APPLICATION OF LIPOLYTIC ENZYMES OF MICROBIAL ORIGIN AS BIOCATALYSTS

Theses of the doctoral dissertation of

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1. INTRODUCTION

Biocatalysis has emerged as an important tool in the industrial synthesis of bulk chemicals, pharmaceutical and agrochemical intermediates, active pharmaceuticals, and food ingredients. However, the number and diversity of the applications are modest, perhaps in part because of perceived or real limitations of biocatalysts, such as limited enzyme availability, substrate scope, and operational stability. Recent scientific breakthroughs in genomics, directed enzyme evolution and the exploitation of biodiversity should help to overcome these limitations.

Worldwide, chemists are vigorously taking on the challenge of developing synthetic methodology and „green" processes that meet the criteria of a sustainable, environmentally conscious development. The biocatalytic transformations today are routinely considered by synthetic organic chemists, and by process engineers, as an economically and ecologically competitive technology, and as a matter of fact for the development of new production routes to fine chemicals, pharmaceuticals, agrochemicals, and even bulk commodities. Market demands are now pushing for individual tailoring of biocatalysts for specialized applications, driven by the new opportunities that have emerged for the discovery of novel enzymes and the fine-tuning, and even redesign of their properties.

Industry continues to demand more selective and efficient catalysts and processes for the manufacture of fine chemicals. Here, enzyme catalysts often have a „natural" advantage that will increasingly be exploited as the demand for enantiomerically pure drugs continues to rise. High economical and low ecological impact, it is adopted for industrial large-scale conversions.

Hydrolases predominate for several obvious reasons, the range of enzyme classes considered for technical processes is growing remarkably broad, and a steadily increasing number of different bioprocesses are being successfully transplanted from the laboratory to the manufacturing plant. To a large degree, this growth is a result of improved access to stable biocatalysts having customized activities and selectivities. This reflects the recent progress in the fields of molecular biology, high-throughput screening techniques, and advanced engineering.

The use of biocatalysis for industrial synthetic chemistry is on the way of significant growth. Biocatalytic processes can now be carried out in organic solvents as well as aqueous environments, so that apolar organic compounds as well as water-soluble compounds can be modified selectively and efficiently with enzymes and biocatalytically active cells. As the use of biocatalysis for industrial chemical synthesis becomes easier, several chemical companies have begun to increase significantly the number and sophistication of the biocatalytic processes used in their synthesis operations.

The application of biocatalysts offers a remarkable arsenal of highly selective transformations for modern preparative organic chemistry. Biocatalysis can provide environmentally friendly processes for all life science related industries (pharma, food, feed, agro).

The development of technologies using lipases for the synthesis of novel compounds will result in their expansion into new areas and increase in number of industrial applications

The economic synthesis of the increasing number of biologically active molecules is one of the biggest challenges on the field of organic chemistry. The preparation and the use of large purity enantiomers are obviously necessary for different industries (mainly pharmaceutical, plastic, cosmetic and food industry). The presence of the other enantiomer besides the active one is a real danger in effective drugs. The most known example for that is the (*R*)-Thalidomide (α-phtalimido-glutaramide, *Contergan*), which has a sedative effect, while its enantiomer pair, the (*S*)-Thalidomide is teratogenic even in small amount. Besides the traditional chemical methods, the growth of the biocatalytical methods both in laboratory and industrial scale are occurred due to environmental aspects and the increased demand toward stereoselective synthesis. As a result of this process the world leading pharmaceutical companies are spending huge amount of money to research projects finding solution to these questions.

Continuous processes could provide environmental friendly and economically efficient solutions; however integrated flow technologies that could cover from lab scale to pilot and production scale exist only scattered today. There are only a few examples of hydrolase-catalyzed enantioselective processes carried out in continuous-flow systems. Although the immobilized biocatalysts can be utilized ideally in continuous-flow systems, most of the continuous-mode biocatalytic syntheses of chiral pharmaceutical intermediates were performed on a relatively large scale using immobilized lipases in a packed-bed reactor so far. It was found that stainless-steel continuous-flow packed-bed bioreactors could be effectively used to study the effects of temperature, pressure and flow rate on lipase-catalyzed kinetic resolution.

In the 21st century mankind has to battle /be taken the field against chronic diseases such as obesity, high blood pressure and cholesterol level, cardiovascular diseases, diabetes, colon cancer etc. Certainly it can be stated that besides genetic factors and lifestyles nutrition and diet play an important role in evolving diseases. Thus the main focus has to be taken rather on prevention than on the treatments of the already existing diseases.

Nowadays, functional foods which have beneficial physiological effects on human body and/or can reduce the risk of chronic diseases, have an emerging role. Food industry is also facing with new challenges due to rapid growth of market for functional and nutraceutical foods and consumer demand for more healthy fats and has to turn toward implementation of mild processes. Applying biocatalysis methods can be proper tool to provide the solution for these challenges.

2. OBJECTIVES

The objective of this research work is to provide biocatalytic methods for enzymatic one-pot synthesis of specific structured lipids and phytosterol esters and the systematic study of sol-gel immobilization method. Furthermore, a novel unconventional enzyme immobilization technique for lipase preparation as food and fine chemical application was presented.

The main objectives of this thesis can be summarized as follows:

- to develop integrated enzymatic methods starting from natural vegetable oil, a medium-chain fatty acid and phytosterol which result in a similar "value-added" blend of structured lipids and phytosterol esters that would be useful as a beneficial food additive. With special emphasis on using starting materials from natural sources and using solely enzymatic methods to avoid the increase of the unwanted *trans*-fatty acid content.
- to find the best process variant for the synthesis of structured lipids and phytosterol esters mixture
- to perform a systematic study of the sol-gel immobilization of a Celite-supported lipase using ternary silane precursor systems of various alkyltriethoxysilanes (alkylTEOS), phenyltriethoxysilane (PhTEOS) and tetraethoxysilane (TEOS).
- to reveal the effects of adsorption / entrapment in composite immobilization.
- to evaluate the effects of binary and ternary precursor systems in composite sol-gel immobilization.
- to investigate the imprinting effect of rationally selected molecules (substrate mimics in experimental X-ray structures of lipases and their structural analogues) in sol-gel process.
- to evaluate the biocatalytic efficiency of the developed enzymes using multisubstrate mixtures in continuous-flow reactor.
- to develop robust immobilized biocatalysts for continuous-flow kinetic resolutions

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Biocatalysts

Novozym 435 (lipase B from *Candida antarctica* – recently named as *Pseudozyma antarctica* – immobilized on acrylic resin; non-specific), lipase from Candida sp. *recombinant*, expressed in *Aspergillus niger* (Novozym CaLB L), Novozym carrier and Lipozyme TL IM (lipase from *Thermomyces lanuginosus* immobilized on silica; 1,3-specific) were donated by Novozymes A/S (Bagsvćrd, Denmark). CaLA-T2-150 (lipase A from *Candida antarctica* (CaLA, registered trademark from Novozymes Bagsværd, Denmark) covalently attached to dry acrylic beads) was the product of Chiral Vision BV.

Amano Lipase PS (lipase from *Burkholderia cepacia (*syn.: *Pseudomonas cepacia)*; >30.000 U/g; non-specific) and Amano Lipase AK (lipase from *Pseudomonas fluorescens*; >20.000 U/g; non-specific) were donated by Amano Pharmaceutical Co. Ltd. (Nagoya, Japan). Lipase A from *Candida antarctica* – recently named *Pseudozyma antarctica* – (Cat. No. 62287; 2 U/mg; 2-specific) and lipase from *Candida cylindracea* – recently named *C. rugosa* – (Cat. No. 62316, 2 U/mg; non-specific) were products of Fluka. Novozym® 435 (Lipase acrylic resin from *Candida antarctica*, recombinant, expressed in Aspergillus niger), Lipozyme, immobilized lipase from *Mucor miehei* – recently named as *Rhizomucor miehei* – Cat. No. 62350; 30 U/g; 1,3-specific) was purchased from Sigma.

The biocatalysts with sterol esterase activity in this study were the air-dried mass of solid state fermentation (SSF) of *Aspergillus oryzae* NRRL 6270 (*Ao*SSF) and *Aspergillus sojae* NRRL 6271 (*As*SSF).

3.1.2 Chemicals, solvents, standards, raw materials

Tricaprylin, 2-Capryloyl-1,3-dioleoyl-gylcerol (LML*),* capryloyl residue at *sn*-1 and *sn*-3 positions (MLM), capryloyl residue at sn-1 / sn-3 position (LLM), capryloyl residue at *sn*-1/*sn*-2 or *sn*-2/*sn*-3 positions (MML), hexan-2-yl-acetate, heptan-2-yl-acetate, octanol-2-yl-acetate, nonan-2-yl-acetate, decan-2-yl-acetate, dodecan-2-yl-acetate, tetraethylene glycol dilauryl ester (L-TEG-L),1-(thiophen-2-yl)ethanol and 1-(thiophen-2-yl)ethyl acetate were synthesized in our laboratory.

The solvents, chemicals were products of Sigma -Aldrich, Fluka, Merck and Alfa Aesar.

3.2 Instrumentation

The following instruments were used during the work: The purity of the synthetic standards and regioisomeric composition of the triglycerides in the final reaction products were analyzed on Alliance

Waters 2690 HPLC equipped with an evaporative light scattering detector (PL-ELS 1000, Polymer Laboratories, operating at 40 °C) and an Ultra C-18 column (250 x 4.6 5μm, Restek) using a binary solvent gradient, The conversion of the phytosterol acylation and triacylglycerol transesterification reactions were checked on a Hewlett Packard 6890 GC equipped with FID detector, on-column injector and HP-1 column (25 m × 200 μm i.d. × 0.11 μm film thickness, Agilent Technologies) and a non-polar fused silica precolumn (0.53 mm i.d., Supelco), in case of sol-gel immobilization and bioimprinting the conversion of the reactions were checked on a Hewlett Packard 6890 GC equipped with FID detector, and Hydrodex β-6TBDM [25 m × 0.25 mm × 0.25 μm film of heptakis-(2,3-di-O-methyl-6-O-*t*-butyldimethylsilyl)-β-cyclodextrin] column, Scanning electron microscopy (SEM), NMR spectrum was recorded in $CDCl_3$ on a Bruker DRX-500 spectrometer (at 500 MHz for ^{1}H- and 125 MHz for ^{13}C-spectra).

3.3 Experimental

3.3.1 Enzymatic processes for one-pot production of SSLs and PSEs compositions

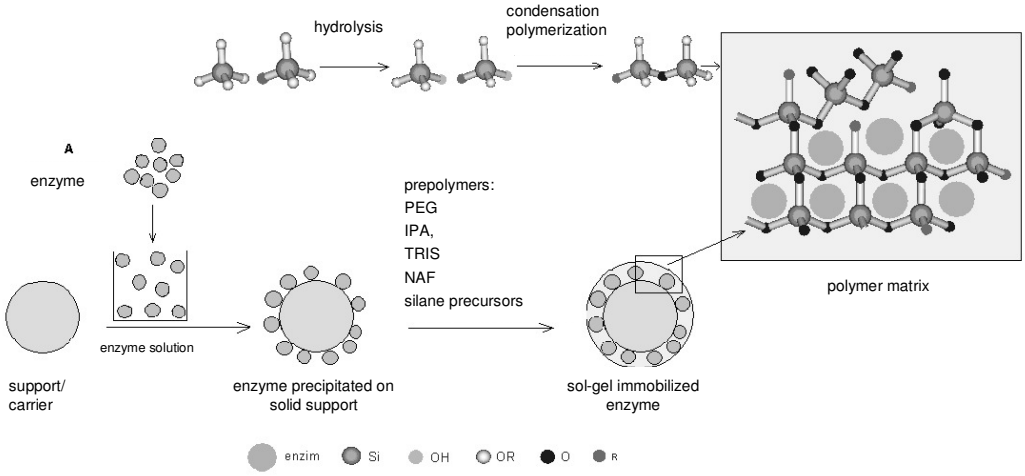
Two different biocatalysts, *Ao*SSF (preparation of *Aspergillus oryza*e NRRL 6270, for sterol ester formation) and Lipozyme (immobilized lipase from *Mucor miehei*, for structured lipid formation), were used in the enzymatic processes. Six process variations with these biocatalysts were tested for integrated enzymatic one-pot processes for production of SSLs and PSEs on 1 mmolar scale (based on PSs) using standard reaction conditions with 0.12w/w% added water (based on the total mass of the starting compounds). Each reaction type was performed in three parallel series using three different compositions of phytosterol, sunflower oil and caprylic acid, in molar ratios of 1:2:12, 1:2:18 and 1:2:24. In each case, aliquots (50 μl, dissolved in 1 ml dichloromethane to stop the reaction) were taken daily and the progress of formation of structured lipids and phytosterol ester was monitored by TLC, GC and HPLC.

3.3.2 Sol-gel immobilization of lipase AK deposited on Celite® 545 using binary or ternary silane precursor systems

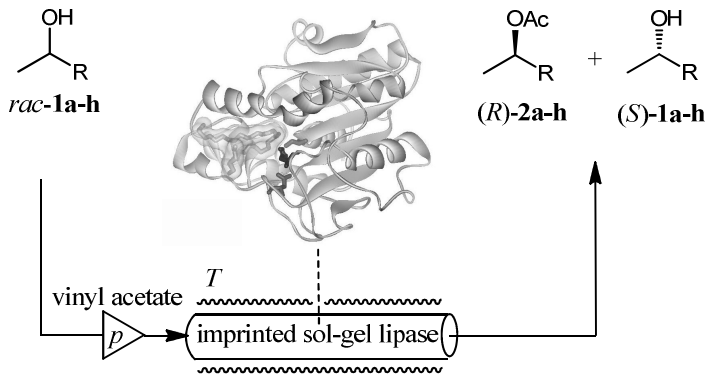
A two-step procedure was applied for sol-gel entrapment of Lipase AK on Celite 545.

Step 1: The lipase AK powder (50 mg for the standard procedure; 50, 125, 250, 375 or 500 mg for the enzyme loading tests) was added to TRIS-HCl buffer (0.1 M, pH 7.5, 780 μl) at 4°C with stirring for 10 minutes followed by addition of Celite 545 (500 mg). Acetone (10 ml) was added to the well stirred Celite-enzyme mixture at 10 ml min^{-1} rate at -18°C. The resulting solid was filtered off and left in air at room temperature for 12 hours for drying. The Celite-enzyme preparations were then used for sol-gel immobilization.

Step 2: TRIS-HCl buffer (0.1 M, pH 7.5, 390 µl), PEG solution (4% w/v, 200 µl), NaF solution (1M, 100 µl) and IPA (200 µl) were mixed in a 20 ml glass vial and the resulting solution was shaken at 1000 cycles per minute at room temperature for 10 minutes. During the continuous shaking, the corresponding silane precursors alkylTEOS-TEOS (1.5 mmol alkylTEOS and 1.5 mmol TEOS) or the mixture of alkylTEOS : PhTEOS : TEOS (3 mmol; the alkylTEOS : PhTEOS : TEOS molar ratio varied from 0.1 : 0.9 : 1 to 0.9 : 0.1 : 1 in 0.1 steps) and Celite-enzyme (250 mg) were added to the vial resulting in a sol suspension. To complete the polymerization, the mixture was shaken for 12 hours at room temperature. The formed solid was washed with IPA (7 ml), distilled water (5 ml), IPA (5 ml) and n-hexane (5 ml). The resulting white powder was dried in a vacuum dessicator for 5 h (until 0.4 mmHg final level of vacuum). The sol-gel lipase AK preparations were stored at room temperature.



3.3.3 Activity tests of the lipase preparations in multisubstrate systems in continuous-flow bioreactors



4. RESULTS

4.1 Enzymatic one-pot synthesis of specific structured lipids and phytosterol esters

As a new application of biocatalysts one-pot synthesis is attractive for many reasons including the minimization of handling procedures, consequent reduction in reagent use, and gains in volumetric productivity.

As it already has been demonstrated, *trans*-free fat alternatives for various food applications by enzymatic routes are possible. Thus, it can be assumed that a fully enzymatic process that avoids chemical interesterification may be superior to the existing methods. Consequently, the physical and physiological properties of enzyme-made "value-added" products are expected to be identical or superior to the blend of chemically made structured lipids and phytosterol esters.

Our major goal was to develop a solvent-free, purely enzymatic process for manufacturing SSLs and PSEs mixtures starting from naturally occurring starting compounds. PSs (mixture of rapeseed and sunflower phytosterols, composed of brassicasterol, campesterol, stigmasterol and β-sitosterol) are readily available because they are by-products of edible oil processing. Caprylic acid, a medium-chain fatty acid found in coconut, and sunflower oil as triglyceride which is rich in linoleic acid (48-74% of total FA), were used in our processes.

Screening lipases and sterol esterases as biocatalysts for enzymatic one-pot processes to produce mixtures of specific structured lipids and phytosterol esters starting from a mixture of phytosterol, caprylic acid and sunflower oil revealed that this valuable food component could be prepared purely enzymatically in organic-solvent-free medium. The process applied an esterification reaction of phytosterol with free fatty acids catalyzed by sterol esterase of *Aspergillus oryzae* and lipase catalyzed transesterification with immobilized lipase from *Mucor miehei* (Lipozyme) resulted in 92.1% conversion to phytosterol ester and 44.1% conversion to MLM / MML triacylglycerols. The final product could be obtained by simple filtration of the biocatalysts from the reaction product followed by removal of the free fatty acids by vacuum distillation. Because chemical transesterification has been avoided, this process resulted in no increase in the initial *trans*-fatty acid content of the mixture.

As the integrated enzymatic process developed for the production of structured lipid - phytosterol ester blends applies only food-grade biocatalysts, suitable industrial applications of the fully enzymatically produced compositions in foods, beverages, pharmaceuticals and nutraceuticals could provide an alternative way to combat obesity by improving the quality of lipid intake.

4.2 Improvement of the catalytic properties of biocatalysts, Fine-tuning the precursor composition for sol-gel immobilization of lipases

According to our previous results, the robust sol-gel entrapment method was chosen for further improvement and optimization. The sol-gel immobilization was systematically studied (precursor systems / support / conditions / additives) on *Pseudomonas fluorescens* lipase (lipase AK). The method (normal and combined) was extended to other enzyme (lipase PS) and support (silica) also.

The sol-gel polimer matrices were performed using binary and ternary systems. Binary preparations were made from alkyltriethoxy- (alkylTEOS) and tetraethoxysilane (TEOS) mixtures at constant molar ratio, and ternary preparations were performed using various alkyltriethoxysilanes [R'-$Si(OEt)_3$ / alkylTEOS], phenyltriethoxysilane [$PhSi(OEt)_3$ / PhTEOS] and tetraethoxysilane (TEOS) precursors. Eight different triethoxysilanes (PrTEOS, HexTEOS, OctTEOS, PFOctTEOS, DecTEOS, DodTEOS, OctdTEOS, PhTEOS) were investigated. The sol-gel encapsulation of lipases combined with adsorption on a solid support enhances the catalytic activity of the biocatalyst and the size distribution of solid support remains constant. The combined sol-gel method, deposition of enzyme on Celite® 545 and sol-gel encapsulation was used in all cases. Supported lipaseAK preparations were made at different enzyme-Celite ratios (10:1, 10:2.5, 10:5, 10:7.5 and 10:10). The preparations were visualized by SEM investigations. According to the GC analysis and the morphology studies with SEM, the 1:10 enzyme-Celite ratio provided the best Celite-supported lipase and was selected for all the further investigations. The effect of the silane precursor composition on enantiomer selectivity and catalytic ability were investigated in the kinetic resolution of racemic secondary alcohols. were also studied. The ternary and binary sol-gel lipase preparations were evaluated by their catalytic behavior in enantiomer selective acetylation of racemic 1-phenylethanol. For comparison, the acylation of racemic 2-heptanol exhibiting moderate enantiomer selectivity was also investigated. Native lipases and lipases immobilized by simple sol-gel entrapment were used as references. To evaluate the efficiency of the immobilization and biocatalysts, the following parameters were compared: specific activities (U_B), and activity yields (Y_A) and enantiomer selectivities (E) and enantiomeric excess (ee). In most cases, alkyltrimethoxysilanes (alkylTMOS's) were preferred for encapsulation of lipases. However, it was indicated that there is no significant difference in properties of encapsulated lipase biocatalysts prepared from alkylTMOS or alkylTEOS silane precursors. Because alkylTEOS's gelation time is longer and more controllable than with alkylTMOS's, the use of R'-$Si(OEt)_3$ and $Si(OEt)_4$ as silane precursors is preferable.

Among the binary systems the PhTEOS:TEOS=1:1 composition resulted in optimal properties regarding both activity and selectivity. These results were used in the fine-tuning of the ternary systems. In ternary systems alkylTEOS:PhTEOS molar ratio was varied from 0.1 to 0.9 in 0.1 steps while keeping the trialkoxysilane (alkylTEOS:PhTEOS) : tetraalkoxisilane (TEOS) molar ratio at 1:1. In general, the best ternary composition can be prepared from HexTEOS, OctTEOS, PFOctTEOS precursors, while enantiomer selectivities were sufficient for almost all the longer alkylTEOS precursors (HexTEOS, OctTEOS, PFOctTEOS, DecTEOS, DodTEOS, OctdTEOS). Among all the ternary systems, the perfluorinated chain containing PFOctTEOS series exhibited the best overall performance. Taking the price of PFOctTEOS also into account, however, the OctTEOS:PhTEOS:TEOS system provided the best performance / price result in the kinetic resolution of 1-phenylethanol.

In our further study the sol-gel encapsulation of two different lipases from *Pseudomonas fluorescence* (lipase AK) and *Pseudomonas cepacia* (lipase PS) and the influence of the porosity of the supports (Celite® 545 or Silica gel) were investigated. Two different enzyme/support ratio (1/5 and 1/10) were also studied. The corresponding enzymes were immobilized using octyltriethoxy- (OcTEOS) and tetraethoxy (TEOS) silane precursors in 1:1 molar ratio. Interestingly, the conversions (c), specific biocatalyst activities (U_B) and selectivities (*E*) depended only slightly from the amount of lipase AK in sol-gel immobilization. On the other hand, the specific enzyme activities (U_E) were much higher at 1/10 lipase AK/support ratio than at the 1/5 ratio. Using lipase PS, the best results were obtained at 1/5 lipase/support ratio with Celite® 545, and 1/5 lipase/support ratio with silica gel without preadsorption.

The supported sol-gel lipases prepared by our methods showed higher productivities, enantioselectivities and conversions then the commercial sol-gel lipase AK or PS preparations in almost all cases.

4.3 Preparation of novel sol-gel lipases by designed bioimprinting

Bioimprinting effect in sol-gel immobilization of lipases was studied to develop efficient novel immobilized biocatalysts with significantly improved properties for biotransformations in continuous-flow systems. The substrate mimics within the active site in experimental lipase structures (PDB) or structurally related compounds were selected systematically as bioimprinting candidates. Four lipases (from Lipase AK, Lipase PS, lipase B from *Candida antarctica* and lipase *Candida rugosa*) were immobilized by sol-gel process with nine bioimprinting candidates using various combinations of tetraethoxysilane (TEOS), phenyltriethoxysilane (PhTEOS), octyltriethoxysilane (OcTEOS) and dimethyldiethylsilane (DMDEOS) as silica precursors. The biocatalytic properties of the immobilized lipases were characterized by enantiomer selective acylation of various racemic secondary alcohols in multisubstrate systems (tests with a two component system: *rac*-**1a**,**b** and a five component system: *rac*-**1c-g**). The biocatalytic usefulness of

the best preparations was demonstrated by the kinetic resolution of racemic 1-(thiophen-2-yl)ethanol (*rac*-**1h**) in batch and continuous-flow systems.

The sol-gel entrapment can „freeze" the conformation of the enzyme; it is thus understandable that the pre-immobilization conformation of the lipase is very important. Furthermore the conditions selected should be sufficient to result in a more stable conformation on the enzyme molecule. If the lid can be kept open, lipase is expected to have a high activity in organic solvents

The study on bioimprinting effects of substrate-mimicking molecules selected systematically with the aid of experimentally verified cases in sol-gel immobilization of lipases with binary and ternary silane precursor compositions indicated that independently from the nature of sol-gel matrix, the most pronounced imprinting effects were found with such additives which were found mimicking the substrates in the experimental structures of the lipases. Our case study revealed, that lauric acid being the most effective imprinting additive for lipase PS (the crystal structure of lipase from *Burkholderia cepacia* included stearic acid) while in case of *Candida* species (CaLB, CrL) tetraethylene glycol dodecyl ether (BRIJ 30) exhibited the most significant imprinting effect (the porcine pancreatic lipase resembling similarity to CrL included tetraethylene glycol octyl ether and the crystal structure of CaLB included methylpenta(oxyethyl)heptadecanoate).

The high efficiency of sol-gel systems containing trialkoxysilanes OcTEOS or PhTEOS may be explained by assuming these silanes or their partially hydrolyzed forms having imprinting effect as well. This can rationalize also why the most efficient biocatalysts from Lipase AK (lipase from *Pseudomonas fluorescens*) did not contain imprinting additives.

The rational selection of imprinting molecules can be combined with proper compositions of silane precursors in large scale production of sol-gel immobilized lipases for various applications. The robust sol-gel entrapped forms of the selected four lipases (from Lipase AK, Lipase PS, lipase B from *Candida antarctica* and *Candida rugosa* lipase) proved to be ideal biocatalysts in biotransformations such as kinetic resolution of a racemic alcohols in batch and continuous-flow systems.

5. NEW SCIENTIFIC ACHIEVEMENTS (THESES)

I. Enzymatic one-pot synthesis of specific structured lipids and phytosterol esters.

T1. It was revealed that specific structured lipid and phytosterol ester mixture as valuable food component could be prepared starting from natural compounds in an enzyme catalyzed one-pot synthesis under solvent-free conditions.

T2. It was stated/concluded that the best process variant using a sequence of sterol esterase (*Ao*SSF)-catalyzed esterification reaction of the free fatty acids and phytosterols, followed by water removal in vacuum and lipase-catalyzed transesterification with immobilized lipase from *Rhizomucor miehei* (Lipozyme) resulted in 92.1% conversion to phytosterol esters and 44.1% conversion to triacylglycerols containing two caprylic esters (MLM/MML). Furthermore as chemical transesterification had been avoided, this process resulted in no increase in the initial trans-fatty acid content of the mixture.

II. Fine-tuning the precursor composition for sol-gel immobilization of lipases

T3. It was revealed by SEM investigations that preparations of lower Lipase AK:Celite ratio (1:10 and 1:4) had uniform and thin enzyme coverage, whereas higher enzyme loading (1:2, 3:4 or 1:1 ratios) resulted in thick enzyme layers containing large crystal-like particles. The enzyme-containing layer remained uniform and thin after the sol–gel entrapment of the Celite-supported Lipase AK of low (1:10) resulting in significant increases in activity yields (Y_A>200%) by lowering the diffusion limitations which are more pronounced in the larger aggregates of free Lipase AK.

T4. The importance of fine tuning was confirmed by the ternary sol-gel systems as the catalytic properties of the best ternary compositions were superior to any of the binary systems.

It was indicated that among binary systems the TEOS:PhTEOS=1:1 composition resulted in optimal properties regarding both activity and selectivity. Thus these results were used in the fine-tuning of the ternary systems.

It was revealed the best ternary composition can be prepared from HexTEOS, OctTEOS, PFOctTEOS precursors, while enantiomer selectivities were sufficient for almost all the longer alkylTEOS precursors (HexTEOS, OctTEOS, PFOctTEOS, DecTEOS, DodTEOS, OctdTEOS). Among all the ternary systems, the perfluorinated chain containing PFOctTEOS series exhibited the best overall performance. Taking the price of PFOctTEOS

also into account, however, the TEOS: OcTEOS:PhTEOS system provided the best performance / price result in the kinetic resolution reactions of 1-phenylethanol.

T5. Successful improvements in the supported sol-gel immobilization of Lipase AK were developed. The new biocatalysts prepared by our immobilization methods showed better catalytic properties then the commercial sol-gel lipases. The activity yields (Y_A) of our biocatalysts showed upto 1.4-fold activity yield increase (Y_A were varied between 109-242 %) compared to the commercial ones.

III. Preparation of novel sol-gel lipases by designed bioimprinting

T6. It was verified that rationally selected substrate mimicking molecules resembling to those found in experimental X-ray structures of lipases showed the most pronounced imprinting effects. Lauric acid was the most effective imprinting additive for Lipase PS (the crystal structure of lipase from *Burkholderia cepacia* included stearic acid) while in case of *Candida* species (CaLB, CrL) tetraethylene glycol dodecyl ether (BRIJ 30) exhibited the most significant imprinting effect (the porcine pancreatic lipase resembling similarity to CrL included tetraethylene glycol octyl ether and the crystal structure of CaLB included methylpenta(oxyethyl)heptadecanoate).

T7. It was concluded that the high efficiency of sol-gel systems containing trialkoxysilanes OcTEOS or PhTEOS may be explained by assuming these silanes or their partially hydrolyzed forms have an imprinting effect as well. This assumption can explain why Lipase AK (lipase from *Psudomonas fluorescens*) required no imprinting additives for maximal performance.

T8. A novel method for testing the biocatalytic properties of the immobilized lipases was developed based on enentioselective GC analysis of the lipase-catalyzed acylation reactions of racemic multisubstrate mixtures in batch and in continuous-flow reactors. It was the first application of multisubstrate systems for comparison of productivity and selectivity of sol-gel entrapped enzymes.

T9. The biocatalytic usefulness of the two most selective sol-gel entrapped lipases (Lipase AK, 2× loading in TEOS:PhTEOS:DMDEOS 4:1:1 system without additive and CaLB, 2× loading in TEOS:PhTEOS:DMDEOS 4:1:1 system with BRIJ) was confirmed by the kinetic resolution of racemic 1-(thiophen-2-yl)ethanol (*rac*-**1h**) in batch and continuous-flow systems reactors on preparative scale.

6 PUBLICATION LIST

Publications in international journals

A. Szydlowska-Czerniak, Gy. Karlovits, **G. Hellner**, Cs. Dianóczki, E. Szłyk, Effect of Enzymatic and Hydrothermal Treatments of Rapeseeds on Quality of the Pressed Rapeseed Oils PART I: Antioxidant Capacity and Antioxidant Content, *Process Biochemistry* 45, 2010, 7-17 (impact faktor: 2.648)

A. Szydlowska-Czerniak, Gy. Karlovits, **G. Hellner**, E. Szłyk, Effect of Enzymatic and Hydrothermal Treatments of Rapeseeds on Quality of the Pressed Rapeseed Oils PART II: Oil Yield and Oxidative Stability, *Process Biochemistry* 45, 2010,247-258 (impact faktor: 2.648)

G. Hellner, E.R**.** Tőke, V. Nagy. Gy. Szakács**,** L. Poppe, Integrated enzymatic production of specific structured lipid and phytosterol ester compositions *Process Biochemistry* 45, 2010, 1245-1250 (impact faktor: 2.648)

A. Tomin, D. Weiser, **G. Hellner**, Zs. Bata, L. Corici, F. Péter, B. Koczka, L. Poppe, Fine tuning the second generation sol-gel lipase immobilization with ternary alkoxysilane precursor systems *Process Biochemistry* 46, 2011,53-58 (impact factor: 2.648)

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