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Effect of two series ionic liquids based on non-nutritive sweeteners on catalytic activity and stability of the industrially important lipases from *Candida rugosa* and *Rhizopus delemar*

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ABSTRACT

This is the first study on the effect of ionic liquids (ILs) based on non-nutritive sweeteners on the catalytic efficiency and the structural and the thermal stability of lipases from *Candida rugosa* (CRL) and *Rhizopus delemar* (RhDL). Initially, we synthesized two series of ILs, namely 1-akyl-3-methylimidazolium saccharinates {[CnC1im][Sac]} and 1-akyl-3-methylimidazolium acesulfamates {[CnC1im][Ace]}, where the alkyl substituent was butyl, hexyl, octyl or decyl. The activities of the two enzymes were tested in two hydrolytic reactions using walnut oil and 4-nitrophenyl acetate as substrates. The activity of CRL toward walnut oil was strongly influenced by the chain-length of the N-alkyl substituents at the imidazolium cation of the compounds. The ILs with short alkyl substituents (butyl or hexyl) activated moderately (up to 30%) or had no effect on CRL activity, while those with medium chain-length substituents strongly inhibited CRL. In the case of CRL, we observed the same behavior pattern for the two tested series of ILs, while for RhDL the same tendency was displayed only for the series of saccharinates. The coating of RhDL with 0.1 M [C8C1im][Sac] or [C8C1im][Ace] resulted in up to 1.5-fold increase in the enzyme initial activity. In contrast, CRL was strongly inhibited by these two ILs. We found that the effect of [C8C1im][Ace] on enzyme activity adopted concentration- and time-dependent manner for the two lipases.

We observed a moderate increase in hydrolytic activity (by 1.2-fold factor) and good storage stability of RhDL (more than 30 days at 25 °C) when small quantities of all ionic liquids (<0.04 M), were added to the enzyme solutions. At the same time, by adding of 0.02 M [C_4C_1im][Sac] or [C_4C_1im][Ace], CRL was also stabilized and its activity was preserved for more than 25 days at room temperature. For comparison, the activities of the stored at 25 °C non-treated with ILs soluble CRL and RhDL were reduced by half for 7 days and 5 days, respectively. In contrast, we observed rapid inactivation at higher temperatures for all ionic liquid-coated enzymes. For example, half-lives of 25 min and 57.3 min at 50 °C were estimated for the pre-treated with [C_4C_1im][Ace]-CRL and for the non-treated-CRL, respectively. In addition, applying Fourier transform infrared spectroscopy, we monitored the changes in the secondary structure of the two enzymes in presence of the tested ILs which explained the altered stability and the activities of the two enzymes. Interestingly, coating with small amounts of ILs prevents aggregation of the two lipases even at high protein concentration.

1. Introduction

Lipases (EC 3.1.1.3, triacylglycerol ester hydrolases) are widespread enzymes, which catalyze the hydrolysis of ester bonds in triacylglycerols with subsequent release of fatty acids, monoacylglycerols, diacylglycerols, and glycerol. They also catalyze the reversed reaction, i.e. esterification in non-aqueous media. Lipases are industrially applied in food, cosmetic, pharmaceutical, and detergent industries for modification of fats and oils, flavor and aroma synthesis, biodiesel production, racemic mixture resolution, etc. [1,2]. Lipases differ in their substrate specificity (chain length of the fatty acid moiety) and positional specificity (regiospecificity). In addition, it has been found recently that some lipases exhibit promiscuous catalytic activity and are able to

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catalyze also carbon–carbon, carbon–nitrogen, and carbon–sulfur bond-forming, oxidation reactions, etc., which makes them the most applicable enzymes in laboratory scale [3]. All lipases hold high degree of structural and functional similarity, regardless of their origin or low amino acid sequence homology [4]. They belong to the α/β fold family and their catalytic center consists of serine, histidine and aspartic acid/glutamic acid residues located in a conservative penta peptide motif. Most of the lipases have an α-helical fragment ("lid") which covers and thus protects the active site from the environment. Upon contact with a hydrophobic surface or a lipid–water interface, the "lid" undergoes conformational changes and moves away allowing the substrate to access the lipase active site. This typical for most of the lipases phenomena is known as interfacial activation [5].

In general, the advantages of enzymes over conventional catalysts are: their higher specificity and activity under mild reaction conditions, as well as their biodegradability and their production from renewable sources. Nevertheless, biocatalysts are much more expensive than conventional catalysts and scientists employ various methods to improve their activity and stability. The most popular strategies applied for enzyme stabilization and/or to alter enzyme specificity and enhance enzyme activity are immobilization, protein or media engineering [6,7].

Many additives such as polyethylene glycols, carbohydrate derivatives (Span, Tween, Triton-X), polyols are able to protect enzyme activity from the environment and in some cases to act even as an enzyme activators [8,9]. In recent years, ionic liquids (ILs) have attracted attention as novel enzyme coating agents, enzyme reaction media, solvent for extraction of biomolecules, etc. ILs are considered as environmentally friendly solvents due to their low vapor pressure, low melting temperature (<100 °C) and high thermal stability. Usually, they are composed of organic cation and organic or inorganic anion and their physicochemical properties are tunable with respect to polarity, viscosity, hydrophobicity, solvent miscibility through appropriate modification of the cation or anion. There are many literature data on enhanced thermal stability and storage stability of pre-treated with ionic liquids proteins and enzymes [10]. In addition, Yamamoto et al. reported that N-alkylpyridinium chlorides and N-alkyl-N-methylpyrrolidinium chlorides are able to induce refolding of denaturated hen egg white lysozyme and a single-chain antibody fragment ScFvOx [11]. Some imidazolium-based ILs are good media for oxidative folding of cysteine-containing peptides [12], at the same time triethyl ammonium phosphate was found to act as a refolding additive for chemically denaturated α-chymotripsin and succinylated concanavalin A [13]. In addition, it was shown that many proteases, lipases, oxido-reductases, galactosidases, etc., exhibit enhanced activity and enantioselectivity in ILs [10,14]. There are also data on the preparation of robust biocatalysts by coating of the enzymes with ionic liquids [9]. For example, coated with imidazolium-based or ammonium-based bis(tri(fluoromethyl)sulfonyl)imides {(NTf_2^-)} lipase from *Candida antarctica* B exhibited enhanced activity and enantioselectivity in the of acylation of 1-phenylethanol [15]. Extremely high enantioselectivity in reactions of acylation of 1,2-cyclic amino alcohols (*E* value 290–1143) was also estimated for the coated with ionic liquids lipase from *Burkholderia cepacia* while the native enzyme did not demonstrate preferences toward one of the enantiomers [16]. In addition, *C. antarctica* B lipase demonstrated very good stability in methanol and the lipase-catalyzed methanolysis of sunflower oil proceeded with 98% conversion of the substrates in presence of [C_4C_1im][NTf_2^-] and [C_2C_1im][NTf_2^-][17]. As a whole, enzymes are usually active in ILs containing tetrafluorborate [BF_4^-], hexafluorphosphate [PF_6^-] and bis[trifluoromethyl sulfonyl]imide [NTf_2^-] anions but they are not stable in ILs containing strongly coordinating anions [14,18].

There are many other examples of successful application of ILs as reaction media in biocatalysis [18,19]. However, the effect of ILs on enzyme activity and stability vary from one species to another, and it is either unpredictable or can be opposite for another enzyme. In recent years, many efforts have been focused toward the design of novel ionic liquids that are biodegradable, less toxic, and environmentally friendly and that have various applications. Novel ionic liquids that are able to maintain the enzyme activity and to ensure good storage stability and selectivity of the biocatalysts are still on demand.

The effect of some ionic liquids based on non-toxic organic anions, e.g. lactate and acetates, on enzyme activity also has been tested. In general, most of lipases were inactivated in such ionic liquids, while the activity of other enzymes such as dehydrogenases was enhanced [14,20,21]. Up to date, however, the effect of ionic liquids containing heterocyclic aromatic anions on the structure, stability and activity of lipases has not been established. It is worthy to be mentioned that saccharinate {[Sac]} and acesulfamate {[Ace]} have structural similarities to thiosaccharinate anion which was well known substituent of [NTf_2^-] in the chemistry of ionic liquids, and that imidazolium-based saccharinates and imidazolium-based acesulfamates are water miscible and has viscosity and melting points similar to these of ILs containing larger perfluoro anions which prove to be good reaction media for some lipase-catalyzed reactions [22]. Based on their appropriate physicochemical characteristics, we assume that imidazolium-based saccharinates and imidazolium-based acesulfamates have a potential to improve the catalytic efficiency of lipases.

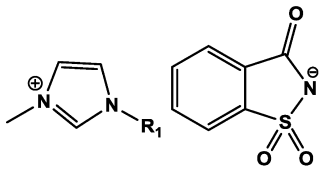
In addition, these two series of ILs are more "green" solvents. On one hand, the imidazolium-based ILs with short chain alkyl substituents at the cation are able to undergo fast biodegradation in 28 days. On the other hand, the non-nutritive sweeteners such as saccharinate and acesulfamates are environmentally more acceptable anions in comparison to the halogenated which are known to be persistent organic pollutants [23]. Moreover, it is known that the toxicity and the biodegradability of the ionic liquids depend on the good combination between the cation and the anion.

To the best of our knowledge, however, there is no systematic research on the effect of imidazolium-based saccharinates and acesulfamates on enzymes and proteins, in particular lipases. Therefore, the overall objective of the present study is to assess the effect of two series ionic liquids on the activity and the stability of two industrially important lipases. In the focus of this paper are the lipases from *Candida rugosa* and *Rhizopus delemar*, which are widely used in detergent formulations and diary industry [1]. We synthesized two series of 1-alkyl-3-methylimidazolium-ionic liquids in which the alkyl substituent varied in chain-length from butyl to octyl. The lipase activity was assayed in two test reactions. We estimated also the thermal- and the conformational stability of the enzymes in presence of the studied ionic liquids.

2. Experimental

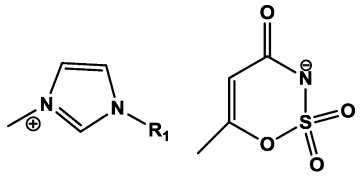
2.1. Materials

Lipase from *C. rugosa* (CRL) (MW 64 kDa, 30 U/mg (olive oil as a substrate), 10% (w/w) protein content) was provided by Amano Pharmaceutical Co., Japan. Lipase from *R. delemar* (RhDL) (MW 32 kDa 100 U/mg, olive oil as substrate) was purchased from Kerry Bio-Science. Walnut oil was purchased from the local market in Bulgaria and has similar lipid composition to sunflower oil. Gum arabic from acacia tree and 4-nitrophenyl acetate were obtained from Sigma. The ILs used were based on 1-alkyl-3-methylimidazolium ion in combination with saccharinate {[$C_nC_1$im][Sac]} and acesulfamate {[$C_nC_1$im][Ace]} as anions (see Scheme 1), and were



3-alkyl-1-methylimidazolium saccharinates

$R_1 = C_4H_9$; $[C_4C_1im][Sac]$
C_6H_{13}; $[C_6C_1im][Sac]$
C_8H_{17}; $[C_8C_1im][Sac]$
$C_{10}H_{21}$;$[C_{10}C_1im][Sac]$



3-alkyl-1-methylimidazolium acesulfames

$R_1 = C_4H_9$; $[C_4C_1im][Acs]$
C_6H_{13}; $[C_6C_1im][Acs]$
C_8H_{17}; $[C_8C_1im][Acs]$
$C_{10}H_{21}$;$[C_{10}C_1im][Acs]$

Scheme 1. Structures of the tested ionic liquids.

synthesized, purified and characterized by the authors as previously described [24,25]. The structure and the purity of ILs were proven by means of ^{1}H and ^{13}C NMR. The NMR spectra were consistent with that previously reported and silver nitrate test showed no residual chloride anions.

2.2. Lipolytic activity assays

2.2.1. Hydrolytic activity assay using the chromogenic substrate 4-nitrophenyl acetate

We applied a fast continuous method to assess the activity of lipases from *C. rugosa* and *R. delemar* in presence of ionic liquids at 25 °C. Prior to be tested, the lipase stock solutions (in 0.05 M sodium phosphate buffer, pH 7.0) were incubated with various concentrations (0.01–0.1 M in water) of the ILs at 25 °C for 1 min to 24 h. Then, aliquots of 0.01 mL CRL (5 mg/mL) or RhDL (5 mg/mL) were withdrawn and were added to a reaction mixture consisting of 1.64 mL sodium phosphate buffer (0.05 M, pH 7.0) and 0.05 mL 0.02 M 4-nitrophenyl acetate in DMSO. Immediately after the enzyme addition, we recorded the increase in absorbance at 410 nm spectrophotometrically which corresponded to the amount of the liberated 4-nitrophenol with time. To monitor the spontaneous hydrolysis of the substrate, control experiments without enzyme were carried out in presence and in absence of ionic liquids.

The relative activities (%) of the ionic liquids pre-treated CRL and RhDL were calculated in comparison to the activity of their non-treated counterparts. The activities of CRL and RhDL without additives were taken as 100%.

2.2.2. Lipolytic activity assay using walnut oil as a substrate

We tested also the activities of the lipases from *C. rugosa* and *R. delemar* in presence of the studied saccharinates and acesulfamates toward their natural substrates, namely the long-chain triacylglycerols that present in plant oils. In a typical reaction, 0.1 g walnut oil (approx. 0.1 M triglycerides) was added to 1.5 mL phosphate buffer (0.05 M, pH 7.0) containing 0.3% (w/v) gum arabic, and then the mixture was emulsified by vigorous magnetic stirring for 5 min at 40 °C. The reaction started by adding 0.05 mL of CRL (10 mg/mL) or RhDL (5 mg/mL). The reaction mixtures were stirred (250 rpm) at 40 °C for 1 h. For each reaction, the rate of hydrolysis of walnut oil was determined by titration of the released free fatty acids with an accurately standardized sodium hydroxide (0.01 M). Control experiments without enzyme were carried out.

To assess the effect of the ionic liquids on the activity of the two lipases, 0.1 M of saccharinates and acesulfamates were added to the emulsified substrate. Then, the activity was expressed as relative activity with respect of the activity estimated for CRL (or RhDL) in the control/reference experiment without additives, which was taken for 100%. The spontaneous hydrolysis of walnut oil in presence of ILs was also taken into account.

2.3. Thermal stability of C. rugosa lipase and R. delemar lipase in presence of ionic liquids

Stock solutions of CRL (10 mg/mL) and RhDL (10 mg/mL) were prepared in sodium phosphate buffer (0.05 M, pH 7.0). Then, the ILs were added to 1 mL of the lipase solutions in such quantities so their final concentration to be 0.1 M. The mixtures were gently stirred at 50 °C for different time intervals. The residual activity of the thermally treated biocatalysts was assessed in a reaction of hydrolysis of 4-nitrophenyl acetate as described in Section 2.2.1. The half-life time was calculated according to the following equations [26]:

$$\alpha = \frac{E}{E_0} = e^{(-kdt)} \quad \text{and} \quad t_{1/2} = \frac{\ln 2}{k_d}$$

where α is deactivation, E and E_0 are the specific activities of the biocatalysts in (U/mg) at given and zero times, k_d deactivation constant. The slope in the plot of $\ln(\alpha)$ vs time gives the deactivation constant (k_d).

The same experiments were carried out also at 25 °C and 40 °C.

2.4. Infrared spectroscopy

FTIR spectra of the CRL (40 mg/mL) and RhDL (40 mg/mL) dissolved in distilled water or in 5% (w/v) ionic liquid (in water) were recorded on Bruker Tensor 27 spectrometer, equipped with a detector of deuterated triglycine sulphate (DTGS). The FTIR spectra were collected by direct deposition of the samples on attenuated total reflectance (ATR) element (diamond crystal) in frequency region 4000–600 cm^{-1} (ATR) with 128 scanning and at resolution of 1 cm^{-1}. The spectra of the proteins were referenced to the respective spectra of water or aqueous solution of the corresponding ionic liquid (5% w/v in water) in order to subtract their absorptions.

In order to describe quantitatively the changes in the enzyme secondary structure induced by the saccharinates and acesulfamates, the ATR-FTIR spectra were treated in accordance with the established methods given in the literature [27,28]. ATR-FTIR spectra were Fourier deconvoluted by Opus software version 5.5 using band width of 14 cm^{-1}, 2.9 resolution enhancement factor, and Lorentzian lineshape. Second derivative spectra were obtained using the Savitzky–Golay algorithm based on 25 smoothing points. Then, the relative contribution of each band component of the Amide I band was determined by curve fitting following the procedure of OPUS program. In the fitting, the number of components and the initial values of their position were set as determined from

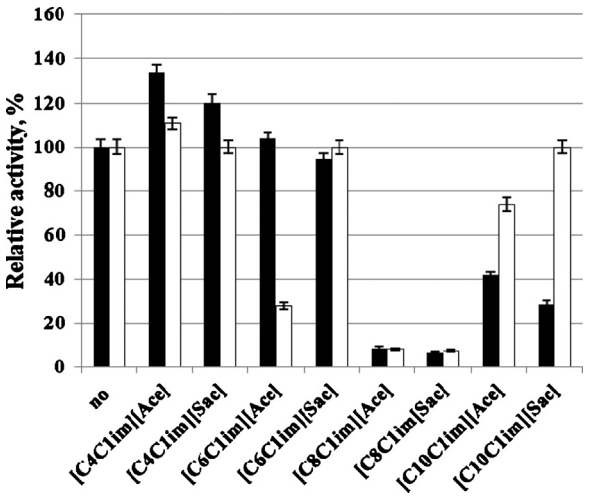


Fig. 1. Lipase-catalyzed hydrolysis of walnut oil in presence of imidazolium-based saccharinates and imidazolium-based acesulfamates. CRL (filled bars); RhDL (open bars). Reaction conditions: triglycerides (0.1 M, approx. 0.1 g walnut oil), ionic liquid (0.1 M), 0.05 M Na_2HPO_4 buffer (1.5 mL, pH 7.0), gum arabic (0.3% (w/v)), CRL (0.05 mL of 10 mg/mL) or RhDL (0.05 mL of 5 mg/mL) – stirring (250 rpm) at 40 °C for 60 min.

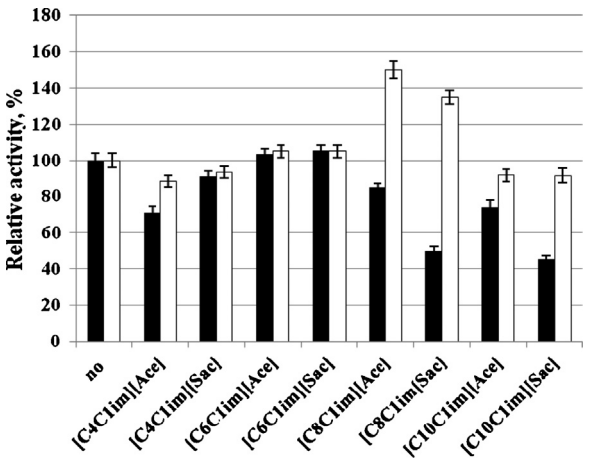


Fig. 2. Effect of pre-treatment of CRL and RhDL with saccharinates and acesulfamates on their catalytic activity in the reaction of hydrolysis of 4-nitrophenylacetate. CRL (filled bars); RhDL (open bars). Conditions: lipase stock solutions (0.1 mL, 5 mg/mL) were incubated with ionic liquids (0.1 M in water) at room temperature for 5 min. Then the activities of all samples were determined in reaction of hydrolysis of 4-nitrophenylacetate at 25 °C. The activities of the non-treated CRL and RhDL were taken for 100%.

the second derivative spectra. The initial bandwidth of all components was set to 14 cm^{-1} and the components were approximated by mixed Lorentzian/Gaussian functions. The curve-fitting was performed according to the Local Least Squares algorithm.

2.5. Statistical analysis

All kinetic experiments were performed in triplicate: the average values were reported along with Standard deviation.

3. Results and discussion

3.1. Effect of 3-alkyl-1-methylimidazolium saccharinates and 3-alkyl-1-methylimidazolium acesulfamates on catalytic activity of C. rugosa lipase and R. delemar lipase

In the reactions of hydrolysis of walnut oil, the substrate and the ionic liquids were mixed at equimolar concentrations (100 mM) before adding the lipases. The structure of the tested compounds is depicted in Scheme 1. In presence of ionic liquids the catalytic activity of CRL was altered comparing with the reactions carried out in conventional (aqueous) reaction media. We observed that CRL efficiency depends on the type of the alkyl substituents at the nitrogen atoms of the imidazolium cation rather than the structure of the anion of the tested ILs (Fig. 1). Most of the ionic liquids with short alkyl substituents of the imidazolium ring (butyl or hexyl) activated or preserved the activity of CRL. At the same time the efficiency of CRL was deteriorated in presence of ILs containing longer alkyl substituents in the cation. In the case of CRL, we observed the same behavior pattern for the two tested series of ILs, while for RhDL the same tendency was displayed only for the series of saccharinates.

Many authors reported on higher sensitivity of lipase activity and selectivity to the variations of the anions rather than to the type of the substituents in the cation [29–31]. In general, the more compact anions such as $[BF_4^-]$, $[PF_6^-]$, $[NO_3^-]$, $[CH_3COO^-]$, etc. possesses more localized negative ion charge, which suggests strong Coulomb interactions between them and the charged residues of the proteins. In contrast, saccharinate and acesulfamate anions are larger in size and their negative charges are delocalized in a considerably greater extent over. This is in agreement with the observed by us modest effect of the anion structure on the activity of the two lipases. Yet, there are some exceptions which are discussed in more details below. In general, the slight differences in activity of the ILs with the same cation are probably due to different interactions between saccharinate or acesulfamate anions and lipase molecules. For example, Nockemann et al. showed that despite of the structure similarities between the above discussed anions, they exhibit different charge distribution thereby offering different type of possible hydrogen bonding [32].

On the other hand, we were not able to find strong correlation between the structure of cation or anion of the ionic liquids and the enzyme activities for the pre-treated with ILs lipases from *C. rugosa* and *R. delemar*, tested in the reaction of hydrolysis of 4-nitrophenyl acetate at room temperature (Fig. 2). The incubation of the two lipases with $[C_4C_1im][Ace]$ and $[C_4C_1im][Sac]$ resulted in slight decrease in their activity in comparison to the activity of the non-treated enzymes. According to Klähn et al. the butyl residues of $[C_4C_1im]^+$ cations easily diffuse into the active site of *Candida antarctica* B lipase [33]. We also assume that these small molecules can interact with non-polar amino acid residues in the vicinity of the binding subside and consequently to induce a conformational change of the protein molecules.

In contrast to the observed low activity of RhDL in presence of 1-octyl-3-methyl imidazolium ILs, tested in hydrolysis of walnut oil (Fig. 1), we found that the pre-treatment with $[C_8C_1im][Sac]$ and $[C_8C_1im][Ace]$ resulted in activation of RhDL. The effect of $[C_8C_1im][Ace]$ on enzyme activity adopted concentration- and time-dependent manner for the two lipases (Fig. 3). Initially, for RhDL we observed up to 50% increase in enzyme activity for few minutes, and then the activity was reduced, moreover the effect was strongest for the highest IL concentrations. These observations can be explained on the basis of RhDL structure. According to the literature the acyl binding site of the lipase from *R. delemar* consists of a shallow hydrophobic hollow located near the protein surface and together with the catalytic center is buried beneath a surface loop ("lid") consisting of short amphipathic helix. Upon contact to the lipid–water interface, the loop undergoes a conformational change and the active site become accessible for the substrate molecules [34]. We assume that the hydrophobic octyl residue of $[C_8C_1im]$ cation, which resemble the acyl moieties of the lipids are able to induce a significant conformational change, and thus to alter the RhDL activity. Similarly, Raynova et al. observed an initial increase

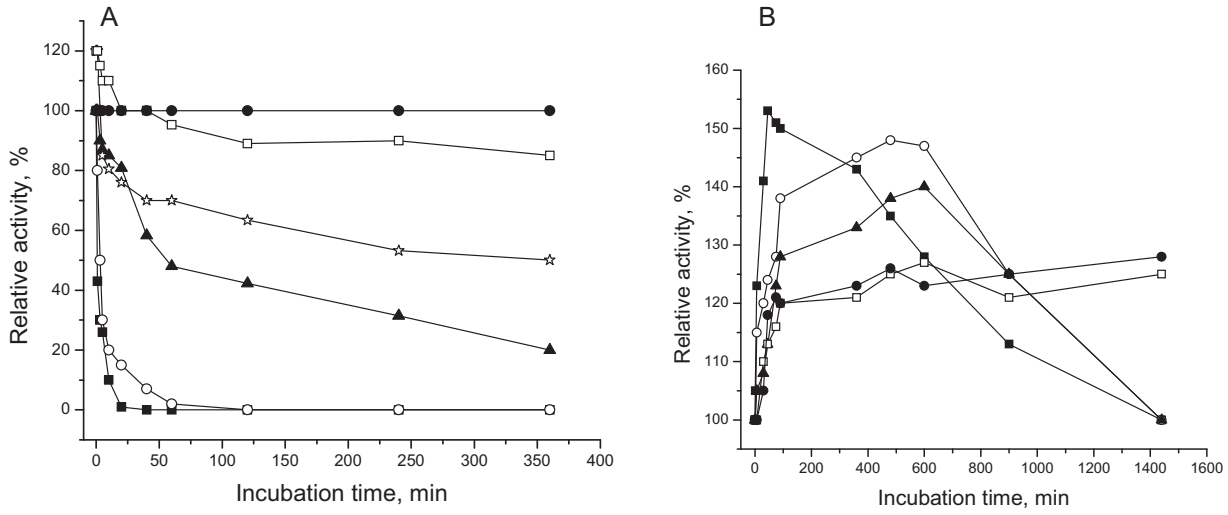


Fig. 3. Dependence of the activities of CRL (A) and RhDL (B) on the concentration of [C_8C_1im]Ace and the incubation time. Conditions: lipase stock solutions (0.1 mL, 5 mg/mL) were incubated with dissolved in water [C_8C_1im]Ace {0.01 M (filled circles); 0.04 M (open squares); 0.05 M (open asterisk); 0.06 M (filled triangles); 0.08 M (open circles) and 0.1 M (filled squares)} at room temperature for 1 min to 24 h. Then the activities of all samples were determined in reaction of hydrolysis of 4-nitrophenylacetate at 25 °C. Activities of the non-treated CRL and RhDL were taken for 100%.

in phenoloxidase activity of the treated with sodium dodecyl sulphate hemocyanin from *Helix aspersa* maxima, which they ascribed to changes in the secondary structure of the protein that caused an opening of the channel to the di-copper center and facilitated the access of the bulky phenolic substrates to the enzyme catalytic site [35]. However, the authors assume that at the final state of the conformational changes, the catalytically active conformation of the binuclear copper active site is affected, which is a possible explanation of the reduced phenoloxidase activity of the hemocyanin with the time [35].

In contrast, at high concentrations of the tested ionic liquid CRL was strongly inhibited, while at lower concentration (<0.02 M) the enzyme activity was preserved. According to the crystallographic data, the substrate binding pocket of CRL is tunnel-like (22 Å length, 4 Å width) and comprises of several hydrophobic residues which closely interact with the aliphatic chains of triglycerides, fatty acids, inhibitors, detergents, etc., especially with alkyl residues located from C4 to C7 and C12 to C14 in the acyl moiety [36]. Probably similar strong interactions occur between CRL molecules and the long alkyl chain substituent at the nitrogen atom of the imidazolium cations, therefore the access of the substrate to the enzyme active center was hindered which is a plausible explanation for the loss in activity.

3.2. Thermal stability of the pre-treated with ionic liquids lipases

The enhanced thermal stability is a desired characteristic of enzymes. Some of the advantages of performing the chemical reactions at higher temperatures are: better homogenization of the reagents, especially viscous lipid substrates; the reaction rate approximately doubles at 10 °C increase in reaction temperature; the risk of bacterial contamination is minimized, etc.

Very efficient and simple method for improving the enzyme thermal stability is immobilization [37]. Protein engineering is another successful strategy to enhance the enzyme stability [38]. Some additives like polyols, carbohydrates, etc. effectively protect enzymes from thermal denaturation [39].

We found that at 50 °C the inactivation was rapid for all ionic liquid-pretreated enzymes (Table 1). The stability of CRL and RhDL strongly depended on the substituents in the cation. In addition, a half-life of 18.3 min and 20.9 min at 40 °C were estimated for [C_8C_1im][Sac] and [C_8C_1im][Ace] incubated RhDL. The reduced

Table 1
Thermal stability of CRL and RhDL in presence of ILs at 50 °C.

Ionic liquid	$\tau_{1/2}$ (min)	
	CRL	RhDL
No additive	57.3	3.75
[C_4C_1im][Ace]	25.0	1.65
[C_6C_1im][Ace]	1.23	0.77
[C_8C_1im][Ace]	–	1.23
[$C_{10}C_1$im][Ace]	–	1.15
[C_4C_1im][Sac]	25.0	0.98
[C_6C_1im][Sac]	1.35	0.57
[C_8C_1im][Sac]	–	0.83
[$C_{10}C_1$im][Sac]	–	0.84

Reaction conditions: mixtures containing 1 mL CRL or RhDL (10 mg/mL) and ionic liquids (0.1 M) were incubated at 50 °C. The residual activity of the thermally treated biocatalysts was assessed in a reaction of hydrolysis of 4-nitrophenyl acetate.

thermal stability of RhDL in presence of these two ILs can be also a reasonable explanation for the lower relative activity of the enzyme in the reaction of hydrolysis of walnut oil (Fig. 1). In accordance to our expectations, the ILs with shorter alkyl substituents in the cation slightly affected the RhDL thermal stability at 40 °C with a half-life of approximately 40 min for [C_6C_1im]-derivatives, while [C_4C_1im]-derivatives have no effect on the stability at this tested temperature. Interestingly, we observed a moderate activation (by 1.2-fold factor) and good storage stability of RhDL (more than 30 days at 25 °C) when small quantities of all ionic liquids (<0.04 M), were added to enzyme solutions. In comparison the non-treated with ILs soluble RhDL (pH 7.0) lost 50% of its activity for 5 days at 25 °C. These results are consistent with the observation of other authors on the effect of ionic liquids containing [C_4C_1im] cations on the storage stability and activity of other lipases. For example, Vidya et al. reported that after 20 days of incubation the activity of the pre-treated with [C_4C_1im][NTf_2] and [C_4C_1im][PF_6] *Pseudomonas cepacia* lipase was 1.8- and 1.6-fold higher as compared to the non-incubated enzyme. The long-term stability of *P. cepacia* lipase was also preserved in [C_4C_1im][BF_4] for the same period [40]. *Candida antarctica* B lipase exhibited much greater stability in both 1-ethyl-3-methylimidizolium bis(trifluoromethylsulfonyl)-imide [C_4C_1im][NTf_2] and butyltrimethylammonium bis(trifluoromethylsulfonyl)imide [btma][NTf_2], [($t_{1/2}$)] 170 and 207 days, respectively [41]. In

Table 2
Main secondary structure elements of *Candida rugosa* lipase and *Rhizopus delemar* lipases obtained by FTIR-spectroscopy in water and in 5% (w/v) ILs.

Solvent	Relative area (%)				
	α-Helix	β-Sheet	β-Turn	Random coils/unordered structures/α-helix	Tyr-residues
CRL					
Water	27.17	34.88	19.99	–	5.13
[C_4C_1im][Ace]	11.55	43.99	18.5	16.46	–
[C_4C_1im][Sac]	16.67	30.55	30.12	17.00	–
[C_8C_1im][Ace]	21.10	13.20	33.25	13.53	4.75
RhDL					
Water	18.16	22.77	19.33	22.75	5.87
[C_4C_1im][Ace]	14.01	38.76	19.51	18.25	–
[C_4C_1im][Sac]	18.9	26.82	18.62	25.01	–
[C_8C_1im][Ace]	36.4	30.33	23.53	–	–

Table 3
Main secondary structure elements of *Candida rugosa* lipase in 50% (w/v) ILs obtained by FTIR-spectroscopy.

Solvent	Relative area (%)				
	α-Helix	β-Sheet	β-Turn	Random coils/unordered structures/α-helix	Aggregated strands
[C_4C_1im][Ace]	15.16	NA	NA	78.23	6.61
[$C_{10}C_1$im][Ace]	31.5	NA	NA	69.25	NA
[C_4C_1im][Sac]	18.62	28.36	14.22	NA	37.93
[$C_{10}C_1$im][Sac]	14.14	30.75	16.03	NA	39.07

addition, CALB exhibited an exclusive thermal stability in ammonium-based IL – half-life of 200 days at 40 °C; however at higher temperatures a fast decline in activity was found [42].

We found that small amounts of [C_4C_1im][Sac] and [C_4C_1im][Ace] (less than 0.02 M) were also good stabilizers for CRL and preserved its activity for more than 25 days at room temperature, while the activity of the non-treated soluble CRL was reduced by half for 7 days. At the same time, even small quantities of the other ILs added to the enzyme solutions completely deactivated the enzyme under the same conditions.

3.3. *Effect of the tested ionic liquids on the secondary structure of CRL and RhDL*

For the lipases, it is well known that the enzyme activity strongly depends on their conformations which in turn are sensitive to the reaction media [43–45].

We applied a single pass attenuated total reflection Fourier transform infrared spectroscopy to estimate the effect of the studied ionic liquids on the secondary structure of the two enzymes. The results were compared with those for CRL and RhDL in water. For each sample, the content of secondary structure elements was calculated by the analysis of the amide I band absorption using second derivative and curve fitting procedure as given in Section 2. For few of the ionic liquids, the data are summarized in Table 2. For the native lipases from *C. rugosa* and *R. delemar* we observed the main secondary structure elements in the following frequency intervals: α-helical structures at 1648–1658 cm^{-1}, organized β-sheets at 1623–1640 cm^{-1}, random coils and unordered α-helical structures at 1640–1648 cm^{-1}, disordered structures (including β-turns, loose, β-strands) at 1660–1695 cm^{-1}, aggregated strands and anti-parallel β-sheets at 1610–1620 cm^{-1}, and side-chain of the tyrosine residue at 1603–1609 cm^{-1} [46]. We observed that all ionic liquids induced a conformational change in the lipases molecules. The decrease in the α-helix content observed in the spectra of CRL in presence of [C_4C_1im][Ace] and [C_4C_1im][Sac], and in the spectrum of RhDL in presence of [C_4C_1im][Ace] is consistent with the observed enhanced activity of the two enzymes in these ionic liquids. Lowering of the α-helices content in the secondary structure of lipases in hydrophobic solvents or ionic liquids is supposed to correspond to the occupation of the molecules in a higher "open" conformation and facilitated access of the substrate to the active center and is in agreement with the results of other authors for lipases from *C. rugosa*, *Chromobacterium viscosum* and *Thermomyces lanuginosa*, *B. cepacia*, etc. in pure ionic liquids or water-ionic liquid emulsions [30,47]. We found very close similarities between the structure of RhDL in 5% (w/v) [C_4C_1im][Sac] and that in water, which can explain the same activity of the lipase in the two solvents (Fig. 2). At the same time, in presence of [C_8C_1im][Ace] (5%, w/v) the lipase from *R. delemar* underwent a significant conformational change and adopted more ordered secondary structure which resulted in increase in α-helical content and β-structures on behalf of the percentage of the unordered structures by 2-fold and 1.3–1.4 fold, respectively. In this more compact structure the tyrosine residues from the protein surface were buried, and thus the band at 1603 cm^{-1}, corresponding to the side-chain of the tyrosine residues exposed to solvent, observed in the spectrum of RhDL in water have disappeared. There are literature data, reporting that the more ordered protein structures are less heat-stable [48,49] which is a logical explanation to the observed by us decrease in thermal stability for RhDL in [C_8C_1im][Ace].

Unexpectedly, we had difficulties to record a reliable ATF-FTIR spectra of the two enzymes in [C_8C_1im][Sac] which we attribute to lower miscibility of the ILs with water and lower solubility of the proteins in the resulted water-IL emulsion.

In addition, to assess the effect of the concentration of the ionic liquids on the secondary structure of CRL, FTIR spectra of the enzyme dissolved in 50% (w/v) ionic liquids in water were recorded. We found that the higher concentration of the ionic liquids caused a serious damage to the protein structure (Table 3). We observed a correlation between the type of the anion and the content of the structural elements. In presence of high amount of saccharinates, CRL was partially unfolded and the share of β-structures and anti-parallel β-sheets prevailed. Furthermore, under these conditions the protein aggregation was strongly promoted. In contrast, only bands for α-helical components and high concentration of unfolded/random segments were of observed in the spectra of CRL in 50% (w/v) acesulfamate-based ILs. These results are consistent with the observed by us deleterious effect of higher [C_8C_1im][Ace] on the CRL activity (Fig. 3).

In summary, all tested ionic liquids had an impact on the secondary structure of the two lipases which subsequently resulted in

change of their catalytic efficiency, thermal- and storage stability. The type of the cation and the concentration of the ILs were the main factors influencing the activity of CRL, while in the case of RhDL, interactions between saccharinate and acesulfamate anions and lipase molecules are also plausible and indirectly evidenced. When ionic liquids containing [C_4C_1im] or [C_6C_1im] cations were added at a low concentration to the reaction mixture they induced a conformational change in which the lipase molecules underwent from "close" to "open" conformation which can explain their enhanced activities in these cases. In presence of ILs with longer substituents at the imidazolium cation, however, the lipase molecules underwent dramatic conformational change and the molecules were proved to be partially unfolded and prone to aggregation. Their active sites become inaccessible to the substrate and the enzyme heat stability was also affected.

4. Conclusions

Catalytic efficiencies and stabilities of tested lipases from *C. rugosa* and *R. delemar* were altered in presence of ionic liquids containing saccharinate or acesulfamate anions. All ILs induced also significant changes in the secondary structure of the lipases. Within the studied series of ILs, we observed stronger effect of the cation than the anion on the lipolytic activities of the two enzymes. Also, we observed a moderate increase in hydrolytic activities, and at the same time a significant increase in storage stability at 25 °C, for the two tested lipases in 5% (w/v) [C4C1im][Sac] and [C4C1im][Ace]. In addition, small quantities of all ILs prevented CRL and RhDL molecules from aggregation, which can be concluded from the lack of bands corresponding to aggregates in FTIR spectra. We found that RhDL hydrolytic activity was improved in water-ionic liquid emulsions containing either [C8C1im][Sac] or [C8C1im][Ace]. However, these ILs have negative effect on RhDL thermal stability, therefore they can be applied only in lipase-catalyzed reactions at ambient temperature.

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