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Biocatalysis – From modeling to application

PhD Thesis

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Tomin A., Tőke E., Bara N., Poppe L.: Aminoxi csoportot tartalmazó átmeneti állapot analogonok szintézise PAL és TAL enzimekhez (Synthesis of aminoxy group containing transition state analogons for PAL and TAL enzymes), *13rd International Conference of Chemistry*, 11th - 13rd November **2007**, Cluj-Napoca, Romania, ISSN: 1843-6293 (Poster)

Tomin A., Kupai K., Hornyánszky G., Poppe L.: Enantiomer szelektív enzimatikus acilezés vizsgálata gyűrűs alkoholokon (Examination of the enzyme catalyzed enantiomer selective acetylation on cyclic alcohols), *Centenáriumi Vegyészkonferencia (Centennial Conference on Chemistry)*, 29th May - 1st June **2007,** Sopron, Hungary (Poster)

Poppe L., Pilbák S., Tomin A., Paizs Cs., Rétey J.: A MIO-csoportot tartalmazó ammónia-liáz /aminomutáz család. Mechanizmusvizsgálat és biokatalitikus felhasználás (The MIO group containing ammonia-lyase/aminomutase family), *Centenáriumi Vegyészkonferencia (Centenary Conference on Chemistry)*, 29th May - 1st June **2007,** Sopron, Hungary (Oral presentation co-author)

Tomin A.: Enantiomer szelektív enzimatikus acilezés vizsgálata gyűrűs alkoholokon (Examination of the enzyme catalyzed enantiomer selective acetylation on cyclic alcohols), *Doktoráns Konferencia (Conference for Doctoral Candidates), Budapest University of Technology and Economics*, 7th February **2007,** Budapest, Hungary (Oral presentation)

Pilbák S., Tomin A., Rétey J., Poppe L.: A fenilalanin ammónia-liáz vizsgálata számítógépes módszerekkel (Examination of phenylalanine ammonia-lyase with calculation methods), *11th International Conference of Chemistry*, November 11th - 13rd November **2005**, Cluj-Napoca, Romania, ISBN: 973-7840-07-0 (Oral presentation co-author)

Poppe L., Pilbák S., Tomin A.: Conformation of the Y100 loop in phenylalanine ammonia-lyase. Loop correction of the x-ray structures by modeling and molecular dynamics, *Molecular Modeling in Chemistry and Biochemistry, Workshop*, 21st - 23th April **2005**, Cluj-Napoca, Romania (Oral presentation co-author)

Poppe L., Pilbák S., Tomin A.: A fenilalanin ammónia liáz enzim Y110 bejárati hurok és a szubsztrát kötődés modellezése (Modeling of Y110 loop in phenyalalanine ammonia-lyase), *MKE QSAR, Kemometria és Molekulamodellezési Szakcsoportok Előadóülése (Proceedings of Specialised Group of Chemometry and Moleculamodeling)*, 7th - 8 th April **2005**, Szeged, Hungary (Oral presentation co-author)

Pilbák S., Tomin A., Poppe L., Rétey J., Lerchl A.: Homológia modellezés enzimmechanizmusok vizsgálatára (Homology modeling for examination of enzyme mechanisms), *MTA Terpenoidkémiai és Elemorganikus Munkabizottság előadóülése (Hungarian Academy of Sciences, Terpenoid Chemical and Element Organic Committee)*, 2th April, **2004**, Budapest, Hungary (Oral presentation co-author)

1. Introduction

The economic synthesis of the increasing number of biologically active molecules is one of the biggest challenge on the filed of organic chemistry. The preparation and the use of large purity enantiomers are obviously necessary for different industries (mainly like the pharmaceutical, plastic, cosmetic and food industry).[1,2,3,4] 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, despite it's enantiomer pair, the (S)-Thalidomide what is teratogenic even in small amount.[5]

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.

For instance a $65 million R&D contract was signed between the Novartis and the MIT to develop continuous-flow technologies for the drug industry. Furthermore test-works was built with continuous-flow reactors by one of the leading chemical industry company, the Degussa. The importance of the biocatalysis is shown in the industrial usage of the enzymes (eg. in the (S)-phenylethylamine production of the BASF or the oxiranyl methanol production of the DSM).

The new and well usable stereoselective processes are studied comprehensively in my PhD Thesis applying the prospects of the biocatalysis.

Our aim was to increase the knowledge about the mechanism of the ammonia- lyases with modeling work, the synthesis and enzyme kinetic examination of substrate analogues and inhibitors. The examination of batch and continuous-flow proccesses were planned in connection with lipase-catalyzed stereoselective acetylations as new synthetic application of highly selective biocatalyst systems. The dynamic kinetic resolution of racemic compounds were also planned in continuous-flow reactor system. In addition, sol-gel enzyme immobilization method was studied with lipases what increased the stability, activity and the selectivity of these enzymes.

2. Backgroud

2.1. The role of ammonia-lyases

Ammonia lyases catalyse the deamination of α-amino acids to α,β-unsaturated bonds (Scheme 1). Phenylalanine ammonia lyase (PAL, EC 4.3.1.24)[6] is an important plant enzyme which is the precursor of a great variety of phenylpropanoids. The related enzyme histidine

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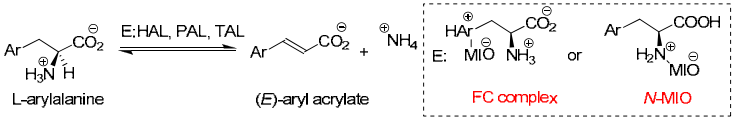
[3] Poppe, L., Novák, L. *Selective Biocatalysis: A Synthetic Approach* **1992** (VCH: Weinheim-New York).
[4] Faber, K. *Biotransformations in Organic Chemistry* 4. kiadás (Springer: Berlin) **2004**.
[5] Nishimura, H., Tanimura, T. *Clinical Aspects of The Teratogenicity of Drugs* **1976** (American Elsevier Publishing Company - New York).
[6] Ritter, H., Schulz, G. E. *Plant Cell* **2004**, *16*, 3426-3436.

ammonia lyase (HAL, EC 4.3.1.3)[7] catalyzes a very similar reaction, converting L-histidine into (*E*)-urocanic acid. Two competing mechanisms − both supported by experimental data − were suggested for the reaction of these enzymes. One is the Friedel-Crafts type reaction in which the MIO methylene is covalently bounded to the substrate aromatic moiety (*Retey* and *co-workors*)[8] and the other which involves an *N*-MIO intermediate (*Hanson* and *Havir*) (Scheme **1**).[9]



Scheme 1. Reaction and mechanism of ammonia lyases

The stereoconstructive nature of the reverse reaction (the enantioselective addition of ammonia to α,β-unsaturated acids) made this approach more attractive for the preparation of non-natural amino acids by biotransformations.

Due to the role of PAL and HAL in human organism, their abnormal activity play key role in inborn and deficiency diseases.

2.2. Lipase-catalyzed kinetic resolution

Hydrolases are the most widely used enzymes in synthetic organic chemistry.[10,11] Hydrolases, especially lipases, are particularly suited to performing stereoselective biotransformations, such as kinetic resolutions, deracemizations and dynamic kinetic resolutions.[12] The vast majority of the enzymatic enantioselective processes was performed in batch mode. Consequently, the main advantages of the flow-through approach − such as facile automation, reproducibility, safety, and process reliability − are not much exploited at research phase. Continuous-flow lipase-catalyzed kinetic reaolution allow the rapid preparation of compounds with minimum workup.[13]

Esterification and transesterification reactions are commonly employed in industry using acids as catalysts at high temperature (100–300 8C) and pressure, which result in poor reaction selectivity, undesirable side products and low yields. The use of triacylglycerol lipases in chemical processes offers better quality of products, and the process can be more effective due to higher selectivity and fewer environmental problems.[14]

2.3. Dynamic kinetic resolution in continuous-flow mode

[7] Poppe, L., Rétey, J. *Angew. Chem. Int. Ed.* **2005**, *44*, 3668-3688.
[8] a) Rétey, J. *Naturwissenschaften* **1996**, *83*, 439-447.; b) Langer, M., Pauling, A., Rétey, J. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1464-1465.; c) Schuster, B., Rétey, J. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 8433-8437.
[9] a) Givot, J. L., Smith, T. A., Abeles, R. H. *J. Biol. Chem.* **1969**, *244*, 6341-6353.; b) Hanson, K. R., Havir, E. A. Arch. Biochem. Biophys. **1970**, *141*, 1-17.; c) Hodgins, D. S. J. Biol. Chem. **1971**, *246*, 2977-2985.
[10] Bornschauer, U. T., Kazlauskas, R. J. *Hydrolases in Organic Synthesis: Regio- and Stereoselective Biotransformations* **2006** (Wiley-VCH: Weinheim-New York).
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[12] Turner, N. J. *Curr. Opin. Chem. Biol.* **2004**, *8*, 114-119.
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V. Tomin A., Weiser D., Hellner G., Bata Zs., Corici L., Péter F., Koczka B., Poppe L.: Fine tuning the second generation sol-gel lipase immobilization with ternary alkoxysilane precursor systems, *Process Biochem.*, **2010**, in press. (IF: 2,444) (doi:10.1016/j.procbio.2010.07.02)

VI. Tomin A., Weiser D., Bata Zs., Corici L., Péter F., Poppe L.: Entrapment of lipases in novel sol-gel systems, *Stud. Univ. Babes-Bolyai Ser. Chem.*, **2010**, in press. (IF: 0,086)

Further publications:

Molnár P., Thorey P., Bánsághi Gy., Székely E., Poppe L., Tomin A., Kemény S., Fogassy E., Simandi B.: Resolution of racemic *trans*-1,2-cyclohexanediol with tartaric acid, *Tetrahedron: Asymmetry,19*, **2008**, 1587-1592. (2,796)

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Tomin A., Boros Z., Szigeti M., Weiser D., Kovács P., Darvas F., Ürge L., Poppe L.: Lipase-catalysed asymmetric acylation of prochiral 1,3-diols in continuous-flow bioreactors, *BioTrans*, 5-9. July **2009**, Bern, Switzerland (Poster)

Tomin A., Dorkó Z., Hornyánszky G., Weiser D., Darvas F., Ürge L., Poppe L.: Lipase-catalysed kinetic resolution of cyclic secondary alcohols in continuous-flow bioreactors, *BioTrans*, 5-9. July **2009**, Bern, Switzerland (Poster)

Tomin A., Weiser D., Bata Z., Corici L., Péter F., Poppe L.: Entrapment of lipases in novel sol-gel systems, *15th International Conference in Chemistry*, 12th-15 th November **2009**, Marosvásárhely, Romania, ISSN 1843-6293 (Oral presentation)

Boros Z., Csajági C., Szatzker G., Szigeti M., Tomin A., Ürge L., Darvas F., Poppe L.: Selective biotransformations in continuous flow reactors, *15th International Conference in Chemistry*, 12 th -15 th November **2009**, Marosvásárhely, Romania, ISSN 1843-6293 (Oral presentation co-author)

Csajági C., Szatzker G., Szigeti M., Tomin A., Tőke ER., Pilbák S., Ürge L., Darvas F., Poppe L.: Selective biotransformations in continuos flow reactors, *4th Central European Conference*: *Chemistry towards Biology*, 8th -11th September **2008** Dobogókő, Hungary (Oral presentation co-author)

Tomin A., Corici L, Ősze M., Wootsch A., Peter F., Poppe L.: Investigation of esterification reaction catalysed by sol-gel immobilized enzymes*, International Congress on Biocatalysis*, 31st August - 4th September **2008** Hamburg, Germany, ISBN: 978-3-930400-74-4 (Poster)

Csajági C., Szatzker G., Szigeti M., Tomin A., Tőke ER., Pilbák S., Ürge L., Darvas F., Poppe L.: Selective biotransformations in continuos flow reactors, *International Congress on Biocatalysis*, 31st August - 4th September **2008** Hamburg, Germany, ISBN: 978-3-930400-74-4 (Oral presentation co-author)

Pilbák S., Holczinger A., Sztancsik K., Tomin A., Rétey, J., Poppe, L.: Phenylalanine and tyrosine ammonia-lyase. Enzyme mechanism and stability, *International Congress on Biocatalysis*, 31st August - 4th September **2008** Hamburg, Germany, ISBN: 978-3-930400-74-4 (Poster)

Tomin A., Holczbauer T., Tőke E., Bara N., Poppe L.: L-Fenilalanin aminoxi analogonjainak új szintézise (Novel synthesis of aminoxy analogons of L-phenylalanine),

4. The main points of the thesis

1. It was verified, that in the reactions catalyzed by the ammonia-lyasas the *N*-methyl-L-phenylalanine is a week substarte, the *N*-methyl- and the *N,N*-dimethyl-4-nitro-L-phenylalanines are strong competitive inhibitors.[I]
2. The role and the active conformation of the Tyr-loop in the PAL reaction was interpreted. The stability of bacterial PAL was evaluated by molecular mechanics and dynamics studies.[II]
2. It was revealed, that the course of the lipase-catalyzed acylation reaction was influenced by the ring size, the size of the substituent adjacent to the asymmetric center and the hybrid state a of the neighboring carbon also.[IV]
3. It was proved, that the selectivity of lipase-catalyzed acylation reaction in batch mode (shake flask) was similar to the results in continuous-flow mode. It was demonstrated, that the productivities of the lipase-catalyzed kinetic resolution of secondery alcohols were significantly higher than in the usual batch mode reactions.[IV]
4. A novel dynamic kinetic resolution method was performed in a cascade system with continuous-flow reactors. The 1-phenylethanol, the simplest aromatic secondary alcohol and the 1-phenylethylamine, a typical racemic secondary amine were chosen as model compounds.[III]
5. Imporvement of the sol-gel immobilization method of lipases were succsessfully developed. The new biocatalysts prepared by our immobilization methods showed better catalytic properties then the commercial lipases prepared by similar methods.[V, VI]

5. Application

Application of enzyme-catalyzed processes can been widely utilized for synthetic purposes without production of toxic side-products. Hydrolases are preferable biocatalysts because their wide substrate specifity. Consequently, replacement of synthetic organic chemistry methods can be achieved with biotransformations.

The main advantages of the flow-through approach: facile automation, reproducibility, safety, and process reliability.

6. Publications

List of publications

I. Viergutz S., Poppe L., Tomin A., Rétey J.: Mechanistic Investigation of Phenylalanine Ammonia Lyase by Using *N*-Methylated Phenylalanines, *Helv. Chim. Acta*, **2003**, *86*, 3601-3612. (IF: 1,861)

II. Pilbák S., Tomin A., Rétey J., Poppe L.: The essential Tyr-containing loop conformation and the role of the C-terminal multihelix region in eukaryotic phenylalanine ammonia-lyases, *FEBS J.,* **2006**, *273(5)*, 1004-1019. (IF: 3,033)

III. Poppe L., Tomin A., Boros Z., Varga E., Ürge L., Darvas F.: Új dinamikus kinetikus reszolválási eljárás, (Novel method for dynamic kinetic resolution) *Hungarian Patent Application*, P0900720, **2009**.

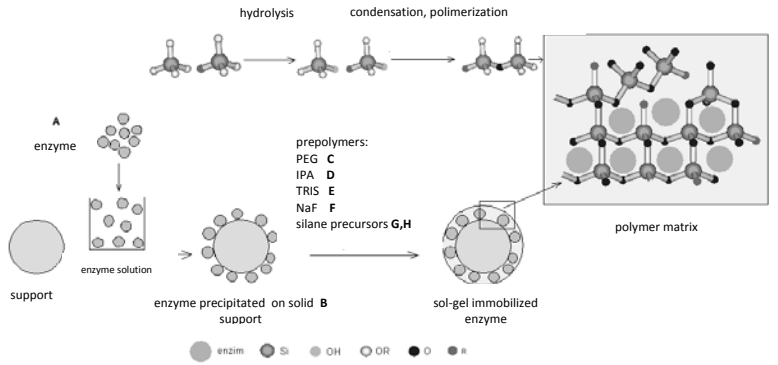
IV. Tomin A., Hornyánszky G., Kupai K., Dorkó Z., Ürge L., Darvas F., Poppe L.: Lipase-catalyzed kinetic resolution of 2-methylene-substituted cycloalkanols in batch and continuous-flow modes*, Process Biochem.*, **2010,** *45*, 859-869. (IF: 2,444)

In the point of the resolution the dynamic kinetic resolution (DKR) is a modern and effective method. This is a combination of a resolution and an *in situ* racemization. As a result of this process the desired enantiomer can be prepared with quantitative yield. The optimal parameters (eg. temperature, pressure, the amount of the catalyst, reaction time, mixing parameters, mass transfer) can be different in continuous-flow mode. The methods in a same reactor chamber can be partially optimized (until the system reaches the mutual optimum), the catalysts can be changed only together (the lifetime of the catalysts could be highly different).

So far the single steps of the continuous-flow DKR (resolution and racemization) were operated in the same circumstances, in the same chamber of the reactor.[15,17] In that case the optimization possibilities were strongly restricted by each other. The authors also experimented with the spacial separation of the two DKR steps, however, continuous-flow reactors were only applied for the racemization process and the enantiomer separation was performed in a backflow reactor.[18]

2.4. Improvement of the sol-gel encapsulation of lipases

Sol-gel encapsulation has proven to be a particularly easy and effective way to immobilize purified enzymes, whole cells, antibodies and other proteins. Sol-gel immobilization of lipases can enhance their thermostability, long-term operational stability and storage life (Scheme 2).[19,20,21,22]



Scheme 2. The sol-gel encapsulation process

[15] Itoh, N., Nakamura, M., Inoue, K., Makino, Y. *Appl. Microbiol. Biotech.* **2007**, *75*, 1249-1256.
[16] Lozano, P., De Diego, T., Gmouh, S., Vaultier, M., Iborra, J. L. *Int. J. Chem. React. Eng.* **2007**, *5*, A53.
[17] Truppo, M. D., Pollard, D. J., Moore, J. C., Devine, P. N. *Chem. Eng. Sci.* **2008**, *63*, 122-130.
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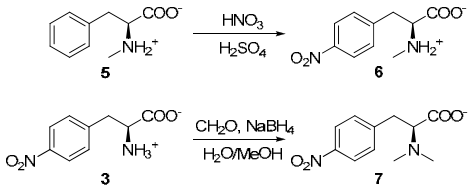
A typical sol-gel immobilization process involves acid-or base-catalyzed hydrolysis, then polycondensation of alkoxysilane precursor [$Si(OR)_4$] in the presence of additives to form a matrix in which the enzyme is encapsulated (Scheme 2). The improvement of entrapment of lipases in hydrophobic sol-gel materials results highly active, stable and reusable heterogeneous biocatalysts.[23]

3. Results and discussion

3.1 Mechanistic investigation of ammonia lyases

Our aim was to prepare phenylalanine analouges and investigate as substrates or inhibitors to provide further experimental evidence for the mechanism of ammonia lyases.

N-Methyl-L-phenylalanine (**5**), *N*-methyl-4-nitro- -phenylalanine (**6**), and *N,N*-dimethyl-4-nitro-L-phenylalanine (7) were synthesized by conventional nirtation and reductive alkylation methods (
Scheme **3**).



Scheme 3. Synthesis of *N*-methyl-4-nitro-L-phenylalanine (**6**), and *N,N*-dimethyl-4-nitro-L-phenylalanine (**7**)

According to the enzymekinetic measurements *N*-Methyl-L-phenylalanine (**5**) behaved as substrate, *N*-methyl-4-nitro-L-phenylalanine (**6**) and *N,N*-dimethyl-4-nitro-L-phenylalanine (7) behaved as competitive inhibitors.[24] Despite of these intresting results, the mechanism can not be elucidated by these results.

The structure and behavior of PAL were investigated by different calculation methods in its active site also.[25] The recently published X-ray structures of PAL revealed that the catalyticly important Tyr110-loop was either missing or far from the active site („loop-out", for *Petroselinum crispum*) (
., A).[6] Based on these preliminaries, a Tyr110-loop-in conformation („loop-in") of the *P. crispum* PAL structure was constructed by partial homology modeling. The static and dynamic behavior of the loop-in / loop-out structures and binding properties (*N*-MIO or Ar-MIO) of the substrates were investigated by molecular dynamics studies. As expected, the Tyr110-loop-in model turned out to be conformationally stable. To study the role of the C-terminal multi-helix domain, Tyr-loop-in / loop-out model structures of two bacterial PALs lacking this C-terminal domain were also built (Scheme 4., B). Results indicated, that Tyr-loop-in conformation was more rigid without the C-terminal multi-helix domain. On this basis it is hypothesized that a role of this C-terminal extension is to decrease the lifetime of eukaryotic PAL by

[23] Péter, F., Zarcula, C., Kiss, C., Csunderlik, K., Poppe, L. *J. Biotechnol.* **2007**, *109*, 131.
[24] Viergutz S., Poppe L., Tomin A., Rétey J. *Helv. Chim. Acta*, **2003**, *86*, 3601-3612.
[25] Pilbák S., Tomin A., Rétey J., Poppe L. *FEBS J.*, **2006**, *273(5)*, 1004-1019.

catalytic behavior in enantiomer selective acetylation of racemic 1-phenylethanol (*rac*-**20a**). For comparison, the acylation of racemic 2-heptanol (*rac*-**35**) 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 *rac*-**20a**.

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

[28] Tomin A., Weiser D., Bata Zs., Corici L., Péter F., Poppe L. *Stud. Univ. Babes-Bolyai Ser. Chem.*, **2010**, nyomdában.

In the system built up to racemic 1-phenylethanol the A_1 and A_5 resolution units were contained 3-3 cascade CaLB-CatCart^{TM} columns (70°C). The A_2, A_3, A_4 units were contained only 2-2 columns. 1-1 V_2O_4- CatCart^{TM} (65°C) column was used at the B_1-B_4 units. After obtaining steady state the fractions were collected at 0.5 ml×min^{-1} flow rate. In these fractions bis-(1-phenylethyl)ether was isolated as a byproduct (23%) besides the 10% remaining (*S*)-1-phenylethanol [(*S*)-**20a**]. The (*R*)-phenylacetate was obtained with 63% yield after purification by column chromatography. Differ from the others the first resolution unit (3 cascade CaLB columns) was heated to 30°C at this test. When these units were heated to 70°C, the enatiomer purity of the main product [(*R*)-**21a**] was decreased as expected (61% conversion, ee(*R*)-**21a**=91,5%). Based on the experience of the 1-phenylethanol (*rac*-**20a**) the dynamic kinetic resolution of the racemic 1-phenylethylamine (*rac*-**33**) was performed successfully with the cascade system. The A_1 resolution contains three, the $A_{2\text{-}5}$ units involve two-two CaLB^{TM} columns connected in line, all operated at 70°C. Raney-nickel CatCart^{TM} columns operated at 60°C were used as racemization units. The best results were obtained with toluene solvent, the flow rate was set to 0.2 mL/min according to prior experiments. When the steady state was achieved, the collected fractions gave 61% (*R*)-*N*-(1-phenylethyl)acetamide [(*R*)-**34**, ee(*R*)-**34**=94.5%] as main product. The same product was obtained with lower conversion values [(*R*)-**34**, 59% ee(*R*)-**34**=96.5%] when the resolution units were operated at 30°C.

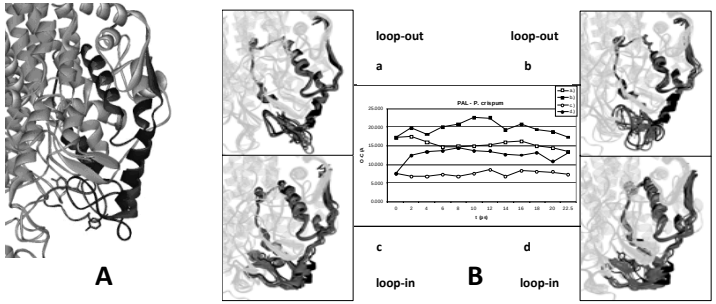
3.4 Improvement of the catalyctic properties of biocatalysts

According to our previous results,[23] 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 biner and ternary systems.[27] Binary preparations were made from alkyltriethoxy- (alkylTEOS) and tetraethoxysilane (TEOS) mixtures at constant molar ratio, and ternary preparations were preformed 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 enhance the catalytic activity of the biocatalyst and the size distribution of solid support remain 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 és 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

[27] Tomin A., Weiser D., Hellner G., Bata Zs., Corici L., Péter F., Koczka B., Poppe L. *Process Biochem.*, **2010**, doi:10.1016/j.procbio.2010.07.02

destabilization, which might be important for the rapid responses in the regulation of phenylpropanoid biosynthesis.



Scheme 4. A) The modified P. crispum PAL structure (1W27mod, grey) overlaid on *P. crispum* PAL crystal structure (1W27; black) B) Molecular dynamics calculations on the Tyr110-loop region of *P. crispum* PAL structures; Comparison of the Tyr110 region in loop-out *P. crispum* PAL (1W27) A) at 300 K, B) at 370 K, and in the modified loop-in *P. crispum* PAL (1W27mod) C) at 300 K and D) at 370 K.

For further investigation of the mechanism, the synthesis of 2-aminooxy or 2-sulfanil-3-phenylpropanoic acid as target inhibitors (bound *via* nitrogen or sulfur to MIO) of PAL were also worked out. The purification and cloning of PAL is in progress.

3.2 Lipase-catalyzed kinetic resolution in batch and continuous-flow system

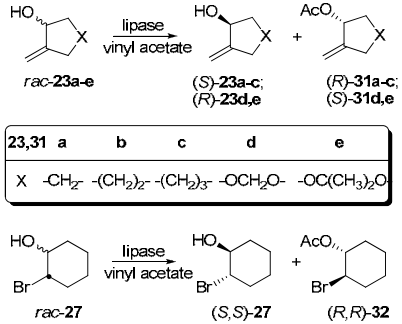
Our major aim was to synthesize cyclic racemic secondary alcohols (2-methylenecyclopentan-1-ol *rac*-**23a**, 2-methylenecyclohexan-1-ol *rac*-**23b**, 2-methylenecycloheptan-1-ol *rac*-**23c**, 6-methylene-[1,3]dioxepan-5-ol *rac*-**23d**, 2,2-dimethyl-6-methylene-[1,3]dioxepan-5-ol *rac*-**23e** and trans-2-bromocyclohexan-1-ol *rac*-**27**) to investigate the effect of the size and bulkiness of the ring on enantiomer selectivity on kinetic resolution reactions in batch and continuous-flow mode (Scheme **5**).[26]

The lipase-catalyzed acetylations of secondary alcohols (*rac*-**23b**, *rac*-**23d** and *rac*-**27**) were compared in batch mode and continuous-flow reactions.**Hiba! A könyvjelző nem létezik.** To demonstrate the synthetic applicability of these processes, the best performing lipase-catalyzed kinetic resolutions were performed on a preparative scale in batch and continuous-flow modes. The racemic secondary alcohols were screened with different lipases in 1:1 molar ratio using vinyl acetate as the acyl donor in hexane-THF solvent in batch mode. The effects of conversion, enantiomeric selectivity and specific reaction rate on the batch and continuous-flow mode biotransformations were investigated.

Most of the hydrolases exhibited the highest enantiomer selectivity in kinetic resolution of the five- and six-membered ring substrates. In the case of the seven-membered ring substrate the activity and enantiomer selectivity decreased as the ring size increased. Interestingly,

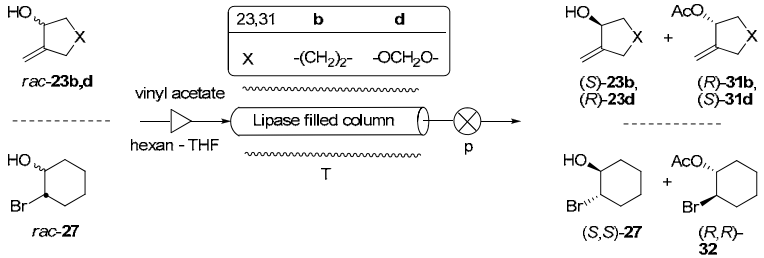
[26] Tomin A., Hornyánszky G., Kupai K., Dorkó Z., Ürge L., Darvas F., Poppe L. *Process Biochem*, **2010,** *45*, 859-869.

introduction of oxygen atoms into the sevenmembered ring increased the rate and the stereoselectivity of the reaction. Further increases in the bulkiness of the seven-membered ring as an isopropylidene ketal in *rac*-**23e** resulted in a significant decline in the activity and enantiomer selectivity of the enzymatic acylation. Comparison of the acylation reactions of the 2-methylene-substituted six-membered cyclic alcoholto *trans*-2-bromocyclohexane-1-ol indicated, that the methylene-substituted compound *rac*-**23b** reacted significantly faster, while the stereoselectivities were almost the same for both. In conclusion, the course of the lipase-catalyzed reactions was influenced not only by the size of the substituent adjacent to the asymmetric center, but also by the hybrid state of the neighboring carbon.



Scheme 5. Lipase-catalyzed kinetic resolution of racemic cyclic allylic alcohols *rac*-**23a–e** and trans-2-bromocyclohexan-1-ol ***rac*-27**

The continuous biotransformations were performed in enzyme-filled, heat- and pressure-resistant stainless steel columns (CatCart^{TM}) using a continuous-flow bench-top lab reactor system (X-Cube^{TM}) (Scheme **6**). This system allows the precise control and variation of flow rate, temperature and back pressure. It had been shown earlier that pressure has only a negligible effect on lipase-catalyzed kinetic resolutions therefore, only the temperature (20–60°C) and flow rate (0.1–0.3 ml×min^{-1}) were varied. The main advantages of packed bed continuous system over the batch reaction: enantiopure samples can be obtained much faster and ther is no need for further filtration of the enzyme.

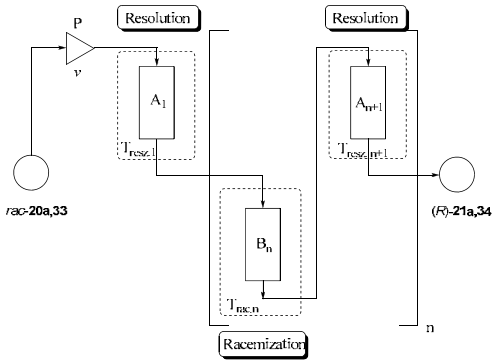


Scheme 6. Lipase-catalyzed kinetic resolution of racemic 2-methylenecyclohexan-1-ol (*rac*-**23b**), 6-methylene-[1,3]dioxepan-5-ol (*rac*-**23d**) and trans-2-bromocyclohexan-1-ol (*rac*-**27**) in a continuous-flow reactor system [▷: pump; ∼∼: temperature control unit; ⊗: pressure regulation valve].

Results indicated that the selectivity in batch mode (shake flask) was similar to the results in continuous-flow mode (enzyme-filled stainless steel bioreactor). The temperature (20–60 °C) and flow rate (0.1–0.3 ml×min^{-1}) mainly affected the productivity (*r*) of the continuous-flow acetylations (*rac*-**23b**, *rac*-**23d** and *rac*-**27**), without significant alteration of the enantiomeric selectivities (*E*), which were similar in the continuous-flow and batch modes. Under proper continuous-flow mode conditions in the preparative scale reactions (50 °C, 0.2 ml×min^{-1}) the productivities (*r*) of the biotransformations were significantly higher than in the usual batch mode reactions (room temperature, 6–24 h) in all cases.

3.3 Development of a novel dynamic kinetic resoltuion method

The dynamic kinetic resolution (DKR) experiments were performed in a cascade system with continuous-flow reactors (n=1,2,...∞) operated at various temperatures ($T_{resz,1}$ - $T_{resz,n+1}$ és $T_{rac,1}$ - $T_{rac,n}$) at the same flow rate (*v*). The resolution took place in the units A_1-A_{n+1}, while the racemization of the substrate was carried out in units B_1-B_n, as depicted in Scheme 7. In order to achieve steady state, eight times higher solvent volume must flow through the reactors than the total dead volume of the system. The 1-phenylethanol (*rac*-**20a**), the simplest aromatic secondary alcohol and the 1-phenylethylamine (*rac*-**33**), a typical racemic secondary amine were chosen as model compounds in our experiments. The stereoselective acetylation of the substrates (*rac*-**20a**,**33**) was performed *via* enzyme [Candida antartica lipase B (CaLB)] catalysis, the substrate residues were racemized through chemical routes.



Scheme 7. Dynamic kinetic resolution in continuou-flow cascade system

The resolution and the racemization can be separately heated. That way independent blocks were made for the better optimization. The solution of the racem starting materials (*rac*-**20a**, *rac*-**33**) and the acylation agents were flown through the system. The main products are the acetate [(*R*)-**21a**] and the acetamide [(*R*)-**34**)] with (*R*)-configuration.
In the case of the 1-phenylethanol the DKR was made with CaLB and V2O4 catalysts, whilst the CaLB and Ra-Ni catalysts were used at the 1-phenylalanine. Hexane:Ethyl Acetate=9:1 was used as a solvent (the EtOAc was the acylation agent too).