Journal of Molecular Catalysis B: Enzymatic 122 (2015) 125–130

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Tuning of hydrophilic ionic liquids concentration: A way to prevent enzyme instability

Mina Solhtalab[a], Hamid Reza Karbalaei-Heidari[a,*], Ghodratollah Absalan[b]

[a] Molecular Biotechnology Lab., Department of Biology, Faculty of Science, Shiraz University, Shiraz 71454, Iran
[b] Professor Massoumi Laboratory, Department of Chemistry, Faculty of Science, Shiraz University, Shiraz 71454, Iran

ARTICLE INFO

Article history:
Received 28 April 2015
Received in revised form 2 September 2015
Accepted 2 September 2015
Available online 7 September 2015

Keywords:
Hydrophilic ionic liquids
Lipase
Thermoanaerobacter
Thermohydrosulfuricus
Thermostability

ABSTRACT

Ionic liquids (ILs) are considered as green alternatives to organic solvents in various enzymatic biotechnological processes. A wide variety of hydrophilic and hydrophobic ionic liquids have been synthesized in order to investigate their effects on both enzyme activity and stability. In most cases, hydrophobic ILs such as those containing $PF6^-$ anion have shown better performances in enzymatic reactions. In the present work, the effect of alkyl chain length of $[C_nMIM][Br]$ ($n = 2, 4, 6, 12$) on hydrolytic activity of *Thermoanaerobacter thermohydrosulfuricus* lipase (TTL), as a model enzyme, was evaluated and compared with $[C_4MIM][PF_6]$ as a hydrophobic ionic liquid. Moreover, effect of hydrophilic ILs on the activity and thermal stability of TTL in different concentrations of ionic liquids was studied. The results showed that TTL had the highest lipase activity in the presence of 0.3 M $[C_2MIM][Br]$, 1 M $[C_4MIM][Br]$ and 0.3 M $[C_6MIM][Br]$. However, no significant effect was revealed in the presence of $[C_{12}MIM][Br]$. A comparison between the effects of $[C_4MIM][PF_6]$ and $[C_4MIM][Br]$ on the TTL thermostability at 85 °C and 90 °C revealed that the hydrophobic anion PF_6^- provided a good stabilizing effect for TTL; although the influence of hydrophilic functions is also promising. According to the findings, it could be concluded that the TTL and probably similar enzymes can be used for several biotransformation reactions by tuning the hydrophilic ionic liquids concentrations as solvents.

1. Introduction

Applying temperatures higher than 60 °C during industrial bioprocesses provides a great deal of advantages such as higher substrate solubility, lower viscosity, higher diffusion coefficients of organic compounds, higher reaction rates and less contamination [1]. Also, organic solvents provide numerous advantages for industrial reactions such as increasing solubility of hydrophobic substrates, change of thermodynamic preference in reactions, preventing water-dependent side-reactions and contamination [2]. But, one of the major obstacles facing the application of enzymes in industrial biotechnology is their low stability at process conditions such as high temperatures and presence of organic compounds. Organic compounds are present in most bioprocess reactions such as esterification, transesterification, and resolution of racemic mixtures. However, these conditions make most of the enzymes inactive or considerably destabilize them. To solve these problems, different strategies have been performed, such as isolation of thermostable species [3] or using various molecular engineering methods [4]. Ionic liquids (ILs), as green alternatives for organic solvents, recently emerged as novel effective solvents for a wide variety of biocatalytic processes. Their physicochemical properties are finely tunable by altering their cationic and/or anionic constituents. The appropriate combination of the cationic and anionic parts in an IL might significantly increase substrate specificity, improve enzyme selectivity or enhance the enzyme activity and/or stability. Several studies have been carried out to investigate the effect of anionic [5,6] and cationic constituents as well as the alkyl chain length [7,8] of ILs on enzymes activity and stability. To the best of our knowledge, less attention has been devoted to hydrophilic ILs, such as those with bromide constituent, due to their destabilizing effect [9]. Moreover, the effect of ILs concentration on enzyme activity and stability is of great concern in biotransformation process.

In this work, *Thermoanaerobacter thermohydrosulfuricus* lipase (TTL) has been checked for its activity and thermostability in different ILs media. TTL is a novel enzyme lipase candidated for industrial applications such as producing enantiopure drugs [10]. The enzyme

* Corresponding author at: Molecular Biotechnology Lab., Department of Biology, Faculty of Sciences, Shiraz University, P. O. Box: 71467-13565, Shiraz 71454, Iran. Fax: +98 71 32280926.
E-mail address: karbalaei@shirazu.ac.ir (H.R. Karbalaei-Heidari).

http://dx.doi.org/10.1016/j.molcatb.2015.09.002
1381-1177/

is originated from an archaebacterium which has been isolated, characterized, and cloned by Royter [11]. Due to its thermostability and high S-enantioselectivity toward esters of secondary alcohols, TTL has many biotechnological applications. The effect of different concentrations of four imidazolium-based ILs, having different alkyl chain lengths, on TTL hydrolytic activity has been investigated and discussed. Also, the effect of nine different ILs: ([C_2MIM][Br], [C_4MIM][Br], [C_6MIM][Br], [C_{12}MIM][Br], [C_4MIM][PF_6], [C_5Py][Br], and [C_{12}Py][Br]) on TTL thermostability at elevated temperatures has been reported to compare the effect of their anionic, cationic parts as well as their alkyl chain lengths on TTL stability.

2. Materials and methods

2.1. Materials

All chemicals were purchased from commercial sources and were of analytical grade. 4-Nitrophenyl palmitate was purchased from Sigma. All UV and visible measurements were carried out by a Shimadzu UV–visible spectrophotometer (Type: UV-120).

2.2. Bacterial strains, plasmid and culture conditions

Escherichia coli XL1-Blue (Stratagene La Jolla, CA) and BL21 (DE3) (BD Biosciences, San Jose, CA) strains were used as host strains for cloning and expression, respectively. pQE-TTL construct was kindly gifted by Prof. Budisa, TUHH, Germany. The *E. coli* strains were grown at 37 °C in Luria–Bertani (LB) medium in the presence of 100 μg/ml ampicillin and the LB plates were prepared and solidified with 2% agar.

Starter cultures were prepared by growing a single colony of *E. coli* BL21 (DE3) cells harboring the recombinant plasmid overnight at 37 °C in flasks containing LB broth supplement with 100 μg/ml ampicillin. Then 50 ml medium inoculated with starter culture (1% (v/v)) was incubated to an OD_{600} of 0.9. The expression was induced with 1 mM isopropyl-β-D-tiogalactopyranoside under incubation at 30 °C with aeration rate of 180 rpm for 6 h.

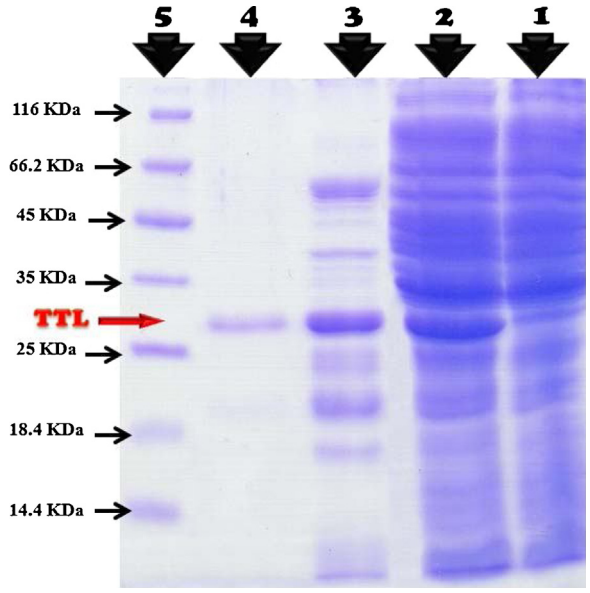

Fig. 1. The SDS-PAGE (12.5%) purification steps of TTL: Lane 1, cell lysate before IPTG induction; Lane 2, cell extract 6 h after IPTG induction; Lane 3, protein contents after heat precipitation; Lane 4, purified TTL after Q-Sepharose column; Lane 5, molecular mass markers.

2.3. Lipase purification

The crude intracellular TTL was isolated by lysing bacterial cells *via* freeze–thawing, treating with lysozyme, and subsequent sonication [10]. The collected crude lipase was purified using a two-step procedure. Heat precipitation was performed at 65 °C for 40 min and further purification was achieved on a Q-Sepharose anion exchange column chromatograph by applying a stepwise NaCl gradient.

2.4. Determination of hydrolytic activity of TTL

The enzyme activity was measured using 4-nitrophenyl palmitate as substrate. Cleavage of *p*NPP was determined at 65 °C in 50 mM Tris–NaCl buffer, pH ~8.0; containing 1% Triton X-100 according to Winkler and Stuckman [12]. A 10 mM stock solution of pNPP was prepared in acetonitrile and 40 μl of it was diluted to 400 μl with the assay mixture to reach a final concentration of 1 mM. The change in absorbance was monitored for 5 min at 410 nm. All measurements were duplicated and corrected for auto hydrolysis. In this text, one unit (1 U) of lipase activity is defined as the amount of enzyme needed to liberate one micromole of *p*-nitrophenol per minute at the experimental conditions described above.

2.5. Preparation of ionic liquids

2.5.1. General procedures for preparation of ionic liquids

All tested ionic liquids were routinely synthesized according to the procedure reported in the literatures [13,14] and their chemical structures were certified by NMR spectroscopy (Supplementary Figs. 1 and 2). The requisite aliphatic bromide (R-X, 200 mmol) was added dropwise to a stirring solution of methylimidazole (15 ml, 189 mmol). The reaction mixture was stirred and refluxed for 24 h at 50 °C. The mixture was then washed with diethyl ether for removing impurities, and then was heated at 50 °C for evaporating the solvent. Ionic liquids with various alkyl chain lengths (n = 2, 4, 6, 12) of methylimidazolium bromide were synthesized**.** For preparation of Ionic liquids with PF_6^- anion, the equal moles of NH_4PF_6 and [C_nMIM][Br] were applied. NH_4PF_6 was dissolved in deionized water and was added dropwise to a stirring solution of [C_nMIM][Br]. The reaction mixture was stirred for 24 h at room temperature to complete the substitution reaction.

2.6. Effect of ILs on TTL activity

A reaction mixture containing buffer, substrate and the selected IL with a final concentration in the range of 0.0–1.5 M was prepared. The hydrolytic activity of TTL was measured by monitoring the absorbance of the solution at 410 nm while adding suitable amount of the enzyme into the reaction mixture. To calculate the real hydrolytic activity of the enzyme, the auto hydrolysis of *p*NPP in the presence of each concentration of IL was corrected using the blank solution. Reproducibility of the data presented was confirmed by repeating the experiments at least thrice.

2.7. Effect of ILs on TTL thermostability

In order to investigate the thermal stability of TTL, 5.2 μg of the lyophilized enzyme powder was added to 600 μl of 50 mM Tris–HCl buffer, pH ~8.0, containing [C_4MIM][Br] at the final concentration of 1.0 M and/or was suspended directly in [C_4MIM][PF_6]. The samples were incubated at 85 °C and 90 °C in hot block. At regular time intervals (0, 15, 30, 45, 60 and 90 min), a fraction of the enzyme solution was withdrawn and cooled on ice for 30 min to allow renaturation of the reversibly denatured proteins. The remaining activity