Archives of Advances
in Biosciences

Original Article:

The Effect of 1-Ethyl-3-methylimidazolium Acetate Ionic Liquid on the Structural and Functional Features of a Representative α-amylase

Reza Farahmand Rad[1], **Mehdi Ebrahimi**[1*]

1. Department of Biology, Faculty of biological sciences, Varamin-Pishva branch, Islamic Azad University, Pishva, Iran.

Cite this article as: Farahmand R, Ebrahimi M. The Effect of 1-Ethyl-3-methylimidazolium Acetate Ionic Liquid on the Structural and Functional Features of a Representative α-amylase. Archives of Advances in Biosciences. 2022; 13:E38397. https://doi.org/10.22037/aab.v13i.38397

https://journals.sbmu.ac.ir/aab/article/view/38397

Article info:
Received: 11 May 2022
Accepted: 05 Jun 2022
Published: 25 Dec 2022

*** Corresponding author:**

Mehdi Ebrahimi, PhD

Address: Department of Biology, Faculty of biological sciences, Varamin-Pishva branch, Islamic Azad University, Pishva, Iran.

E-mail:
ebrahimi@iauvaramin.ac.ir

Abstract

Introduction: α-Amylases catalyze the starch hydrolysis reaction and are widely used in starch processing industries. Despite the high demand for the use of α-amylase in various industries, most α-amylases do not have the required functional characteristics such as proper activity, temperature and pH compatibility, as well as temperature stability. In this regard, ionic liquids are widely used today as co-solvents to improve enzymes' activity, stability, and selectivity.

Materials and Methods: Activity, optimum temperature, optimum pH, and thermal stability of B. subtilis α-amylase were investigated in the presence of 1-ethyl-3-methylimidazolium acetate. Structural changes in the presence of [EMIm][Ac] were examined using intrinsic fluorescence spectroscopy.

Results: The activity of α-amylase and Vm increased in the presence of 0.4 M of [EMIm][Ac] while Km decreased. The optimum temperature and pH did not change in the presence of [EMIm][Ac] compared to its absence. At 40 and 50 °C, the inactivation rate of α-amylase in the presence of both concentrations of 0.4 and 1 M of [EMIm][Ac] is the same as in its absence. However, the rate of inactivation at 60 and 70 °C is higher than that in the absence of [EMIm][Ac]. The results of intrinsic fluorescence spectroscopy confirmed the structural changes in the presence of [EMIm][AC].

Conclusion: [EMIm][Ac] can be used to increase the activity of B. subtilis α-amylase without affecting the optimal temperature and pH parameters. Moreover, [EMIm][Ac] relatively improves the thermal stability of the α-amylase.

Keywords: Activity, α-amylase, *Bacillus subtilis*, Ionic liquids, 1-Ethyl-3-methyLimidazolium acetate, Stability, Structure.

1. Introduction

α-Amylases catalyze the hydrolysis reaction of α-1,4-glycosidic bonds of starch and produce a variety of products such as dextrin, smaller oligosaccharides, maltose, and glucose [1]. Since starch is known as one of the most abundant polysaccharides in nature, it is widely used as a raw material in various industries such as the food industry, high fructose corn syrup (HFCS), ethanol, and paper production. One of the most important and widely used enzymes in the starch processing industries is α-amylase, which accounts for approximately 25% of the total industrial enzyme market [2]. Since the liquefaction and saccharification steps in the starch biodegradation process are performed at relatively high temperatures and acidic pH, α-amylases used in these industries must be compatible with this condition [3]. To increase the efficiency of starch processing processes, extensive research has been conducted over the past decade to explore α-amylase with the desired properties. Various

amylases have been isolated from plants, animals, and microorganisms, but most of them do not have the characteristics required for use in industrial processes. As a result, only a small number of discovered amylase have reached the commercial scale [4]. Best sources for the production of commercial amylase include *B. subtilis*, *A. niger*, *A. oryzae*, and *B. licheniformis* [5].

Since most wild-type enzymes do not have appropriate characteristics for industrial processes, enzyme engineering approaches have been deployed to improve the characteristics of existing enzymes. In recent years, the use of ionic liquids (ILs) as additives or co-solvents in the enzymatic reaction medium has attracted enormous interest [6, 7]. Ionic liquids are solvents that only consist of cations and anions. These solvents have unique properties such as melting point of less than 100 °C, non-flammable temperature, high solvent capacity, and chemical stability as well as ionic conductivity, recyclability, and reusability [8, 9]. Since these properties can be changed by combining different cations and anions, ionic liquids are also known as "designer solvents" [10]. In addition, studies have shown that the stability of biomolecules increases in the presence of ionic liquids. These benefits have led to the use of ionic liquids as alternatives to conventional organic solvents to improve the efficiency of existing processes [11].

To date, a variety of ionic liquids have been produced from a combination of different cations and anions and have been used in enzymatic catalysis as a solvent or additive. These ionic liquids can be miscible, partially miscible, or immiscible with water, and most of them affect the activity of enzymes [12]. Moreover, it has been shown that the solubility of carbohydrates with sucrose, lactose, cellulose, glucose, cyclodextrin, and alcohol groups in ionic liquids is higher than that in conventional organic solvents [13]. The reason is the formation of hydrogen bonds between the anions of these ionic liquids and carbohydrates, which facilitates the dissolution and conversion of these compounds into products through enzymatic reactions [14].

Although numerous studies have been conducted to appreciate the mechanism of protein denaturation in the presence of ionic liquids, there is still no general rule in this regard. For this reason, the activity and stability of enzymes in the presence of ILs are still being studied and are considered as one of the attractive strands of research. In this study, the activity, stability, and structural changes of α-amylase from *Bacillus subtilis* in the presence of 1-Ethyl-3-methylimidazolium acetate ([EMIm][Ac]) were investigated.

2. Materials and Methods

Materials

B. subtilis α-amylase and 3, 5-Dinitrosalicylic acid (DNS) were purchased from Sigma. Starch and [EMIm][Ac] were purchased from Aldrich. All chemicals were analytical grades. Double distilled water was used to prepare the required solutions and buffers. The chemical structure of [EMIm][Ac] is shown in Figure 1.

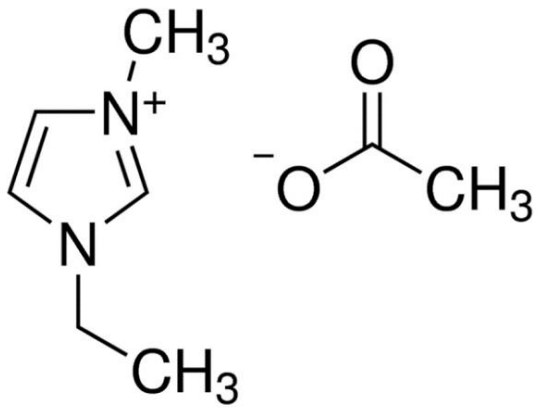

Figure 1. The chemical structure of [EMIm][Ac] ionic liquid

Determination of the activity parameters of *B. subtilis* α-amylase activity assay was performed according to the Miller method [15]. In this method, sugars produced from starch hydrolysis reduce DNS to 3-amino-5-nitrosalicylic acid, which has a light absorption at a wavelength of 540 nm. The starch solution was prepared by dissolving 1% w/v starch in 20 mM sodium phosphate buffer with 6.7 mM sodium chloride (pH 6.9). To perform this procedure, 0.5 ml of α-amylase (1 unit/ml) in the presence or absence of ionic liquid was added to 1 ml of starch solution. The reaction solution was incubated for 15 minutes at 20 °C.

DNS solution was prepared by desolvation of 3,5-dinitrosalicylic acid (96 mM) and sodium-potassium tartrate (5.3 M) in water. Then, 1 ml of DNS solution was added to the reaction solution. The reaction was stopped by incubating reaction solution in a boiling water bath for 15 minutes. And then it was placed on ice for 10 minutes. The light absorption of the solution was recorded at 540 nm. The same volume of distilled water without enzyme was used as the blank solution. One unit of α-amylase activity was defined as the amount of enzyme that releases 1 mM of reducing sugars (with maltose as standard) per minute under

specified assay conditions.The α-amylase activity was measured in the presence of [emim][Ac] (1 and 0.4 M) and its absence in different concentrations of starch (from 2 to 10 mg/ml). Lineweaver-Burk diagrams were drawn and Km and Vm were determined by extrapolating the line at different concentrations of ionic liquid.

To determine the optimum temperature, the α-amylase (in the presence or absence of ionic liquid) and starch solution were incubated separately for 5 minutes at 25 to 80 °C (5 °C intervals) and then the enzyme activity was measured.

To determine the optimal pH, α-amylase solution (in the presence and absence of ionic liquid) was prepared in glycine-HCl, sodium-citrate, sodium-acetate, sodium-phosphate, and Tris-HCl buffers with pH from 3 to 10 (0.5 unit intervals). Then, the enzymatic activity of the α-amylase solution was determined.

Thermal inactivation

The α-amylase solution was measured in the presence of [emim][Ac] (0.4 and 1 M) and in its absence at 40, 50, 60, and 70 °C at 5, 10, 15, and 20 minutes. Residual activity versus time diagram was used to compare the thermal denaturation status of the enzyme.

Fluoresce spectroscopy

The α-amylase solution (0.1 mg/ml) was incubated in the presence of ionic liquid (0.2, 0.4, and 1 M) and its absence for 5 minutes at room temperature. Excitation was then performed at 295 nm using a Perkin-Elmer fluorescence spectrophotometer (USA). Light emission was then recorded in the range of 300 to 400 nm. Both excitation and emission slit were set at 5 nm. An enzyme-free solution of each ionic liquid concentration was used as a blank to remove the fluorescent effects of the imidazolium ring in [EMIm][Lac]. The emission of each sample was subtracted from the blank and the result was employed for emission spectra.

3. Results

Effect of [EMIm][Ac] on α-amylase activity

The activity of *B. subtilis* α-amylase in the presence of different concentrations of [EMIm][Ac] is shown in Figure 2. According to this graph, the highest α-amylase activity is obtained at a concentration of 0.4 M [EMIm][Ac], while the lowest activity is obtained at a concentration of 0.8 M [EMIm][Ac].

Effect of [EMIm][Ac] on Km and Vm of a-amylase

To determine the Km and Vm parameters, *B. subtilis* α-amylase activity was measured at different concentrations of starch in the presence and absence of [EMIm][Ac], and a Lineweaver-Burk diagram was drawn (Figure 3). By extrapolating the line obtained in this diagram, the values of Km and Vm parameters were determined. The results are shown in the following Table 1. According to these results, Vm of *B. subtilis* α-amylase increased in the presence of both ionic concentrations [EMIm][Ac]. However, the Km values of this enzyme in the presence of both concentrations [EMIm][Ac] decreased compared to its absence. In addition, both Vm and Km parameters in the presence of one molar concentration [EMIm][Ac] increased relative to the 0.4 M concentration.

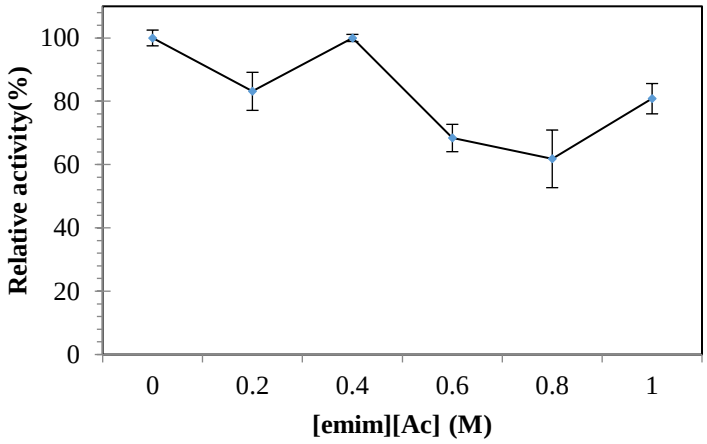

Figure 2. a-amylase relative activity in presence of [EMIm][Ac]

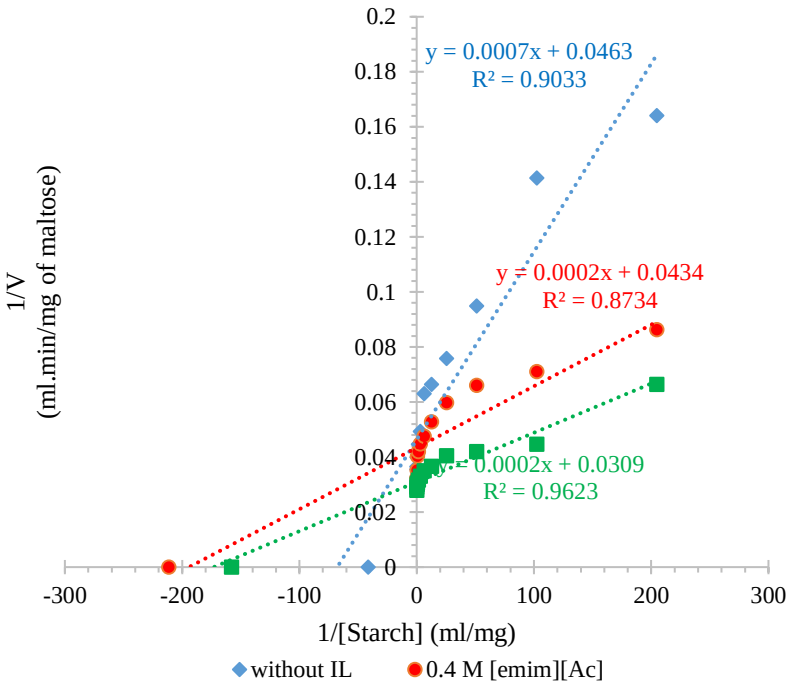

Figure 3. The Lineweaver-Burk plot of *B. subtilis* α-amylase in the presence (0.4 and 1 M) and absence of [emim][Ac]

Table 1. Vm and Km of *B. subtilis* α-amylase.

	without IL	0.4 M [emim][Ac]	1 M [emim][Ac]
Vm	21.60±0.013	23.04±0.35	32.36±0.054
Km	0.024±0.004	0.004±0.0004	0.006±0.0006

Optimum pH and temperature

The optimal temperature and pH factors of α-amylases are of great importance in the starch processing industry. *B. subtilis* α-amylase activity in the presence of [EMIm][Ac] and its absence at different pHs are shown in Figure 4. Based on the results, the highest α-amylase activity was determined in the presence and absence of [EMIm][Ac] at pH 7.5.

Figure 5 shows the α-amylase activity at different temperatures in the absence and presence of [EMIm][Ac]. According to the obtained results, the highest α-amylase activity was determined in the absence and the presence of [EMIm][Ac] at 40 °C. α-Amylase activity decreases to 80% in the absence of [EMIm][Ac] at 45 °C. However, α-amylase activity in the presence of [EMIm][Ac] gradually decreases from 45 to 55 °C, so that at 55 °C, the activity of the enzyme decreases to 80%. Therefore, it can be stated that in the presence of [EMIm][Ac], the optimal temperature of α-amylase is similar to that of the enzyme without ionic liquid, but the activity of the enzyme in the presence of [EMIm][Ac] decreased slightly at 45 and 50 °C. In the absence of ionicliquid, a sharp decrease is observed.

Thermal stability

One of the most important applications of ionic liquids in the field of biotransformation is to improve the temperature stability of enzymes. Other advantages of using ionic liquids in biotransformation include ease of preparation, improved enzyme solubility in the reaction medium, and cost-effectiveness [26].

In the present study, the stability of α-amylase enzyme in the presence of two concentrations of 0.4 and 1 M [EMIm][Ac] and also in its absence at temperatures of 40, 50, 60, and 70 °C at 5, 10, 15 and 20 minutes were evaluated (Figure 6). The slope of the line obtained in each case indicates the rate of inactivation of the enzyme. The rate of inactivation at 40 and 50 °C in the presence of both 0.4 and 1 M [EMIm][Ac] is similar to its absence. While the rate of inactivation in the presence of both concentrations of [EMIm][Ac] is higher than in its absence at 60 and 70 °C.

Intrinsic fluorescence spectroscopy

Fluorescence spectroscopy is a common method for studying structural changes, folding/unfolding status, and binding of a substrate or ligand to proteins [37]. This technique can be used both intrinsically and externally, but intrinsic fluorescence is a priority in the study of proteins. In intrinsic fluorescence, without the use of external fluorescence compounds,

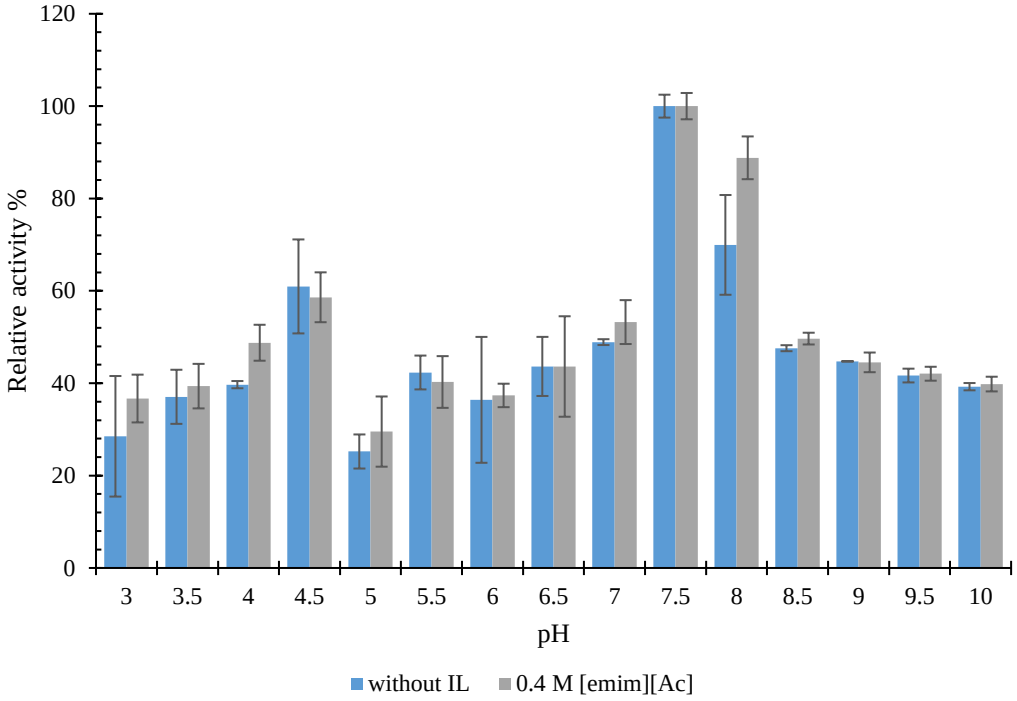

Figure 4. The activity of α-amylase at different pH in the presence and absence of [emim][Ac]

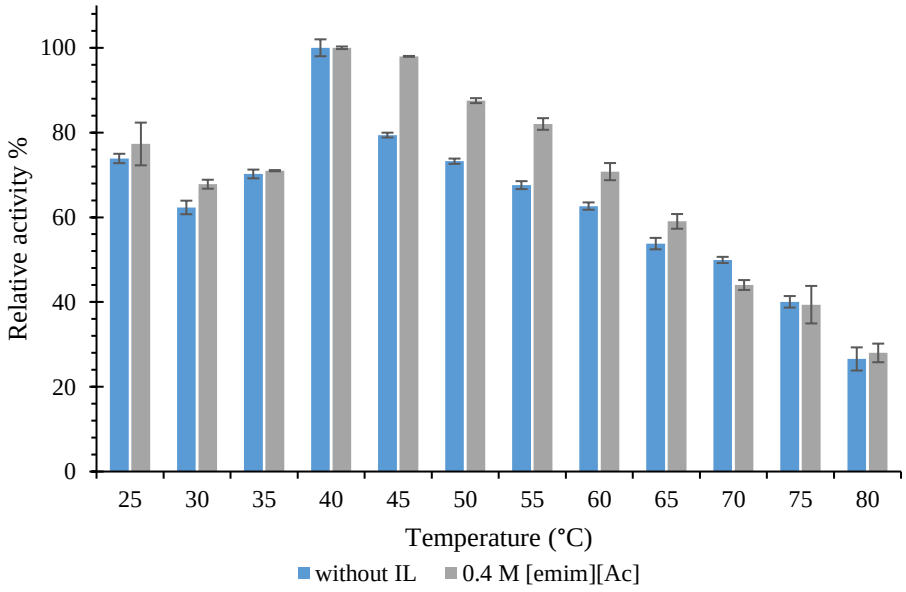

Figure 5. The α-amylase activity at different temperatures in the presence and absence of [EMIm][Ac]

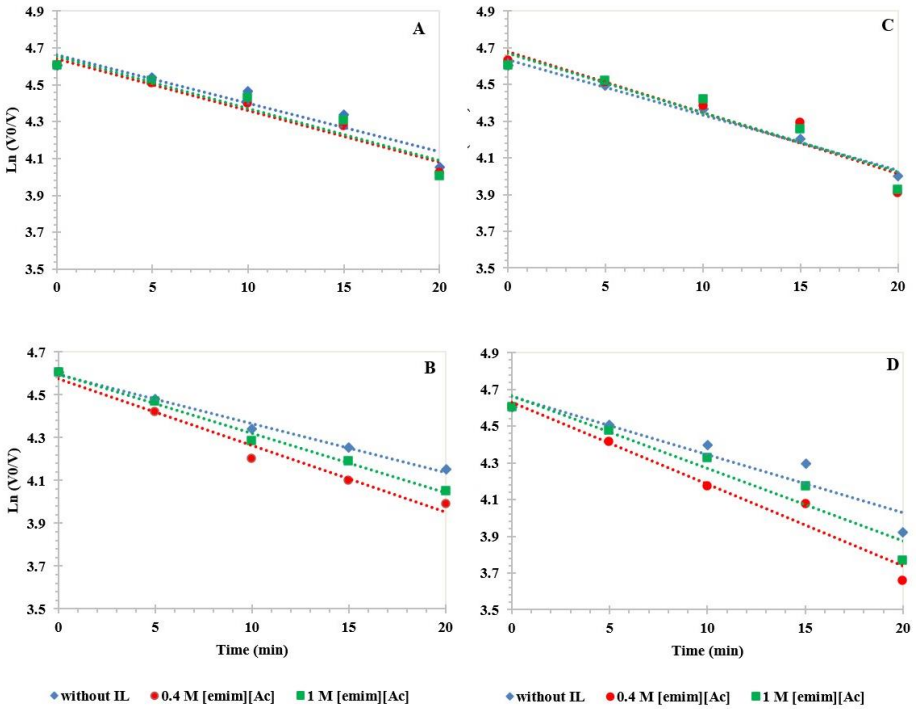

Figure 6. Thermal inactivation of α-amylase in the absence (●) and presence of 0.4 M of [EMIm][Ac] at 40 (A), 50 (B), 60 (C), and 70 °C (D)

the fluorescence capability of the amino acids phenylalanine, tyrosine, and tryptophan is used to study structural changes in proteins. In the meantime, the amino acid tryptophan is more useful due to its longer excitation and diffusion wavelengths (in the range close to UV) as well as longer diffusion times. Tryptophan λ_{max} depends on its microenvironment as well as solvent exposure [38]. Accordingly, in the present study, the structural changes of α-amylase in the presence of [EMIm][Ac] were investigated using the intrinsic fluorescence of the tryptophan (Figure 7).

The λ_{max} changed from 330-350 nm in the absence of [EMIm][Ac] to the range of 430-450 nm and led to the creation of another peak in the range of 570-580 nm in

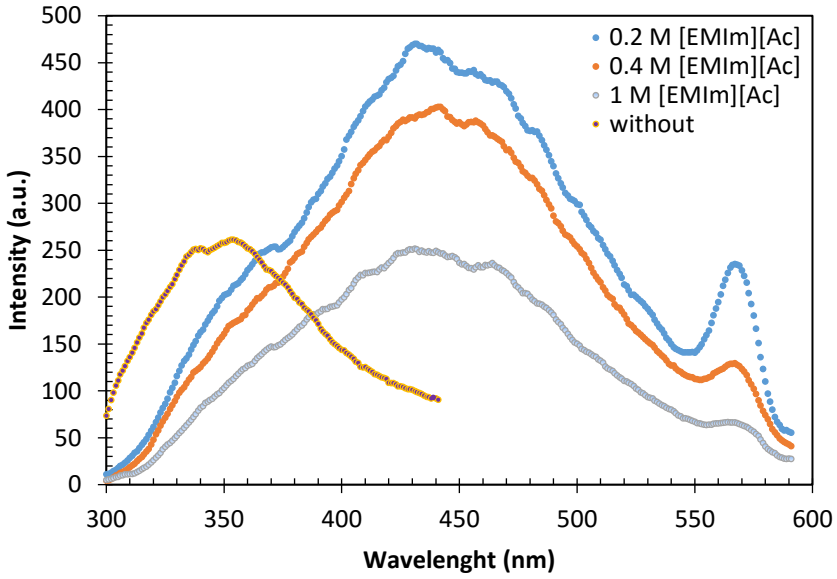

Figure 7. Fluorescence spectra of α-amylase with and without [EMIm][Ac]

the presence of ionic liquid. Thus, a redshift in the tryptophan wavelength occurred in the presence of ionic liquid. A redshift in the fluorescence spectrum occurs when tryptophan is transferred from a non-polar medium to a polar medium [39]. In the absence of [EMIm][Ac], thus, tryptophan is located inside an α-amylase structure and away from the polar environment. In the presence of [EMIm][Ac], due to the induction of structural changes in the enzyme, tryptophan is transferred to the vicinity of water molecules, which increases wavelength, i.e., a redshift occurred. Sequence analysis revealed that there were a total of 12 tryptophan amino acids (Figure 8 A), 4 of which (W14, W144, W191, and W287) were buried inside the structure (Figure 8 B). Therefore, structural changes due to the presence of [EMIm][Ac] can change the position of these four residues to surface areas resulting in the observed changes in the fluorescence spectrum.

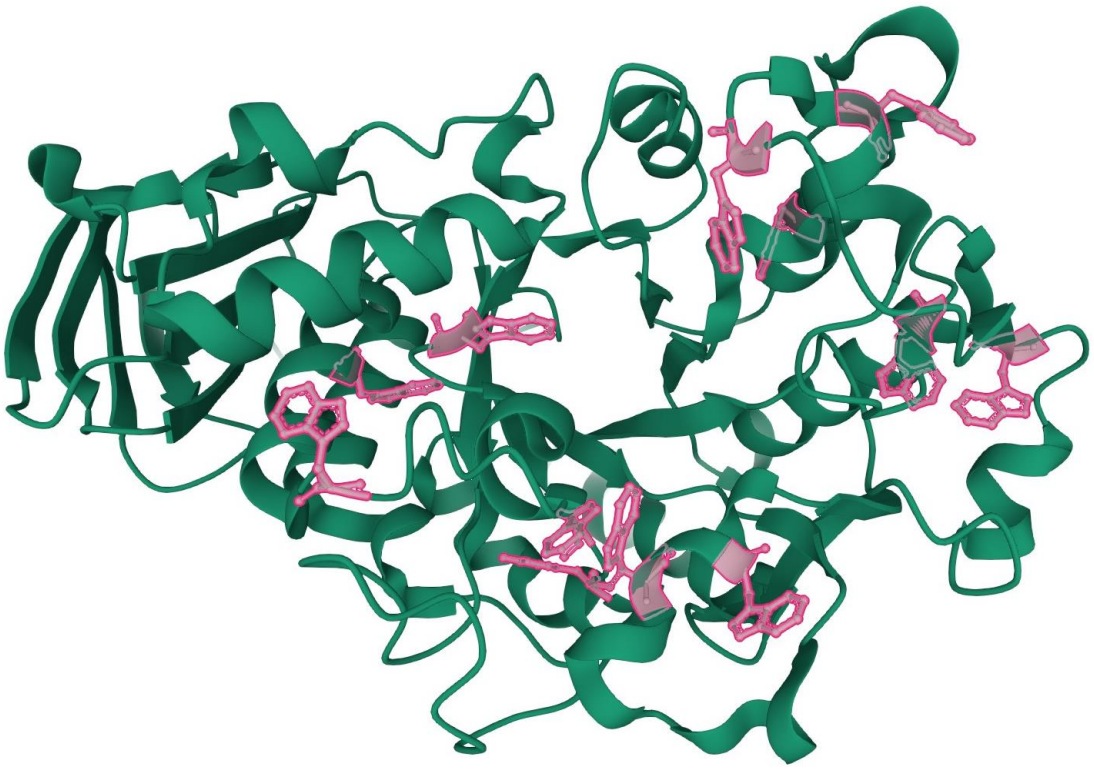

Figure 8. (A) The amino acid sequence of α-amylase from *B. subtilis* (PDB=1UA7). (B) The related natural 3D structure of *B. subtilis* α-amylase. Tryptophan residues represented in pink

4. Discussion

The activity of most enzymes in a mixture of ionic liquids and aqueous solvents decreases with an increase in ionic liquid concentration [16-19]. In this study, the activity of *B. subtilis* α-amylase was decreased with increase in concentration of [EMIm][Ac]. Results of our previous study showed that the activity of *A. oryzae* a-amylase decreased in the presence of [EMIm][Lac] with the lowest activity at 0.8 M [20]. Sate *et al* (2007) reported a similar effect of different ionic liquids on different enzymes. *Candida antarctica* lipase activity decreased in the presence of ionic liquids [emim][$EtOSO_3$], [emim][NO_3], and [emim][$N(CN)_2$] due to enzyme aggregation in the presence of ionic liquids [21]. However, the activity of the luciferase enzyme decreased in the presence of [TMG][Ac], [TMG][Pro] but increased in the presence of [TMG][Lac] [17, 21, 22]. Therefore, it is important to evaluate the activity of each enzyme in the presence of ionic liquids. Acetate anion (OAc^-) in ionic liquid forms hydrogen bonds with carbohydrates, including starch, and therefore its solubility in water increases. At the same time, the acetate anion forms strong hydrogen bonds with the enzyme molecule and makes the enzyme denatured [6]. Therefore, a decrement in *B. subtilis* a-amylase activity can be due to the formation of hydrogen bonds by the acetate anion and thus its denaturation.

The results of Vm and Km measurement indicate that

the solubility of starch increases in the presence of [EMIm][Ac] and thus the availability of α-amylase enzyme to starch also increases. However, according to the results reported by Akhavan et al. (2020), Km of α-amylase increases in the presence of [EMIm][Lac] while Vm decreases [20]. The presence of a higher hydroxyl group in lactate anion compared to acetate makes lactate anion more capable of forming hydrogen bonds and thus dissolving starch. However, this [EMIm][Lac] property can inactivate the α-amylase enzyme, resulting in a decrease in Vm in the presence of [EMIm][Lac]. Similar effects have been reported in other studies. Ebrahimi et al. (2012), reported an increase in the activity of luciferase enzyme in the presence of lactate anion but a decrease in the presence of propionate anion. However, the Km of luciferase enzyme in the presence of lactate anion is slightly higher than that of propionate anion in the absence of ionic liquid. Therefore, increasing the ability of hydrogen bond formation by ions of ionic liquids and thus increasing the dissolution of substrates can weaken the structure and activity of the enzyme while interfering with the natural patterns of hydrogen bonding.

Investigation of optimum pH in the presence of [EMIm][Ac] was shown no change. A change in pH, changes the ionization state of the ionizable side chain of the amino acid residues in the proteins. This change can weaken or strengthen the natural structure of the protein by affecting the electrostatic interactions involved in the formation of the natural structure of the protein. In addition, altering the ionization states of amino acid residues at the active site of the enzyme can alter enzyme activity. Thus, pH, or put differently the concentration of protons in solution, can act as an activator or inhibitor of enzyme activity [23]. Ionic liquids alter the microenvironment around the residues of the active site of the enzyme through dehydration mechanisms, columbic interactions, and hydrogen bonds. As a result, the optimal pH of the enzyme changes with the change in pKa of the groups participating in the reaction [24, 25].

The optimum temperature of *B. subtilis* α-amylase was not improved in the presence of [EMIm][Ac]. The similar result was reported in the presence of [EMIm][Lac] [20]. According to these results, it can be stated that the decrease of hydrogen bond formation capacity in acetate anion (due to the absence of hydroxyl group) compared to lactate anion has improved enzyme performance at higher temperatures.

Although the effect of ionic liquids on the stability of all enzymes is not the same, the results of several studies show that in most cases ionic liquids have improved the thermal stability of proteins [17, 22, 27-36]. According to the thermal stability investigation, 0.4 M concentration of [EMIm][Ac] was not improve thermal stability of *B. subtilis* α-amylase. While according to the results reported by Akhavan et al. (2020), [EMIm][Lac] does not affect the inactivation rate of α-amylase at 40 and 50 °C, and only at 60 °C, the rate of α-amylase inactivation decreases. The reason for these differences, in addition to the presence of different anions, may be due to differences in the amino acid composition and structure of the enzymes studied. Therefore, in evaluating the function of proteins in ionic liquids, it is necessary to pay attention to their structural and functional properties to select the appropriate combination of cation and anion to achieve the desired goal [31].

5. Conclusion

Based on the results obtained in the present study, the presence of [EMIm][Ac] as a co-solvent causes structural changes in B. subtilis α-amylase. These structural changes increase its activity without affecting the optimal temperature and pH parameters, as well as by improving the thermal stability of the α-amylase.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this study. No human or animal was used.

Funding

The paper was extracted from the MSc. thesis of the first author, Department of microbial biotechnology, Faculty of Biological science, Islamic Azad University.

Author's contributions

The authors equally contributed to preparing this article.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This study was part of the first author's thesis. We would like to thank the Faculty of Biological science,

Varamin-Pishva Branch of Islamic Azad University, for their help with data collection.

References

[1] Yang Y, Xu RM, Song J, Wang WM. High throughput biotechnology in traditional fermented food industry. Recent Pat Food Nutr Agric. 2010; 2(3):251-7. [DOI:10.2174/2212798411002030251] [PMID]

[2] Elyasi Far B, Ahmadi Y, Yari Khosroshahi A, Dilmaghani A. Microbial Alpha-Amylase Production: Progress, Challenges and Perspectives. Adv Pharm Bull. 2020; 10(3):350-8. [DOI:10.34172/apb.2020.043] [PMID] [PMCID]

[3] Saini R, Singh Saini H, Dahiya A, Harnek Singh Saini C. Amylases: Characteristics and industrial applications. J Pharmacogn Phytochem. 2017; 6(4):1865-71. https://www.phytojournal.com/archives?year=2017&vol=6&issue=4&ArticleId=1586

[4] Jujjavarapu SE, Dhagat S. Evolutionary Trends in industrial production of α-amylase. Recent Pat Biotechnol. 2019; 13(1):4-18. [DOI:10.2174/2211550107666180816093436] [PMID]

[5] Sundarram A, Murthy TPK. a-Amylase production and applications: a review. Appl Environ Microbiol. 2014; 2(4):166-75. [DOI:10.12691/jaem-2-4-10]

[6] Welton T. Ionic liquids: a brief history. Biophys Rev. 2018; 10(3):691-706. [DOI:10.1007/s12551-018-0419-2] [PMID] [PMCID]

[7] Yang Z. Natural deep eutectic solvents and their applications in biotechnology. Adv Biochem Eng Biotechnol. 2019; 168:31-59. [DOI:10.1007/10_2018_67] [PMID]

[8] Vanda H, Dai Y, Wilson EG, Verpoorte R, Choi YH. Green solvents from ionic liquids and deep eutectic solvents to natural deep eutectic solvents. C R Chim. 2018; 21(6):628-38. [DOI:10.1016/j.crci.2018.04.002]

[9] Vekariya RL. A review of ionic liquids: Applications towards catalytic organic transformations. J Mol Liq. 2017; 227:44-60. [DOI:10.1016/j.molliq.2016.11.123]

[10] Lei Z, Chen B, Koo YM, MacFarlane DR. Introduction: ionic liquids. Chem Rev. 2017; 117(10):6633-5. [DOI:10.1021/acs.chemrev.7b00246] [PMID]

[11] Egorova KS, Gordeev EG, Ananikov VP. Biological activity of ionic liquids and their application in pharmaceutics and medicine. Chem Rev. 2017; 117(10):7132-89. [DOI:10.1021/acs.chemrev.6b00562] [PMID]

[12] Zhao H. Protein stabilization and enzyme activation in ionic liquids: specific ion effects. J Chem Technol Biotechnol. 2016; 91(1):25-50. [DOI:10.1002/jctb.4837] [PMID] [PMICD]

[13] Zhao H, Baker GA, Song Z, Olubajo O, Crittle T, Peters D. Designing enzyme-compatible ionic liquids that can dissolve carbohydrates. Green Chem. 2008; 10(6):696-705. [DOI:10.1039/B801489B]

[14] Rabideau BD, Ismail AE. Mechanisms of hydrogen bond formation between ionic liquids and cellulose and the influence of water content. Phys Chem Chem Phys. 2015; 17(8):5767-75. [DOI:10.1039/C4CP04060K]

[15] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959; 31(3):426-8. [DOI:10.1021/ac60147a030]

[16] Dabirmanesh B, Daneshjou S, Sepahi AA, Ranjbar B, Khavari-Nejad RA, Gill P, et al. Effect of ionic liquids on the structure, stability and activity of two related α-amylases. Macromolecules. 2011; 48(1):93-7. [DOI:10.1016/j.ijbiomac.2010.10.001]

[17] Ebrahimi M, Hosseinkhani S, Heydari A, Khavari-Nejad RA, Akbari J. Controversial effect of two methylguanidine-based ionic liquids on firefly luciferase. Photochem Photobiol Sci. 2012; 11(5):828-34. [DOI:10.1039/c2pp05389f]

[18] Yang Z, Yue YJ, Huang WC, Zhuang XM, Chen ZT, Xing M. Importance of the ionic nature of ionic liquids in affecting enzyme performance. J Biochem. 2009; 145(3):355-64. [DOI:10.1093/jb/mvn173] [PMID]

[19] Zhang Y, Cremer PS. Interactions between macromolecules and ions: The Hofmeister series. Curr Opin Chem Biol. 2006; 10(6):658-63. [DOI:10.1016/j.cbpa.2006.09.020]

[20] Akhavan T, Ebrahimi M, Hekmat A. Structural evidence for kinetic and thermal stability changes of α-amylase due to exposure to [emim][lactate] ionic liquid. Turkish J Biochem. 2020; 45(6):785-91. [DOI:10.1515/tjb-2019-0270]

[21] Sate D, Janssen MHA, Stephens G, Sheldon RA, Seddon KR, Lu JR. Enzyme aggregation in ionic liquids studied by dynamic light scattering and small angle neutron scattering. Green Chem. 2007; 9(8):859-67. [DOI:10.1039/B700437K]

[22] Ebrahimi M, Hosseinkhani S, Heydari A, Khavari-Nejad RA, Akbari J. Improvement of thermostability and activity of firefly luciferase through [TMG][Ac] ionic liquid mediator. Appl Biochem Biotechnol. 2012; 168(3):604-15. [DOI:10.1007/s12010-012-9803-8] [PMID]

[23] Tipton KF, Dixon HBFF. Effects of pH on enzymes. Methods Enzymol. 1979; 63:183-234. [DOI:10.1016/0076-6879(79)63011-2] [PMID]

[24] Pace CN, Grimsley GR, Scholtz JM. Protein ionizable groups: pK values and their contribution to protein stability and solubility. J Biol Chem. 2009; 284(20):13285-9. [DOI:10.1074/jbc.R800080200] [PMID] [PMCID]

[25] Xu P, Liang S, Zong MH, Lou WY. Ionic liquids for regulating biocatalytic process: Achievements and perspectives. Biotechnol Adv. 2021; 51:107702. [DOI:10.1016/j.biotechadv.2021.107702] [PMID]

[26] Patel R, Kumari M, Khan AB. Recent advances in the applications of ionic liquids in protein stability and activity: A review. Appl Biochem Biotechnol. 2014;

172(8):3701-20. [DOI:10.1007/s12010-014-0813-6] [PMID]

[27] Bisht M, Venkatesu P. Influence of cholinium-based ionic liquids on the structural stability and activity of α-chymotrypsin. New J Chem. 2017; 41(22):13902-11. [DOI:10.1039/C7NJ03023A]

[28] Defaei M, Taheri-Kafrani A, Miroliaei M, Yaghmaei P. Improvement of stability and reusability of α-amylase immobilized on naringin functionalized magnetic nanoparticles: A robust nanobiocatalyst. Int J Biol Macromol. 2018; 113(2017):354-60. [DOI:10.1016/j.ijbiomac.2018.02.147] [PMID]

[29] Dey TB, Kumar A, Banerjee R, Chandna P, Kuhad RC. Improvement of microbial α-amylase stability: Strategic approaches. Process Biochem. 2016; 51(10):1380-90. [DOI:10.1016/j.procbio.2016.06.021]

[30] Iyer P, Ananthanarayan L. Enzyme stability and stabilization-Aqueous and non-aqueous environment. 2008; 43(10):1019-32. [DOI:10.1016/j.procbio.2008.06.004]

[31] Naushad M, Alothman ZA, Khan AB, Ali M. Effect of ionic liquid on activity, stability, and structure of enzymes: a review. Int J Biol Macromol. 2012; 51(4):555-60. [DOI:10.1016/j.ijbiomac.2012.06.020]

[32] Persson M, Bornscheuer UT. Increased stability of an esterase from Bacillus stearothermophilus in ionic liquids as compared to organic solvents. J Mol Catal. 2003; 22(1-2):21-7. [DOI:10.1016/S1381-1177(02)00294-1]

[33] Rodríguez O, Cristóvão RO, Tavares APM, MacEdo EA. Study of the alkyl chain length on laccase stability and enzymatic kinetic with imidazolium ionic liquids. Appl Biochem Biotechnol. 2011; 164(4):524-33. [DOI:10.1007/s12010-010-9154-2] [PMID]

[34] Zhao H. Protein stabilization and enzyme activation in ionic liquids: specific ion effects. J Chem Technol Biotechnol. 2016; 91(1):25-50. [DOI:10.1002/jctb.4837]

[35] Zhao H, Jones CL, Cowins JV. Lipase dissolution and stabilization in ether-functionalized ionic liquids. Green Chem. 2009; 11(8):1128-38. [DOI:10.1039/B905388C]

[36] Zhao H, Olubajo O, Song Z, Sims AL, Person TE, Lawal RA, et al. Effect of kosmotropicity of ionic liquids on the enzyme stability in aqueous solutions. Bioorg Chem. 2006; 34(1):15-25. [DOI:10.1016/j.bioorg.2005.10.004] [PMID]

[37] Muginova SV, Galimova AZ, Polyakov AE, Shekhovtsova TN. Ionic liquids in enzymatic catalysis and biochemical methods of analysis: Capabilities and prospects. J Anal Chem. 2010; 65(4):331-51. [DOI:10.1134/S1061934810040027]

[38] Lakowicz JR. Principles of fluorescence spectroscopy. Springer science & business media; 2013. [DOI:10.1007/978-0-387-46312-4]

[39] Vivian JT, Callis PR. Mechanisms of Tryptophan Fluorescence Shifts in Proteins. Biophys J. 2001; 80(5):2093-109. [DOI:10.1016/S0006-3495(01)76183-8] [PMID] [PMCID]