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Investigation of Activity, Stability, and Structural Changes of *Aspergillus Oryzae* α-Amylase in the Presence of Water 1-Ethyl-3-methylimidazolium Acetate Mixture

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ABSTRACT

α-Amylases catalyze the hydrolysis of starch and are widely used in various industries, especially in the food industry. Despite the high demand for the use of α-amylase in various industries, most α-amylases do not have the temperature and pH stabilities for use in industrial processes. Ionic liquids are broadly used as co-solvents to improve the activity, stability, and selectivity of enzymes. In this study, the effect of 1-ethyl-3-methylimidazolium acetate on the activity, temperature and pH optimum, thermal stability, and structure of an α-amylase from *Aspergillus oryzae* were investigated. The activity of α-amylase was decreased with increments in [EMIm][Ac] concentration. V_m of α-amylase in the presence of [EMIm][Ac] was increased while Km was decreased. Same as the absence of [EMIm][Ac], the optimal pH and temperature in the presence of [EMIm][Ac] were 7.5 and 45 °C, respectively. The inactivation rate of α-amylase at temperatures of 40, 50, and 60 °C in the presence of both 0.4 and 1 M [EMIm][Ac] is less than in its absence. Intrinsic fluorescence spectroscopy revealed structural changes in the presence of [EMIm][Ac]. These results indicate the improvements in the activity, optimum temperature, and thermal stability of α-amylase due to the structural changes induced with [EMIm][Ac].

Keywords: Ionic liquids, [EMIm][Ac], Fluorescence spectroscopy

INTRODUCTION

α-Amylases catalyze the hydrolysis reaction of α-1,4-glycosidic bonds in starch and produce a variety of products such as dextrin, smaller oligosaccharides, maltose, and glucose [1]. α-Amylases occupy approximately 25% of the industrial enzyme market by widely use in various industries such as ethanol production, high fructose corn syrup (HFCS), the food industry, paper production, textiles, and detergents [2]. The use of α-amylase in these industries requires the α-amylases which are stable in process conditions. For example, the two stages of liquefaction and saccharification in the biodegradation process of starch require the use of high temperature and acid resistant amylase [3]. For this reason, extensive research has been done to discover new sources of α-amylases. Several amylases were isolated from various plants, animals, and microorganisms. Most of the obtained amylases do not have the necessary thermal and operational stabilities and regio- or enantioselectivities for use in industrial processes. Therefore, only a small number of discovered amylases have reached to commercial scale production [4]. Sources were used in the commercial production of amylase include *Bacillus subtilis*, *Aspergillus niger, Aspergillus oryzae*, and *Bacillus licheniformis* [5].

The other attractive approach to improve the functions such as activity and stability is using ionic liquids (ILs) as additives or co-solvents [6,7]. Factors such as ionic nature, polarity, hydrogen bonding, basicity, and anion nucleophilicity are responsible for improvement of enzymatic activity and stability in the presence of ILs [8]. Ionic liquids are solvents that just consist of ions. These solvents have unique properties like as melting point less than 100 °C, non-flammable, high solvent capacity, temperature and chemical stability, ion conductivity, reusability, and recyclability [9,10]. Because these properties can be altered for different purposes by combining different cations and

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anions, ionic liquids are also referred to as "designer solvents" [11]. These prominent features as well as increasing the stability of biomolecules in the presence of ionic liquids have led to the consideration of these solvents in various fields, especially in biotechnology, as an alternative to organic solvents to improve process efficiency and improve existing processes [12].

To date, various ionic liquids have been produced by combining different cations and anions and used as co-solvents or enzyme additives. These ionic liquids can be water-miscible, partially miscible, and immiscible and most of them have affected the activity of enzymes [13]. The solubility of carbohydrates in ionic liquids is higher than in conventional organic solvents [14]. This is due to the formation of hydrogen bonds between the anions of ionic liquids and carbohydrates in which facilitates the dissolution and conversion of these compounds into products through enzymatic reactions [15].

Some ionic liquids can denature enzymes, which is a reversal feature in the development of the use of ionic liquids in enzymatic reactions [16]. Despite extensive studies were done to clarify the reasons for the denaturation of enzymes in the presence of ionic liquids, there is still no general rule in this regard. Therefore, the effect of ionic liquids on the activity and stability of enzymes is still being studied. Imidazolium-based ionic liquids are considered one of the most important classes of ionic liquids due to their advantages such as safety, low cost, non-toxic, biodegradable, and solubility in water. One of the most important properties of the imidazolium ring is the h-bonding tendency of aromatic hydrogens present in the C-H bonds [17]. The acetate anion is small and polar, possessing a hydrophobic methyl tail in addition to a hydrophilic carboxylate headgroup. Due to these propetiesionic liquids consisting of acetate anions have received much attention in some industrial processes such as carbohydrate processing [18]. Taking into account the advantages of small imidazolium cation and acetate anion and with the aim to improve the activity and thermal stability of *Aspergillus oryzae* α-amylase, [EMIm][Ac] was used as a co-solvent. In addition, with the aim of better understanding the mechanism of the observed effects, some kinetic and structural changes were evaluated.

MATERIALS AND METHODS

Materials

α-Amylase, 3,5-dinitrosalicylic acid (DNS), starch, and [EMIm][Ac] were purchased from Sigma-Aldrich. All chemicals were analytical grade. Double distilled water was used to prepare the required solutions and buffers.

α-Amylase Assay

The activity of *Aspergillus oryzae* α-amylase was determined according to the method described by the Miller [19]. In this method, reducing sugars liberated from starch cause DNS reduction. Changes in absorbance was recorded using a UV spectrophotometer at 540 nm. Starch solution (1% w/v) was prepared in sodium phosphate buffer (20 mM) with sodium chloride (6.7 mM, pH 6.9). α-amylase (0.5 ml, 1 U ml^{-1}) with or without [EMIm][Ac] was added to 1 ml of starch solution. After 15 min of incubation at 20 °C, one ml of DNS solution (96 mM 3,5-dinitrosalicylic acid and 5.3 M sodium potassium tartrate) was added to the reaction. Then, enzymatic reaction was stopped by incubating in a boiling water bath (15 min). After 10 min of incubation at room temperature, the light absorption was recorded at 540 nm. A starch substrate solution without α-amylase enzyme (equal volume of distilled water) was used as control. The amount of the enzyme that releases 1 mM of reducing sugars (with maltose as the standard) per minute under the assay conditions was considered as one unit of α-amylase activity [20].

Determination of K_m and V_{max}

To determine the K_m and V_{max} of α-amylase, the activity was measured in different concentrations of the starch substrate from 2 to 10 mg ml^{-1}. The activities were used to obtain the Lineweaver-Burk plot. K_m and V_m were determined by extrapolating the line for without and presence of [EMIm][Ac] (1 and 0.4 M).

Optimal Temperature and pH

To obtain the optimal temperature, the enzyme solution (in the presence and absence of 0.4 M [EMIm][Ac]) and the starch solution were incubated separately for 5 min at 25 to 80 °C (with 5 °C intervals). Then the enzyme activity was measured by adding appropriate amounts of the enzyme

solution to the starch solution.

To determine the optimal pH, α-amylase solution in the presence of 0.4 M [EMIm][Ac] and its absence was prepared with pH from 3 to 10 (0.5 unit intervals) using glycine-HCl, sodium-citrate, sodium-acetate, sodium-phosphate, and Tris-HCl buffers . The α-amylase solution was used to determine activity.

Thermal Inactivation

Thermal inactivation of the α-amylase enzyme in the presence of 0.4 and 1 M [EMIm][Ac] and its absence was determined by incubation of enzyme solution at temperatures of 40, 50, 60, and 70 °C. Then, the α-amylase activity at 5, 10, 15, and 20 min was measured. The effect of temperature on the inactivation of α-amylase was compared between the absence and presence of [EMIm][Ac] using residual activity versus time plots.

Fluoresce Spectroscopy

Intrinsic fluorescence spectroscopy was performed using the 0.1 mg ml^{-1} α-amylase solutions and the Perkin-Elmer fluorescence spectrophotometer apparatus (LS45, USA). The α-amylase solutions in the presence of [EMIm][Ac] (0.2, 0.4, and 1 M) and its absence were incubated for 5 minutes at room temperature. Excitation was made at 295 nm and the emission was recorded in the range of 300 to 400 nm with a 5 nm slit. A solution without an enzyme was used as a control to remove the noise of the Imidazolium ring of [EMIm][Ac]. The final emission spectra resulted from the subtraction of the emission of each sample from the blank.

RESULTS AND DISCUSSION

Effect of [EMIm][Ac] on Amylase Activity

The effect of different concentrations of [EMIm][Ac] on *A. oryzae* α-amylase activity is shown in Fig. 1. α-amylase activity was decreased with increasing concentration of [EMIm][Ac]. The lowest enzyme activity was observed in the presence of 0.6 M [EMIm][Ac] at 28%. With increasing concentration of [EMIm][Ac] from 0.6 M, no change in enzyme activity was observed.

Enzymatic activity in the mixture of water and most ionic liquids decreases with increasing ionic liquid concentration [21-24]. According to our previous study, the activity of

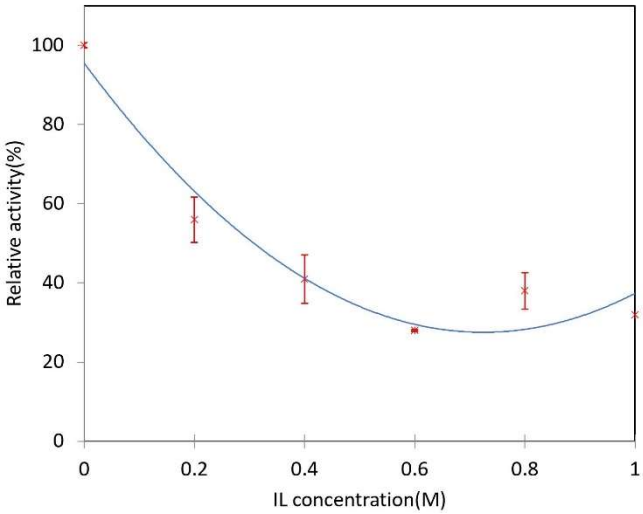

Fig. 1. Relative activity of α-amylase in the presence of [EMIm][Ac].

A. oryzae α-amylase decreased in the presence of [EMIm][Lac], so the lowest activity was obtained at a concentration of 0.8 M [20]. Zhao *et al.* reported the strong deactivationg effect at 17 and 34% and complete inactivation at 51% of [EMIm][Ac] [25]. However, there are exceptions when the activity of the luciferase enzyme decreases in the presence of [TMG][Ac], [TMG][Pro] but increases in the presence of [TMG][Lac] [22,26,27]. There are several other reports that imiduzolium based ILs increase enzymatic activity. Activity of cellulase from *Stachybotrys microspora* was enhanced to 115.5% and 114.5% in the presence of 5% (v/v) of 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM][DEP] and 1-allyl-3-methylimidazolium chloride [AMIm][Cl] [28]. Moreover, an increase in the activity of commercial proteases (Neutrase 0.8 l, Flavourzyme 500 l, and Alcalase 2.4 l) in the presence of ILs choline chloride, tetramethylammonium bromide, and [EMIm][Br] has been reported (20, 15 and 150%, respectively) compared to the control samples [29]. Therefore, the activity of each enzyme in the presence of ionic liquids should be evaluated. Acetate anion (OAc^-) in ionic liquid has a high ability to form hydrogen bonds with carbohydrates, including starch, and therefore increasing its solubility in water. The hydrogen bond ability of OAc^- induce opposite effect on the activity of α-amylase [30]. The hydrogen bond ability of iones leads to interactions with hydrogen bonds of the catalytic triad of

amino acids, conformational changes within the active sites of enzymes and therefore decrease in α-amylase activity [6,31].

Effect of [EMIm][Ac] on K_m and V_m of α-Amylase

The activity of the *A. oryzae* α-amylase was determined in different concentrations of starch and the presence and absence of [EMIm][Ac]. Using the linear equation obtained in the Lineweaver-Burk diagram (Fig. 2), the values of the K_m and V_m parameters were obtained (Table). According to these results, V_m increases in the presence of [EMIm][Ac] while K_m values in the presence of both concentrations (0.4 and 1 M) of [EMIm][Ac] decrease compared to its absence. In addition, in the presence of 1 M of [EMIm][Ac], V_m increased compared to 0.4 M. However, in both concentrations of [EMIm][Ac] no important change in Km was observed. These results may be due to the increase in the solubility of starch in the presence of [EMIm][Ac] and as a result, the increase in the number of accessible starch molecules to α-amylase. However, it seems that this property of [EMIm][Ac] simultaneously affects the structure of the enzyme, inactivating it and thus reducing the V_m of the enzyme [8]. Therefore, increasing the tendency of hydrogen bonding by anions of ionic liquids can weaken the structure and so the lose of the enzyme activity.

Kudou *et al.* (2014) explained that decrease in K_m and increase in V_m parameters of TmBgl1A in the presence of [BMIm][Ac] is due to an increase in enzyme-substrate affinity [30]. Ions of ionic liquids in an aqueous solution, bind directly with the enzyme or change the microenvironment around the enzyme [32] and affect the flexibility of an enzyme's active site which is essential in its substrate affinity. The kosmotoropic anions such as [Ac] induce high rigidly of enzyme, while chaotropic anions like as [Cl] induce high flexibility [30]. Optimum pH and temperature of α-amylase

Optimal temperature and pH are important factors for the use of α-amylase in carbohydrate processing industries. *A. oryzae* α-amylase activity in the presence of [EMIm][Ac] and its absence at different pH is shown in Fig. 3. The highest α-amylase activity was unchanged in the range of 7.5 to 8. In other words, [EMIm][Ac] at a concentration of 0.4 M does not affect the optimal pH of the enzyme. Changes in pH can change the ionization state of the ionizable side chain of

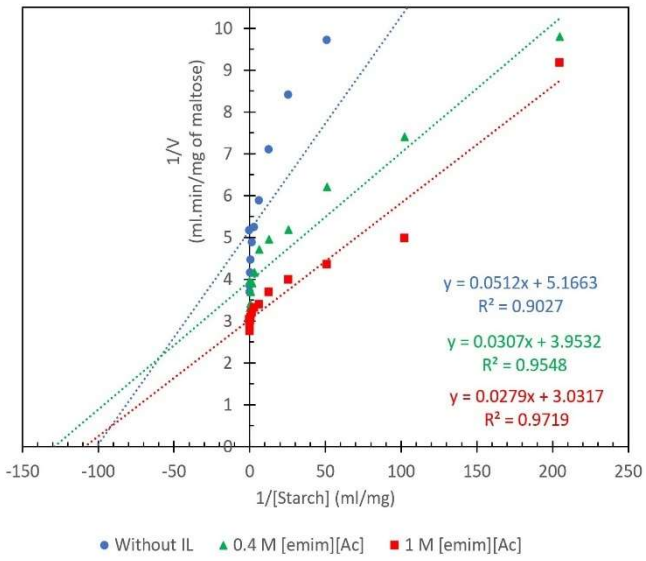

Fig. 2. The Lineweaver-Burk plot to determine K_m and V_m.

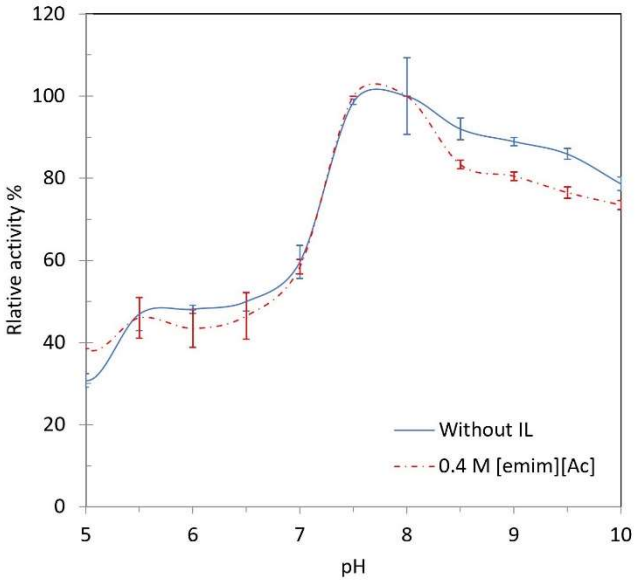

Fig. 3. The activity of α-amylase with and without [EMIm][Ac] at different pH from 5 to 10.

amino acid residues in the protein. This change affects the electrostatic interactions involved in the formation of the natural structure of the protein and can weaken or strengthen the natural structure of the protein. In addition, changing the ionization status of the residues in the active site of the enzyme can change the activity of the enzyme [33]. Ionic

liquids change the microenvironment around the residues of the active site of the enzyme through dehydration mechanisms, coulombic interactions, and hydrogen bonds [34]. As a result, the pKa of the groups participating in the reaction and finally the optimal pH of the enzyme changes [34,35].

Results of the study of α-amylase activity at different temperatures in the presence of [EMIm][Ac] are shown in Fig. 4. According to this graph, the highest α-amylase activity in the absence of [EMIm][Ac] was determined at 45 °C and a slight decrease at 50 °C. Similarly, in the presence of [EMIm][Ac], the highest α-amylase activity was obtained at 45 °C. Therefore, it can be stated that in the presence of [EMIm][Ac], the optimal temperature range of α-amylase was associated with an increase of 5 degrees. However, the same concentration of [EMIm][Lac] was not cause a significant change in the optimal temperature of α-amylase [20]. The same results were reported by Kudou *et al.* (2014), where the optimal temperature of *Tm*Bgl1 in the presence of [BMIm][Ac] and its absence was determined at 58 °C [30].

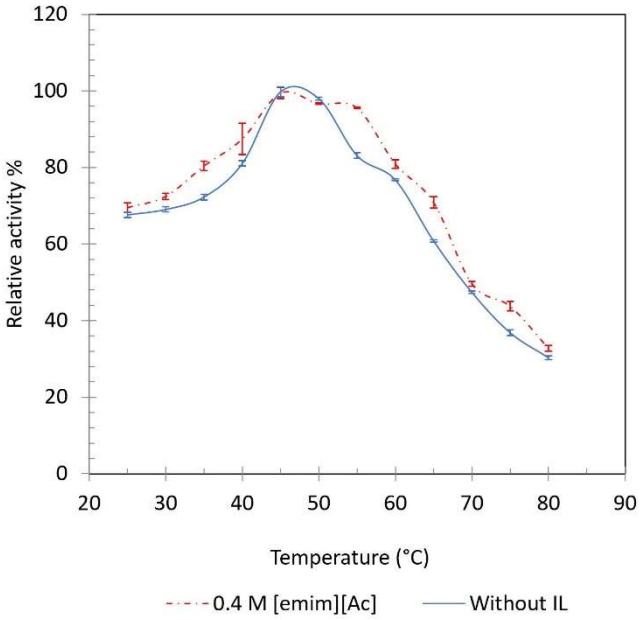

Fig. 4. The activity of α-amylase with and without [EMIm][Ac] at different temperatures from 25 to 80 °C.

Thermal Stability

One of the most important applications of ionic liquids in the field of biotransformation is to improve the thermal stability of enzymes for use in industrial processes. Several studies show that ionic liquids increase or improve the thermal stability of proteins [22,26,36-45]. However, in some cases, ionic liquids decreased the thermal stability of enzymes and so due to the differences in the amino acid composition and structure of enzymes, the effect of ionic liquids on the thermal stability is not the same [40].

In the present study, the thermal stability of *A. oryzae* α-amylase in the absence and presence of two concentrations (0.4 and 1 M) of [EMIm][Ac] at 40, 50, 60, and 70 °C for 5, 10, 15, and 20 min was evaluated. The slope of the line obtained in each case shows the rate of inactivation of the enzyme (Fig. 5). The rate of inactivation at 40, 50, and 60 °C in the presence of 0.4 and 1 M [EMIm][Ac] was less than in its absence. While at 70 °C in the presence of [EMIm][Ac] and its absence, the slope of the lines was almost the same, and therefore the rate of inactivation of the α-amylase was the same. In addition, there was not important difference between the rate of enzyme inactivation at the concentrations of [EMIm][Ac] at the studied temperatures. Zhao explained that high concentrations of strongly hydrated ions such as acetate tends to dehydrate and decrease enzyme flexibility which means more enzyme stability [46]. Intrinsic fluorescence spectroscopy

Fluorescence spectroscopy is one of the most widely used techniques for examining the folding/unfolding state and the binding of a substrate or ligand to proteins [47]. Due to the lack of external interfering factors, the use of intrinsic fluorescence is a priority in the study of structural changes in proteins. In proteins, the three amino acids Phenylalanine, Tyrosine, and Tryptophan can be used for the intrinsic fluorescence technique. The amino acid Tryptophan is more widely used due to its higher excitation and emission wavelengths (in the near UV range) as well as its longer emission time. The λ_{max} of Tryptophan depends on the environment and the exposure to the solvent [48]. Accordingly, in the present study, the structural changes of amylase in the presence of [EMIm][Ac] based on the fluorescence of the amino acid tryptophan were evaluated (Fig. 6). The λ_{max} is changed from 350 in the absence of ionic liquid to the range of 430-450 in the presence of all concentrations of [EMIm][Ac]. In the other words, in the presence of ionic liquid, a red shift in the tryptophan λ_{max} has

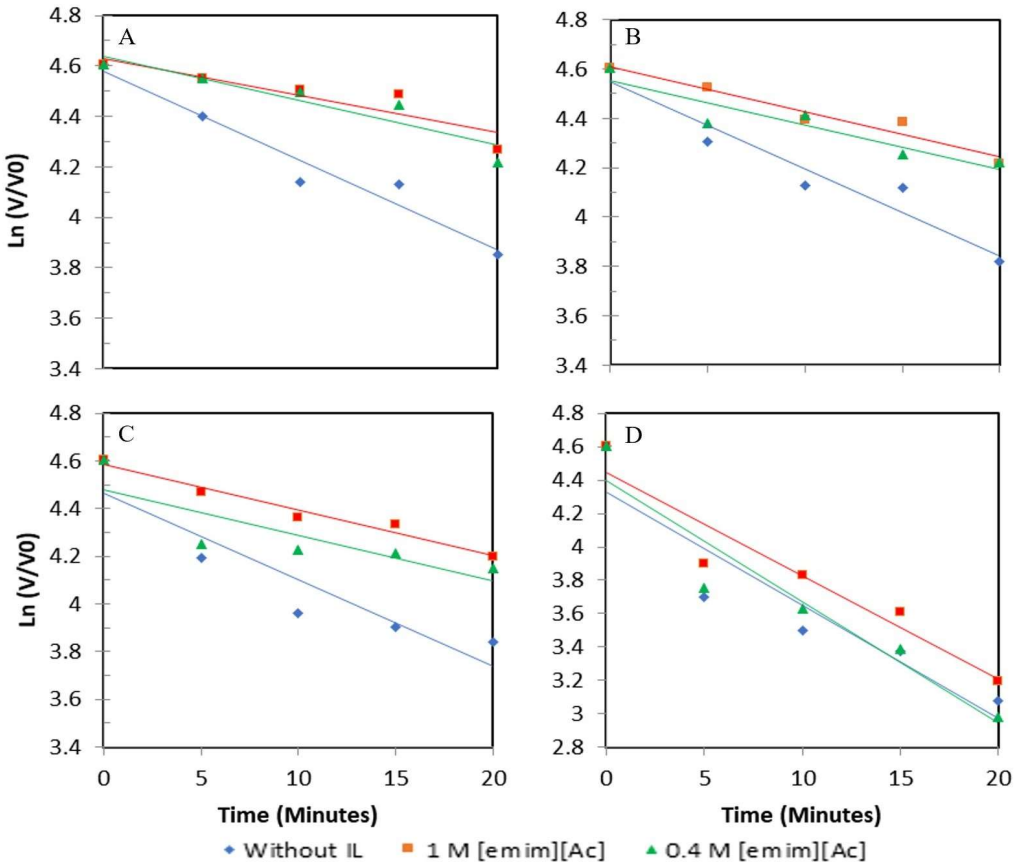

Fig. 5. Percentage of relative remaining activity against time to investigate the thermal inactivation of α-amylase in the absence and presence of 0.4 M of [EMIm][Ac] at 40 (A), 50 (B), 60 (C), and 70 °C (D).

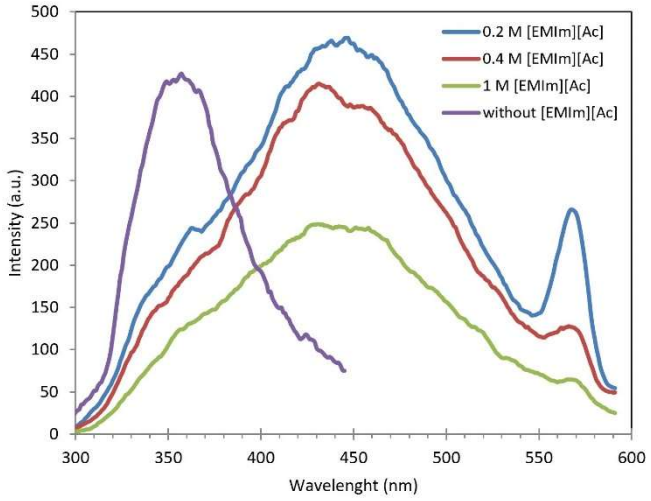

Fig. 6. Fluorescence spectra of amylase with and without [EMIm][Ac].

occurred. The intensity and shift of λ_{max} reflects the atmosphere surrounding tryptophan [30]. The redshift in the fluorescence spectrum occurs when tryptophan is transferred from a non-polar medium to a polar medium [49]. Thus, in the absence of [EMIm][Ac], tryptophan is located in the interior of the α-amylase and far from the polar environment. In the presence of [EMIm][Ac] due to induction of structural changes in the enzyme, tryptophan is moved to the vicinity of water molecules. For this reason, an increase in wavelength, in other words, a redshift has occurred.

CONCLUSIONS

Based on the results obtained in the present study, the presence of [EMIm][Ac] as a co-solvent induces structural changes in the *Aspergillus oryzae* α-amylase in a way that in spite of decrease in the activity causes a slight decrease in K_m

and an increase in V_m. In addition, due to the lack of change in the optimal pH, it can be expected that there will be no change in the ionization states of residues in the presence of [EMIm][Ac]. However, increasing the optimum temperature and temperature stability indicates a change in the ion network of the solvent and the formation of new interactions in the solvent and between the solvent and the enzyme molecule. Therefore, the combination of cation and anion in [EMIm][Ac] can facilitate the access of α-amylase to the substrate and increase the enzyme activity by inducing changes in the solvent network and α-amylase structure while improving the functional properties of α-amylase.

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