Esterification of phenolic acids catalyzed by lipases immobilized in organogels

M. Zoumpanioti, E. Merianou, T. Karandreas, H. Stamatis, A. Xenakis

Section: Microbial and Enzyme Technology

Esterification of phenolic acids catalyzed by lipases immobilized in organogels

M[1] Zoumpanioti, E[1,2] Merianou, T[1] Karandreas, H[2] Stamatis, A[1,*] Xenakis

[1]Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, Athens, Greece.

[2]Biological Applications and Technologies Department, University of Ioannina, Greece.

[*]Corresponding Author: Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48, Vassileos Constantinou Ave., 11635 Athens, Greece
e-mail: arisx@eie.gr, tel. +302107273762, fax +302107273758

Keywords

Biocatalysis; antioxidants; hydroxypropylmethyl cellulose.

Abstract

Lipases from *Rhizomucor miehei* and *Candida antarctica* B were immobilized in hydroxypropylmethyl cellulose organogels based on surfactant-free microemulsions consisting of n-hexane, 1-propanol and water. Both lipases kept their catalytic activity, catalyzing the esterification reactions of various phenolic acids including cinnamic acid

derivatives. High reaction rates and yields (up to 94%) were obtained when lipase from *C. antarctica* was used. Kinetic studies have been performed and apparent kinetic constants were determined showing that ester synthesis catalyzed by immobilized lipases occurs via the Michaelis-Menten mechanism.

Introduction

Phenolic acids are natural antioxidants found in fruits, vegetables and cereals and are common constituents of plant tissues. Apart from their antioxidant capacity they present several biological and pharmacological properties such as anti-inflammatory, anti-carcinogenic and neuroprotective effects.

Phenolic acids have very low solubility in water or oil, prohibiting their use in most media. Thus, their conversion through the esterification of the carboxylic acid function with medium- and long-chain alcohols to obtain an amphiphilic molecule, can be used to alter their solubility in fats and oils (Figueroa-Espinoza and Villeneuve 2005), enhancing their use as food antioxidant additives as well as their application to cosmetics.

Various organic solvents have been tested as reaction media for the enzymatic esterification of phenolic acids (Priya and Chada 2003; Widjaja *et al*. 2008). The direct esterification of phenolic acids with aliphatic alcohols catalyzed by lipases in anhydrous solvents (Compton *et al*. 2000; Stamatis *et al*. 2001) or solvent-free systems (Stamatis *et al*. 1999; Weitkamp *et al*. 2006) have been reported, however, the reaction rate and the esterification yield were significantly low. Their esterification in water-in-oil microemulsions has been achieved with high yield (Giuliani *et al*. 2001). However, the relatively polar phenolic acids present low solubility in such systems and the

application of microemulsions in industry is hindered by the presence of high concentrations of surfactants. As an alternative, surfactant free microemulsions (Topakas *et al*. 2003; Zoumpanioti *et al*. 2006) and ionic liquids (Katsoura *et al*. 2006; Ambika *et al*. 2008) have been proposed.

Immobilized enzymes are still good options to carry out esterification of phenolic acids, due to the facility of recovering the enzyme and the product separately (Zoumpanioti *et al.*, 2010). Moreover, the cost of the procedure can be low due to small amount of enzyme used to achieve the catalysis as compared to the use of free enzyme. In the present study, we report the use of lipases immobilized in hydroxypropylmethyl cellulose (HPMC) organogels (MBGs) based on surfactant-free microemulsions and the potential of these MBGs to be used as alternative media for the enzymatic esterification of relatively large amounts of various phenolic acids with 1-octanol in a solvent-free system. The selected HPMC-surfactant free microemulsion system has been chosen among other immobilization systems as it presents several advantages over other immobilization systems, such as easy catalyst separation, good storage stability and reusability as well as easy operation and effective use under mild conditions (Delimitsou *et al*. 2002, Zoumpanioti *et al*. 2006, Zoumpanioti *et al*. 2010).

Materials and Methods

Materials

Lipase from *Rhizomucor miehei* (as lipase from *Mucor miehei,* RmL) and lipase B from *Candida antarctica* (CaL-B) were supplied by Fluka, Switzerland. The enzyme preparations used in the various experiments had a specific activity of 7.5 U/mg protein (1 U corresponds to the amount of RmL which liberates 1 μmol butyric acid per min at

pH 7.5 and 40 ºC using tributyrin as substrate) and 9.2 U/mg protein (1 U corresponds to the amount of CaL-B which liberates 1 μmol butyric acid per min at pH 8.0 and 50 ºC using tributyrin as substrate). *p*-Hydroxyphenylpropionic acid (*p*-HPP), *p*-hydroxybenzoic acid (*p*-HBA), *p*-hydroxyphenylacetic acid (*p*-HPA) and *o*-, *m*-, *p*-coumaric acids were obtained from Sigma, *m*-hydroxyphenylacetic acid (*m*-HPA) was from Aldrich; *o*-hydroxyphenylacetic acid (*o*-HPA), cinnamic and ferulic acids were from Fluka, Switzerland. Structures of the phenolic acids used are given in figure 1. Hydroxypropylmethyl cellulose (HPMC) was from Sigma. All other reagents were of the highest commercially available purity.

(Insert figure 1 about here)

Preparation of microemulsions and organogels

Surfactant-free microemulsions were prepared by mixing n-hexane/1-propanol/buffer (47.2:50.8:2 by vol.) in 1 ml. The buffer solution was 200 mM Tris/HCl, pH 7.5 containing the lipase. The mixture was vigorously shaken for several seconds until a stable, transparent solution was obtained.

Lipase-containing organogels were prepared by introducing appropriate amounts of surfactant free microemulsions containing lipase to a second solution of HPMC in water in a similar manner as described elsewhere (Delimitsou *et al*. 2002).

Lipase-catalyzed reactions

3.5 g of the HPMC organogel containing 0.6 mg of lipase, was cut into regularly sized sections and placed into a reaction vial. The reaction was initiated by adding 10 ml 1-octanol containing the appropriate amount of the phenolic acid (solvent-free system). The reaction system was stirred at 150 rpm at room temperature. At intervals, samples

of 40 μl were withdrawn and analyzed by GC as described elsewhere (Stamatis and Xenakis 1999).

The determination of lipase activity in the various organogels was based on the measurement of the reaction velocity of the esterification of 1-octanol with 100 mM of cinnamic acid, *o*-, *m*-, *p*-HPA, *p*-HPP and *p*-HBA, 50 mM *o*-, *m*-, *p*-coumaric acid, or 70 mM ferulic acid.

For the determination of the apparent kinetic constants of the esterification reactions studied, the acid concentrations varied within the range of 5-100 mM for *p*-HPP, 40-90 mM for *o*-HPA, 40-100 mM for *p*-HPA and 10-90 mM for *m*-HPA, respectively. The concentrations were kept below 100 mM as there were solubility limitations. All kinetic measurements were carried out in duplicate.

Parametric identification of maximum velocity and Michaelis-Menten constants was used from the equation for reaction velocity. The program of identification was based on minimization of quadratic errors using a Gauss-Newton algorithm.

Results and Discussion

Effect of phenolic acid on the reaction rate and conversion

The ability of HPMC organogels based on surfactant free microemulsions to serve as a reaction medium for the lipase catalyzed esterification of various phenolic acids with 1-octanol was determined. The solubility of phenolic acids is low in non polar organic solvents as well as in water. The main advantage of these systems is that as they contain a relatively polar solvent (1-octanol), they have the ability to solubilize larger quantities of phenolic acids, with respect to water and various non polar organic solvents, such as hexane.

In Table 1, the reaction rates as well as the reaction yields of the esterification of various phenolic acids with 1-octanol are presented. These systems constituted an effective medium for the esterification reactions of phenolic acids. Both lipases catalyzed the esterification reactions of several phenolic acids; however, esterification reactions catalyzed by CaL-B had much higher rates and conversion yields in comparison to those catalyzed by RmL. This is in agreement with previously reported work (Weitkamp *et al*. 2006; Vosmann *et al*. 2006; 2008), where immobilized CaL-B (Novozym 435) exhibited by far higher transesterification activity comparing to the immobilized RmL (Lipozyme RM IM) and *T. lanuginosus* (Lipozyme TL IM) as well as previous work of our group (Zoumpanioti *et al*. 2006) where CaL-B lipase exhibited much higher esterification activity compared to RmL lipase in surfactant-free microemulsions. However, the reversed phenomenon took place in the ionic liquid [bmim]PF_6, as, in this case, immobilized RmL exhibited higher esterification rates and conversion yield towards several phenolic acids as compared to immobilized CaL-B (Katsoura *et al*. 2009).

(Insert table 1 about here)

Table 1 also shows that the esterifications of cinnamic and ferulic acids as well as the isomers of coumaric acid, presented very low conversion yields or no conversion at all. This phenomenon may be due to the fact that these phenolic acids have an unsaturated bond close to the carboxylic function (unsaturated side chain). On the other hand, when the acid has a saturated side chain - as in the case of HPP and the isomers of HPA - the esterification presents high reaction yield. It seems that the double bond causes a steric or an electronic effect that inhibits the esterification. This is in agreement with the findings of Guyot *et al*. (1997) where the esterification of ferulic and caffeic acid catalyzed by *C. antarctica* lipase (Novozyme 435) did not take place, while the

esterification of dihydrocaffeic acid gave a yield of 78%. They attributed this to the simultaneous presence of a double bond on the side chain that conjugates with the cycle and the presence of a *p*-hydroxyl that totally inhibits the lipase. The same conclusion has been drawn by Buisman *et al*. (1998) for benzoic acid derivatives and by Stamatis *et al*. (2001) for cinnamic acid derivatives.

For the esterification reaction of the three isomers of HPA, catalyzed by CaL-B, the reaction with the higher rate was the one of *p*-HPA, followed by *m*-HPA and *o*-HPA. Hence, when the hydroxyl group is in *p*- position, and thus in a higher distance from the carboxyl group, the hindrance caused by hydroxyl group is lower, leading to effective enzyme-substrate contact and higher reaction rate. This behavior, attributed to the reduced steric hindrance that the molecule of *p*-HPA exhibits in comparison to *o*-HPA, also applies to RmL. These results are similar to the ones obtained for the esterification of the three isomers of HPA with 1-propanol in relative surfactant-free microemulsions (Zoumpanioti *et al*. 2006).

When the isomers of coumaric acid were used for the esterification of 1-octanol catalyzed by RmL, no ester production was observed. In the case of catalysis by CaL-B, *o*-coumaric acid reacted, although at a low rate, and the reaction with *p*-coumaric acid gave only a trace of product while the reaction with *m*-coumaric acid gave no product at all. This is in contrast to what was observed for the isomers of HPA and could be attributed to an electronic rather than a steric effect. The behaviour observed in this work is different from the one observed by Weitkamp *et al*. (2006). They tested the transesterification activity of immobilized CaL-B towards the synthesis of several medium- or long-chain alkyl cinnamates and hydroxycinnamates and observed that the transesterification activity of the lipase increased in the order meta>para>ortho, with respect to the position of the hydroxy substituents at the phenyl moiety.

Comparing *p*-HPA to *p*-HPP (Figure 1, the phenolic ring of the former should cause a higher steric hindrance as it is closer to the carboxylic function. However, the esterification of *p*-HPA takes place at a much higher rate and yield than the esterification of *p*-HPP. This is in agreement to what was found for the esterification of these acids with 1-propanol in relative surfactant-free microemulsions (Zoumpanioti *et al*. 2006). The difference in the reaction rates of the esterification of these particular phenolic acids cannot, though, be attributed to the higher steric hindrance of *p*-HPA. Subsequently, this can be attributed to the high stereoselectivity of lipases which plays an important role to the integration of each reaction.

Kinetic studies

(Insert table 2 and figure 2 about here)

Figure 2 shows the reaction rate of the esterification of phenolic acids catalyzed by CaL-B, as a function of the acid concentration in solvent-free systems. The maximum acid concentration depended on the maximum solubility of each acid. The results of the kinetic analysis indicate that a Michaelis-Menten mechanism occurs in the system. Apparent kinetic constants (K_m^{app} and V_{max}^{app}) are presented in Table 2. The kinetic analysis of the esterification of *p*-HPP with 1-propanol catalyzed by CaL-B encapsulated in related surfactant free microemulsions showed that K_m^{app} is of the same order of magnitude as the one calculated for the organogels (K_m^{app}=54 mM), however, V_{max}^{app} in microemulsions seems to be much higher (V_{max}^{app}=0.26 mM/min) (data not published). The same observation has been made for RmL immobilized in HPMC MBGs based on lecithin microemulsion catalyzing propyl laurate synthesis, where K_m^{app} for the acid was of the same order of magnitude (K_m^{app}=74 mM), while V_{max}^{app} was much higher (V_{max}^{app}=0.125 mM/min) (Delimitsou *et al*. 2002).

Conclusions

Lipases from CaL-B and RmL, immobilized on HPMC MBGs efficiently catalyze the esterification of polar phenolic acids in a solvent-free system. Hydroxylated acids with a saturated side-chain were esterified with high rates and reaction yields (up to 94%) when CaL-B was used. Kinetic studies have revealed that a Michaelis-Menten mechanism applies in the system.

References

Ambika P, Singh P, Chauhan SMS (2008) Chemoselective esterification of phenolic acids in the presence of sodium bicarbonate in ionic liquids. Synthetic Comm 38:928-936

Buisman GJH, van Helteren CTW, Kramer GFH, Veldsink JW, Derksen JTP, Cuperus FP (1998) Enzymatic esterifications of functionalized phenols for the synthesis of lipophilic antioxidants. Biotechnol Lett 20:131-136

Compton DL, Laszlo JA, Berhow MA (2000) Lipase-catalyzed synthesis of ferulate esters. J Am Oil Chem Soc 77:513-519

Delimitsou C, Zoumpanioti M, Xenakis A, Stamatis H (2002) Activity and stability studies of *Rhizomucor miehei* lipase immobilized in novel microemulsion-based organogels. Biocatal Biotransform 20:319-327

Figueroa-Espinoza MC, Villeneuve P (2005) Phenolic acids enzymatic lipophilization. J Agric Food Chem 53:2779-2787

Giuliani S, Piana C, Setti L, Hochloepper A, Pifferi PG, Williamson G, Faulds CB (2001) Synthesis of pentylferulate by a feruloyl esterase from *Aspergillus niger* using water-in-oil microemulsions. Biotechnol Lett 23:325-330

Guyot B, Bosquette B, Pina M, Graille J (1997) Esterification of phenolic acids from green coffee with an immobilized lipase from *Candida antarctica* in solvent-free medium. J Biotechnol Lett 19:529-532

Katsoura MH, Polydera AC, Tsironis L, Tselepis AD, Stamatis H (2006) Use of ionic liquids as media for the biocatalytic preparation of flavonoid derivatives with antioxidant potency. J Biotechnol 123:491-503

Katsoura MH, Polydera AC, Tsironis LD, Petraki MP, Rajačić SK, Tselepis AD, Stamatis H (2009) Efficient enzymatic preparation of hydroxycinnamates in ionic liquids enhances their antioxidant effect on lipoproteins oxidative modification. New Biotechnol 26:83-91

Priya K, Chada A (2003) Synthesis of hydrocinnamic esters by *Pseudomonas cepacia* lipase. Enzyme Microb Technol 32:485-490

Stamatis H, Xenakis A (1999) Biocatalysis using micromulsion-based polymer gels containing lipase. J Mol Catal B: Enzym 6:399-406

Stamatis H, Sereti V, Kolisis FN (1999) Studies on the enzymatic synthesis of lipophilic derivatives of natural antioxidants. J Am Oil Chem Soc 76:1505-1510

Stamatis H, Sereti V, Kolisis FN (2001) Enzymatic synthesis of hydrophilic and hydrophobic derivatives of natural phenolic acids in organic media. J Mol Catal B: Enzym 11:323-328

Topakas E, Stamatis H, Biely P, Kekos D, Macris BJ, Christakopoulos P (2003) Purification and characterization of a feruloyl esterase from *Fusarium oxysporum*

catalyzing esterification of phenolic acids in ternary water-organic solvent mixtures. J Biotechnol 102:33-44

Vosmann K, Weitkamp P, Weber N (2006) Solvent-free lipase-catalyzed preparation of long-chain alkyl phenylpropanoates and phenylpropyl alkanoates. J Agric Food Chem 54:2969-2976

Vosmann K, Wiege B, Weitkamp P, Weber N (2008) Preparation of lipophilic alkyl (hydroxy)benzoates by solvent-free lipase-catalyzed esterification and transesterification. Appl Microb Biotechnol 80:29-36

Weitkamp P, Vosmann K, Weber N (2006) Highly efficient preparation of lipophilic hydroxycinnamates by solvent-free lipase-catalyzed transesterification. J Agric Food Chem 54:7062-7068

Widjaja A, Yeh TH, Ju YH (2008) Enzymatic synthesis of caffeic acid phenethyl ester. J Chinese Inst Chem Eng 39:413-418

Zoumpanioti M, Karali M, Xenakis A, Stamatis H (2006) Lipase biocatalytic processes in surfactant free microemulsion-like ternary systems and related organogels. Enzyme Microb Technol 39:531-539

Zoumpanioti M, Stamatis H, Xenakis A, (2010) Microemulsion-based organogels as matrices for lipase immobilization. Biotech Adv 28:395-406

Table 1. Reaction rate of the esterification of various phenolic acids with 1-octanol catalyzed by CaL-B and RmL immobilized in HPMC and conversion yield after 26 days, in solvent-free systems.

Phenolic acid	**Reaction rate (mM/min) x 10^3**		**Conversion (%)**	
	CaL-B	RmL	CaL-B	RmL
p-HPP	1.5	0.1	61	3*
p-HPA	9.5	0.1	94	4*
m-HPA	2.7	0.1	78	3*
o-HPA	1.9	0.1	48	4*
p-HBA	2.3	0.1	12	2
o-coumaric acid	1.7	nr	9	nr
m-coumaric acid	nr	nr	nr	nr
p-coumaric acid	traces	nr	traces	nr
Cinnamic acid	1.1	nr	8	nr
Ferulic acid	nr	nr	nr	nr

nr: no reaction

* : not final conversion

Table 2. Effect of the phenolic acid on the apparent kinetic constants of their esterification with 1-octanol catalyzed by CaL-B immobilized in HPMC organogels, in solvent-free systems.

Phenolic Acid	V_{max}^{app} (mM/min) x 10^5	K_m^{app} (mM)
p-HPP	7 ± 0.7	72 ± 12
p-HPA	30 ± 5	95 ± 33
m-HPA	30 ± 5	83 ± 27
o-HPA	3 ± 0.6	95 ± 32

p-HPP: *p*-hydroxyphenylpropionic acid, *o, m, p*-HPA: *o, m, p*-hydroxyphenylacetic acid

Figure 1. Structures of the phenolic acids that have been used: A: cinnamic acid, B: *p*-coumaric acid, C: ferulic acid, D: *p*-hydroxybenzoic acid (*p*-HBA), E: *p*-hydroxy-phenylacetic acid (*p*-HPA) and F: *p*-hydroxyphenylpropionic acid (*p*-HPP).

Figure 2. Effect of phenolic acid concentration on the reaction rate of the esterification with 1-octanol catalyzed by CaL-B immobilized in HPMC organogels, in solvent-free systems for A: *p*-HPP, B: *p*-HPA, C: *m*-HPA and D: *o*-HPA. The inserts represent the respective double reciprocal plots.

Figure 1.

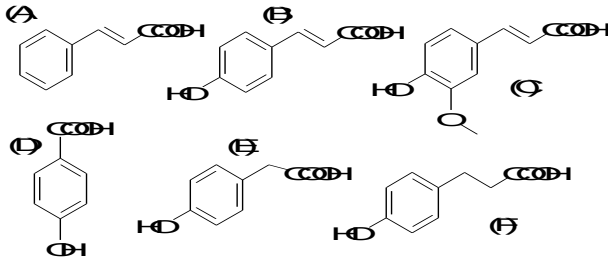
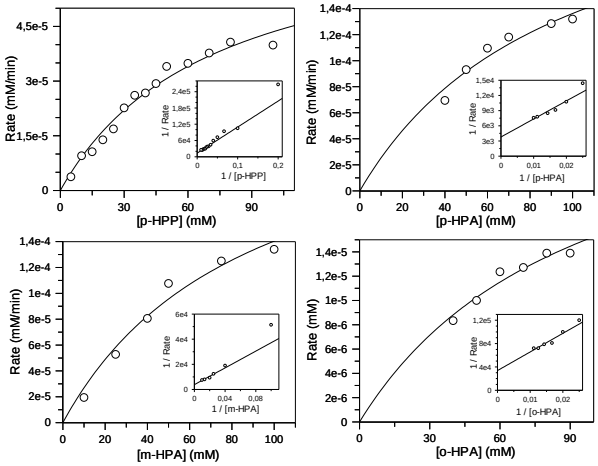


Figure 2.


Rate (mM/min)
[p-HPP] (mM)
1 / Rate
1 / [p-HPP]
Rate (mM/min)
[p-HPA] (mM)
1 / Rate
1 / [p-HPA]
Rate (mM/min)
[m-HPA] (mM)
1 / Rate
1 / [m-HPA]
Rate (mM)
[o-HPA] (mM)
1 / Rate
1 / [o-HPA]