Study of Organic Solvent Hydrophobicity on Lipase Catalyzed Reaction Esterification

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Dedicated to Prof. Dr. Đurđa Vasić-Rački on occasion of her 60th birthday

The paper studies the effect of hydrophobicity of nonpolar organic solvents (cyclohexane, *n*-hexane and isooctane) on lipase-catalyzed esterification of glycerol with oleic acid catalysed by immobilized 1,3-specific *Mucor miehei* lipase. The esterification was carried out with and without molecular sieves in a batch stirred-tank reactor (BSTR).

Enzyme selectivity was in function of solvent hydrophobicity and related to the system with molecular sieves, where the equilibrium was shifted toward the production of diolein.

Key words:
Solvent hydrophobicity, lipase-catalyzed esterification, molecular sieves, long-chain fatty acid esters

Introduction

The use of enzymes in organic media with low water content has been one of the most exiciting facets of enzymology in recent times.[1–5]

Water is necessary for the catalytic function of enzymes, because it participates in all noncovalent interactions that maintain the conformation of the catalytic site of enzymes. Removal of all water from enzyme surroundings should drastically distort its conformation and results in enzyme deactivation. The role of water becomes even more important when one focuses on enzyme in low water media. It has been observed that the water present on the enzyme correlated well with the reaction rates in different solvents. The nature of the solvents is crucial for maintaining the layer of essential water around enzyme. The most hydrophobic solvents, such as hydrocarbons, are suitable for this purpose, because the less hydrophobic the solvent, the higher its affinity for water and essential water is stripped from the enzyme.[6–9] Furthermore, the effects of solvents on enzyme selectivity also differ for a given substrate with different enzymes, and for a given enzyme with different substrates.[10] The nature of the solvent media affected both the enantio- and regioselectivity of enzymes.

It is also possible to shift the hydrolysis-esterification equilibrium towards ester formation because most employed lipases are quite stable in organic solvents. The control of water activity during the reaction is crucial. The yield of lipase-catalyzed esterification can be increased as follows: a) by adding adsorbents such as molecular sieves, alumina, silica gel, and zeolites;[11,12] b) by increasing solvent hydrophobicity.

Hydrophobicity is generally measured by the log *P* of the partition coefficient of the organic solvent in an octanol/water two-phase system (log *P*). The solvents with log $P > 3$ are more suitable for esterification of glycerol and oleic acid because of good solubility of reactant (oleic acid) and products (glycerides).

In this work, we studied the effect of hydrophobic organic solvents on enzyme selectivity in esterification catalyzed by 1,3-specific immobilized lipase from *Mucor miehei* in a batch stirrer-tank reactor (BSTR), and the effect of molecular sieves in cyclohexane, *n*-hexane and isooctane on monoolein (MO) and diolein (DO) production. Under optimum conditions, the maximum yield of MO, DO and the residual amount of oleic acid were determined chromatographically.

Experimental

Materials

Commercial immobilized lipase from *M. miehei*, *Lipozyme* (Fluka (Buchs, Switzerland) was selected to catalyze the reactions. *Lipozyme* is the registered Trademark of Novo Nordisk AS, which was measured lipase activity. The activity of *Lipozyme* used for our syntheses was 62 batch interesterification units (BIU) g. One BIU corresponds to the amount of enzyme which liberates 1

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μmole oleic acid per minute at pH 8.0 and 40 °C (triolein was used as substrate).[13,14]

Glycerol was purchased as a technical product of w = 95 % purity from Kemika (Zagreb, Croatia); *N*,*N*-dimethylformamide (DMF), acetone, *n*-butanol chloroform, cyclohexane, *n*-hexane, isooctane, oleic acid (w = 65 %), and silylating mixture according to Sweeley (Ψhexamethyldisilazane/trimethylchlorosilane/pyridine) = 2:1:10 [15] were purchased from Fluka (Buchs, Switzerland); 5Å molecular sieves (activated) were from Supelco (PA, Bellafonte, USA). Anhydrous sodium sulphate in form of powder was purchased from Baker (Deventer, Holland).

Oleic acid, nonadecanoic acid, 1-monooleyl--*rac*-glycerol, 2-monooleylglycerol and diolein (mixed isomers of χ = 85 % 1,3-and χ = 15 % 1,2-isomer), as standards, were purchased from Sigma (St.Louis, MO, USA). The standard solutions in chloroform were used for calibration of the individual retention time, and the stock solution for the determination of relative retention times of lipolysis products in relation to nonadecanoic acid. Nonadecanoic acid (γ = 1.3 mg ml^{-1}) was added into the sample before silylating and used as the internal standard.

Equipment and procedure

LR-A 250 IKA laboratory reactor with DTM 11 IKATRON temperature control was filled with an equimolar concentration of reactants. Total reaction volumes were 75 ml.

Reactions were carried out at T = 25, 37 and 50 °C for 45 h under atmospheric pressure, γ = 6.67 mg ml^{-1} of *Lipozyme* and χ = 0.32 mol mol^{-1} *n*-hexane. The effects of various organic solvents such as *N*,*N*-dimethylformamide (DMF), acetone, *n*-butanol, chloroform, cyclohexane, hexane and isooctane (log P: –1.01 to 4.00) on the enzymatic esterification reactions with 6.67 mg ml^{-1} of *Lipozyme* and 0.32 mole mole^{-1} of solvents were investigated in preliminary examinations. Initial rates of esterification reactions by *Lipozyme* in solvent system were determined after 2 h of starting reaction expressed in ν / mmol L^{-1} h^{-1}.

Molecular sieves (40 mg ml^{-1}) were added into reactions which was carried out at 37 °C during 45 h under atmospheric pressure, 6.67 mg ml^{-1} of *Lipozyme* and 0.32 mol mol^{-1} cyclohexane, hexane and isooctane. The assay of enzyme reaction was monitored by the determination of free oleic acid and MO with chromatographic methods in organic solvents phase. Free oleic acid and monoolein were converted into their trimethylsilylether (TMS) derivatives and analyzed by gas chromatography.

Chromatographic analyses of MO and free oleic acid were performed using an Autosystem XL (Perkin Elmer, Norwalk, CT, USA) gas chromatograph with flame-ionization detector and a split injection system (split ratio 1:100). SPB-5 capillary column (Supelco, Inc., Bellafonte, PA, USA), 30 m long, 0.53 mm i.d., 0.5 μm film thickness was used. The analyses were carried out in the programmed temperature condition from 180 °C to 270 °C, with rate temperature change of 8 °C min^{-1} and then isothermal for 65 min. Detector temperature was 300 °C and injector temperature was 290 °C. Chromatography software from Perkin Elmer Nelson (Turbochrom 4 – rev. 4.1.) was used for data acquisition from the FID. Hydrogen was obtained by the Claind hydrogen generator. The results were expressed as the concentration of oleic acid and monoolein. The concentrations of diolein were calculated by molar balance using the measured oleic acid and 2-monoolein concentrations.

The calculated concentrations of diolein were confirmed by high-performance liquid chromatographic (HPLC) analyses using an HPLC TSP Spectra System with P2000 gradient binary pump, SCM1000 vacuum membrane degasser, Rheodyne injector 7725i, loop 20 μl, refractive index detector Spectra System RI-150. Chromatography software from TSP (PC 1000) was used for data acquisition from RI detector.

Results and discussion

This study examined the effect of organic solvent hydrophobicity on the lipase selectivity in the production of mono– and diolein in esterification in a batch stirred-tank reactor (BSTR). In order to show the effect of solvent hydrophobicity on this kind of reaction, a model lipase-catalyzed reaction esterification of glycerol with oleic acid in a solvent system with 1,3-specific lipase from *Mucor miehei*, was chosen.

For 1,3-selectivity, we supposed that triolein was not produced during esterification. Chromatographic analyses confirmed the enzyme 1,3-selectivity and the absence of 2-monoolein. Taking the above into consideration, the scheme of our reactions, in equimolar ratio of glycerol and oleic acid, can be shown as major:

i) glycerol + oleic acid $\rightleftharpoons$ 1(3) monoolein (+H_2O)

ii) 1(3) – monoolein + oleic acid $\rightleftharpoons$ 1,3 – diolein (+H_2O)

iii) glycerol + 1,3 – diolein $\rightleftharpoons$ 2 (1(3) – monoolein)

Lortie et al. postulated that, when 2-monoolein is present in the mixture, a 1,3-specific enzyme in

esterification reactions is able to esterify this kind of molecule in the free 1 and 3 positions leading to triolein synthesis.[16]

The major advantage of the use of organic media is the increase of efficiency in the conversion of hydrophobic substances, it can be accomplished due to the increased substrate solubility in organic solvents compared to aqueous solutions. In an organic reaction medium enzymes indicate several interesting properties like enhanced (thermal) stability and different substrate and stereospecificities.[17] Organic solvents modify the catalytic activity of an enzyme through direct molecular solvent-enzyme interactions.

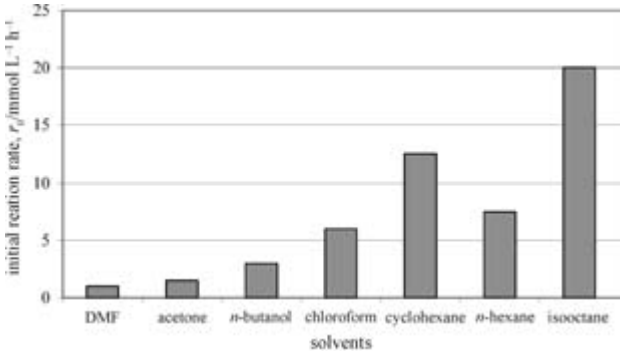
The decreased intramolecular mobility of enzymes in anhydrous organic solvents is often used to explain increased thermal stability and the maintenance of conformations inducted by high concentrations of ligands in aqueous solution.[18] The lower activity of enzymes in anhydrous media might be the result of the restricted flexibility.[19] The activation of lipases has been ascribed to interfacial activation that involves a conformational change of lipase at the water-lipid or water-insoluble-substrate interface in reaction media.[20,21]

In order to select appropriate conditions under which to use lipases as catalysts for ester synthesis, it seemed necessary to study the effect of solvent hydrophobicity on lipase activity.

The following physical properties were considered as potential solvent descriptors: solvent-accessible non-polar saturated area, solvent-accessible non-polar unsaturated area, solvent-accessible polar saturated area, polarizability, dipole moment, log *P*, density, molecular volume, dipolarity/polarizability (π^*), hydrogen bond donor ability (α), and hydrogen bond donor ability (β).[22]

Preliminary examinations were carried out in solvent system; the use of solvent log *P* values lower than 3 (–1.0 for DMF, –0.23 for acetone, 0.80 for *n*-butanol, 2.0 for chloroform) and solvents higher than 3 (log *P* for cyclohexane is 3.35, for *n*-hexane is 3.86, for isooctane is 4.00). Initial rate measured after 2 h increased in the solvent system with higher log *P* than 3, as shows Fig.1. In a heterogeneous system, in our experiments, solvents with higher log *P* increased one of the substrate solubility (oleic acid) and products solubility (mono- and diacylglycerols). Better substrate and products solubility led to lower internal pore diffusional limitation and influenced the kinetics of immobilized enzyme.[23] We used these solvents in our further experiments.

Moreover, the effect of reaction temperature, 25 °C, 37 °C and 50 °C, was tested in order to find the optimal reaction temperature using *n*-hexane.



F i g . 1 – *Intitial rate measured after 2 h in solvent system with different solvents*

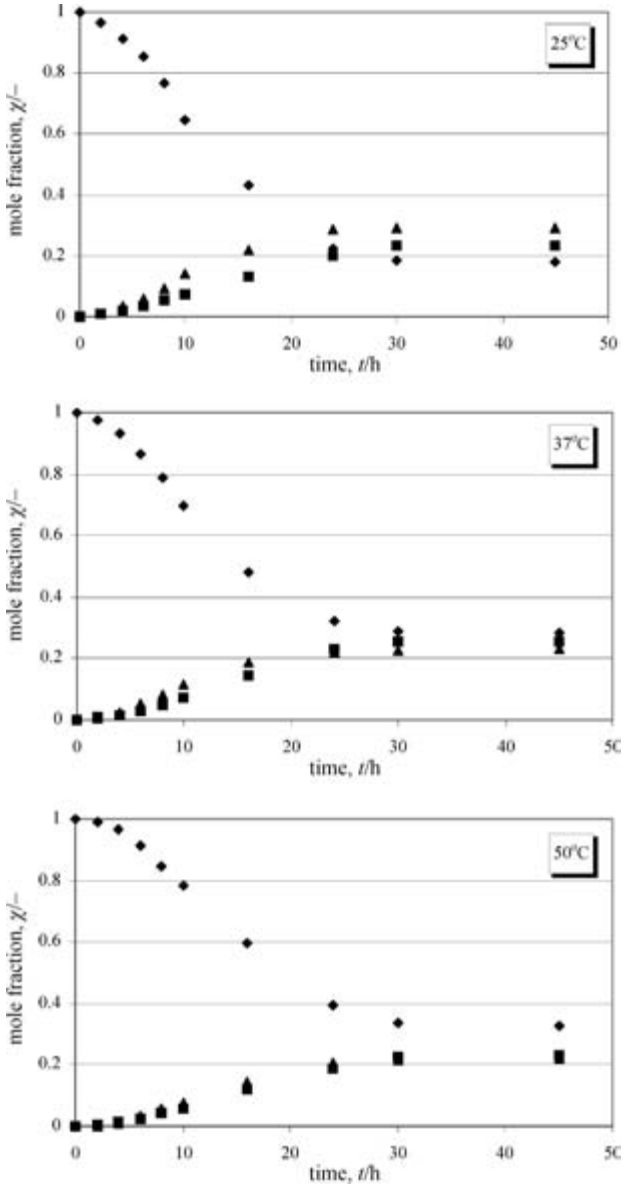
n-hexane was chosen on the basis of physical properties of used nonpolar solvents and substrate and products solubilities.[24,25,26]

New examples of temperature effects on enantioselectivity in the range of 20–60 °C clearly show that this quantity is a simple instrument with which a given lipase-catalyzed reaction can be optimised.[27,28,29] The conventional expectation that enantioselectivity increases upon lowering the temperature. It was speculated that at higher temperatures, the larger flexibility of the lipase favors preferred orientations of its amino acid side chains so as to associate more effectively with the phenyl ring of the substrate, the basis of such associations being OH···π and/or CH···π interactions[30].

The temperature effects on lipase-catalysed resolution were expanded to include the resolution of secondary alcohols, as well. *Rhizomucor miehei lipase* (RML) is a good enzyme for this resolution, yielding enantioselectivities as high as 102. In most lipase catalyzed reactions studied the E values increased by a factor of 2–3 when the temperature was lowered from 40 °C to –5 °C.[31]

Fig. 2 shows the time course of the lipase-catalyzed esterification of glycerol and oleic acid in solvent system using *n*-hexane at 25 °C, 37 °C and 50 °C. The results indicated lower residual oleic acid and higher diolein concentration at 25 °C compared to other examined reaction temperatures. These results were in consideration with mentioned literature.

When applying lipases in non-aqueous medium, it is important to consider the degree of residual water fraction, because this influences activity, thermostability, and stereoselectivity.[2,32–38] The key factor that distinguishes molecular-level details in different media is the partitioning of hydration water between enzyme and bulk solvents. The enzyme surface and the active site region are well hydrated in aqueous media, whereas with increasing polarity of organic solvent the hydration water is stripped. In contrast, water stripping in octane allows effi-



F i g . 2 – *The experimentally determined residual oleic acid, monoolein and diolein concentration in the esterification of oleic acid and glycerol catalyzed by immobilized lipase from Mucor miehei in the solvent system using n-hexane at different temperatures.*◆ *– oleic acid;* ■ *– monoolein;* ▲ *– diolein*

cient hydration of the active site uniformly by mobile and weakly bound water and some structural water similar to that in aqueous solution. These differences in the active site hydration are consistent with the inverse dependence of enzymatic activity on organic solvent polarity and indicate that the behaviour of hydration water on the enzyme surface and in the active site is an important determinant of biological function especially in low water media.[39,40] Some authors suggest that a single physicochemical parameter such as log *P* is incapable of satisfactorily predicting the biocompatibility of organic solvents, given complexity of enzyme deactivation in biphasic systems. Therefore, they recommend that instead of hydrophobicity of the solvent (log *P*), its functionality should be considered in previously conducted screening for a solvent.[41] Although, the organic solvents employed had very similar log *P*, the differences in the formation of 1(3)-monoolein and 1,3 diolein were observed.

On the basis of these results we chose cyclohexane, *n*-hexane and isooctane, and tested importance of the used solvents on enzyme selectivity in the presence of molecular sieves, for the purpose of water reduction as the esterification product.

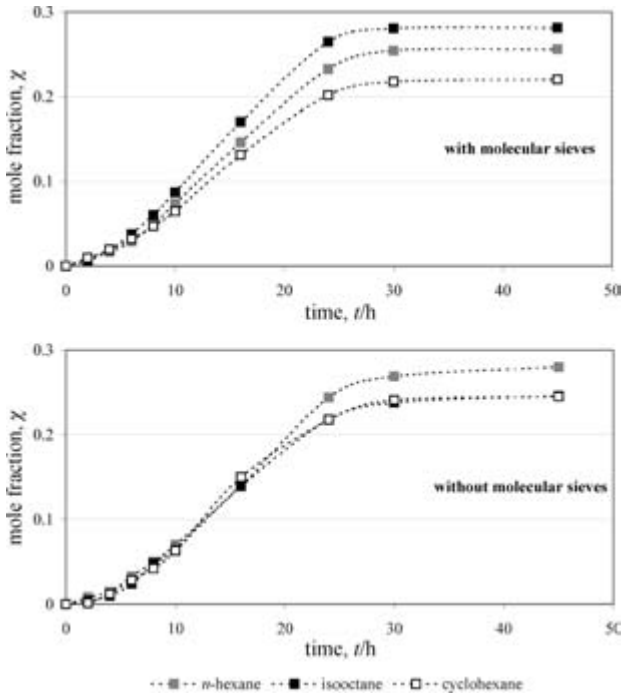
The water fraction of the system can significantly affect the equilibrium shift. Adding molecular sieves or solid anhydrous salts in the reaction mixture could also improve the product yield by controlling the water activity. Some authors dry solvents over molecular sieves prior to use,[42] while others insert molecular sieves at starting reaction mixture[12,43] or add them into reaction mixture 8 h after starting in order to higher yield.[44] In our experiments, we added molecular sieves at reaction start.

We wished to investigate the lipase selectivity of MO and DO production, which depended on the solvent hydrophobicity and the presence of molecular sieves. For this purpose, enzyme activities were performed at fixed substrates, lipase, and solvents concentration.[45]

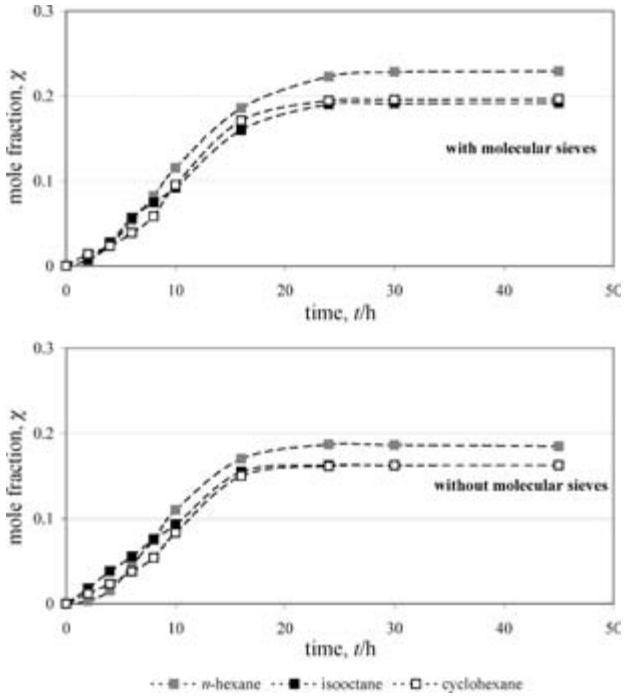
The effect of organic solvents on the monoolein concentration with and without molecular sieves, is shown in Fig. 3. The initial yields were approximately equivalent in both experiments, but not at equilibrium where monoolein yields were higher in the system with molecular sieves working with cyclohexane and *n*-hexane. Lower yield was found in the system with isooctane and without molecular sieves. The differences in the monoolein concentration, obtained in the system with molecular sieves, increased by using chosen solvents with a higher log *P* value. By comparison of results presented in Fig. 3, it is evident that only the system with molecular sieves followed the increase of log *P* values in the monoolein production.

This traceability was not noticed in the diolein production, as show Fig. 4, although the system with molecular sieves had higher final conversion for all the used solvents.

We believed that the solvents contained a feasible low water fraction. The comparison of Figs. 3 and 4 showed in the system with molecular sieves, solvent hydrophobicity affected on enzyme selectivity in monoolein production. That can be explained by the fact, that hydrophobicity depended on anhydrous conditions of organic solvents and affected the final monoolein concentrations.

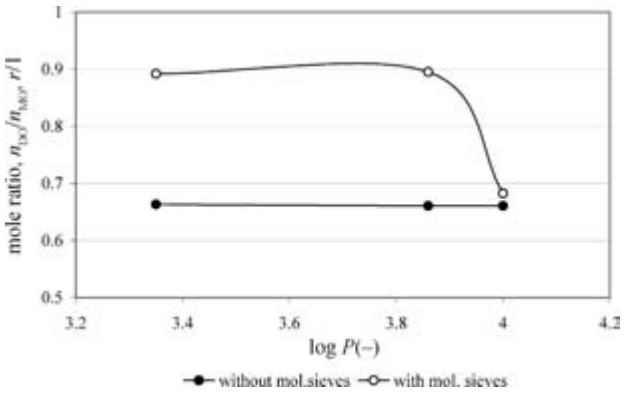


F i g . 3 – *Concentrations of monoolein in the system with molecular sieves and without molecular sieves after 45 h followed reaction esterification at 37 °C, in the solvent system in the presence of organic solvents*



F i g . 4 – *Concentrations of diolein in the system with molecular sieves and without molecular sieves after 45 h followed reaction esterification at 37 °C, in the solvent system in the presence of organic solvents*

The enzyme selectivity for the formation of MO increased with log *P* value in the examined range with the presence of molecular sieves (Fig. 5).



F i g . 5 – *Effect of hydrophobicity (versus log P value) on DO/MO ratio after 45 h followed esterification, in the solvent system with cyclohexane (3.35), n-hexane (3.85) and isooctane (4.00) with and without molecular sieves at 37 °C*

In the system without molecular sieves, the selectivity of MO did not depend on log *P* as presented in Fig. 3. That is in accordance with the initial hypothesis that water has a crucial role in glyceride production and low water media is appropriate. When water was removed with molecular sieves, the rate of reaction depended on log *P* value.

The enzyme selectivity was higher in the production of MO with the presence of molecular sieves (up to 50 %).

On the basis of these results, we can conclude that both solvent hydrophobicity and the presence of molecular sieves affected the glyceride composition during the lipase-catalyzed esterification. The glyceride composition depended on enzyme selectivity, reaction rate and the MO production.

The results on oleic acid esterification may reflect not only intrinsic enzyme selectivity but also the relative solubility of oleic acid and the effect on the enzyme activity due to the alcohol polarity. This work clearly shows that both the water control with molecular sieves and partition coefficient, log *P* are one of key parameters to control a glyceride synthesis reaction catalyzed by immobilized 1,3-specific lipase from *Mucor miehei*. The results of this work allowed a better understanding of the effects of organic solvents and water control on enzymatic synthesis of oleic acid esters and, therefore, it could help define the ways to improve the performance of the synthetic reaction by immobilized lipase.

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List of symbols

c – concentration, mol l^{-1}
n – amount of substance, mol
P – hydrophobicity
r – reaction rate, mmol l^{-1} h^{-1}
ν – mole ratio, n_{DO}/n_{MO}, mol mol^{-1}
t – time, h
T – temperature, °C
w – mass fraction, %
χ – mole fraction, –
γ – mass concentration, mg l^{-1}
Ψ – volume ratio, –

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