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The influence of two imidazolium-based ionic liquids on the structure and activity of glucose oxidase: Experimental and theoretical studies

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ABSTRACT

The search for ionic liquids (ILs) with biochemical and biomedical applications has recently gained great attention. IL containing solvents can change the structure, stability and function of proteins. The study of protein conformation in ILs is important to understand enzymatic activity. In this work, conformational stability and activity of the enzyme in two imidazolium-based ILs (1-butyl 3-methyl-imidozolium and 1-hexyl 3-methyl-imidozoliumbromides) were investigated. We treated glucose oxidase as dimer-active enzyme in different IL concentration and seen that GOx activity was inhibited in the presence of ILs. Our experimental data showed that inhibition of activity and reduction of enzyme tertiary structure are more for hexyl than butyl derivative. These experimental results are in agreement with foregoing observations. To find a possible mechanism, a series of molecular dynamics simulation of the enzyme were performed at different IL concentration. The structure parameters obtained from MD simulation showed that conformational changes at the active site and FAD-binding site support the hypothesis of enzyme inhibition at the presence of ILs. Root mean square deviation and fluctuation calculations indicated that the enzyme has stable conformation at higher IL concentration, in agreement with experimental observation. But hexyl derivative has a much stronger stabilization effect on the protein structure. In summary, the present study could improve our understanding of the molecular mechanism about the ionic liquid effects on the structure and activity of proteins.

1. Introduction

Ionic liquids (ILs) are a class of solvents that consist of salts that do not crystallize at room temperature. ILs have low volatility, low flammability and high thermal stability compared to some of the most common organic solvents, leading to their frequent classification as environment friendly green solvents. In addition, ILs are "designer solvents", because it is easier to change their structure and thus their solvent properties than for normal organic solvents [1]. All these interesting combinations of properties make ILs widely useful in several applications. ILs have been used to solvate biomaterials such as cellulose [2–4], inhibit or enhance enzymatic activity [5–7] enhance antibiotic efficacy and act as antimicrobial agents [8–10]. ILs are applicable for extraction [11], separation [12], organic synthesis and catalysis [13,14], inorganic synthesis [15], nanomaterial synthesis [16–19] and enzymatic reactions [20–25].

ILs has both stabilizing and destabilizing effect on protein, depending on its physico-chemical properties [26]. It was reported that [Bmim][Cl] and [Hmim][Cl] reduce the stability and activity of α-amylase using fluorescence and differential scanning calorimeter techniques [27]. Similarly, it was suggested destabilizing effect of [Bmim][Br] and [Chol][Cl] on the protein RNase A [28]. In contrast, a great number of experiments have indicated that ILs can stabilize the native structures of proteins [2, 29–37]. Another study showed that imidazolium-based ILs keep the native structure of lysozyme intact [38]. Some experiments have showed that ILs can be even used for refolding proteins [39,40]. Silva et al. also reported that longer alkyl group of imidazolium-based ILs (more hydrophobic) are associated with a higher destabilization effect on HSA than short alkyl group (less hydrophobic) [41]. ILs with long decyl chain on imidazolium cation has more destabilizing impact on the BSA than short chain ILs [42]. It was observed that [bmim]BF_4 did not affect the conformation of glucose oxidase using UV–Vis and CD spectra [43]. The Raman spectra indicated that there is no interaction between glucose and [Bmim]BF_4. Kudou et al. suggested that the improvement in the activity of β-glucosidase induced by [Bmim][OAc] could be linked to the flexibility of the conformation of protein [44]. Enzyme inhibition is extensively analyzed due to its great interest both in the study of

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enzyme mechanisms [45] and in pharmacological investigations [46], while enzyme activation is subject of a less detailed presentation. The role of alkyl chain length in the inhibitory effect on enzyme activity was also studied [47]. It was found that increasing the length of the hydrophobic tail leads to a decrease of the inhibition constant (K_i) for cresolase inhibition and an increase of the K_i value for catecholase inhibition.

Glucose oxidase (GOx) is a unique model enzyme because the oxidation of glucose by GOx is most widely studied among the enzymatic reactions due to its crucial applications in food and fermentation analysis, in medical diagnosis and in chemical analysis [48]. GOx is a dimeric enzyme containing two identical subunits (named as subunit A and B) and two tightly, but non-covalently, bound flavin adenine dinucleotide (FAD) as cofactors [49].

Molecular dynamics (MD) simulations on biomolecules could provide detailed information of the structural changes on the atomic level. Simulations can explore the relationships between the function and conformational structure of macromolecule [50]. Recent reports have shown that MD simulations can help characterize IL effects on proteins and elucidate stabilization and/or destabilization mechanisms [51–56].

Though the interactions of ILs with proteins/enzymes have been studied, understanding is not yet complete and does not provide a universal mechanism related to the influence of ILs on protein stability and activity [25,57,58]. Therefore, this study attempts to move forward in investigating IL-enzyme system to provide more details on how ILs can alter enzymatic activity. We recently reported the effect of $CaCl_2$ on the conformational stability and activity of GOx [59]. We noticed that divalent salt at high concentration could alter the conformational structure resulting in enzyme inactivation. In this study, efforts are made to investigate the effect of 1-butyl 3-methyl-imidozolium bromide ([C_4mim]Br) and 1-hexyl 3-methyl-imidozolium bromide ([C_6mim]Br) on the structure and function of glucose oxidase using combination of theoretical and experimental approaches.

2. Experimental

2.1. Materials

Glucose oxidase from *Aspergillus niger* (EC 1.1.3.4), peroxidase from *horseradish* (EC 1.11.1.7) and β-D-glucose were purchased from Sigma. 4-aminoantipyrine was bought from Merck. All other chemicals for the preparation of ILs were also purchased from Sigma-Aldrich-Merck Chemical Co., and were of the highest purity available. Double distilled water was used for all the measurements.

2.2. Synthesis of ionic liquids

Specific procedures to prepare 1-butyl 3-methyl-imidozolium bromide (named as IL1) and 1-hexyl 3-methyl-imidozolium bromide (named as IL2) were similarly to the procedures described for [C_4mim]Cl and [C_6mim]Cl, but using 1-bromobutane and 1-bromohexane, respectively [60,61]. The structures of two studied ionic liquids are shown in Scheme 1.

IL1 IL2

Scheme 1. Structure of studied ionic liquids.

2.3. Enzymatic activity measurement

GOx activity was determined using colorimetric method. The system involves two enzymatic reactions. First, GOx catalyzes the oxidation of β-D-glucose to produce gluconic acid and H_2O_2 in the presence of molecular oxygen. In a second step, peroxidase catalyzes the reaction of a dye precursor with hydrogen peroxide to produce quinonimine. The activity was estimated from the absorbance change at 525 nm because of the formation of quinoneimine dye. GOx in 10 mM phosphate buffer, pH 6.5, was incubated in the presence of two ionic liquids of IL1 (0.10, 0.20, 0.30, 0.40, 0.61, 0.81 M) and IL2 (0.12, 0.24, 0.37, 0.49, 0.74, 0.99 M) for 2 h at 25 °C before the spectra were recorded.

Briefly, 50 mL of the deionized water, 0.5 g of β-D-glucose, 0.45 g 4-aminoantipyrine, 0.47 g p-hydroxybenzene and 0.3 mL horseradish peroxidase were taken. Finally, the solution of GOx on incubation with and without ILs is mixed with the above-mentioned solution. Temperature was adjusted at 25 °C and determination of enzyme activity in the presence of two ionic liquids in above concentrations was done [62].

2.4. Fluorescence spectroscopy

Intrinsic fluorescence spectra of GOx were monitored using Hitachi F-2700 spectrophotometer (Tokyo, Japan). For monitoring tryptophan the excitation wavelength of 295 nm was used and the spectra were recorded between 300 and 400 nm. Various solutions of ionic liquids, IL1 (0.20, 0.40 M) and IL2 (0.24, 0.49 M), were prepared. The solutions of GOx (3 μM) were prepared in phosphate buffer at 25 °C (pH 6.5). Samples containing GOx and different concentration of ionic liquid solutions were mixed. Fluorometric experiment of 3 μM enzymes in phosphate buffer and various concentrations of ionic liquid were performed.

2.4.1. The inner filter effect

It is well known that optical absorption of the quencher at the excitation wavelength can make the effective intensity of the exciting light beam decrease and absorption in the emission wavelength can make the measured fluorescence intensity decrease. This is called the inner filter effect [63]. Therefore, the inner filter effect must be considered. Fluorescence intensities of GOx samples were corrected for respective absorbance of IL1 and IL2 according to the following Eq. [64]:

$$F_c = F \times anti\log\left\{\frac{A_{ex} + A_{em}}{2}\right\} \quad (1)$$

where F_c is the corrected fluorescence intensity, F is the intensity observed with the spectrofluorimeter, and A_{ex} and A_{em} are the absorbance of ligand at the emission and excitation wavelengths, respectively.

2.5. Circular dichroism spectroscopy

All the circular dichroism (CD) spectra were recorded using Aviv spectropolarimeter model 215 (proterion Corp., USA) at 25 °C. The far-UV region (190–260) using 1 mm path length was used to investigate the changes in the secondary structures of GOx upon treatment of both ILs. The enzyme concentration in the experiments for far-UV region was 3 μM. The CD software was used to predict the secondary structure of the protein according to the statistical method [65,66].

2.6. Molecular dynamics simulation

We performed MD simulations of GOx with two different concentrations of two ionic liquids, IL1 (0.40, 0.81 M) and IL2 (0.49, 0.99 M). Additionally, we simulated GOx in the absence of ILs. The simulations were also performed in solutions of ionic liquids. In addition, one more simulation was carried out with pure water to obtain dynamical behavior of pure water and of these ionic liquids in the limit of infinite dilution. All MD calculations were conducted using GROMACS 5.0.4 package with a