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Evaluation of the toxicity of ionic liquids on trypsin: A mechanism study

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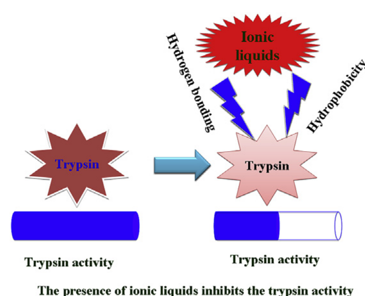
HIGHLIGHTS

- The presence of ionic liquids (ILs) inhibited the trypsin activity.
- The IL-trypsin interaction was mainly driven by hydrogen bonding.
- Besides hydrogen bonding, hydrophobicity was also an important parameter.
- Relationship of IC_{50}, hydrogen bonding ability and hydrophobicity was established.

GRAPHICAL ABSTRACT



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ABSTRACT

The toxicity of ionic liquids (ILs) was evaluated by using trypsin as biomarker. Experimental results indicated that the trypsin activity was inhibited by ILs and the degree of inhibition highly depended on the chemical structures of ILs. Primary analysis illustrated that hydrophobicity of ILs was one of the driven forces ruling the ILs-trypsin interaction. Thermodynamic parameters, Gibbs free energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) were obtained by analyzing the fluorescence behavior of trypsin in the presence of ILs. Both negative ΔH and ΔS suggested hydrogen bonding was the major driven force underlying the IL-trypsin interaction. To assess the toxicity of ILs, it should be considered the combination of the hydrogen bonding ability and hydrophobicity of ILs. A regression based model was established to correlate the relationship of the inhibitory ability, hydrophobicity and hydrogen bonding ability of ILs.

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1. Introduction

Over the past decade, ionic liquids (ILs) have attracted much attention because of their unique physicochemical properties such as extremely low vapor pressure, excellent thermal and chemical stability and good solubility for a wide range of organic and inorganic compounds (Hernández-Fernández, 2015). Therefore, ILs have been regarded as a good alternative to conventional solvents and widely used in chemical engineering, biocatalysis and nanotechnology fields (Ajloo et al., 2013). As the environment grew in importance, people come to realize that the use of ILs on a large scale inevitably causes the release of these chemicals into

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environment. The high chemical stability of ILs may lead to their accumulation in the circumstance, thus causing damage to the ecosystem and affecting living organisms. Therefore, it is worth investigating the toxicity and ecotoxicity of ILs systematically to understand their impact on human life comprehensively. Up to now, many works have reported the biological effects of ILs on aquatic organisms like fishes (Dong et al., 2013; Wang et al., 2010; Li et al., 2012), algae (Ma et al., 2010; Das and Roy, 2014; Chen at al., 2014), and marine invertebrates (Shi et al., 2013; Kumar et al., 2011; Pretti et al., 2009; Costello et al., 2009). The obtained results indicate that ILs do exhibit some toxicity. Furthermore, there are many other testing models used to evaluate the toxicity of ILs, such as bacteria (Yan et al., 2015; Costa et al., 2015), crops (Liu et al., 2015, 2013, 2014), mice/rats (Dumitrescu et al., 2014; Jodynis-Liebert et al., 2010) and cell lines (Samori et al., 2010; Radošević et al., 2013; Kaushik et al., 2012; Mikkola et al., 2015).

Enzymatic inhibition assay was another popular method for evaluating the biological toxicity and environmental impact of the most commonly encountered ILs. In this context, enzymes like acetylcholinesterase (Stolte et al., 2012; Schaffran et al., 2009; Torrecilla et al., 2009), lipase (Klähn et al., 2011), carboxylesterase (Laicure et al., 2013; Costa et al., 2014), catalase (Pinto et al., 2011; Saadeh et al., 2009), cellulase (Wang et al., 2011; Adsul et al., 2009; Ilmberger et al., 2012; Bose et al., 2012), and adenosine deaminase (Ajloo et al., 2013) are usually used. It is demonstrated that the alkyl chain length on the IL cation and the types of IL anions are the key parameters determining the toxicity of ILs (Pinto et al., 2011). An IL with a longer alkyl chain is more toxic; anions species have little effect on the enzyme activity (Pham et al., 2010).

Despite the progress made in this field, knowledge about the molecular interaction mechanisms between ILs and enzymes are still limited. Generally, the active sites of enzymes usually locate in their hydrophobic regions. Hydrogen bonding is also obligatory for maintaining the enzyme activity. The hydrophobic and hydrogen bonding interactions between ILs and enzymes may have negative influence on the enzyme stability (Fan et al., 2013a, 2012). These findings suggest that studies on the molecular mechanism of IL-enzyme interaction are extremely important to better understand the toxicity of ILs.

Trypsin is a common digestive protease excreted by the pancreas and takes part in the digestion of food proteins and other biological processes. If the pollutants enter gastrointestinal tract of humans or animals, they may interact with trypsin and affect the trypsin activity, causing pathological changes. Therefore, trypsin can be used as a biomarker to evaluate the toxicity of pollutants. Just recently, two papers have reported the use of trypsin as biomarker to evaluate the potential toxicity of dimethyl phthalate (Wang et al., 2015) and an azo dye (acid yellow) (Wang et al., 2012).

The aims of this work were therefore to investigate the molecular mechanism of IL-trypsin interaction with the use of fluorescence technique. The thermodynamic parameters of IL-trypsin interaction were characterized and a model for predicting the toxicity of ILs on trypsin was established.

2. Experimental

2.1. Materials

Trypsin from bovine pancreas ($\geq$2500 units mg^{-1}) was purchased from Aladdin Reagent Co. (Shanghai, China). Benzoyl-DL-arginine *p*-nitroanilide (BAPA) was purchased from Sigma–Aldrich Co. (St. Louis, USA). 1-Butyl-3-methylimidazolium chloride (99%, [C_4mim]Cl), 1-butyl-3-methylimidazolium bromide (99%, [C_4mim]Br), 1-butyl-3-methylimidazolium trifluoromethanesulfonate (99%, [C_4mim]TfMs), 1-butyl-3-methylimidazolium tetrafluoroborate (99%, [C_4mim]BF_4), 1-butyl-3-methylimidazolium acetate (99%, [C_4mim]Ac), 1-butyl-3-methylimidazolium nitrate (99%, [C_4mim]NO_3), 1-hexyl-3-methylimidazolium chloride (99%, [C_6mim]Cl), 1-hexyl-3-methylimidazolium bromide (99%, [C_6mim]Br), 1-octyl-3-methylimidazolium chloride (99%, [C_8mim]Cl), 1-octyl-3-methylimidazolium bromide (99%, [C_8mim]Br), 1-decyl-3-methylimidazolium chloride (99%, [C_{10}mim]Cl), and 1-decyl-3-methylimidazolium bromide (99%, [C_{10}mim]Br) were obtained from Lanzhou Institute of Chemical Physics of the Chinese Academy of Sciences (Lanzhou, China). All the other chemicals are analytical grade unless stated otherwise. Ultrapure water (18.2 MΩ cm) produced by an Aquapro purification system (Aquapro International Co., Ltd., Dover, DE, USA) was used throughout the experiments.

The stock solution of trypsin (0.5 g L^{-1}) was prepared by 1.0 mM HCl aqueous solution, Tris–HCl buffer (50 mM, pH 8.2) containing 0.02 M $CaCl_2$ was prepared by dissolving 6.05 g of tris(hydroxymethyl)aminomethane and 2.94 g of $CaCl_2 \cdot 2H_2O$ in 1.0 L of water; the substrate solution of BAPA was prepared as follows: dissolving 40 mg of BAPA in 1.0 mL of dimethyl sulfoxide and then diluting to 100 mL with Tris–HCl buffer prewarmed to 37 °C. Solutions of ILs (0.10 – 2.5 M) were prepared by Tris–HCl buffer and all the stock solutions were stored in the dark at 0–4 °C.

2.2. Trypsin activity assays

Trypsin activity, with BAPA as substrate, was measured via the method reported in the literature with minor modification (Ministry of Agriculture of the People's Republic of China, 2006). In brief: 0.1 mL of trypsin solution (0.5 g L^{-1}), 4.9 mL of Tris–HCl buffer and 5 mL of BAPA solution (0.4 g L^{-1}) were added into a 10-mL glass-stoppered tube. Before addition, all the solutions were incubated at 37 °C for 20 min at least. After incubation at 37 °C for 10 min, the absorbance of the resulting solution was measured by a spectrophotometer (model TU-1810, Purkinje General Instrument Co., Beijing, China) at 400 nm, wherein the trypsin activity was defined as the absorbance variation for a 10-min duration (ΔA_0 min^{-1}).

To study the effects of ILs, a specified amount of an IL was added into the above enzymatic reaction system, the trypsin activity in the presence of an IL was registered as ΔA min^{-1}; the relative activity of the enzyme with and without the presence of an IL was thus expressed as $\Delta A/\Delta A_0$. The half maximal inhibitory concentration (IC_{50}), indicating the concentration of each IL needed to inhibit trypsin activity by half was used to quantitatively evaluate the inhibition effectiveness of an IL on the trypsin activity.

2.3. Fluorescence measurements

Fluorescence spectroscopic analysis was performed on a Cary Eclipse fluorescence spectrophotometer (Agilent, Santa Clara, CA, USA) equipped with 1.0 cm quartz cells and a thermostatic bath. Typically, 5.0 mL of trypsin solution (0.5 g L^{-1}), and a known concentration of an IL were added to a 10.0 mL standard flask and diluted with Tris–HCl buffer solution to the volume. Fluorescence emission spectra of trypsin were measured in the range of 292–498 nm with excitation wavelength at 280 nm. The slit widths for both excitation and emission were 5 nm.

2.4. Measurements of the octanol–water partition coefficients (P) of ILs

The *P* values of the twelve ILs, used in this work, were measured per the methods already reported (Fan et al., 2014). Typically, the solutions of ILs (0.1 M for each) were prepared with Tris–HCl buffer saturated by octanol; 10.0 mL of the solution of a specific IL and

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