Journal of Molecular Catalysis B: Enzymatic 60 (2009) 50–56

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Lipases in water-in-ionic liquid microemulsions: Structural and activity studies

Ioannis V. Pavlidis[a], Dimitrios Gournis[b], George K. Papadopoulos[c], Haralambos Stamatis[a,*]

[a] Laboratory of Biotechnology, Department of Biological Applications and Technologies, University of Ioannina, 45110 Ioannina, Greece
[b] Department of Materials Science & Engineering, University of Ioannina, 45110 Ioannina, Greece
[c] Laboratory of Biochemistry and Biophysics, Faculty of Agricultural Technology, Epirus Institute of Technology, 47100 Arta, Greece

ARTICLE INFO

Article history:
Received 19 January 2009
Received in revised form 6 March 2009
Accepted 18 March 2009
Available online 27 March 2009

Keywords:
Lipase
Ionic liquids
Microemulsions
Stability
FT-IR
Circular dichroism

ABSTRACT

Water-in-ionic liquid (w/IL) microemulsions formulated with non-ionic surfactants, (Tween 20 or Triton X-100) in 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6), were used as media for lipase-catalyzed esterification reactions. The catalytic behavior and stability of lipases from *Candida rugosa, Chromobacterium viscosum* and *Thermomyces lanuginosa* in these novel microemulsions were investigated and compared to other microheterogeneous media used so far for enzyme-catalyzed reactions. The catalytic behavior of the enzymes depends strongly on the surfactant concentration and the water content. The dependence of the esterification activity of lipases on molar ratio of water to surfactant (w_o) follows a bell-shaped profile, presenting a maximum at $w_o \approx 5$. The operational stability of lipases in w/IL microemulsions, especially at high incubation temperature (50 °C), was significantly increased compared to that observed in other microheterogeneous media. The highest half-life times (>100 h) were obtained in w/IL microemulsions with low water content. Conformational studies via Fourier transform-infrared (FT-IR) and circular dichroism (CD) spectroscopy indicated that lipases entrapped in w/IL microemulsions in most cases retain their native structure or adapt a more rigid structure compared to other microheterogeneous media, which correlated well with the stability results. A simple procedure suitable for ester separation and enzyme reuse was developed. *T. lanuginosa* lipase retained 90% of activity after ten reaction cycles in w/IL microemulsions formulated with Tween 20.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Reverse micelles formed in non-polar organic solvents (water-in-oil, w/o microemulsions) are thermodynamically stable, nanometer-sized water droplets dispersed in an organic phase by means of surfactant. Reverse micelles are capable of hosting proteins (including enzymes) in their so-called water pool. The protein molecules can be entrapped in the polar core of the micelles avoiding direct contact with the organic solvent. Reverse micelles have been used as a model system for biological studies, ranging from basic biochemical research [1–3] to numerous applications in biotechnology and particularly in biocatalytic transformations. Reverse micelles have been associated with the idea of micro-reactors for enzymic reactions, when substrates and/or products are lipophilic and low water content is desired [4–7].

Recently, ionic liquids (ILs, organic salts consisting only of ions, liquid at or near room temperature) have received growing attention as an alternative to organic solvents used for the enzymatic transformation of various compounds [8–10]. It was reported that enzymic activities in such organic salts are generally comparable with, or higher than, those observed in conventional organic solvents [10,11]. Furthermore, the use of ionic liquids in many cases enhanced the enzyme's thermal and operational stability as well as the regio- or enantioselectivities [10,12].

More recently it was reported that ionic liquids can be used instead of organic solvents for the formation of nano-sized aqueous droplets, similar to that in w/o microemulsions [13–16]. Gao et al. [13,14] characterized [bmim]PF_6/Triton X-100 or Tween 20/H_2O systems by various spectroscopic techniques. They recognized three types of microstructures formed in these systems: water in [bmim]PF_6 (w/IL) microemulsions, [bmim]PF_6 in water (IL/w) microemulsions and bicontinuous systems. These new IL-based microemulsion systems combine the advantages of the ILs and those of the organized nano-structured media and could be used as a novel medium for biocatalytic reactions. The application of these micro-heterogeneous systems as new type of reaction environment is currently under investigation [16–18].

In the present study we investigated the use of w/IL microemulsion systems, stabilized by non-ionic surfactants, such as Tween 20 and Triton X-100, in [bmim]PF_6 as a novel reaction system for lipase-catalyzed reactions. The ability of lipases from *Candida rugosa*, *Chromobacterium viscosum* and *Thermomyces lanuginosa,*

* Corresponding author at: Laboratory of Biotechnology, Department of Biological Applications and Technologies, University Campus, 45110 Ioannina, Greece. Tel.: +30 26510 97360; fax: +30 26510 97343.
E-mail address: hstamati@cc.uoi.gr (H. Stamatis).

1381-1177/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2009.03.007

which are largely employed in various biocatalytic processes, to keep their catalytic activity in these novel micro-heterogeneous media was studied. The catalytic behavior and the stability of lipases in w/IL microemulsion systems were compared with those obtained in other water-restricted media used so far for biocatalytic reactions, such as reverse micellar system formulated with AOT (bis-(2-ethylhexyl) sulfosuccinate sodium salt) in organic solvents, as well surfactant-free microemulsion-like ternary systems (SLM) [19]. Fourier transform-infrared (FT-IR) and circular dichroism (CD) spectroscopy were employed to investigate potential changes in enzyme structure, in an attempt to explain the stabilization phenomena observed upon their entrapment in these novel ionic liquid-based microheterogeneous reaction systems. Moreover, a simple procedure suitable for the separation of product and the reuse of the entrapped-enzyme was developed.

2. Materials and methods

2.1. Materials

Lipases from *Candida rugosa* lipase (Crl) and *Chromobacterium viscosum* lipase (Cvl) were purchased from Fluka. Novozyme TL 100 L (*Thermomyces lanuginosa* lipase, Tll) was a generous gift from Novozymes. The ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6) was purchased from Alfa Aesar. Tween 20 (polyethylene glycol sorbitan monolaurate) was purchased from Fluka. Bis-(2-ethylhexyl) sulfosuccinate sodium salt (AOT) and Triton X-100 (*t*-octylphenoxypolyethoxyethanol) were purchased from Sigma. All other reagents were of analytical grade. The aqueous lipase solutions were prepared by dissolving lipase in 25 mM Tris–HCl buffer (pH 7.5).

2.2. Ternary phase diagrams

The ternary phase diagrams were constructed on the basis of the isotropic properties of various compositions of the ionic liquid ([bmim]PF_6), surfactant (Tween 20 or Triton X-100) and water at various temperatures (30–50 °C). Solutions of ionic liquid and surfactant were prepared, and then titrated with 25 mM Tris–HCl buffer (pH 7.5). The solutions were incubated in an orbital shaker for 1 day at predetermined temperature, and then they were centrifuged at 12,000 rpm for 2 min. Visual observations were made until phase separation, which determined the phase limiting line.

2.3. Preparation of microemulsion systems

The w/IL microemulsion systems were prepared by mixing the required volumes of [bmim]PF_6 and surfactant according to the phase diagram in Fig. 1 (systems composition are included in the Supporting Information, Table SI-1). The mixture was left in an orbital shaker at 30 °C in order to get a clear homogeneous solution. Then, the aqueous phase containing the enzyme was added and the final water content of the system was adjusted by addition of the required amount of buffer. The mixture was shaken vigorously, because of the high viscosity of the system, until a transparent single-phase solution was obtained.

The AOT formulated w/o microemulsions (AOT/RM) were prepared in a similar manner as described elsewhere [20]. The AOT concentration was 100 mM. In the present work microemulsions of $w_o = 6$ ($w_o = [H_2O]/[AOT]$) were used. Preliminary results showed that at $w_o = 6$ lipases exhibit high activity and thermal stability.

The surfactant-free microemulsion-like ternary systems (also known as surfactantless microemulsions, SLM), composed by *n*-hexane, 2-propanol and Tris–HCl buffer, were prepared in a similar manner as described by Zoumpanioti et al. [19]. Preliminary tests showed that lipases exhibit high activity when entrapped in the system with 64.4% *n*-hexane, 33.4% 2-propanol and 2.2% aqueous phase (v/v/v). This system forms similar micro-regions as the w/o microemulsions [21] and it was chosen for comparative studies with other microheterogeneous systems.

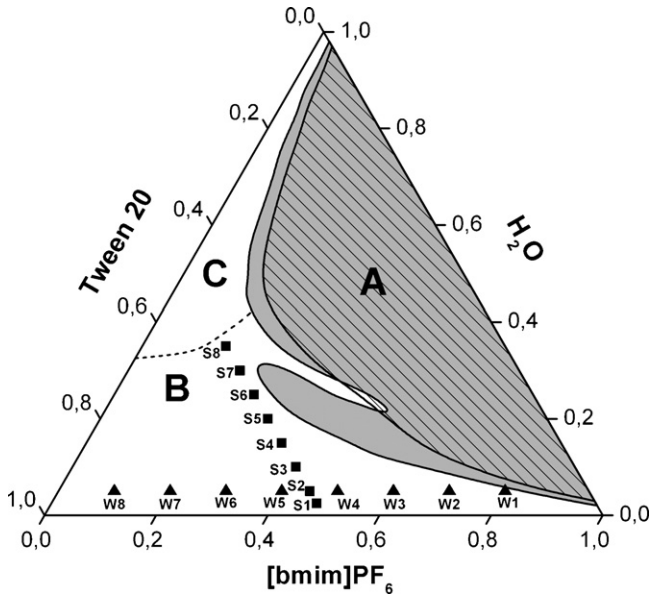

Fig. 1. Phase diagram (w/w) of the system water/Tween 20/[bmim]PF_6 at 30 °C (light grey area) and 50 °C (sparse line pattern). (A) Two phase systems, (B) w/IL microemulsions, (C) bicontinuous and IL/w microemulsions. S systems (■), W systems (▲).

2.4. Activity of lipases in microemulsion systems

The lipases entrapped in the above water-restricted media were used to catalyze the esterification of natural fatty acids with various aliphatic alcohols, using 50 mM fatty acid and 100 mM alcohol as substrate. Lipase-catalyzed reactions took place in capped vials placed in an orbital shaker at 30 °C at 200 rpm. The enzyme concentration varied from 5.2 μg/ml to 46.2 μg/ml. Aliquots were withdrawn at selected time intervals and extracted with *n*-hexane in the case of w/IL microemulsions or acetonitrile in the case of the other two microheterogeneous systems, and the upper organic phase was analyzed for ester content by gas chromatography (GC). All experiments were carried out in triplicate.

GC analysis was performed by a Shimatzu GC 17A device (Tokyo, Japan), which was equipped with a β-DEX™ 120, fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness, Supelco) and a flame ionization detector (FID) with helium as the carrier gas.

2.5. Enzyme stability

The thermostability of the lipases was studied in various microheterogeneous systems in a range of temperatures from 30 °C to 50 °C. The residual lipase activity was measured by spectrophotometric assay, by monitoring the hydrolysis of 0.5 mM *p*-nitrophenyl butyrate (*p*NPB) at 30 °C [22]. After incubation, the samples were withdrawn at regular time intervals in order to measure the remaining enzyme activity by the addition of substrate (*p*NPB) in the form of a concentrated solution in DMSO. The formation of *p*-nitrophenol was detected at 340 nm [22]. In the case of w/IL microemulsions, 20 μl of the microemulsion is injected in 1 ml of Tris–HCl buffer-containing substrate (*p*NPB). In the homogeneous mixture formed, the remaining enzyme activity was determined by monitoring the formation of *p*-nitrophenol at 405 nm.

Table 1
Thermomyces lanuginosa lipase synthetic activity (in propyl laurate synthesis) when entrapped in various water restricted media (standard deviation in all cases was up to 8%).

System	Initial rate (mmol h^{-1} mg^{-1} enzyme)		Conversion after 24 h (%)	
	Tween 20 microemulsions	Triton X-100 microemulsions	Tween 20 microemulsions	Triton X-100 microemulsions
S2	0.877	0.077	75.1	19.1
S4	0.583	0.150	85.1	6.1
S7	0.713	0.051	69.9	3.2
W1	0.419	0.213	100.0	14.2
W3	0.616	0.043	94.5	13.7
W7	0.923	0.077	55.7	5.3
[bmim] PF_6[a]	0.063		55.3	
AOT/RM	0.257		74.1	
SLM	0.008		7.3	

[a] Water saturated ionic liquid.

The experimental results were elaborated with the "one-step" deactivation model and the series type deactivation model which was introduced by Henley and Sadana [23]. The non-linear Levenberg–Marquadt regression method was applied to fit the residual activity data. The fitting of the experimental data was better in the case of the series type deactivation model and this model was chosen for the elaboration of the data. The Newton–Raphson method was used to calculate the half-life times ($t_{1/2}$) from the two-step deactivation equation [23], using the parameter values calculated from the experimental data.

2.6. FT-IR spectroscopy

IR spectra were measured using a Shimadzu FT-IR 8400 spectrometer (Tokyo, Japan), equipped with a deuterated triglycine sulfate (DTGS) detector, in the region of 4000–400 cm^{-1}. The spectrum acquisition (all samples were overlaid on a zinc selenide attenuated total reflectance (ATR) accessory), the data analysis of the amide I region and the band assignment were performed as described in previous work [24]. The data were manipulated using WinSpec software (LISE-Facultеїs Universitaires Notre-Dame de la Paix, Namur, Belgium). The band assignment is described in Supporting Information (SI) section.

2.7. Circular dichroism spectroscopy

CD far-UV (200–260 nm) spectra were obtained on a Jasco J-815 spectropolarimeter (Tokyo, Japan) equipped with a Peltier system for temperature control. For the aqueous samples a quartz cell with path-length 1.0 cm was used, while for the microemulsion samples we used a 0.1 cm path-length quartz cell. All spectra were obtained at 25 °C, with a 2 nm bandwidth and a scan speed of 10 nm/min. For every medium a baseline was recorded and subtracted from the protein spectrum. The protein concentration varied from 1.3 μg/ml to 46.2 μg/ml. The secondary structure element content was estimated using the DICHROPROT application package [25] based on the SELCON 2 algorithm described by Sreerama and Woody [26].

3. Results and discussion

3.1. Phase diagram of w/IL microemulsions

In the present work, two monophasic ternary systems consisting of a hydrophobic ionic liquid such as [bmim]PF_6, water and a non-ionic surfactant were investigated as potential media for lipase-catalyzed reactions; the first system was composed of [bmim]PF_6, Tween 20 and water (25 mM buffer Tris–HCl, pH 7.5) whereas in the second type Tween 20 was replaced by Triton X-100. The phase diagram of ternary system consisting of Tween 20, at 30 °C and 50 °C, is depicted in Fig. 1. Region A corresponds to unstable systems that tend to separate rapidly into two phases upon standing. Compositions that constitute the regions B and C of the phase diagram are homogeneous, optically transparent and show no phase separation. It is interesting to note that as the temperature increases from 30 °C to 50 °C the monophasic area of the system also increases. An analogous phase diagram was determined for the ternary system formed with Triton X-100 (data not shown). The phase behavior of these systems is similar to that reported by Gao et al. [13,14]. According to these works, when the water content of the system is less than about 34% (region B), water-in-[bmim]PF_6 (w/IL) micro-regions were identified using various spectroscopic techniques [13,14], while at higher water content (region C) the ionic liquid microdroplets were dispersed in a continuous water medium or bicontinuous microstructure were formed. All systems used correspond to w/IL micro-regions. Systems indicated as S systems correspond to w/IL microemulsions (B region) with constant surfactant concentration (50%, w/w), and systems indicated as W systems have constant water content (5%, w/w).

3.2. Lipases esterification activity in w/IL microemulsions

The ability of Crl, Cvl and Tll to keep their catalytic activity in various w/IL microemulsion systems formulated with either Tween 20 or Triton X-100 was determined using the esterification of 1-propanol with lauric or caprylic acid as model reactions. All three lipases kept their ability to catalyze the esterification of lauric acid with 1-propanol in all w/IL microemulsion systems tested (see Table SI-1). In most cases, the conversion of the esterification reaction is up to 80% after 24 h of incubation. Similar behavior was observed for the esterification of 1-propanol with fatty acids of varying chain length (from 8 to 18 carbon atoms).

Table 1 shows the reaction rates and % conversions for the esterification of lauric acid with 1-propanol catalyzed by Tll in different w/IL microemulsion systems formulated with either Tween 20 or Triton X-100, as well as in other microheterogeneous systems used so far for similar biocatalytic reactions, such as AOT-based reverse micellar system (AOT/RM), surfactant-free microemulsion-like ternary systems (SLM) as well as in water-saturated [bmim]PF_6. In most cases the reaction rates and % conversions observed in Tween 20-based w/IL microemulsions are significantly higher than those observed in other microheterogeneous media, as well as in w/IL microemulsion systems formulated with Triton X-100. It was reported that the catalytic behavior of lipases in the presence of Tweens is better than in the presence of Triton X-100 [27]. Moreover, the better catalytic behavior of lipases observed in w/IL microemulsion systems compared to water-saturated [bmim]PF_6 indicates that the formation of microemulsions is beneficial. The biocatalytic performance of lipases observed in Tween-based w/IL microemulsion systems was superior to other microheterogenous

media independently of the composition and the chain length of the fatty acid used (data not shown).

It is interesting to note that in some reaction systems (e.g. S7 or W7), the % conversion is relatively low regarding the high initial reaction rates observed. This unexpected behavior could be attributed to the different stability of the enzyme in various w/IL microemulsions (as discussed in Section 3.5). Moreover, since short chain alcohols can act as cosurfactants, their depletion (due to esterification reaction) could affect the biocatalytic behavior of the entrapped enzyme [6]. It is expected that this effect depends on the composition of the microemulsion systems.

The use of Tween 20 formulated w/IL microemulsions as media for the esterification of caprylic acid with various aliphatic alcohols was also investigated. Fig. 2 shows the effect of the alcohol chain length on the rate of their esterification catalyzed by Cvl and Tll in w/IL microemulsions (system W3). As it can be seen, the alcohol chain length affects significantly the catalytic activity of the lipases. Both enzymes show a maximum reaction rate for the esterification of medium chain alcohols (up to 6 carbon atoms) and a profound decrease of their activity when decreasing chain length of the alcohols. The lower reaction rates were observed when ethanol was used as substrate. This is in accordance to that observed for various lipases in other w/o microemulsions [20] and could be attributed to the inhibitory effect of this alcohol due to its high solubility on the aqueous microphase of the systems and therefore on the enzyme microenvironment. However, factors such as the different partitioning of various alcohols on the enzyme microenvironment and the cosurfactant role of alcohol in the microemulsion systems can also affect the enzyme catalytic behavior [19,20].

3.3. Effect of w_o

One of the most important factors which affect the catalytic behavior of enzymes in various microheterogeneous media is the water content (expressed as the molar ratio of water to surfactant, w_o). In the present work the dependence of lipases activity on w_o has been investigated at a fixed percentage of water (5%, w/w, W systems) by injecting the same volume of water into Tween 20/[bmim]PF_6 solutions of different concentrations. As it can be seen in Fig. 3, w_o has a strong effect on the lipases activity. The esterification activity of Crl, Cvl and Tll follows a rather sharp bell-shaped profile, presenting a maximum at $w_o = 5.3$ for Crl and Cvl and $w_o = 4.6$ for Tll. Similar bell-shaped dependence of activity on w_o is also observed for these lipases in other w/o microemulsion systems [28,29]. However the optimum w_o values observed in w/IL systems are lower than those observed in other w/o microemulsions. It is known that the use of non-ionic surfactants, such as Tweens, for the formation of reverse micelles in organic solvents leads to significant decrease of the optimum w_o value for Cvl compared to that observed in reverse micellar systems formulated with ionic surfactants such as AOT [30].

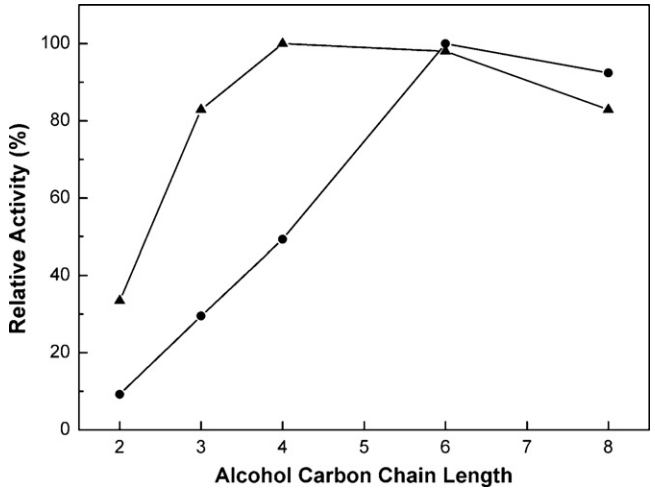

Fig. 2. Effect of alcohol chain-length on the esterification of caprylic acid catalyzed by Cvl (▲) and Tll (●) when entrapped in W3 w/IL microemulsion system.

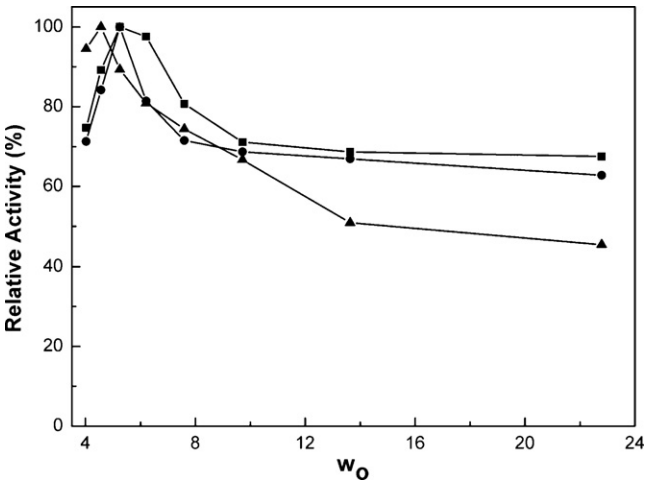

Fig. 3. Effect of w_o on propyl laurate synthesis catalyzed by Crl (■), Cvl (●) and Tll (▲) in w/IL microemulsions.

The optimum w_o value for enzymatic activity depends on several factors such as the microemulsion composition, the enzyme concentration as well as its molecular dimensions [4,7]. It was proposed that the maximum catalytic activity is observed in systems where the size of microdroplets becomes comparable to the protein's dimensions [1,2]. In this case, spontaneous fluctuations of the protein structure, that usually disturb its catalytically active conformation, are reduced [1,2,5]. More specifically, reverse micelles with relatively low water content do not provide sufficient space to completely encapsulate enzyme molecules in the water microdroplets, presumably forcing enzymes into a less active conformation. On the other hand, the reduced enzyme activity observed in high w_o systems could be related to the decreased stability of enzymes observed in these systems, as discussed in Section 3.4. However, there is an ongoing discussion in the literature as to whether the w_o dependence of the activity is related to the size of the enzyme molecule or not [1,2,4,6,31].

3.4. Effect of the surfactant concentration

It is well known that the addition of surfactants can enhance the activity of lipases in aqueous solutions due to the interfacial activation and due to the emulsification of hydrophobic substrates [32]. In microemulsion systems the concentration of surfactant, at a constant water-to-surfactant molar ratio (w_o), is a parameter which determines the concentration of micelles and therefore the size of the interfacial area in the system. Fig. 4 shows the effect of this parameter at constant value of $w_o = 5$ (which is circa the optimum w_o value for all lipases studied) on the activity of three lipases in w/IL microemulsion systems. As it can be seen, as the surfactant concentration increases, the activity of the lipases goes through a maximum value and then decreases. Similar decrease on the enzymic activity under high surfactant concentration in w/o microemulsions was reported for lipases from *C. viscosum* [29,33], *Rhizopus arrhizus* [34] and porcine pancreatic lipase [35].

As indicated before, when the surfactant concentration is increased at constant w_o, the microdroplets population also increases. In this case, the interaction of droplets with mem-

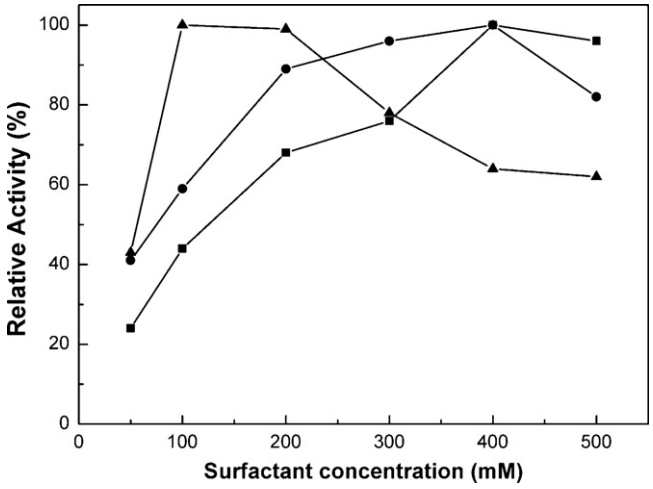

Fig. 4. Effect of surfactant concentration on propyl laurate synthesis catalyzed by Crl (■), Cvl (●) and Tll (▲) in w/IL microemulsions at $w_o = 5$.

brane active enzyme molecules, such as lipases, is increased. It was reported that these interactions reduce lipase activity due to inhibition by surfactant molecules [34,35]. Moreover, the effect of surfactant concentration on the substrate distribution in micellar interface must also be taken into consideration. The increase of surfactant concentration will affect the adsorption of amphiphilic substrates (as fatty acids and alcohols) on the interface, and therefore their availability to the enzyme microenvironment [4,29].

3.5. Stability of lipases

The thermal stability of Crl, Cvl and Tll entrapped in various w/IL microemulsion systems compared to that observed in other microheterogeneous media (AOT-formulated w/o microemulsions or surfactantless microemulsions), as well as in water, was investigated. The stability of the lipases was studied over a 5-day period at 30 °C, 40 °C and 50 °C in the absence of substrates. The experimental results were elaborated with the one-step deactivation model and the series type deactivation model [23], but the series type deactivation model was chosen for further elaboration of the data, because the best fitting was achieved with this model (in all cases RMSD was higher than 0.900). The half-life times, calculated from the two-step deactivation kinetics, for the three lipases used are given in Table 2 (deactivation kinetics are presented in Supporting Information Table SI-2). As it can be seen from Table 2 (see also Table SI-2), in most cases Crl and Tll stability was higher in w/IL microemulsions, compared to that observed in other microheterogeneous media as well as in water, indicating that w/IL microemulsion systems provide a protective environment for these lipases, especially at high incubation temperatures. In the case of Cvl, the stability exhibited in all microheterogeneous systems was higher than that exhibited in water. The entrapment of the lipases in the w/IL microemulsions has a stabilizing effect, referring to the stability exhibited in water, as well as in water-saturated [bmim]PF_6 (data not shown). It is interesting to note that no direct correlation between the surfactant or the ionic liquid concentration of the w/IL microemulsions and the stability of lipases was observed. However, in most cases studied, higher stability of lipases was observed in systems with low water content. In w/IL microemulsions with constant surfactant concentration (S systems), lipases exhibited higher stability when the w_o is low (S2 system). This is in accordance to that observed for various enzymes in other micro-heterogeneous media and w/o microemulsions [4,6,41]. The higher stability of lipases observed in systems with low w_o values could be related to the higher rigidity of the protein molecules in the water microdroplets, as indicated in Section 3.3. It must be noted that the correlation between the w_o value and the enzyme stability mentioned in S systems is not observed in W systems, which indicates that the composition of the w/IL microemulsion systems also affects enzyme's stability. A number of reports show that lipases in ionic liquids maintain their activity over a much longer period than in organic solvents and often at a relatively higher temperature [11,36–38]. The stabilization effect is often attributed to various factors including electrostatic interactions between ionic liquids and enzyme molecules [39]. Sheldon et al. [10,40] suggested the protecting effect of the ionic liquid on the essential water surrounding the enzymes which leads to enzyme stabilization. They also suggested that the high viscosity of ionic liquids slows the migration of protein domains from the active conformation into the inactive one. In the case of w/IL microemulsions, it is expected that a small amount of ionic liquid will be soluble on the aqueous microphase of the system and therefore on the enzyme microenvironment facilitating in this way such protective interactions between ionic liquid and protein molecules.

3.6. Structural studies

The conformational changes of the lipases upon entrapment in w/IL microemulsion systems compared to other microheterogeneous media were investigated using two well-established techniques for conformational analysis of proteins, specifically CD and FT-IR spectroscopy. It must be noted that CD spectroscopy cannot be applied in w/IL liquid microemulsion systems because of low signal intensity and excessive background noise, due to the presence of the imidazolium ring of the ionic liquid used [42]. On the other hand, the ATR-FT-IR technique renders feasible the study of a protein's secondary structure in liquids including ILs-based media [43]. Proteins absorb in infrared in the amide regions, due to the peptide bond vibrations. The amide I region (mainly due to the C=O stretching vibration) found at approximately 1600–1700 cm^{-1} is mostly used in protein secondary structure determination due to its sensitivity in conformational changes and the significantly higher signal intensity than in other amide bands. The band consists of several overlapping components that are assigned to different secondary structure elements (see SI, figure SI-1). The second

Table 2
Half-life constants ($t_{1/2}$, h) for three lipases in various systems.

Enzyme	T (°C)	Aqueous	AOT/RM	SLM	W1	W3	W7	S2	S4	S7
Crl	30	2.5	0.2	0.3	12.6	23.5	8.5	30.1	1.7	1.5
	40	1.3	0.1	0.2	2.0	8.3	7.1	13.3	1.0	1.2
	50	0.7	<0.1	<0.1	0.9	6.8	4.7	9.9	0.7	0.1
Cvl	30	6.5	>100	>100	58.8	>100	6.1	>100	>100	24.4
	40	2.2	90	>100	40.5	48.0	2.9	22.7	24.2	20.7
	50	0.9	4.9	19.6	28.4	41.3	1.8	4.3	5.9	10.9
Tll	30	75.0	0.9	32.6	>100	>100	>100	>100	24.0	17.6
	40	53.4	0.8	17.1	36.9	47.7	>100	67.9	22.6	17.4
	50	6.4	0.6	8.8	3.4	31.2	64.9	38.9	20.7	16.0

Table 3
Quantitative estimation (%) of the secondary structure elements of lipases in water and in S4 microemulsion system calculated by FT-IR analysis in amide I region (β-sheet content includes the antiparallel β-sheet content).

Enzyme	Medium	α-Helix	β-Sheet	β-Turns	Random	Antiparallel β-sheet
Crl	Aqueous	25.3	30.7	23.3	20.7	13.0
	S4	23.5	44.2	20.2	12.1	13.7
Cvl	Aqueous	22.0	37.5	22.8	17.7	25.9
	S4	25.1	39.6	20.1	15.1	11.5
Tll	Aqueous	32.6	26.7	34.1	6.6	7.3
	S4	26.7	46.9	15.1	11.4	10.5

derivative method is a reliable and commonly used method to identify and separate these components [44].

The secondary structure elements of the lipases in aqueous solution and in w/IL microemulsion system S4 are presented in Table 3. Increased β-sheet content was observed for lipases in aqueous solution, referring to previous works [45–49]. This difference can be attributed to the high enzyme concentration used in FT-IR experiments (1.5 mg/ml), which can lead to protein aggregation [50]. The content of antiparallel β-sheets is an index for intermolecular interactions, which lead to the formation of protein aggregates [51].

As it can be seen in Table 3, lipases entrapped in w/IL microemulsions (system S4) undergo some alterations in their structure. Crl shows a decrease in random coil when entrapped in S4 systems, while Tll exhibits a decrease in α-helix and β-turns content and an increase in β-sheet and random coil. Moreover, an increase in β-sheet content was observed in all lipases when entrapped in S4 system. This increase can be attributed to a more rigid structure that the lipases tend to adapt when entrapped in water-restricted environment [52,53]. This observation is in accordance with the high enzymes stability observed in w/IL microemulsion systems (Table 2).

The effect of the system composition onto enzyme structure was investigated with the Cvl entrapped in various w/IL microemulsions (Table 4). In most cases studied, no significant change in the α-helix content was observed. Though a decrease of the antiparallel β-sheet content is observed in most cases (except for W7 system), the total β-sheet content was not reduced. This observation is in accordance with the assumption that the lipases adopt a rigid form in the w/IL microemulsion systems, which is more stable than the native one (as discussed also in Sections 3.3 and 3.5). However, in systems S7 and W7, where the Cvl stability was significantly lower than in other w/IL systems (Table 2), there was a significant decrease in the α-helix content. In system S7, the increase of the random coil is characteristic for enzyme unfolding which leads to deactivation. In the case of the system W7 an increase in antiparallel β-sheet content was observed, which can be attributed to a loss of hydrogen-bonding interactions between the water molecules and the surface of the protein [52]. The water stripping from the

Table 4
Quantitative estimation (%) of the secondary structure of Cvl in various systems calculated by FT-IR analysis in amide I region (β-sheet content includes the antiparallel β-sheet content).

Medium	α-Helix	β-Sheet	β-Turns	Random	Antiparallel β-sheet
Aqueous	22.0	37.5	22.8	17.7	25.9
W1	24.1	27.6	27.2	21.1	5.1
W3	26.5	47.7	21.0	5.0	5.0
W7	11.7	63.9	12.5	11.7	33.5
S2	25.8	41.4	15.7	17.2	0.7
S4	25.1	39.6	20.1	15.1	11.5
S7	16.3	46.8	10.7	26.1	15.9

Table 5
Quantitative estimation (%) of the secondary structure elements of lipases in water and in various microheterogeneous systems from CD spectra.

Enzyme	Medium	α-Helix	β-Sheet	Other
Crl	Aqueous	26.2	13.4	60.4
	AOT/RM	7.1	33.8	59.1
	SLM	3.0	43.1	53.9
Cvl	Aqueous	33.8	10.0	56.2
	AOT/RM	7.8	44.9	47.3
	SLM	2.1	45.2	52.7
Tll	Aqueous	35.9	5.6	58.5
	AOT/RM	7.3	45.4	47.3
	SLM	1.7	38.9	59.4

enzyme surface leads to intermolecular interactions, such as these observed in lyophilisation process [54] and finally leads to enzyme unfolding.

In order to compare structural changes of lipases after their entrapment in w/IL microemulsions with those occurred in other microheterogeneous media, the secondary structure of the lipases entrapped in w/o microemulsions and ternary surfactantless systems was investigated by far UV-CD spectroscopy. An inconsistency of secondary structure of lipases in water was observed between the results obtained from CD and FT-IR spectroscopy, which can be attributed to the enzyme concentration used in each experimental procedure, as discussed before. From the CD data, presented in Table 5, we observed that the secondary structure of lipases is significantly altered from their native forms when entrapped in w/o microemulsions or surfactantless systems. Specifically, in all cases, a substantial decrease in α-helix (up to 32%) and an increase in β-sheet content (up to 35%) were observed with respect to the lipase's structure in water, while these variations were more prominent in the case of the surfactantless microemulsions. Similar decrease in α-helix content of proteins entrapped in water restricted media was observed was also reported [35,52,55]. These extended structural changes in w/o microemulsions and SLM systems may explain the lower stability of lipases in these microheterogeneous media compared to that observed in w/IL microemulsions.

3.7. Enzyme reuse

Despite the application advantages offered by microemulsion media the applications of this process are hindered because product isolation and repeated use of enzyme. In this study, the ability to repeated use of Tll entrapped in w/IL microemulsion systems

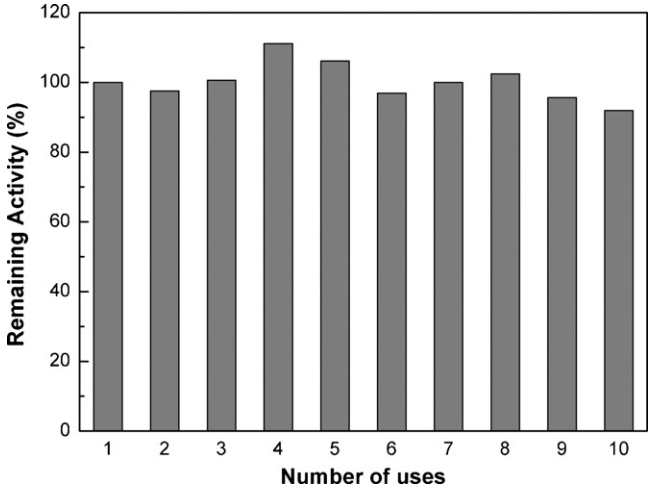

Fig. 5. Reusability of Tll entrapped in W3 w/IL microemulsion system, at 30 °C.

was investigated for the esterification of 1-butanol with caprylic acid at 30 °C. The reaction was performed for 8 h after which the product and remaining substrates were extracted with equal volume of *n*-hexane. The extraction procedure is repeated 5 times in order to remove any trace of the product and reactants. It must be noted that the solubility of ionic liquid, Tween 20 as well as water is negligible in *n*-hexane phase, while the enzyme remains in the ionic liquid-based microemulsion phase and can be used repeatedly. New substrates were added into extracted lipase-contained w/IL microemulsions and the reaction mixture was incubated at 30 °C in a new reaction cycle. When ten reaction cycles were completed (80 h of total operation) the residual activity of the lipase is 90%, as presented in Fig. 5, indicating that the microenvironment of the w/IL microemulsion exerts excellent protection to the entrapped enzyme molecules after repeated uses.

4. Conclusions

In the present work we have shown that w/IL microemulsion systems formulated with a non-ionic surfactant such as Tween 20 are a promising medium for biocatalytic processes. The lipases from *C. rugosa, C. viscosum* and *T. lanuginosa* exhibited higher catalytic performance and operational stability in these novel systems in comparison to other micro-heterogeneous media used so far for various biocatalytic reactions. The retention of catalytic activity is due to the entrapment of enzyme molecules into aqueous microdroplets formed in w/IL microemulsions, indicating that these IL-based reaction systems provide a protective environment for the enzymes. This observation was confirmed by spectroscopic studies which indicate that enzymes entrapped in w/IL microemulsions tend to retain their native structure or adapt a more rigid structure compared to that observed in other reaction media. The enhanced stability of enzymes in w/IL microemulsions, the ability of easily separation of products from the reaction system and good enzyme reusability, as well as the unique solvent properties of ionic liquids compared to conventional organic solvents, indicate that these novel micro-heterogeneous media can be efficiently used as reaction media for various biocatalytic reactions.

Acknowledgments

This work was supported from the Research Committee of the University of Ioannina, Greece. We also want to acknowledge Mr. D. Kyrkas and Dr. E. Macallini for their assistance. I.V.P. is very thankful to the Bodossakis Foundation for the financial support. The Jasco J-815 spectropolarimeter was granted to E.I.T. via the Epirus Regional Development Project of the 3rd Community Support Framework.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2009.03.007.

References

[1] P.L. Luisi, L.J. Magid, CRC Crit. Rev. Biochem. 20 (1986) 409–474.
[2] K. Martinek, N.L. Klyachko, A.V. Kabanov, Y.L. Khmelnitsky, A.V. Levashov, Biochim. Biophys. Acta: Biomembranes 981 (1989) 161–172.
[3] T. Maruyama, L.C. Park, T. Shinohara, M. Goto, Biomacromolecules 5 (2004) 49–53.
[4] C.M.L. Carvalho, J.M.S. Cabral, Biochimie 82 (2000) 1063–1085.
[5] N.L. Klyachko, A.V. Levashov, Curr. Opin. Colloid Interface Sci. 8 (2003) 179–186.
[6] H. Stamatis, A. Xenakis, F.N. Kolisis, Biotechnol. Adv. 17 (1999) 293–318.
[7] K. Tonova, Z. Lazarova, Biotechnol. Adv. 26 (2008) 516–532.
[8] U. Kragl, M. Eckstein, N. Kaftzik, Curr. Opin. Biotechnol. 13 (2002) 565–571.
[9] S. Park, R.J. Kazlauskas, Curr. Opin. Biotechnol. 14 (2003) 432–437.
[10] F. van Rantwijk, R.A. Sheldon, Chem. Rev. 107 (2007) 2757–2785.
[11] J.L. Kaar, A.M. Jesionowski, J.A. Berberich, R. Moulton, A.J. Russell, J. Am. Chem. Soc. 125 (2003) 4125–4131.
[12] E. Fehér, B. Major, K. Bélafi-Bakó, L. Gubicza, Biochem. Soc. Trans. 35 (2007) 1624–1627.
[13] Y. Gao, N. Li, L.Q. Zheng, X.Y. Zhao, S.H. Zhang, B.X. Han, W.G. Hou, G.Z. Li, Green Chem. 8 (2006) 43–49.
[14] Y.N. Gao, S.B. Han, B.X. Han, G.Z. Li, D. Shen, Z.H. Li, J.M. Du, W.G. Hou, G.Y. Zhang, Langmuir 21 (2005) 5681–5684.
[15] M. Moniruzzaman, N. Kamiya, K. Nakashima, M. Goto, ChemPhysChem 9 (2008) 689–692.
[16] Z. Qiu, J. Texter, Curr. Opin. Colloid Interface Sci. 13 (2008) 252–262.
[17] M. Moniruzzaman, N. Kamiya, K. Nakashima, M. Goto, Green Chem. 10 (2008) 497–500.
[18] G.P. Zhou, Y. Zhang, X.R. Huang, C.H. Shi, W.F. Liu, Y.Z. Li, Y.B. Qu, P.J. Gao, Colloids Surf. B: Biointerfaces 66 (2008) 146–149.
[19] M. Zoumpanioti, M. Karali, A. Xenakis, H. Stamatis, Enzyme Microb. Technol. 39 (2006) 531–539.
[20] H. Stamatis, A. Xenakis, M. Provelegiou, F.N. Kolisis, Biotechnol. Bioeng. 42 (1993) 103–110.
[21] Y.L. Khmelnitsky, A. Van Hoek, C. Veeger, A.J.W.G. Visser, J. Phys. Chem. 93 (1989) 872–878.
[22] G.E. Crooks, G.D. Rees, B.H. Robinson, M. Svensson, G.R. Stephenson, Biotechnol. Bioeng. 48 (1995) 78–88.
[23] J.P. Henley, A. Sadana, Enzyme Microb. Technol. 7 (1985) 50–60.
[24] E. Serefoglou, K. Litina, D. Gournis, E. Kalogeris, A.A. Tzialla, I.V. Pavlidis, H. Stamatis, E. Maccallini, M. Lubomska, P. Rudolf, Chem. Mater. 20 (2008) 4106–4115.
[25] G. Deleage, C. Geourjon, Comput. Appl. Biosci. 9 (1993) 197–199.
[26] N. Sreerama, R.W. Woody, Anal. Biochem. 209 (1993) 32–44.
[27] V.M.G. Lima, N. Krieger, D.A. Mitchell, J.D. Fontana, Biochem. Eng. J. 18 (2004) 65–71.
[28] M.L.M. Fernandes, N. Krieger, A.M. Baron, P.P. Zamora, L.P. Ramos, D.A. Mitchell, J. Mol. Catal. B: Enzym. 30 (2004) 43–49.
[29] M. Moniruzzaman, Y. Hayashi, M.M.R. Talukder, E. Saito, T. Kawanishi, Biochem. Eng. J. 30 (2006) 237–244.
[30] M.J. Hossain, T. Takeyama, Y. Hayashi, T. Kawanishi, N. Shimizu, R. Nakamura, J. Chem. Technol. Biotechnol. 74 (1999) 423–428.
[31] B. Orlich, R. Schomacker, in: T. Scheper (Ed.), History and Trends in Bioprocessing and Biotransformations, Springer-Verlag, Berlin, Heidelberg, 2002, pp. 185–209.
[32] M.Y. Lee, J.S. Dordick, Curr. Opin. Biotechnol. 13 (2002) 376–384.
[33] M.M. Zaman, Y. Hayashi, M.M.R. Talukder, T. Kawanishi, Biochem. Eng. J. 29 (2006) 46–54.
[34] E.D. Brown, R.Y. Yada, A.G. Marangoni, Biochim. Biophys. Acta—Protein Struct. Mol. Enzymol. 1161 (1993) 66–72.
[35] A.G. Marangoni, Enzyme Microb. Technol. 15 (1993) 944–949.
[36] L. Gubicza, N. Nemestóthy, T. Fráter, K. Bélafi-Bakó, Green Chem. 5 (2003) 236–239.
[37] P. Lozano, T. De Diego, T. Sauer, M. Vaultier, S. Gmouh, J.L. Iborra, J. Supercrit. Fluids 40 (2007) 93–100.
[38] O. Ulbert, K. Bélafi-Bakó, K. Tonova, L. Gubicza, Biocatal. Biotransform. 23 (2005) 177–183.
[39] Y. Yuan, S. Bai, Y. Sun, Food Chem. 97 (2006) 324–330.
[40] R.A. Sheldon, R. Madeira Lau, M.J. Sorgedrager, F. Van Rantwijk, K.R. Seddon, Green Chem. 4 (2002) 147–151.
[41] A.A. Tzialla, E. Kalogeris, D. Gournis, Y. Sanakis, H. Stamatis, J. Mol. Catal. B: Enzym. 51 (2008) 24–35.
[42] R.M. Lau, M.J. Sorgedrager, G. Carrea, F. Van Rantwijk, F. Secundo, R.A. Sheldon, Green Chem. 6 (2004) 483–487.
[43] K.K. Chittur, Biomaterials 19 (1998) 357–369.
[44] A. Dong, P. Huang, W.S. Caughey, Biochemistry 29 (1990) 3303–3308.
[45] S. Noinville, M. Revault, M.H. Baron, A. Tiss, S. Yapoudjian, M. Ivanova, R. Verger, Biophys. J. 82 (2002) 2709–2719.
[46] C. Otero, M. Fernández-Pérez, J. Pérez-Gil, Enzyme Microb. Technol. 37 (2005) 695–703.
[47] P. Walde, Biochemistry 32 (1993) 4029–4034.
[48] D. Lang, B. Hofmann, L. Haalck, H.J. Hecht, F. Spener, R.D. Schmid, D. Schomburg, J. Mol. Biol. 259 (1996) 704–717.
[49] E.P. Melo, M.A. Taipa, M.R. Castellar, S.M.B. Costa, J.M.S. Cabral, Biophys. Chem. 87 (2000) 111–120.
[50] J.M. Palomo, M. Fuentes, G. Fernández-Lorente, C. Mateo, J.M. Guisan, R. Fernández-Lafuente, Biomacromolecules 4 (2003) 1–6.
[51] S. Mukherjee, P. Chowdhury, F. Gai, J. Phys. Chem. B 111 (2007) 4596–4602.
[52] T. De Diego, P. Lozano, S. Gmouh, M. Vaultier, J.L. Iborra, Biomacromolecules 6 (2005) 1457–1464.
[53] K. Griebenow, A.M. Klibanov, J. Am. Chem. Soc. 118 (1996) 11695–11700.
[54] G. Vecchio, F. Zambianchi, P. Zacchetti, F. Secundo, G. Carrea, Biotechnol. Bioeng. 64 (1999) 545–551.
[55] R.W. McCabe, A. Rodger, A. Taylor, Enzyme Microb. Technol. 36 (2005) 70–74.