Bulgarian Chemical Communications, Volume 49, Special Edition B (pp. 132 – 136) 2017

Structural basis for the inactivation of *Candida rugosa* lipase in the presence of amino acid ionic liquids

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Received February 24, 2017; Revised March 13, 2017

Dedicated to Acad. Bogdan Kurtev on the occasion of his 100th birth anniversary

The aim of this study is to evaluate the interactions between a lipase from *Candida rugosa* (CRL) with ionic liquids (ILs) consisting of cholinium [Chol] or 1-ethyl-3-methylimidazolium [emim] cation, and anions of amino acids with uncharged non-polar or polar side chains. The effect of the ILs on the enzyme activity was followed in a spectrophotometric assay using 4-nitrophenyl acetate as a substrate. The compounds were tested in a wide concentration range. Added to the reaction mixture at concentrations up to 0.025 mM, all cholinium-based ILs, except cholinium glycinate, enhanced or had no effect on CRL activity. Large influence of the anion and a clear trend in the order Leu>Trp≥Thr>Val≥Met>Ile>Gly of decreased activity was observed for the two series. The effects induced of [emim] were stronger than those observed for [Chol]. FTIR spectroscopy was applied to be monitored IL-induced changes in the secondary structure of CRL. A correlation between the activities of CRL in presence of ILs and the changes in the enzyme structure was established.

Key words: *Candida rugosa* lipase; ionic liquids; hydrolytic activity; protein secondary structure

INTRODUCTION

Lipases [EC 3.1.1.3] are hydrolytic enzymes that catalyze the hydrolysis of ester bonds in triacylglycerols, which results in formation of di-, monoacylglycerols, glycerol and fatty acids [1]. Despite of their origin, lipases share common structural organization (α/ß fold) and have the same catalytic centre [1]. They differ in their substrate specificity and biochemical properties, which is a key factor for their versatile applications as biocatalysts for various industrial processes, diagnostic tools or food supplements in medicine, biosensors for pesticide detection, etc. [2–6].

Microbial enzymes are of great commercial interest, which is due to their advantages over the enzymes of plant and animal origin. For example, they are obtained at lower production costs, they can be easily isolated and genetically manipulated, they are relatively more stable, *etc.* [7, 8].

Lipase from *Candida rugosa* (CRL) has a broad specificity and is used as catalyst in biodiesel production, reactions of enantioselective hydrolysis and/or esterification, modifications of natural products, etc. [9–12]. Yet, biocatalysts remain relatively expensive and less stable in comparison to the conventional catalysts and despite of their higher selectivity their industrial usage is not high.

It is noteworthy to be mentioned that most of the bacterial lipases exhibit good activity in organic solvents, while some of enzymes are alkaline-, acid- or salt-tolerant [13, 14]. Media engineering is an easy-to-perform and an effective strategy to enhance activity, improve stability and/or tuning the lipases selectivity [15, 16]. In some cases adding of a surfactant to the reaction media or pre-treatment of lipases with organic solvents may alter their specificity or may have an effect on their activity [17, 18]. In last two decades scientists have focused their attention on the possible application of ionic liquids (ILs) as solvents or co-solvents for enzyme-catalyzed reactions [19]. ILs are mixtures of organic cations and/or anions that melt below 100 °C [20]. Up to date there is no systematic knowledge on the interactions of ILs with lipases, in particular CRL, although many papers have been published on this topic. For example, CRL exhibited higher thermal stability in 1-methyl-3-octyl-imidazolium hexafluorophosphate [omim] [PF_6] than in hexane [21]. Li *et al.* reported that CRL chemically-modified with IL based on PEGylated imidazolium cation and dihydrogen phosphate anion have also enhanced thermal stability catalytic activity and is tolerant toward polar organic solvents in comparison to the native

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CRL [22]. In addition, Cabrera-Padilla *el al.* found that adding of 1-butyl-3methylimidazolium bis-(trifluoromethylsulfonyl) imide [bmim][NTf_2] to the enzyme loading solution facilitated CRL adsorption on eco-friendly organic polymer, which resulted in 3-fold increase of the immobilization yield [23]. Shah and Gupta reported a highly enantioselective transesterification of (±)-1-phenylethanol in [bmim][PF_6] catalyzed by CRL [24]. Enhanced enantioselectivity of CRL in [bmim][PF_6] was reported by other authors [25, 26]. On the other hand, CRL did not exhibit any activity in the reaction of acylation of flavonoid glycosides in 1-butyl-3-methylimidazolium tetra-fluoroborate [bmim][BF_4] and [bmim][PF_6] [27]. Low enantioselectivity was reported also for the CRL-catalyzed esterification of ibuprofen with 1-propanol in ILs containing imidazolium or phosphonium cation and organosulfate anion [28].

The activity of CRL in presence of ILs containing amino acid anions has not been evaluated yet. Such ionic liquids have relatively low toxicity and are considered as biocompatible and easily biodegradable [29]. In this research, we tested the activity of CRL in presence of IL containing 1-ethyl-3-methylimidazolium [emim] or cholinyl [Chol] cation and non-polar amino acids as anions. The results from the enzymatic activity assays are correlated with the induced by ILs changes in protein structure.

EXPERIMENTAL

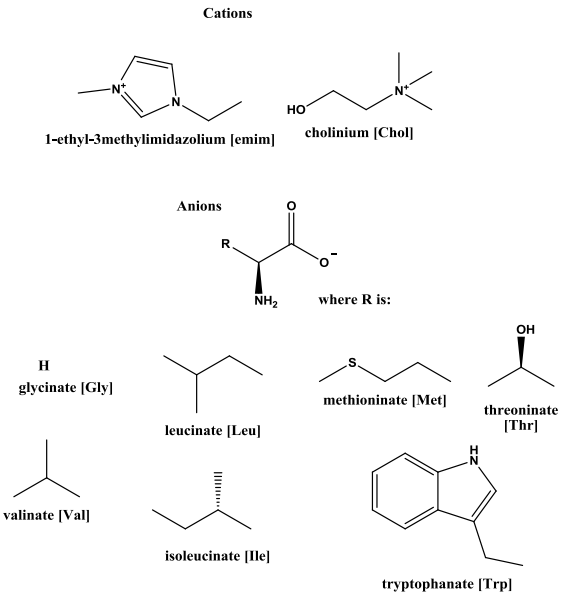
Materials

Lipase from *Candida rugosa* (CRL) (MW 64 kDa, 30 U/mg (olive oil as a substrate), 10% (w/w) protein content) was purchased by Amano Pharmaceutical Co., Japan. 4-Nitrophenyl acetate was obtained from Sigma. 1-Ethyl-3-methyl-imidazolium amino acids [emim][AA] and cholinium amino acids [Chol][AA] (Scheme 1) were synthesized, purified and characterized as previously described [30, 31].

Hydrolytic activity assay

Prior to be tested, 20 μL of CRL stock solutions (20 mg/mL in 0.05 M sodium phosphate buffer, pH 7.0) were mixed with 20 μL of solutions of ILs in water (0.01–0.5 M). Then, aliquots of 0.01 mL IL-treated CRL were withdrawn and were added to a reaction mixture consisting of 1.65 mL sodium phosphate buffer (0.05 M, pH 7.0) and 0.04 mL of 0.02 M 4-nitrophenyl acetate in DMSO. The release of 4-nitrophenol with the time was monitored spectrophotometrically at 410 nm (εmolar = 14 200 M^{-1} cm^{-1}). Spontaneous hydrolysis was taken into account in control experiments without an enzyme but in presence of the corresponding amount of IL (or water). The activity of CRL-IL complexes is expressed as relative activity in comparison to the activity of native CRL, which is taken for 100%.

All experiments were performed in triplicate and the mean values were reported. The relative standard deviation for each experiment was up to 5%.



Scheme 1. Structures of the tested compounds.

Fourier transform infrared spectroscopy (FTIR)

For the measurements, solutions of 40 mg/mL CRL in 5% (w/v) ionic liquid or water were prepared. Infrared spectra of the CRL-IL complexes were recorded on Bruker Tensor 27 spectrometer, equipped with deuterated triglycine sulphate detector (DTGS). Each sample was deposited onto a diamond crystal (ATR element) and a 64 scan interferogram was collected in a single beam mode, with 2 cm^{-1} resolution from 4000–600 cm^{-1}. Reference spectra containing the corresponding IL in the same concentration were recorded. A baseline correction was performed in the amide I region (1600–1700 cm^{-1}) assuming a linear baseline. In order to enhance the component peaks contributing to Amide I band the spectra were treated by Fourier-self deconvolution using Opus software version 5.5. Second derivative spectra were obtained using the Savitzky-Golay algorithm based on 25 smoothing points. In the fitting, the number of components and the initial values of their position were set as determined from the

second derivative spectra. The initial bandwidth of all components was set to 11 cm^{-1} and the components were approximated by mixed Lorentzian/Gaussian functions. The curve-fitting was performed according to the Local Least Squares algorithm. The assignment of the Amide I band positions to secondary structure was done according to the literature data [32].

RESULTS AND DISCUSSION

Activity of CRL in presence of [emim][AA] and [Chol][AA]

ILs containing amino acid anions with uncharged non-polar or polar side chains were selected for this study. All compounds were tested in a concentration range between 0.025 and 0.25 mM. We found that both the structure of the cation and the anion of the ILs have an effect on the CRL hydrolytic activity (Fig. 1A and 1B).

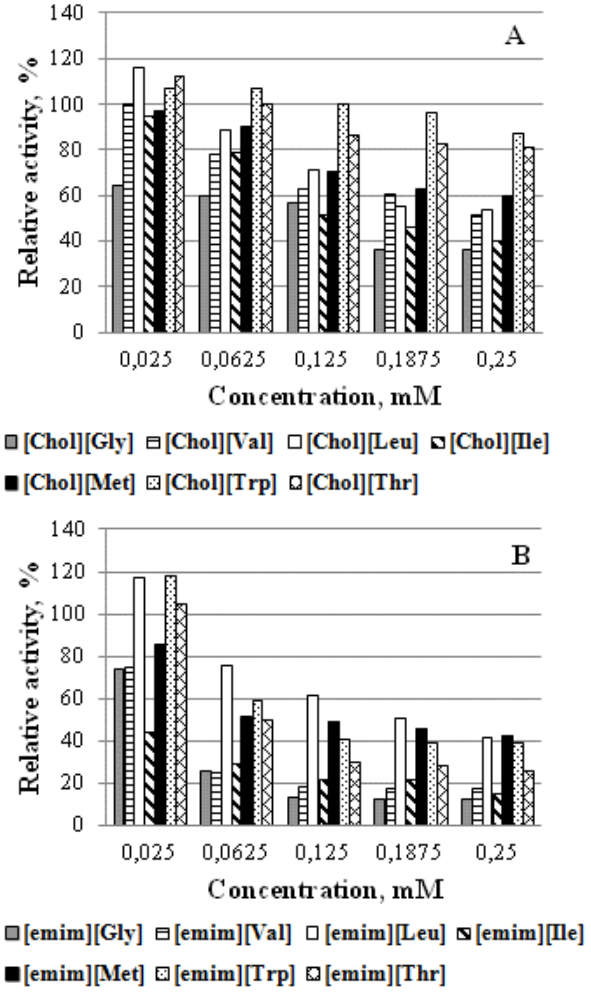


Fig. 1. Hydrolytic activity of *Candida rugosa* lipase in presence of cholinium-based amino acids (A) and 1-ethyl-3-methylimidazolium-based amino acids (B).

Significant decrease of the hydrolytic activity of CRL was observed in presence of all [emim]AAs, except for the most dilute solutions of [emim][Leu], [emim][Trp] and [emim][Thr]. On the other hand, CRL is relatively stable in presence of [Chol][Trp] and [Chol][Thr] at the all tested concentrations. In contrast, the hydrolysis rate of 4-nitrophenyl acetate tends to decrease in presence of the other cholinium amino acids in a dose-dependent manner. In comparison, Deive *et al.* reported for a downward shifts of the thermal unfolding of a lipase from *Thermomyces lanuginosus* in the presence of cholinium alaninate, cholinium glycinate and cholinium lysinate [33]. It was assumed that the examined salts induce also some changes in the lipase conformation, which resulted in its increased lipolytic activity [33]. In addition, higher yield of the target fatty acid esters *i.e.* enhanced esterification activity was reported for CRL pre-coated with tetraethylammonium l-histidinate and tetraethylammonium l-asparaginate [34]. To the best of our knowledge, beside the above two papers, there is no systematic research on the effect of amino acid-based ILs on the activity and/or structure of lipases. Large influence of the anion and a clear trend in the order Leu>Trp≥Thr>Val≥Met>Ile>Gly of decreased activity was observed for the two series.

Conformational changes in CRL molecules in presence of [emim][AA] and [Chol][AA]

We recorded FTIR spectra of the CRL-IL complexes that exhibited an enhanced, unaltered and/or deteriorated catalytic performance in comparison to the activity of the native CRL in order to assess the changes in the enzyme secondary structures that were induced by the amino acid salts. There are several main secondary structures that were observed in the following frequency intervals: α-helical structures at 1650–1659 cm^{-1}, β-sheet structures at 1625–1635 cm^{-1}, random coils at 1640–1647 cm^{-1}, β-turns at 1670–1688 cm^{-1}, as well as aggregated or antiparallel β-sheets at 1609–1614 cm^{-1}, and vibrations of tyrosine residues comprising the protein molecule at 1600–1606 cm^{-1}. The estimated elements of the secondary structure are summarized in Table 1. A decrease in α-helix and β-structures in favor of unordered and aggregated structures has been found in the FTIR spectra of the two CRL-glycinate complexes. In addition, an increase in the proportion of the band, characteristic for the amino acid side-chains (mainly benzene ring of tyrosyl

Table 1. Main secondary structure elements of *Candida rugosa* lipase obtained by FTIR-spectroscopy in water solution of ILs.

Solvent	Relative area, %					
	α-helix	β-sheet	β-turn	random coils/unordered structures	antiparallel β-sheets/aggregated strands	Tyr-residues
water	27.2	34.9	20	–	–	5.1
[Chol][Gly]	16.5	20.6	18.3	26.5	7.8	17.2
[emim][Gly]	16.2	16.9	10.5	19.7	18.4	18.2
[Chol][Met]	25.8	16.5	11.9	28.5	17.3	–
[emim][Met]	25.2	18.6	10.7	25.5	20.1	–
[Chol][Val]	41.7	28.9	16.2	–	13.2	–
[emim][Val]	24.3	21.8	11.3	20.7	21.8	–
[Chol][Thr]	22.0	27.3	12.7	15.6	17.5	–
[emim][Thr]	25.3	20.7	13.1	25.1	–	14.9

Complexes were prepared by mixing of CRL (40 mg/mL, 0.62 mM) with water solutions of 100 mM [Chol][AA] or [emim][AA].

residues) can be seen, which means that these hydrophobic residues, which in the native lipase are buried in the interior of the protein molecule, in presence of [Chol][Gly] and [emim][Gly] become more exposed to the solvent. All this implies a partial unfolding of the CRL in presence of the two glycinates and correlates with the observed reduction in the enzyme hydrolytic activity.

At the lowest tested protein-to-IL ratio, [Chol][Met] and [Chol][Val] have no effect on the CRL activity, while [emim][Met] and [emim][Val] have weak inhibitory effect. No unfolding was noticed in the FTIR spectra of CRL in presence of these four ILs. However, in this case a significant rearrangement in the protein molecule was observed. Interestingly, the two IL with methionine anions induced the same structural changes in the CRL conformation and the effect of the cation is neglectable. On the other hand, both [Chol][Val] and [emim][Val] suppressed the aggregation and/or unfolding and the lipase folds into more coiled and compact conformation. In addition, an 1.5-fold increase in the α-helix content of CRL was observed in the presence of cholinium valinate. This change, however, probably did not occur near the enzyme active centre, thus the enzyme activity remained preserved. In contrast, the two ILs with the threonine anion induced reorganization in CRL molecules which probably makes the active site more accessible to the substrate and resulted in the enzyme activation.

CONCLUSION

Ionic liquids that contain anions of amino acids with medium or large size hydrophobic side-chain residues have a stimulatory effect on *Candida rugosa* lipase, when added to the reaction mixture in quantities up to 100-times higher than the concentration of the lipase. In general, such ILs do not promote unfolding, but induce rearrangement in the protein molecule and possibly the enzyme active site becomes more accessible to the substrate. On the other hand ILs with glycinate anion initiate processes of denaturation and aggregation which results in decreased enzyme activity.

Acknowledgements: *COST action CM1206 "EXIL" is gratefully acknowledged.*

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ОБЯСНЕНИЕ НА РАЗЛИКИТЕ В АКТИВНОСТТА НА ЛИПАЗА ОТ *CANDIDA RUGOSA* В ПРИСЪСТВИЕ НА ЙОННИ ТЕЧНОСТИ НА ОСНОВАТА НА АМИНОКИСЕЛИНИ ЧРЕЗ ПРОМЕНИ В ПРОТЕИНОВАТА КОНФОРМАЦИЯ

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Постъпила на 24 февруари 2017 г.; Коригирана на 13 март 2017 г.

(Резюме)

Във фокуса на настоящото изследване са взаимодействията на липаза, изолирана от *Candida rugosa* (CRL), с йонни течности, съдържащи холин [Chol] или 1-етил-3-метил имидазолиев [emim] катион и аниони – незаредени аминокиселини. Ефектът на йонните течности (ЙТ) върху ензимната активност беше изследван за всички съединения в широк концентрационен интервал в моделна реакция спрямо субстрат 4-нитрофенил ацетат. С изключение на глицината, всички останали ЙТ от холиновата серия нямат ефект или повишават активността на CRL, добавени в ниски концентрации към реакционната смес. За двете серии ЙТ се наблюдава зависимост на ензимната активност от структурата на аниона, макар че тенденцията е по-ясно изразена при серията, съдържаща [emim] катион. С помощта на ИЧ спектроскопия бяха проследени промените във вторичната структура на CRL, индуцирани от ЙТ. Направена е корелация между възникналите промени в структурата на ензима и отчетената активност в присъствие на ЙТ.