehyde had significant effect on particle size and activity of lipase PS CLEA (Table 13).[142] Use of excess of glutaraldehyde made larger particles which led to lower activities.

Table 13. The particle size and V_{max} of lipase PS CLEA.[142]

Glutaraldehyde concentration [mM]	Particle diameter [μm]	V_{max} [mmol $mg^{-1}h^{-1}$]
10	<5	6.51
40	5-10	4.02
60	Large clusters	2.45

Additives have been used to improve the activity and enantioselectivity of immobilized enzymes (See also chapter 2.3.3.1.). In making CLEAs, co-precipitation of additives, such as surfactants or crown ethers, with enzyme followed by cross-linking can lock the enzyme in more favourable conformation.[77,137] Since an additive is not covalently bound to the enzyme, it can be washed from CLEA using, for instance, an appropriate organic solvent to leave the immobilized enzyme locked in the favourable conformation. Slight improvement in activity was obtained with lipases when using SDS, Triton X-100 or dibenzo-18-crown-6 as additives (Table 12).[137] Also the use of SDS and non-ionic surfactant Tween-80 gave higher activities when preparing CLEA from *Thermomyces lanuginosus* lipase.[143]

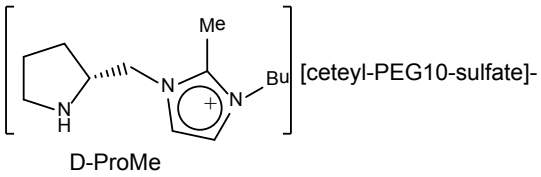
2.3.5. Coating

Ionic liquids with melting points ranging from 50 to 100 °C are used to coat enzymes to combine the benefits of ILs and immobilization. In the procedure, ILs are first heated and melted, and the enzyme is aggregated and dispersed gently through the melted liquid. The obtained mixture is then cooled and cut into small pieces. The reactions catalyzed by IL coated enzymes have markedly higher enantioselectivities than those with free enzymes. Moreover, the activity and stability of enzymes are enhanced.

IL coated enzymes (ILCE) were first reported by Lee and Kim in 2002.[144] Lipase PS was coated with [PPMIM][PF_6] (m.p. 53 °C, PPMIM=1-(3´-phenylpropyl)-3-methylimidazolium), and the ionic liquid coated enzyme obtained was used in transesterification of secondary alcohols with vinyl acetate in toluene. ILCE was more selective than the free enzyme and in the first run the activity was 65% of the activity of the native enzyme. However, the catalytic activity of ILCE increased up to the level of the native enzyme in the second run. After the fifth run, ILCE retained 93% of the activity of the native enzyme. The reduced activity of fresh ILCE was suggested to be due to diffusional limitations.

In another procedure, lipase PS was coated with [BDMIM][ceteyl-PEG10-sulfate] by mixing lipase PS in phosphate buffer with the IL and lyophilizing the mixture.[145,146] The enantioselective acylation of 1-phenylethanol with vinyl acetate in DIPE in the presence of the ILCE obtained showed enhanced enantioselectivity (*E*=96) and activity (c=49%, 2 h) compared to the native enzyme (*E*=17, c=15%, 26 h). Remarkable acceleration was obtained also in enzymatic acylation of other secondary alcohols, the enantioselectivities being excellent. The activation depends on a coating process; the best result was obtained

when ILCE was prepared by using 100 equivalents of IL versus protein. Lyophilization of the enzyme in the presence of IL was essential to activate the lipase. Cationic part of the coating material had significant effect on the enantioselectivity of ILCE. It was suggested that the cationic part might bind with lipase protein.[146] MALDI-TOF mass spectrometric analysis indicated that IL binds to the enzymes and may have impact on lipase flexibility or conformation. When lipase PS was coated with ionic liquid D-ProMe (Scheme 10), the acceleration was even higher compared to coating with [BDMIM] [ceteyl-PEG10-sulfate].[147]



Scheme 10. D-ProMe for PS coating.[147]

Pretreating of a lipase with ILs and coating with polyethyleneimine have also been efficient methods to increase the enantioselectivity of lipases. Lipase from *Mucor javanicus* was suspended in ILs and assayed in the hydrolysis of *p*-nitrophenyl butyrate.[148] The activities of the IL-pretreated lipase were much higher than those of the free lipase. When treating the lipase with [BMIM][PF_6], [EMIM][NTf_2], [BMIM][BF_4] and [EMIM] [BF_4] the activities were 1.8, 1.7, 1.6 and 1.6 times higher than those of untreated lipase, respectively. Polyethyleneimine (PEI) is a polymer with high density of ionized tertiary, secondary and primary amino groups, and this permits strong ionic exchange of polymer on any area of protein surface containing anionic groups. Thus, it has been used to stabilize proteins. Coating of CAL B and CrL immobilized covalently on CNBr-agarose with PEI improved the enantioselectivity of the hydrolysis of 2-hydroxyphenylacetic acid methyl ester.[149] The enantioselectivity of coated CAL B increased from E=1.5 to E>100, and the enantioselectivity of coated CrL from E=8 to E=21 at pH 5.

3. AIM OF THE STUDY

New methods for immobilization of enzymes are needed for developing more efficient enzyme catalysis. The aim of this study was to develop immobilization methods for lipase PS used in the synthesis of enantiopure intermediates and to study the effects of immobilization on activity, selectivity and possible side reactions in enzymatic acylation. The results are compared to those obtained by using lipase PS on celite (P. Hara, unpublished results). Water content in support materials used in immobilization varies notably and must be taken into account to minimize hydrolytic side reactions in transesterification reactions. The use of enzyme catalysis presumes that the ester product is not hydrolyzed due to side reaction. Understanding and elimination of side reactions offer chiral compounds with increased enantiopurity and yield. Thus, side reactions are studied in this work. The main targets in this thesis were

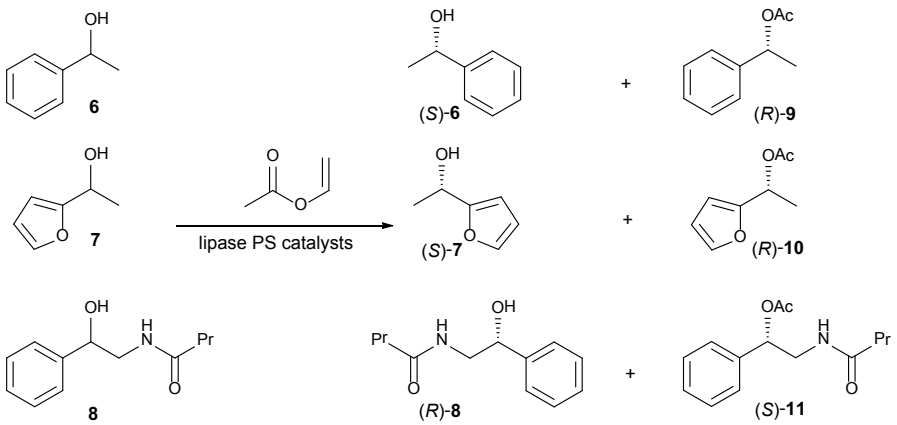
1. To immobilize lipase PS from *Burkholderia cepacia* as sol-gels (xerogels), CLEAs and support/IL/lipase PS (SILE) catalysts (Papers I-III).

2. To study the catalytic properties of the immobilized lipase PS preparations using transesterification between **6-8** and vinyl acetate in organic solvents and in ionic liquids as the model reactions (papers I-III)

3. To study the effect of immobilization on hydrolysis as a side reaction in the acylation reaction (Papers I and II).

4. To develop analytical methods needed for developing immobilization methods, following the acylation reactions and activity of the enzyme preparations.

The acylation of alcohols **6-8** with vinyl acetate (Scheme 11) was chosen as a model reaction to study the activity and selectivity of the immobilized lipase PS preparations. The secondary alcohols used as substrates are common structures in drugs and fine chemicals.



Scheme 11. Kinetic resolution of **6-8** catalyzed by lipase PS preparations.

4. MATERIALS AND METHODS

4.1. Materials

Substrates **6** and **7** were commercially available from Aldrich and Fluka, respectively. Amide **8** was prepared by the reaction of 2-amino-1-phenylethanol with butanoic anhydride (0.9 eqv.).

Ionic liquids [EMIM][NTf_2], [EMIM][BF_4] and [BMIM][PF_6] were prepared following the methods in literature[62,98,150], methyl trioctylammonium trifluoroacetate ([MTOA][TFA]), 1-butyl-4-methylpyridinium tetrafluoroborate ([4MBPy][BF_4]) and 1-butyl-3-methylimidazolium trifluoromethanesulfonate [BMIM][TfO] were obtained from Merck KGaA and used as received.

Silane precursor methyltrimethoxysilane (MTMS) was from Aldrich and tetramethoxysilane (TMOS) from Fluka. The supports applied in this study were active carbon (AC), activated carbon cloth (ACC 507-15, 1500 m^2/g) and activated carbon paper (STV 505, 700 m^2/g) from Nippon Kynol, Japan, and alumina (Versal VGL-25, 63-100 μm).

4.2. Enzymes

Burkholderia cepacia lipase (lipase PS) was studied in immobilization experiments, and the enzyme preparations were applied to the kinetic resolution of **6-8** (Scheme 11). Lipase PS was purchased from Amano Enzyme Inc. (Nagoya, Japan). Two forms of free lipase PS powder were used: Lipase PS "Amano", diluted with diatomaceous earth containing 10% protein according to bicinchoninic acid assay, was used in original paper III. Lipase PS "Amano" SD, diluted with dextrin, containing 3 % protein (bicinchoninic acid assay), was used in original papers I and II as Amano changed the production of the lipase PS. Lipase PS was also adsorbed on celite (20 w/w%) in the presence of sucrose.[6] Both Lipase PS "Amano" and lipase PS "Amano" SD gave similar activity when adsorbed on Celite.

4.3. Analytical methods

Enzymatic acylations were followed by chiral GC chromatography by the separation of the enantiomers and quantification of the enantiomeric excesses. GC analysis was performed with Agilent 6890 Series II Chromatograph equipped with flame ionization detector (FID) and automatic injection. The acylation of **6** and **7** was analyzed with Varian Chrompack CP-Chirasil-DEX CB column (25 m x 0.25 mm) and the acylation of **8** with Varian Chrompack CP-Chirasil-L-valine column (25 m x 0.25 mm). The samples

from the resolution reactions were derivatized with propionic anhydride in the presence of 4,4-dimethylaminopyridine (DMAP) to achieve good baseline separation for the enantiomers of unreacted **6-8** and product enantiomers **9-11** (Scheme 11).

As an example of the chromatographic analysis, the chromatograms of *rac*-**6** derivatized with propionic anhydride (a), *rac*-**6**-acetate (b) and the reaction sample from the kinetic resolution of *rac*-**6** with vinyl acetate in the presence of lipase PS (c) is presented in Fig. 10.

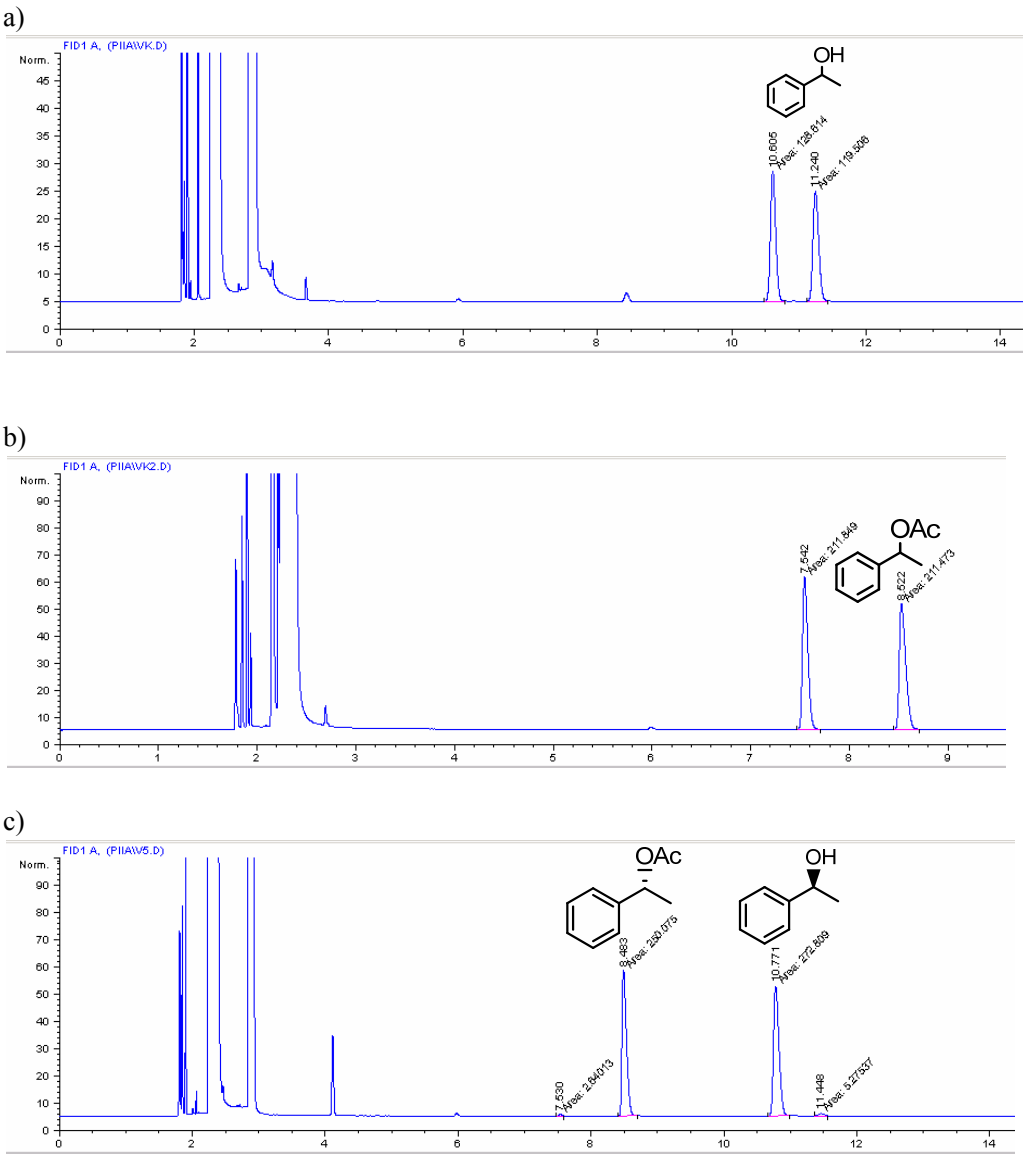


Figure 10. GC chromatograms of a) *rac*-**6** derivatized as the propionate, b) *rac*-**6** derivatized as the acetate and c) sample taken from the kinetic resolution of *rac*-**6** at 50 % conversion. t_r/min.

In the development of CLEAs, the efficiency of the precipitation and cross-linking steps were monitored by following activity yield in the hydrolysis of *p*-nitrophenyl acetate (0.1 M, at pH 4.5) by UV-spectroscopy at λ= 400 nm at 25 °C. The UV measurements were carried out on Perkin Elmer Lambda 650 UV/VIS spectrophotometer. The activity of the enzyme after precipitation and/or the cross-linking step was obtained as enzyme activity (As^{-1}, A=absorbance) and the activity is expressed as v_0 ($\mu M min^{-1}$) calculated based on the calibration curve A vs. concentration. The protein content of lipase PS powder was determined with UV-spectroscopy using bicinchoninic acid assay and bovine serum albumin as the standard protein at λ=562 nm, 25 °C.[151,152]

4.4. Mathematical equations

For the reaction where no side reactions occur, c is conversion of the reaction at a certain time, and enantiomeric ratio *E* the enantioselectivity of an irreversible enzymatic kinetic resolution reaction. Enantiomeric excess (ee) is the absolute difference between the mole fractions of each enantiomer. Enantiomeric excess of the substrate and the product (ee_S and ee_P, respectively) were obtained from the GC chromatograms of the reaction samples (Chapter 4.3., Fig. 10). The correlation between c, *E*, ee_S and ee_P is shown by eqs. (1) and (2). [18,19,158]

(1) $c=ee_S/(ee_S+ee_P)$

(2) $E=\ln[(1-c)(1-ee_S)]/\ln[(1-c)(1+ee_S)]$

In the present work the determination of *E* has been based on equation (2) using linear regression (*E* as the slope of the line $\ln[(1-c)(1-ee_S)]$ *versus* $\ln[(1-c)(1+ee_S)]$). When *E* values are 200 or higher, even minor variation in ee caused by experimental errors leads to significant changes in the value of *E* and, therefore, *E*>200 is given rather than more exact values.

The catalytic efficiency of the enzymes is measured by determining specific activity. Specific activity of the reaction corresponds to the rate at the beginning of the reaction. The specific activity is calculated over the first smallest possible time interval, and is linear over a short period after the start of the reaction. To measure the specific activity, enzyme assays are typically carried out while the reaction has progressed only a few percent towards total completion. In this study specific activity (μmol $min^{-1}g^{-}1$) was determined using linear regression c·Vconversion vs. t·m(enzyme), where m(enzyme) is the mass of the enzyme preparation in conversions less than 10%.

4.5. Preparation of the lipase PS sol-gels, CLEAs and SILEs

Lipase PS sol-gels, CLEAs and SILEs were prepared by straightforward methods without any special equipment and additives. The procedure of immobilization is described in detail in the Experimental parts of the original papers I-III. Despite high

amount of stabilizers in crude lipase PS preparations from Amano (10 % protein in lipase PS "Amano" and 3 % protein in lipase PS "Amano" SD), the crude enzymes were used without further purification. Lipase PS "Amano" solutions in buffer were centrifuged prior to use to remove insoluble material (Paper III).

4.5.1. Lipase PS sol-gels (Paper I and II)

Lipase PS sol-gels were prepared without any additives by following the low-methanol method described in literature [117]. The sol precursor was prepared by using the mixture of methyltrimethoxysilane (MTMS) and tetramethoxysilane (TMOS) (1:4), and the condensation reaction was catalyzed by acidic water (HCl, pH 2.85). Since methanol is known to be harmful for enzymes, it was removed from the sol by evaporation prior adding the enzyme. Lipase PS powder was dissolved in phosphate buffer at pH 7 as the optimum pH of the enzyme is between 7 and 8. After addition of the sol, gelation took place immediately and the obtained sol-gel was aged to finish the condensation and dried. Drying in the air either at room temperature or at 40 °C was not efficient enough for acylation reactions in dry organic solvents. Therefore, the gel was lyophilized to xerogel. The optimum lyophilization time was 5 h after which the activity of the sol-gels decreased.

4.5.2. Lipase PS CLEAs (Paper I)

Lipase PS CLEAs were prepared according to the literature method known for lipases.[135] Since the CLEA method combines the purification and immobilization, lipase PS "Amano" SD was used without further purification in spite of the large amount of dextrin stabilizer (3% protein). Common precipitants, saturated ammonium sulphate, 1,2-dimethoxyethane, acetone and ethanol, were tested to reach the optimal precipitation degree and activity. Dextrin stabilizer caused problems in precipitation when using organic solvents as precipitant, since it formed sticky substance together with the enzyme. As a result, the activity after precipitation and cross-linking was poor. On the contrary, saturated ammonium sulphate gave 100% precipitation and increased activity also after cross-linking. Also dextrin stayed soluble and caused no problems during the procedure. Glutaraldehyde is a common reagent to cross-link proteins via free amino groups in the protein. Optimization of the amount of glutaraldehyde is essential to avoid leaching or excess cross-linking leading to loss of flexibility of the enzyme. Glutaraldehyde amount (100 mM) and cross-linking time (5 h) were optimized to reach the highest activity.

4.5.3. Lipase PS SILEs (Paper III)

Lipase PS SILEs were prepared by modifying the methods in literature.[105,107] In the method used, IL is used as a vector between lipase PS and support material. SILEs were prepared by using lipase PS "Amano", containing 10% PS in celite as a crude powder. Celite stabilizer was centrifuged from the enzyme -buffer mixture (phosphate buffer, pH 7.8) prior to immobilization. pH was in lipase PS optimum range 7-8. Ionic

liquids used ([EMIM][BF_4] and [EMIM][NTf_2]) were chosen as they were known to be suitable for lipase PS.[98] Also few other ILs were tested with alumina as a support, but the reactivities in the acylation of **6** with vinyl acetate in toluene were negligible. Support materials used in the study were active carbon, alumina Versal VGL-25 63-100 μm, Kynol™ ACC 507-15 active carbon cloth, 1500 m^2/g (measured specific surface area 1680 m^2/g, pore volume 0.60 cm^3/g) and Kynol™ STV 505 active carbon paper, 700 m^2/g (measured specific surface area 1223 m^2/g, pore volume 0.43 cm^3/g). Supports were pre-dried and wetted with a solution containing lipase PS in phosphate buffer and dilutant. The SILEs obtained were dried in a rotary evaporator to have seemingly dry enzyme preparations. Dilutant was used to dissolve IL with enzyme mixture. Also the enzyme preparation without any ionic liquid was prepared from ACC 507-15 with the same method to analyze the effects of ILs.

4.6. Enzymatic acylation

Immobilized enzymes were tested in the enzymatic acylation of racemic alcohols **6-8** with vinyl acetate (Scheme 11) to specify the activity and stability of each enzyme preparation. From each acylation reaction, specific activity as μmol $min^{-1}g^{-1}$ was determined. The reactions were further followed 24-48 h to reach 50% or higher conversions when possible.

For enzymatic acylation in the presence of lipase PS sol-gels and CLEAs, as well as AC and alumina powders, a typical reaction was performed by mixing an organic solvent and vinyl acetate (0.2 M) with a lipase preparation. The racemic alcohol (0.1 M) was added to start the reaction. The reactions were shaken at room temperature or at 48 °C. The progress of the reactions was followed by taking samples (50 μL) at intervals and analyzing the ees and conversions by GC. The samples were derivatized with propionic anhydride in the presence of DMAP (1% in pyridine) to achieve a good baseline separation.

For enzymatic acylation in the presence of SILEs, vinyl acetate (0.2 M) was dissolved in an organic solvent and the mixture was stirred (300 rpm) with tailor-made stirrer shaft where the matt-structured SILE cloth was attached as suitable, rectangular pieces. The addition of the substrate (0.1 M) initiated the reaction. Before addition of the substrate, the system was preheated to the desired reaction temperature 25 – 60 °C. The reuse of sol-gels and CLEAs was studied using the enzymatic acylation of **7** and the reuse of SILEs using the enzymatic acylation of **6** as the model reactions. More detailed procedures are described in the experimental parts of the original papers I-III.

5. RESULTS AND DISCUSSION

This section summarizes the results obtained experimentally. More detailed discussion is presented in the original papers I-III. As stated before, this study aims at immobilization of lipase PS as sol-gels, CLEAs and SILEs, and at evaluation of their activity and stability using the enzymatic kinetic resolution of secondary alcohols **6-8** in organic solvents and in ionic liquids as model reactions (Scheme 11). The results were compared to those obtained with lipase PS adsorbed on celite (lipase PS-celite). For immobilization of lipase PS, widely used adsorption on celite, sol-gel and CLEA methods as well as modern SILE method were chosen. The activity of immobilized enzymes, kinetic resolution of **6-8**, the effect of solvents and immobilization method, possibilities to catalyst reuse as well as the effect on hydrolysis side reaction are discussed. The enantiopreference of the acylation reactions of **6-8** is known yielding (*R*)-esters of **6** [62] and **7** [153], and (*S*)-ester of **8** [98]. In lipase-catalyzed reaction, condition engineering (choice of solvent, reaction temperature and acyl donor) is important for obtaining high enantioselectivity. In kinetic resolution of **6-8**, the solvents, reaction temperature and acyl donor were selected on the basis of screening reactions with the lipase PS powder and lipase PS-celite in the acylation of **6-8** (P.Hara, unpublished results).

The demands for enzyme preparations used in organic synthesis are high enantioselectivity, activity and stability. Activity, stability and selectivity of lipase PS xerogels and CLEAs were studied in the acylation of **6-8** with vinyl acetate as an acyl donor (Papers I and II). Organic solvents toluene, DIPE and TBME were used according to solvent screening, and ionic liquids [EMIM][NTf_2], [EMIM][BF_4] and [BMIM][PF_6] were chosen according to the previous results in the laboratory [98]. The results were compared to those obtained with the same amount of lipase PS "Amano" SD powder (100 mg/mL vs. xerogel based on 100 mg/mL and 50 mg/mL vs. CLEA based on 50 mg/mL) to observe the effects of the immobilization. The reuse of xerogels and CLEAs is discussed in chapter 5.3. Peracetylated β-cyclodextrin stabilizes and activates lipase PS sol-gels [128] as well as activates lipase PS when co-lyophilized with the enzyme for the acylation of **7** [154]. In lipase PS "Amano" SD preparation used for sol-gel immobilization, the protein content is only 3% according to bicinchoninic acid assay, diluted to dextrin. In preparing sol-gels, this dextrin used as dilutant may be useful for maintaining the activity during gelation and lyophilization, and it may also have stabilizing effects in acylation.

ACC 507-15 is made from phenolic resins and its surface has acidic and basic sites.[155] These acidic and basic residues can form hydrogen bonds with IL and enzyme thus stabilizing the immobilized enzyme. Activity, stability and selectivity of lipase PS SILEs were studied with ACC 507-15 SILEs (Paper III), since the reactivity of SILEs prepared using active carbon and alumina as supports was negligible (conversions less than 14 % after 24 h) in kinetic resolution of **6** with vinyl acetate in toluene, and the reactivity of STV 505 supports was lower than that of ACC 507-15 SILEs (See below). ACC 507-15 SILE preparations are convenient in use, since they can be cut into suitable pieces

and used as such when shaking the reaction mixture, or they can be attached to stirring blade. In this study, ACC 507-15 SILEs were attached to stirring blade and recycled without further washes between the cycles. The reuse of ACC 507-15 SILEs in different temperatures is discussed in chapter 5.3. Lipase PS preparations on active carbon cloth ACC 507-15 had high activity with both [EMIM][NTf_2] and [EMIM][BF_4] ionic liquids. As the average pore diameter in ACC 507-15 is 1.9 nm [155], and lipase PS is a globular protein with approximate dimensions 30 Å x 40 Å x 50 Å (3 nm x 4 nm x 5 nm) [80], it can be assumed that part of the protein is immobilized on the surface of ACC 507-15, when the stabilization of acidic and basic residues is more important. Alumina and AC were not sufficient supports for lipase PS. Alumina is not adequate support either for immobilization of CAL B (similar size to PS) due to hindered effect of pore diameter (63-200 μm, average pore diameter 60 Å). Accordingly, the enzyme was located in the external surface of alumina and poor enzyme loading was observed.[156] In this study the alumina was even smaller fraction 63-100 μm, indicating that lipase PS stayed on the surface of the support, which caused the low activities. This can be also the reason for low activities when active carbon was used as supports. The wettability of STV 505 was poor causing low loadings and thus low activities.

5.1. Activity of lipase PS preparations (Papers I-III)

The specific activity of the enzyme preparations is expressed in μmol min^{-1} g^{-1}, where g^{-1} refers to rate per gram of lipase PS preparation. Specific activity is the rate of formation of the first few percents of the products such that the product(s) concentrations have not risen to a level to significantly affect the rate. To study the activity of lipase PS preparations, specific activities for the acylation of **6-8** with vinyl acetate in organic solvents, ionic liquids and IL:solvent mixtures were determined and compared to specific activities of free lipase PS powder (Tables 14 and 15).

In organic solvents, the specific activities of lipase PS xerogels in the acylation of **6-8** were generally lower than with the lipase PS powder (Table 15, entries 1-4, 11,12,19, Paper I). This can be due to diffusional limitations caused by xerogel. Lyophilization of xerogels also caused shrinking, and the capillary stress during drying can partially crush the enzyme and cause loss of activity.[121] Specific activities in ionic liquids and in IL:solvent mixtures (Paper II) were lower than in organic solvents with all the substrates studied, since the high viscosity of ILs increase diffusional limitations and mass-transfer limitations. Thus, the activity was increased in mixtures of IL:organic solvent of low viscosity. In [EMIM][NTf_2]:solvent and [BMIM][PF_6]:solvent mixtures the specific activities of xerogels were higher than those of lipase PS powder (entries 6,10,14,18,21,25), whereas in [EMIM][BF_4]:solvent mixtures they were lower (entries 8,23) indicating that hydrophobic ILs are more suitable than hydrophilic [EMIM][BF_4]. This trend can also be seen in literature, where the activity of lipase PS has followed the order [BF_4]<[PF_6]<[NTf_2].[37,54,57,58] Also the possibility of reduced calcium binding in lipase PS due to strong hydrogen bonding ability of BF_4^- has been proposed to lower the activities in BF_4 ionic liquids.[57]

Lipase PS CLEA was significantly more active than lipase PS powder in organic solvents (Table 15, Paper I). Also the specific activity in the acylation of **6-8** with vinyl acetate was higher when CLEA was used in ILs and IL:solvent mixtures rather than lipase PS powder (Paper II). The activities in IL:solvent mixtures were higher than in pure ILs, but considerably lower than in organic solvents. Specific activity of CLEA in the acylation of **6** was higher in hydrophilic TBME than in toluene (Table 15, entries 1-4), whereas xerogel and lipase PS-celite had lower activities in TBME than in toluene. (Table 14, entries 2 and 3, Table 15, entries 1-4). These results show that CLEA is a suitable catalyst also in hydrophilic media, such as hydrophilic organic solvents and ionic liquids. Previously BSA additive yielded high activity CLEAs from lipase PS.[140] In that procedure, the activity was extremely low when BSA was not used. In this case, the activity of lipase PS CLEA was decreased when BSA additive was used (specific activity=7.0 μmol min^{-1} g^{-1} vs. specific activity=3.9 μmol min^{-1} g^{-1} in the acylation of **6** with vinyl acetate in toluene). The differences in activities of lipase PS CLEAs is probably due to different lipase PS preparations used to immobilization, which leads to choice of different precipitant (saturated ammonium sulphate instead of acetone).

The immobilization of lipase PS as ACC 507-15 SILEs with or without ionic liquids clearly improved activity (Table 14, entries 1,4-6, Paper III) in the acylation of **6** with vinyl acetate in toluene compared to free enzyme powder (entry 1). The use of [EMIM][NTf_2] in SILE preparation increased activity (entries 4,5,7,8,10,11), and the use of [EMIM][BF_4] decreased activity (entry 6). This also indicates that the hydrophobic ILs are more suitable for lipase PS than hydrophilic ones as stated before.

The most active lipase PS preparation in the acylation of **6** and **8** was lipase PS-celite (Table 14, entries 2,3,9), however, activity of lipase PS-celite was not studied in ionic liquids, since earlier studies with lipase PS-celite showed very low activity in ILs [98]. Specific activity is expressed by gram of lipase PS preparate. The enzyme loading in ACC 507-15 SILEs was 4%, which leads to lower activities compared to lipase PS-celite (enzyme loading 20% (w/w)). ACC 507-15 SILEs were significantly more active than xerogels and CLEAs. CLEAs had higher activity than xerogels. The activity of lipase PS was increased when immobilized as CLEAs, SILEs and adsorbed on celite. Although specific activity of lipase PS xerogels is lower than with free lipase PS powder, the immobilization had stabilizing effect seen in the kinetic resolution of **6-8** (See Chapter 5.2). With lipase PS powder, specific activity was higher with 50 mg/mL lipase PS powder than with 100 mg/mL in most of the acylation reactions of **6-8** (Table 15). This is due to hydrolysis side reaction, which is faster when more lipase PS powder is used.

Table 14. Specific activities for the acylation of **6-8** with vinyl acetate in organic solvents in the presence of lipase PS powder, lipase PS-celite and ACC 507-15 SILEs at room temperature.

Entry	Compound	Solvent	Lipase preparation	Specific activity [μmol $min^{-1}g^{-1}$]
1	**6**	toluene	lipase PS "Amano" 160 mg/ml[a]	8.8 ± 1.0
2	**6**	toluene	lipase PS-celite 20% (w/w)[b]	103.0 ± 1.0
3	**6**	TBME	lipase PS-celite 20% (w/w)	77.7 ± 1.1
4	**6**	toluene	ACC 507-15/lipase PS	15.7 ± 0.6
5	**6**	toluene	ACC 507-15/[EMIM][NTf_2]/lipase PS	19.5 ± 0.9
6	**6**	toluene	ACC 507-15/[EMIM][BF_4]/lipase PS	12.0 ± 0.6
7	**7**	DIPE	ACC 507-15/lipase PS	27.1 ± 0.01
8	**7**	DIPE	ACC 507-15/[EMIM][NTf_2]/lipase PS	40.0 ± 0.01
9	**8**[c]	TBME	lipase PS-celite 20% (w/w)	87.6 ± 6.5
10	**8**[c]	TBME	ACC 507-15/lipase PS	23.5 ± 1.5
11	**8**[c]	TBME	ACC 507-15/[EMIM][NTf_2]/lipase PS	35.1 ± 2.1

[a] Mechanical stirring instead of shaking used in this experiment. Lipase PS powder amount related to protein amount in ACC 507-15 SILEs.
[b] Lipase PS "Amano" and lipase PS "Amano" SD gave similar activitites on celite
[c] reaction temperature 48 °C

Table 15. Specific activities (μmol $min^{-1}g^{-1}$) for the acylation of **6-8** with vinyl acetate in organic solvents and ionic liquids in the presence of lipase PS "Amano" SD powder, xerogels and CLEAs.

Entry	Comp.	Solvent[a]	Powder[b]	Xerogel[c]	Powder[d]	CLEA[e]
1	**6**	Toluene	2.7 ± 0.3	1.42 ± 0.02	3.4 ± 0.2	7.0 ± 0.9
2	**6**	TBME	2.5 ± 0.2	1.27 ± 0.04	3.2 ± 0.3	8.5 ± 0.3
3	**6**[f]	Toluene	3.8 ± 0.2	2.05 ± 0.06	5.9 ± 0.2	32.3 ± 0.1
4	**6**[f]	TBME	4.4 ± 0.1	1.69 ± 0.02	5.7 ± 0.1	19.7 ± 0.9
5	**6**	[EMIM][NTf_2]		0.31 ± 0.01		0.32 ± 0.01
6	**6**	[EMIM][NTf_2]:toluene	0.08 ± 0.01	0.98 ±0.08	0.13 ± 0.01	1.57 ± 0.47
7	**6**	[EMIM][BF_4]		0.19 ± 0.01		1.65 ± 0.07
8	**6**	[EMIM][BF_4]:toluene	0.57 ± 0.01	0.35 ± 0.03	1.15 ± 0.01	6.60 ± 0.71
9	**6**	[BMIM][PF_6]		0.41 ± 0.01		1.1 ± 0.1
10	**6**	[BMIM][PF_6]:toluene	0.46 ± 0.01	0.72 ± 0.05	0.79 ± 0.01	1.90 ± 0.41
11	**7**	DIPE	3.5 ± 0.2	1.47 ± 0.02	3.8 ± 0.2	30.1 ± 0.4
12	**7**	TBME	3.9 ± 0.1	0.94 ± 0.03	3.6 ± 0.1	18.6 ± 0.2
13	**7**	[EMIM][NTf_2]		0.90 ± 0.01		0.17 ± 0.01
14	**7**	[EMIM][NTf_2]:DIPE	0.21 ± 0.01	0.71 ± 0.04	0.26 ± 0.01	1.80 ± 0.18
15	**7**	[EMIM][BF_4]		0.15 ± 0.02		2.28 ± 0.25
16	**7**	[EMIM][BF_4]:DIPE	1.54 ± 0.01	2.19 ± 0.09	1.27 ± 0.01	4.82 ± 0.13
17	**7**	[BMIM][PF_6]		0.27 ± 0.04		1.34 ± 0.38
18	**7**	[BMIM][PF_6]:DIPE	0.43 ± 0.01	0.68 ± 0.04	0.79 ± 0.01	2.57 ± 0.42
19	**8**[f]	TBME	1.7 ± 0.1	1.54 ±0.03	2.38 ± 0.03	6.7 ± 0.3
20	**8**[f]	[EMIM][NTf_2]		0.03 ± 0.01		0.06 ± 0.01
21	**8**[f]	[EMIM][NTf_2]:TBME	0.12 ± 0.01	0.31 ± 0.01	0.16 ± 0.01	0.35 ± 0.04
22	**8**[f]	[EMIM][BF_4]		0.04 ± 0.01		1.91 ± 0.01
23	**8**[f]	[EMIM][BF_4]:TBME	0.41 ± 0.01	0.30 ± 0.02	0.84 ± 0.01	1.69 ± 0.40
24	**8**[f]	[BMIM][PF_6]		0.08 ± 0.01		0.67 ± 0.07
25	**8**[f]	[BMIM][PF_6]:TBME	0.07 ± 0.01	1.06 ± 0.11	0.79 ± 0.01	3.29 ± 0.38

[a] IL:solvent mixtures 1:2
[b] lipase PS "Amano" SD 100 mg/mL
[c] xerogel based on 100 mg/mL lipase PS "Amano" SD
[d] lipase PS "Amano" SD 50 mg/ml
[e] CLEA based on 50mg/ml lipase PS "Amano" SD
[f] reaction temperature 48 °C

5.2. Kinetic resolution of 6-8 (Papers I-III)

Kinetic resolution catalyzed by lipase PS powder, lipase PS-celite, xerogels, CLEAs and ACC 507-15 SILEs was studied in the acylation of **6-8** with vinyl acetate in organic solvents and ionic liquids (Scheme 11) by following the reactions 24-48 h to reach 50 % or higher conversions when possible. The acylation of lipase PS-celite in ionic liquids was not studied since lipase PS-celite was inactive in the earlier study, probably because the adsorption forces are not strong enough to keep lipase PS immobilized in polar ILs.[98]

The acylation of **6** with vinyl acetate was studied in toluene, TBME, ionic liquids and IL:toluene mixtures in the presence of lipase PS preparations (Tables 16 and 17). In toluene, lipase PS-celite, xerogel, CLEA, ACC 507-15/lipase PS and ACC 507-15/[EMIM][NTf_2]/lipase PS were highly active and enantioselective (E>200, c=50% in 24 h, in 2 h with lipase PS-celite) (Table 16, entries 2,4-6, Table 17, entries 1,3). In toluene, lipase PS powder had also high activity and selectivity, except when mechanical stirring was used instead of shaking (Table 16, entry 1, Table 17 entry 1). The acylation of **6** in the presence of ACC 507-15/[EMIM][BF_4]/lipase PS had lower enantioselectivity (*E*=152, Table 16 entry 6). This is probably due to hydrophilic nature of [EMIM][BF_4] which can strip the essential water layer from the enzyme. In TBME, the acylation of **6** proceeded smoothly and the enantioselectivity was high (>200) in the presence of lipase PS-celite, xerogel and CLEA (Table 16, entry 3; Table 17, entry 2,4). Additionally, the conversion and enantioselectivity were clearly higher than in the presence of lipase PS powder (Table 17, entry 2,4). This indicates the stabilization and activation of lipase PS in hydrophilic solvents like TBME when immobilized as lipase PS-celite, xerogels and CLEAs. The acylation of **6** with vinyl acetate in ionic liquids and IL:toluene mixtures was studied in the presence of xerogels, CLEAs and lipase PS powder (Table 17, entries 5-10, Paper II). In pure ILs (Table 17, entries 5,7,9), the acylation was slow but the enantioselectivity remained high (*E*>200). The conversion was higher with xerogels than with CLEAs. In IL:toluene mixtures (Table 17, entries 6,8,10), the enantioselectivity was high and the acylation in the presence of xerogel was faster, reaching 45% and 46% conversion in 24 h in [EMIM][NTf_2]:toluene and in [BMIM][PF_6]:toluene, respectively. The acylation of **6** in IL:toluene mixtures in the presence of CLEAs led to high enantioselectivities in each IL:toluene mixture. The conversion was highest in [EMIM][BF_4]:toluene (c=41 %, 24 h, entry 8). Xerogel was more stable in ionic liquids and IL:toluene mixtures than CLEA, hydrophobic [EMIM][NTf_2] and [BMIM][PF_6] being most appropriate ILs in the acylation of **6**, which is in line with literature.[54,58] Xerogels were also shown to be stable against storage, since the acylation of **6** with vinyl acetate in toluene proceeded similarly with fresh xerogel and the one stored for 1.5 months at 4°C (unpublished results).

The acylation of **7** with vinyl acetate was studied in DIPE, TBME, ionic liquids and IL:DIPE mixtures in the presence of lipase PS preparations (Tables 16 and 17). Immobilization of lipase PS as xerogel improved the enantioselectivity in DIPE and in TBME compared to lipase PS powder while the conversions were similar (Table 17, entries 11,12). CLEAs had similar conversions to the lipase PS powder in DIPE and

in TBME, but the enantioselectivity was decreased. In the presence of ACC 507-15 SILEs, the acylation of **7** was fast (Table 16, entries 7,8), but enantioselectivity was low. Enantioselectivity was slightly higher when ACC 507/[EMIM][NTf_2]/lipase PS was used instead of ACC 507-15/lipase PS. In ILs and IL:DIPE mixtures, the enantioselectivity in the acylation of **7** in the presence of xerogels was only moderate and lower than in DIPE, although the acylation reached 50% conversion (Table 17, entries 13-18). The enantioselectivities were also lower than those obtained with lipase PS powder. The conversions and enantioselectivities were moderate when the acylation of **7** in the presence of CLEAs was performed in IL:DIPE mixtures (Table 17, entries 13-18). However, in [EMIM][BF_4]:DIPE mixture (Entry 16) the enantioselectivity was higher than in DIPE (*E*=79 vs. *E*=58), and the acylation reached 40% conversion. Interestingly, CLEAs had higher conversions and enantioselectivities in the acylation of **6** and **7** in hydrophilic [EMIM][BF_4]:IL mixtures than in hydrophobic IL:solvent mixtures. In contrast xerogels performed better in hydrophobic IL:solvent mixtures, although generally hydrophobic ILs are considered more suitable for lipase catalysis.[54,58]

The acylation of **8** with vinyl acetate was studied in TBME, ionic liquids and IL:TBME mixtures in the presence of lipase PS preparations (Tables 16 and 17). In TBME, the acylation of **8** in the presence of lipase PS-celite, xerogels and CLEAs reached 50 % conversion after 6 h, 30 h and 48 h reaction, respectively, showing that lipase PS-celite was the most active preparation (Table 16, entry 9, Table 17 entry 19). Enantioselectivity was high in each case. The diminishing of the hydrolysis side reaction could be clearly seen when lipase PS-celite, xerogels and CLEAs were used instead of lipase PS powder (reaction stopped at 25 -33% conversion). When the acylation of **8** in TBME was performed in the presence of ACC 505-15 SILEs (mechanical stirring instead of shaking), the reaction stopped at 32% and 36% conversion (Table 16, entries 10,11) due to low solubility of **8**, which led to crystallization of substrate and product on SILE catalyst during the reaction. In ILs and IL:TBME mixtures, the acylation of **8** in the presence of xerogels and CLEAs was slow. In [BMIM][PF_6]:TBME mixture, both xerogel and CLEA reached 41% conversion, and the enantioselectivity was 102 and 105, respectively. In the earlier study of the acylation of **8** in IL in the presence of lipase PS-celite, the conversions were negligible showing that lipase PS-celite is not suitable catalyst in ILs.[98] These results indicate the stabilization of lipase PS when immobilized as xerogels and CLEAs also in ionic liquids.

The acylation in the presence of lipase PS powder was faster in many reactions when 50 mg/mL lipase PS powder was used than when 100 mg/mL lipase PS powder was used (Table 17, entries 3-4, 12,14,18,19,25). This reveals the effect of the hydrolysis side reaction, which is stronger when more lipase PS powder is used, and depends on the solvent and temperature. Since all acylation reactions performed in the presence of ionic liquid or ionic liquid:solvent mixture were slower and the selectivity did not increase, ionic liquid is not beneficial for the acylation of **6**-**8** in the presence of these lipase PS preparations.

Table 16. Acylation of **6-8** with vinyl acetate in organic solvents in the presence of lipase PS powder, lipase PS-celite and SILEs at room temperature.

Entry	Comp.	Solvent	Preparation	t [h]	c [%]	*E*
1	**6**	toluene	lipase PS "Amano" 160 mg/ml[a]	24	30	112
2	**6**	toluene	lipase PS-celite 20% (w/w)[b]	2	50	>200
3	**6**	TBME	lipase PS-celite 20% (w/w)	6	50	>200
4	**6**	toluene	ACC 507-15/lipase PS	24	44	>200
5	**6**	toluene	ACC 507-15/[EMIM][NTf_2]/lipase PS	24	50	>200
6	**6**	toluene	ACC 507-15/[EMIM][BF_4]/lipase PS	24	48	152
7	**7**	DIPE	ACC 507-15/lipase PS	6	46	25
8	**7**	DIPE	ACC 507-15/[EMIM][NTf_2]/lipase PS	6	51	33
9	**8**[c]	TBME	lipase PS-celite 20% (w/w)	6	51	>200
10	**8**[c]	TBME	ACC 507-15/lipase PS	24	32	>200
11	**8**[c]	TBME	ACC 507-15/[EMIM][NTf_2]/lipase PS	24	36	>200

[a] Mechanical stirring instead of shaking used in this experiment. Lipase PS powder amount related to protein amount in ACC 507-15 SILEs.
[b] Lipase PS "Amano" and lipase PS "Amano"SD gave similar activitites on celite
[c] reaction temperature 48 °C

Table 17. Acylation of **6-8** with vinyl acetate in organic solvents and ionic liquids in the presence of lipase PS "Amano" SD powder, xerogels and CLEAs.

Entry	Comp.	Solvent[a]	Powder[b]		Xerogel[c]		Powder[d]		CLEA[e]	
			t [h]	c [%]/*E*	t [h]	c [%]/*E*	t [h]	c [%]/*E*	t [h]	c [%]/*E*
1	**6**	Toluene	24	50/>200	24	50/>200	24	49/>200	24	50/>200
2	**6**	TBME	27	36/56	24	47/>200	27	36/53	24	51/>200
3	**6**[f]	Toluene	24	49/>200	24	51/>200	24	51/198	24	50/>200
4	**6**[f]	TBME	24	40/77	24	40/174	24	46/112	30	47/>200
5	**6**	[EMIM][NTf_2]			24	30/>200			24	5/>200
6	**6**	[EMIM][NTf_2]:toluene	48	17/51	24	45/>200	48	17/>200	24	10/>200
7	**6**	[EMIM][BF_4]			24	23/>200			24	24/>200
8	**6**	[EMIM][BF_4]:toluene	24	50/>200	24	39/>200	24	47/>200	24	41/>200
9	**6**	[BMIM][PF_6]			24	34/>200			24	18/>200
10	**6**	[BMIM][PF_6]:toluene	48	50/>200	24	46/>200	48	49/>200	24	20/>200
11	**7**	DIPE	24	50/115	24	51/139	24	51/131	24	53/58
12	**7**	TBME	27	42/50	30	40/78	27	46/30	24	56/35
13	**7**	[EMIM][NTf_2]			24	28/38			24	3/-
14	**7**	[EMIM][NTf_2]:DIPE	48	45/88	24	51/66	48	30/51	24	19/49
15	**7**	[EMIM][BF_4]			24	23/46			24	34/40
16	**7**	[EMIM][BF_4]:DIPE	6	50/115	24	54/61	24	51/134	24	40/79
17	**7**	[BMIM][PF_6]			24	41/78			24	21/49
18	**7**	[BMIM][PF_6]:DIPE	24	34/60	24	34/60	24	50/77	24	33/78
19	**8**[f]	TBME	24	25/30	30	49/>200	48	33/14	48	49/>200
20	**8**[f]	[EMIM][NTf_2]			24	5/>200			24	1/>200
21	**8**[f]	[EMIM][NTf_2]:TBME	48	30/30	24	26/>126	48	24/>200	48	7/-
22	**8**[f]	[EMIM][BF_4]			24	5/>200			24	17/-
23	**8**[f]	[EMIM][BF_4]:TBME	24	33/50	24	28/89	48	23/50	48	33/55
24	**8**[f]	[BMIM][PF_6]			24	13/>200			24	10/>200
25	**8**[f]	[BMIM][PF_6]:TBME	48	16/15	24	41/102	48	48/70	48	41/105

[a] IL:solvent mixtures 1:2
[b] lipase PS SD 100 mg/mL
[c] xerogel based on 100 mg/mL lipase PS
[d] lipase PS SD 50 mg/ml
[e] CLEA based on 50mg/ml lipase PS
[f] reaction temperature 48 °C

5.3. Reuse of the lipase PS sol-gels, CLEAs and SILEs (Papers I and III)

Stability of enzyme preparations allowing reuse of the catalyst in repeating reaction batches is important when used in larger scale and especially when the method is used on industrial application. For this reason, the reuse of lipase PS xerogel and CLEAs was studied in the model reactions: acylation of **7** in DIPE (Paper I), **6** in toluene and **8** in TBME (P. Hara, unpublished results). The reuse of ACC 507-15 SILEs was studied in the acylation of **6** by reusing the same SILE at eight different temperatures between 25-60 °C by increasing the temperature 5 °C after each cycle, and then applying the SILE again at 25 °C (Paper III). Xerogels and ACC 507-15 SILEs were proven to be recyclable several times without significant loss of activity or selectivity.

Lipase PS xerogel was used 8 times in the acylation of **7** with vinyl acetate in DIPE (activity loss after 8 cycles 6%, Paper I, Fig. 4) and 5 times in the acylation of **6** with vinyl acetate in toluene (activity loss after 5 cycles 13%, Fig. 11, P. Hara, unpublished results) at room temperature without significant loss of activity or selectivity. This indicates the stabilization of lipase PS during immobilization. In the acylation of **6** with vinyl acetate in toluene at 48 °C, the activity of xerogel started to decrease during third run (24 h, c=34 %, activity loss 33%) although *E* was >200 in each experiment. In the acylation of **8** with vinyl acetate in TBME at 48 °C, the activity decreased after two cycles (14%). Higher temperature (48 °C vs. room temperature) led to faster inactivation of xerogel during cycles.

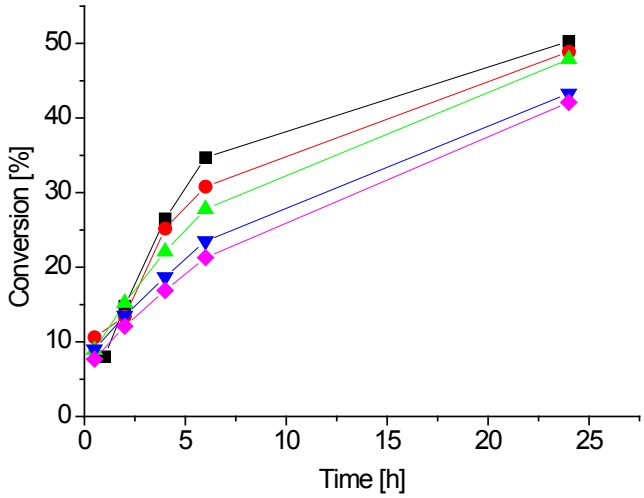


Figure 11. Recycling of the lipase PS xerogel in the acylation of **6** with vinyl acetate in toluene. Cycle 1 (■), cycle 2 (●), cycle 3 (▲), cycle 4 (▼), cycle 5 (♦). *E*>200 in each cycle.

Lipase PS CLEA was highly active, and the acylation of **6-8** smoothly proceeded to 50% conversion with a fresh catalyst in organic solvents. It was not, however, stable enough to be recycled. The reuse did not have effects on enantioselectivity (**7**, Paper I, Fig. 5, **6** and **8** unpublished results), but the activity decreased markedly (12-49%) during the second cycle in each case.

The reuse of lipase PS SILEs was studied using the acylation of **6** with vinyl acetate in toluene as a model reaction (Paper III). The stability towards recycling and changes of temperature were studied by subjecting the same catalyst at temperatures between 25 - 60 °C by increasing the temperature 5 °C after each cycle, and finally performing the acylation again at 25 °C (Table 18). ACC 507-15/lipase PS (no IL) maintained the activity and selectivity through temperature range, the conversion loss being 4% after 9 cycles. However, when applied again at 25 °C the specific activity was clearly lower. The activity and enantioselectivity of ACC 507-15/[EMIM][NTf_2]/lipase PS was high after the temperature range and also when applied again at 25 °C, the conversion decreased only 3% and specific activity 0.7 μmol min^{-1} g^{-1} after 9 cycles, indicating that [EMIM][NTf_2] stabilized the enzyme during these cycles. In case of ACC 507-15/[EMIM][BF_4], the activity started to decrease after 50 °C, and when applied again at 25 °C, the enantioselectivity also decreased. This shows slight destabilization of lipase PS by hydrophilic [EMIM][BF_4]. This is in line with the results that hydrophobic ILs like [EMIM][NTf_2] activate and stabilize enzymes, whereas hydrophilic ILs like [EMIM][BF_4] can even cause decrease of activity by stripping off the essential water.[54,57,129] Also, as water is a common contaminat in organic solvents, especially in higher temperatures, water can cause a partial hydrolysis of [BF_4] anion with formation of HF, which is known to inactivate enzymes.[42,46] This stabilization of [EMIM][NTf_2] and destabilization of [EMIM][BF_4] could be seen when comparing both specific activities and conversions to those of ACC 507-15/lipase PS preparation. As a result, each SILE studied was recyclable several times at varying temperatures, the most active and stable preparation being ACC 507-15/[EMIM][NTf_2]/lipase PS. Since the ACC 507-15 SILEs had high stability and the activity and enantioselectivity remained high after several cycles in various temperatures, and the structure of the immobilized enzyme preparation is convenient and mechanically stable in use, it is possible to use them also in larger scale in batch and continuous flow reactors after optimization. Also other, less harmful ionic liquids e.g. halogen-free Ammoeng and Ecoeng ionic should be tested instead on [EMIM][NTf_2], which is known to be toxic.[40]

Table 18. Reuse of **6** with vinyl acetate in toluene in the presence of Kynol™ ACC 507-15-based SILEs at 25 - 60 °C. s.a.=specific activity.

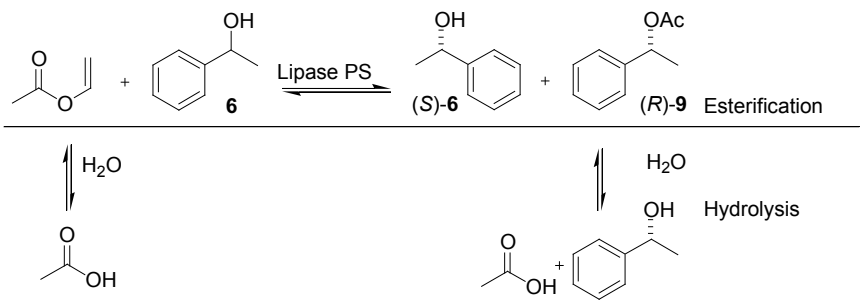
		ACC 507-15/lipase PS		**ACC 507-15/ [EMIM] [NTf_2]/lipase PS**		**ACC 507-15/ [EMIM] [BF_4]/lipase PS**	
Number of use	T (°C)	s.a. [µmol min^{-1} g^{-1}]	Conversion [%][a] / *E*	s.a. [µmol min^{-1} g^{-1}]	Conversion [%][a] / *E*	s.a. [µmol min^{-1} g^{-1}]	Conversion [%][a] / *E*
1	25	15.7 ± 0.6	44 / >200	19.5 ± 0.9	50 / >200	12.0 ± 0.6	48 / 152
2	30	24.9 ± 0.2	50 / >200	36.5 ± 1.6	51 / >200	13.6 ± 0.9	50 / 136
3	35	20.8 ± 0.9	49 / >200	45.5 ± 3.5	51 / >200	24.5 ± 1.0	50 / >200
4	40	31.3 ± 2.9	51 / >200	42.1 ± 1.4	51 / >200	23.0 ± 0.7	50 / >200
5	45	32.3 ± 0.2	51 / >200	47.6 ± 1.9	51 / >200	24.8 ± 0.6	50 / >200
6	50	36.9 ± 1.2	51 / >200	33.9 ± 0.7	51 / >200	21.8 ± 0.2	50 / >200
7	55	31.0 ± 0.8	51 / >200	32.9 ± 1.0	51 / >200	13.9 ± 0.3	45/ >200
8	60	23.8 ± 0.7	50 / >200	36.7 ± 0.9	51 / >200	9.2 ± 0.4	38 / >200
9	25	6.4 ± 0.3	40 / >200	18.8 ± 1.3	47 / >200	3.2 ± 0.2	15 / 117

[a] Conversion after 24 h.

5.4. The effect of immobilization on hydrolysis side reaction (Papers I and II)

As already stated, the acylation of **8** in TBME in the presence of lipase PS powder started to retard after a certain concentration was reached or the reaction stopped at an early stage. For this reason, attention was paid to the possibility of hydrolysis as a side reaction.

Residual water from enzyme preparations, substrates, solvent or atmosphere can lead to the hydrolysis of acyl donors and ester products in dry reaction mixtures. In Scheme 12, possible hydrolysis side reaction in the esterification of **6** is presented as an example. Due to this hydrolysis, the reactions can stop before reaching 50% conversion [157] or the enantioselectivity decreases. The enzyme preparation used plays significant role in hydrolysis in dry conditions. E.g. celite support can be used to decrease undesirable hydrolysis.[99] Xerogels, CLEAs and lipase PS-celite reduce hydrolysis in kinetic resolution, since the acylation of **8** proceeds smoothly to 50% conversion (Table 16, entry 9; Table 17, entry 19), contrary to lipase PS powder. The hydrolysis was studied more detailed in the presence of xerogels.



Scheme 12. Hydrolysis as a side reaction in a lipase-catalyzed esterification of **6**.

The rate of hydrolysis side reaction depends on the reaction conditions, substrate structure and solvent hydrophilicity. In the case of **6-8**, the hydrolysis of **8** took place most rapidly (Table 19, Fig. 12). Also the hydrolysis was faster in more hydrophilic TBME (more water available) than in toluene (entries 1 and 2). Hydrolysis of the product esters **9-11** occurred in all solvents tested with lipase PS powder, conversion being 4-86% after 24 h reaction. The hydrolysis in ILs was tested in [EMIM][BF_4]:solvent mixture, since [EMIM][BF_4] is the most hydrophilic IL used in this study. Hydrolysis is faster in more hydrophilic IL mixture than in organic solvent with **9** and **10** but, interestingly, slower in case of **11**. Xerogel hydrolyzed significantly less of the esters than free lipase PS powder. The suppression of hydrolysis is most evident in the acylation of **8**. When the acylation of **8** in the presence of free lipase PS powder stopped around 30% conversion, in the presence of xerogel the acylation proceeded to 49% conversion (Fig. 12). The same effect could be seen in the acylation of **6** in the presence of free lipase PS in TBME (18% hydrolysis), where conversion was 36% after 27 h, whereas in toluene (4% hydrolysis) the conversion was 50% after the same time. When xerogel was used, the conversions in TBME and toluene were 47% and 50%, respectively. Similar effect can also be seen with CLEA, although there were no more detailed studies of hydrolysis. When the acylation of **8** stopped at 33% conversion with free lipase PS, CLEA acylated **8** smoothly (c=49 %, 48 h, Fig. 12.). The same effect was observed with **6** in TBME. The suppression of hydrolysis effected on enantioselectivity, since *E* of the acylation of **8** improved from 30 to >200 when xerogel was used, and from 14 to >200 in case of CLEA. This improvement occurred also in the acylation of **6** in TBME where *E* increased from 56 to >200 in case of xerogel and from 53 to >200 in case of CLEA. As a conclusion, both xerogel and CLEA clearly suppress the unwanted hydrolytic side reactions, especially in hydrophilic organic solvents like TBME. Xerogel is also catalyst of choice in IL:solvent mixtures.

Table 19. Conversion of the side reaction (%) after 24 h in the hydrolysis of enantiopure esters **9-11** (0.05 M) by the residual water in the enzyme preparation and the solvent system at room temperature.

Entry	Substrate	Solvent	log P	c [%] by lipase PS[a]	c [%] b y Xerogel[b]
1	(*R*)-**9**	Toluene	2.8	4	1
2	(*R*)-**9**	TBME	1.35	18	3
3	(*R*)-**9**	[EMIM][BF_4]:Toluene 1:2		27	2
4	(*R*)-**10**	DIPE	1.9	20	10
5	(*R*)-**10**	TBME	1.35	12	2
6	(*R*)-**10**	[EMIM][BF_4]:DIPE 1:2		22	26
7	(*S*)-**11**[c]	TBME	1.35	86	16
8	(*S*)-**11**[c]	[EMIM][BF_4]:TBME 1:2		35	45

[a] 100 mg mL^{-1} of lipase PS powder

[b] Xerogel based on 100 mg mL^{-1} of lipase PS powder.

[c] Butanoate instead of acetate; reaction temperature 48 ºC

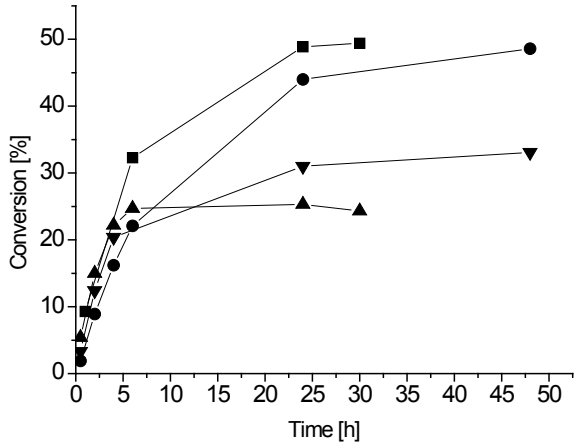


Figure 12. Acylation of **8** (0.1 M) with vinyl acetate (0.2 M) in TBME at 48 ºC in the presence of lipase PS powder (100 mg mL^{-1}; –▲–), (50 mg mL^{-1}; –▼–), xerogel (100 mg of lipase PS; –■–) and CLEA (50 mg lipase PS; –●–).

6. SUMMARY

In this thesis immobilization methods of lipases, mainly *Burkholderia cepacia* lipase, lipase PS, and the effects of immobilization on activity and kinetic resolution in organic solvents and in ionic liquids is described. Lipase PS "Amano" or lipase PS "Amano" SD was immobilized using three different methods. The methods were sol-gel, cross-linked enzyme aggregates (CLEA) and supported ionic liquids enzymes (SILE) methods. These immobilized lipase preparations were used in enzymatic acylation of racemic alcohols **6-8** to study the the effect of immobilization on activity, enantioselectivity and hydrolysis side reactions in organic solvents, ionic liquids, and in their mixtures. The results were compared to those obtained with lipase PS immobilized on celite, the method widely used for lipase immobilization. Moreover, reuse possibilities of these immobilized enzymes were studied.

I have shown that lipase PS xerogels had low specific activity in organic solvents, ILs and in IL:solvent mixtures. However, the kinetic resolution of **6-8** proceeded smoothly in organic solvents. In ILs and IL:solvent mixtures lipase PS xerogel was more stable than free lipase PS powder and lipase PS-celite, and the proceeding of the acylation depends on substrate structure. Xerogel was also reusable 5 times in the resolution of **6** in toluene and 8 times in the resolution of **7** in DIPE. In these reaction conditions, the conversion loss in the acylation of **6** was only 13 % and in the acylation of **7** 6 % after the cycles. Hydrolysis of the product esters was suppressed.

In this thesis lipase PS CLEAs showed higher activity than free lipase PS powder in all solvent systems used. Kinetic resolution proceeded well in organic solvents. However, CLEA preparation rapidly lost its activity in systems containing IL. CLEA seems to be a good choice in hydrophilic solvents, since it maintained the activity and selectivity in kinetic resolution in TBME, although xerogel and lipase PS-celite were slower in TBME than in toluene. CLEA performed well also in hydrophilic [EMIM][BF_4] and in [EMIM][BF_4]:solvent mixtures, although xerogel showed higher activity in hydrophobic solvents. CLEA also suppressed the hydrolysis side reaction. Recyclability of CLEAs was limited.

I have shown that lipase PS ACC 507-15 SILEs, immobilized on active carbon cloth ACC 507-15, were highly active and enantioselective, the most active being ACC 507-15/[EMIM][NTf_2]/lipase PS. This preparation was reusable 9 times in temperatures 25 - 60°C. [EMIM][NTf_2] clearly stabilized lipase PS against inactivation at high temperatures. ACC 507-15 SILEs had also good mechanical resistance in used stirring system.

Lipase PS-celite is highly active and enantioselective catalyst in organic solvents. However, it is not a suitable catalyst in polar solvents like ionic liquids, since adsorption forces are not enough to keep the enzyme attached on the support in polar environments.

When the results are compared to those of lipase PS-celite, lipase PS-celite and ACC 507-15/[EMIM][NTf_2]/lipase PS SILE gave the highest activity. With these catalysts the acylation proceeded smoothly in organic solvents. Xerogel is the choice in ionic liquids, when the product ester is an activated ester and when hydrolysis is possible as a side reaction. CLEA performed well in organic solvents, including hydrophilic TBME. The use of ionic liquids was not beneficial in the acylations of **6-8** in these conditions. SILEs were shown to be suitable especially in larger scale, due to their stability and activity when reused, mechanical resistance and convenient handling. I have shown that there is no "number one" method among the immobilization methods studied, rather the choice of lipase PS preparation depends on the structure of the substrate and the reaction conditions, especially on the solvent used.

7. ACKNOWLEDGEMENTS

The experimental work of this thesis was carried out at the Laboratory of Synthetic Drug Chemistry, Department of Pharmacology, Drug Development and Therapeutics, Faculty of Medicine, University of Turku. The Academy of Finland is gratefully acknowledged for funding this work and COST D25 for funding short term scientific mission to Delft University of Technology.

I want to express my sincere gratitude to my supervisor, Professor Liisa Kanerva for introducing me the world of enzymes and giving me the possibility to do this work. I also want to thank you for the guidance and interesting discussions during the years.

Special thanks to Associate Professor Ulf Hanefeld for giving me the possibility to visit Delft University of Technology and for introducing me sol-gel and CLEA methods. I also want to thank Professor Dmitry Yu. Murzin and Professor Jyri-Pekka Mikkola for the possibility to visit the Laboratory of Industrial Chemistry and Reaction Engineering, Åbo Akademi and to learn the supported ionic liquids catalysts technique.

Professor Kristiina Kruus and Professor László Poppe are acknowledged for reviewing this thesis.

Dr. Arto Liljeblad is acknowledged for all the help during the years and for critical reading of the manuscript of this thesis. I want to thank my colleagues (in alphabetical order) from the Laboratory of Synthetic Drug Chemistry for the nice working environment and for sharing your knowledge with me. Thank you Päivi Alanko, Jürgen Brem, Monica Fitz, Ari Hietanen, Annukka Kallinen, Anu Kiviniemi, Toni Kurki, Niko Laaksovirta, Hanna Launela, Outi Lehtovirta, Dr. Xiang-Guo Li, Tarja Limnell, Dr. Katri Lundell, Otto Långvik, Harri Mäenpää, Tihamér Paál, Päivi Perkiö, Maria Puustinen, Mari Päiviö, Maria Rantapaju, Tiina Saloranta, Elina Siirola, Riku Sundell, Dr. Mihaela Turcu and Marita Vainio. It was a privilege to work with you all.

Sincere thanks to my parents Maire and Timo for their love and encourangement for studying. I also want to thank my friends for joy you bring to my life.

I would like to express my warmest thanks to my husband Mika and my children Iina and Aleksi for their love and support, and directing my thoughts away from chemistry.

Lieto 2011,

Piia Hara

Piia Hara

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