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ORIGINAL ARTICLE

Effect of ionic liquids [HMIm][Cl] on the structure, stability and activity of *α*-amylase *Bacillus licheniformis* (BLA): A molecular Dynamics Simulation

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ABSTRACT
Molecular dynamics simulations of an enzyme, α-amylase from Bacillus Licheniformis (BLA) in the presence of 1-hexyle 3–methylimidazolium chloride, [HMIM][Cl] ionic liquid (IL), is studied in detail. The influence of the IL on BLA activity, stability and structure is investigated at 300 K and 343 K with increasing hydration levels based on conformational flexibility, stability, compactness, transport properties and contact map criteria. Upon changes in IL concentration, both enzymes activity and stability were reduced, in agreement with the reported experimental observations. This effect was however reduced at higher water contents due to the protective thick layer of water molecules surrounding the protein conformation.
Keywords*: Ionic liquid, α-amylase, Molecular dynamic simulation, Diffusion coefficient*

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INTRODUCTION

Bacillus licheniformis α-amylase (BLA) is a remarkably thermostable enzyme that is widely used in industrial starch hydrolysis [1]. BLA provides an interesting model system for investigating the origins of protein thermal resistance due to its high thermal stability. It is not understood what makes BLA a highly thermostable enzyme, despite the wealth of biochemical, mutational and structural data acquired in a number of academic and industrial laboratories [2]. A reason can be that when it is exposed to very high temperatures, BLA unfolds irreversibly and the kinetics of the inactivation reaction can be studied, only. According to Kilbanov and co-workers [3], the main cause of irreversible thermal inactivation of BLA is the conformational scrambliny of unfolded molecules. This mechanism may be lowered by additional stabilizing interactions reducing the rate of enzyme unfolding.

Though science has reached maturity about application of enzymes in organic synthesis on laboratory and industrial scales, introduction of ionic liquids (ILs) based reactions has opened new dimensions to enzyme applications [4]. One of the attracting features of ILs is their designable nature for a variety of potential applications [5]. Room temperature ionic liquids (RTILs) typically consist of bulky and asgmmetric organic cations and inorganic or organic anions [6]. The most characteristic property of ionic liquids is that their melting temperatures are much lower than those of typical salts. RTILs poses slow vapor pressure, non-flammablity, high stability and non-volatility. So, they have the potential to be used as desirable solvents for reactions and processes [7]. Recently, researchers have discovered that ILs are more than just green solvents and they have found several applications such as replacing them with volatile organic solvents, making new materials, conducting heat effectively, supporting enzyme-catalyzed reactions, hosting a media and removal of metal ions [8]. Also, some researchers have been interested to know how ILs stabilize and activate enzymes. In this regard, several groups have explored the structural and conformational dynamics of proteins in variety of catalysts, purification of gases, homogenous and heterogeneous catalysis, biological reactions ILs.

Bahareh [9]. Observed that [BMIm][Cl] and [HMIm][Cl] reduce both enzymatic stabilities and activities of α-amylase from Bacillus amyloliquefaciens and Bacillus licheniformis. Roger and co-workers [10] suggested that a high chloride concentration and activity of 1-butyl- 3-methylimidazolium chloride ([BMIm][Cl]) is responsible for breaking the hydrogen bonding network in polysaccharides and promoting dissolution. Klahn et al [11] observed that the small size of Cl anion allows them to penetrate into the protein-core and destabilize enzymes by their presence in the enzyme-core. Huddleston [12] reported that the chloride anions which are highly hydrophilic and therefore water-miscible strip away more water molecules from the protein surface.

Therefore, the presence of ILs in enzyme microenvironment has consequences on the enzyme. However, the exact relationship between the ILs structures and enzyme activity or stability is not clearly understood, yet. Investigations at atomic level can explain how enzymes are stabilized or destabilized by ILs. By using molecular dynamics (MD) simulations, it is possible to provide useful information about the structural and dynamics properties of biomolecules [13]. In this way, this study applies MD simulations to explore the properties of BLA in the present of water miscible IL of 1-hexyl-3-methylimidazolium chloride [HMIm][Cl] to declare the effect of this IL on stability and activity of BLA.

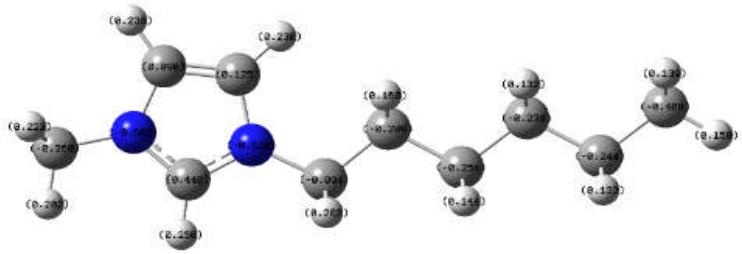


Fig. 1: Optimized structure of [HMIm] [Cl]

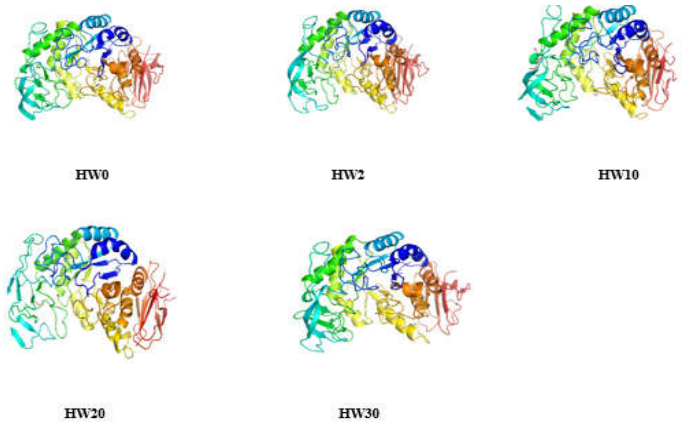


Fig. 2: Snapshots of α-amylase equilibrated structures after 100 ns of simulations in [HMIm] [Cl] systems at 343k

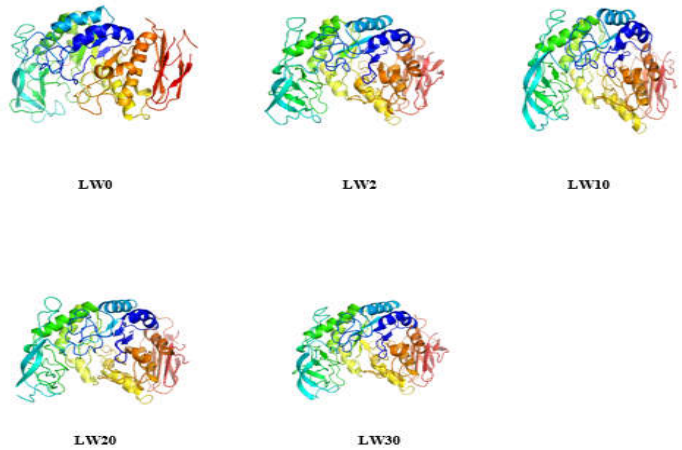


Fig. 3: Snapshots of α-amylase equilibrated structures after 100 ns of simulations in [HMIm] [Cl] systems at 300k

MATERIAL AND METHODS

Molecular dynamics (MD) simulations

The BLA structure [PDB: 1 BLI] was taken from the protein Data bank [14] and single point charge (SPC) model of water was used [15]. The geometric parameters of [HMIm] [Cl] were derived from density functional theory (DFT) [16] geometry optimization on the B_3LYP/6-31G level of theory using Gaussian 98 [17]. The atomic charges of the IL were assigned from the calculation results of electrostatic surface potential fits through CHelpG procedure [18].

The acquired structural and conformational data were transferred to GROMACS 4.5.5 simulation package [19, 20] to perform the MD simulations and the Gromos 96 43A1 force field [21] was selected.

Then, the protein was immersed in five different amounts of water and [HMIm] [Cl] molecules by adjusting number of molecules and physical properties of the simulation systems according to Table 1.Next, LINCS algorithm [22] was employed to fix the chemical bonds between the atoms of the protein and the systems constitutes and finally the main MD simulation was started with a time step of 0.2 fs and periodic boundary conditions in all directions.

To maintain a constant temperature and pressure during the simulations, the Berendsen thermostat [23] and Parrinello-Rahman coupling [24] algorithms were used, respectively. The cut-off values of 1.4 and 1.0 nm were respectively chosen for van der Waals and short-range electrostatic interactions while the particle-mesh Ewald (PME) algorithm [25] was applied to estimate the long-range electrostatic interactions.

Table1. Details of the simulation systems

[HMIm][Cl]-Amylase System‡	Water percentage	Simulation duration (ns)	Simulation box (nm×nm×nm)	Temperature (K)	Pressure (atm)
W0	0%	100	(8×8×8)	300,343	1
W2	2.5%	100	(8×8×8)	300,343	1
W10	10%	100	(8×8×8)	300,343	1
W20	20%	100	(8×8×8)	300,343	1
W30	30%	100	(8×8×8)	300,343	1

‡ Throughout this study, LW and HW systems are donated to simulations at 300 and 343 K, respectively. For example, HW20 refers to a system containing 20% of water and simulated at 343 K.

RESULTS AND DISCUSSION

The stability, structural and dynamics properties of BLA in [HMIm][Cl] at different water percentages were described using MD simulations through analyzing protein and IL compactness, distance between the IL molecules and the protein, protein diffusion coefficient and the protein contact maps at two temperatures. The average values of the evaluated properties along the 100 ns simulations are reported in Table 2 and the corresponding details are discussed in the following subsections.

Table 2: Average values of the evaluated properties over 100 ns of MD simulations

[HMIm][Cl]-Amylase (BLA)	LW0	LW2	LW10	LW20	LW30	HW0	HW2	HW10	HW20	HW30
RMSD (nm)	0.211	0.233	0.248	0.191	0.213	0.212	0.251	0.205	0.221	0.273
Protein Rg (nm)	2.325	2.305	2.309	2.344	2.350	2.349	2.325	2.358	2.367	2.363
IL Rg (nm)	3.431	3.444	3.464	3.496	3.533	3.446	3.450	3.468	3.510	3.557
Protein-IL Distance (nm)	0.158	0.126	0.072	0.114	0.064	0.091	0.144	0.198	0.126	0.228
RMSF(nm)	0.0545	0.0557	0.0635	0.0591	0.0643	0.0625	0.0597	0.0661	0.0779	0.093

Amylase conformational flexibility

Root mean square fluctuations (RMSF) of protein carbons of type alpha were studied to measure the overall flexibility of the systems. RMSF was calculated according to Equation 1, where T is the time over which the property is averaged and x_i is the reference position of particle i.

$$RMSF = \sqrt{\frac{1}{T}\sum_{tJ=1}^{T}\left[x_i(t_J) - \bar{x}_i\right]^2} \qquad [1]$$

As shown in Fig 4 and tabulated in Table 2, higher percentages of water (W30 systems) increase flexibility (higher RMSF results). Also, it is evident that higher temperature increases the conformational flexibility, too.

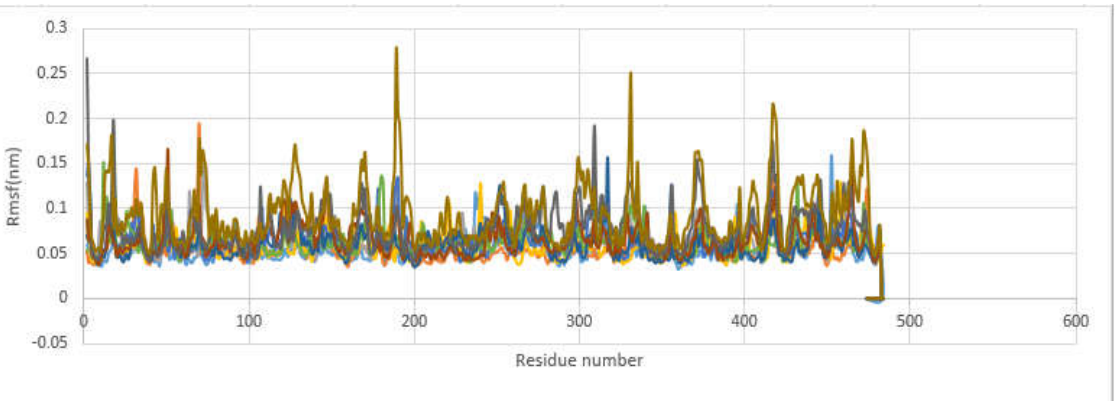


Fig. 4: Alpha-carbon RMSF evolution of BLA Residues

Amylase conformational stability

Conformational stability of α-amylase at different percentages of [HMIm] [Cl] was analyzed through root mean square displacement (RMSD) of protein carbons of type alpha. RMSD values were computed according to Equation 2 and are measures of the average distance between the atoms of the protein for each system. In this equation, δ is the distance between N pairs of equivalent alpha Carbon atoms.

$$RMSD = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\delta_i^2} \qquad [2]$$

The evolution of the RMSD values, see Fig. 5, illustrates equilibrium status for every system since 10 ns of the MD simulations. Since then, the conformational changes are negligible and the RMSD values fluctuate around an average amount.

The average RMSD results, Table 2, imply that higher percentages of water (W20 and W30 systems) provide higher amylase conformational stability (i.e. lower RMSD results) and less conformational changes during the course of simulations, with respect to the initial protein configurations. It means that when higher concentrations of the IL are provided, more chloride anions are available. These anions can penetrate into the BLA core and destabilize it [11]. Also, it is evident that higher temperature is not favorable for protein stability. So that BLA proceeds to irreversible thermal inactivation and unfolds [3].

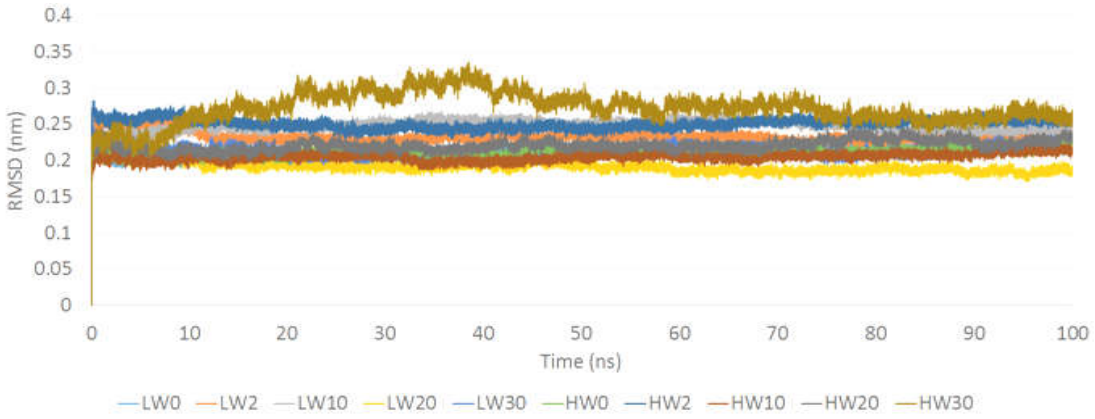


Fig. 5: Alpha-carbon RMSD evolution of the IL-BLA systems with time

Amylaseand [HMIm][Cl] compactness

Activity changes of the enzyme in ILs can be related to the compactness of the enzyme in such systems which is in turn associated with radius of gyration (R g). R g for each system was calculated based on Equation 3, in which I and A are the second moment of area and the total cross-sectional area, respectively. The results are shown in Fig.6 and 7 and reveal equilibrium status for the protein after 10 ns that is in agreement with the RMSD results of Fig. 5.

$$Rg = \sqrt{\frac{I}{A}} \qquad [3]$$

Lower Rg values are attributed to higher degree of compactness. Therefore, according to Table 2 and Fig. 6, order of α-amylase compactness is similar for all systems but is slightly reduced at the presence of more water molecules which correlates to decline activity at lower water contents.

On the other hand, compactness values of the IL masses are more. A reason is that the size of IL molecules is much smaller than the protein and their structures are more rigid and less compact. Hence, it is predictable to observe lower Rg for α-amylase. However, similar trend is exhibited for both the enzyme and IL constitutes. So that higher water percentage and temperature decreases their compactness. It is noteworthy that despite the protein that requires a lag time to establish equilibrium with its microenvironment, the [HMIm] [Cl] molecules acquire equilibrium at the initial stages of the simulations and retain their statuses to the end of the simulation.

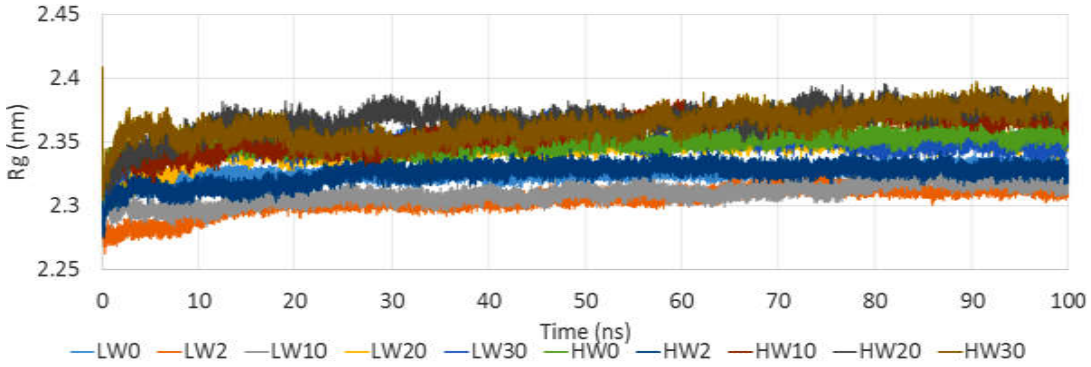


Fig. 6: Protein Rg evolution of the systems with time

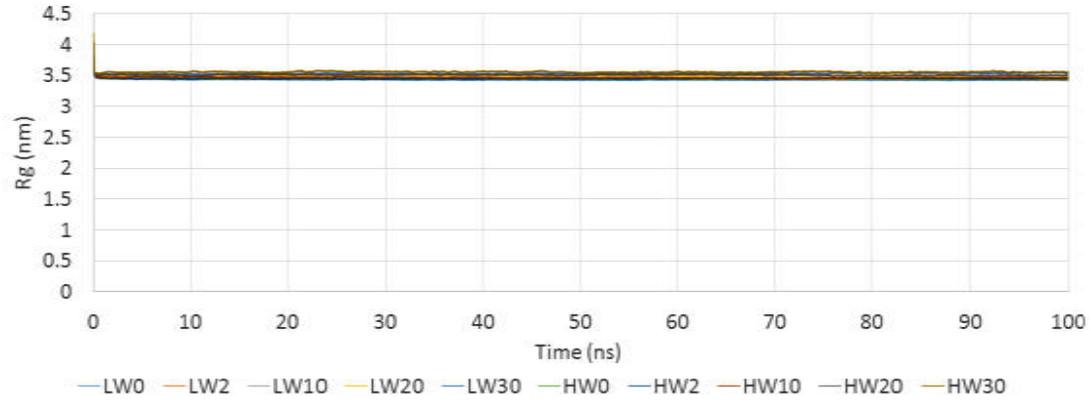


Fig.7: IL Rg evolution of the systems with time

Intermolecular distance between [HMIm] [Cl] molecules and BLA

The average intermolecular distances between the IL molecules and α-amylase are displayed in Fig.8 and the values are averaged in Table 2. The IL-enzyme distances oscillate between 0.0 to 0.5 nm which is a hallmark of dynamic nature of the systems. So that the molecules are not fixed at a definite distance and are free to change their relative positions to an extent of 0.5 nm.

The lowest intermolecular distances refer to the W30 systems. However at elevated water percentages, accumulation of water molecules around the enzyme induces a separation between the IL mass and the enzyme. Furthermore, the smaller size of water compared to [HMIm] [Cl] increases the entropy of water molecules. Therefore, when temperature is raised to 343 K, the additional energy of system is transferred to water degrees of freedom which in turn increases water entropy and makes a very dynamic barrier to IL access to the enzyme. As a consequence, the average protein-IL distance values are greater at 343 K.

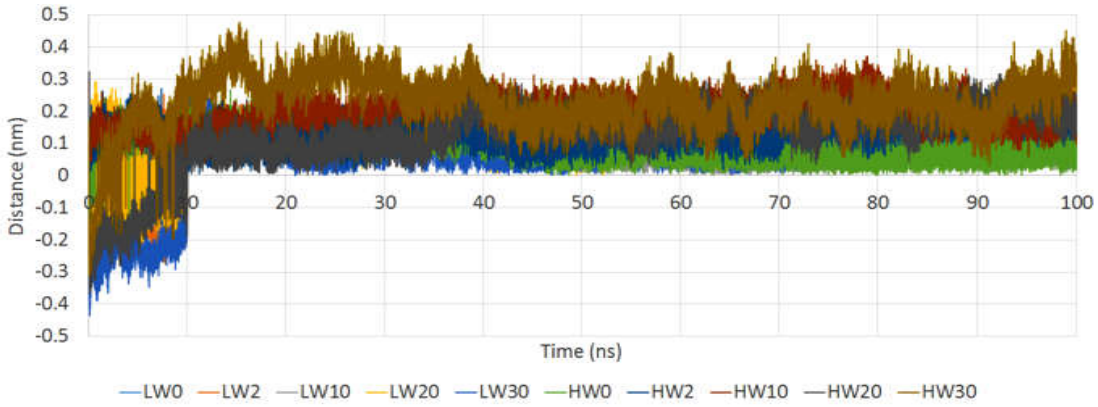


Fig. 8: Distance between the protein and IL of the systems through time

Amylase and [HMIm] [Cl] diffusion coefficients

Among different transport properties, the diffusion coefficients of the enzyme in IL-water systems were studied. The enzyme diffusion coefficients (mean square displacement; MSD) were extracted from the Einstein formulation [26, 27] , i.e. Equation 4.In this equation, $r(t)$ and $r(0)$ are respectively the positions of the α-amylase center of mass at time t and 0, and k is the Einstein's diffusion constant.

$$MSD = \lim_{t\to\infty}\left\langle \left| r(t) - r(0) \right|^2 \right\rangle \qquad (5)$$

The calculated values are plotted in Fig.9 and present an ascending trend with time. It means that the protein moves towards the other system components and regions, continuously. Also, the presence of different water amounts has given various *MSD* values, at each instant of the simulation. So that contribution of more small sized solvent molecules, i.e. water, facilitates protein diffusion. Moreover, as it can be expected, at higher temperature of 343 K, the protein can move readily and the *MSD* values are enhanced. When the conditions of higher temperature and more water contents are provided in systems of HW20 and HW30, the calculated *MSD* starts increasing dramatically.

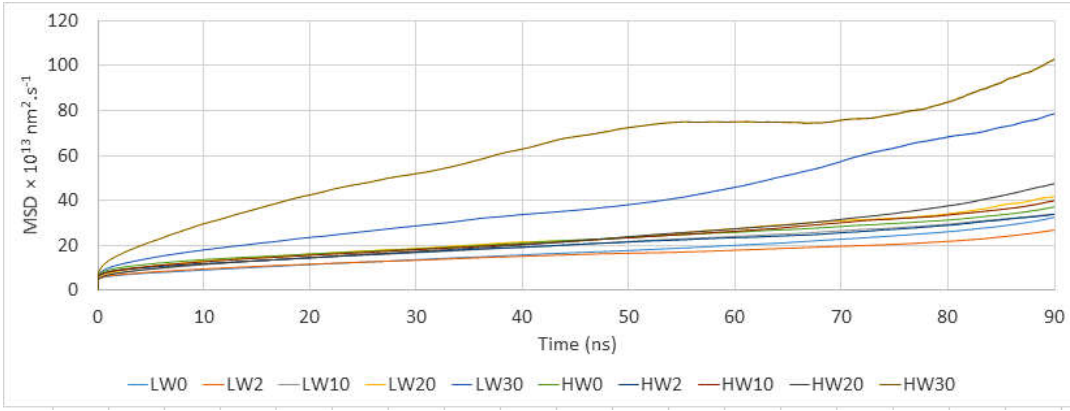


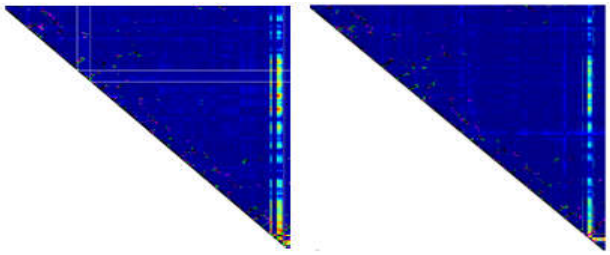
Fig. 9: The evolution of enzyme diffusion coefficients with time

Contact map

The network of contacts between BLA aminoacid residuesin each system were evaluated and visualized by version 0.9.6 of CMView [28], see Fig.10 and Table 3.In this figure, red and blue colors intensities show the difference between structures. Blue sections demonstrate the unchanged contacts between two structures and red sections show the contacts with the most difference between two structures.

Results demonstrate that the number of contactmap red point's decrease at higher water percentages and higher temperature of 343 K causes which mean at higher temperature and water content, the structural changes decrease and the enzyme activity is more preserved.

There is a region with difference between Ala181 and Gln374-Pro432 and also contact between Gly179 and Glu458-Val460at lower water percentages while the HW30 domains include Tyr14-Asn188, Pro 16-Glu189, Tyr14-Glu189, Glu189-Gln330 and Gly191-Gln330 contact.



HW10 LW30

Fig. 10: Sample BLA contact map for the systems with 10% water content at 343 K and 30% at 300 K with respect to the 0% water system

Table 3: Number of contact map in the amylase (BLA) at each water percentage

	Type	LW2	LW10	LW20	LW30	HW2	HW10	HW20	HW30
Number of contacts	CM1 CM2	2620 2548	2559 2548	2546 2548	2617 2548	2623 2531	2552 2531	2516 2531	2512 2531
Number of unique contacts	CM1 CM2	342 270	777 766	437 436	353 284	372 280	407 386	320 335	635 654
Number of common contacts	CM1 CM2	2278 2278	1782 1782	2109 2109	2264 2264	2251 2251	2145 2145	2196 2196	1877 1877
Contact map overlap		78.8%	53.6%	70.7%	78.0%	77.5%	73.0%	77.0%	59.3%

CONCLUSION

The structural and dynamics properties of BLA in a chloride consisting RTIL at different water percentages were described using MD simulations. It is showed that the both stability and flexibility of the BLA structures are more affected by the presence of water molecules, with a minimum RMSD around 20% of water content. The size and water solubility of the IL anion, i.e. chloride is found to eliminate the stability of the protein because chloride anions penetration through the protein-core and change its conformation that leads to instability at low water contents. Also, the IL restrains the movement of both proteins even at regions that are highly flexible in aqueose. Therefore, the diffusion coefficients are influenced and the IL induced constraints decline selectivity of BLA.

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