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Kinetic and Stability Study of the Peroxidase Inhibition in Ionic Liquids

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The activity and stability of peroxidase in aqueous solutions of two ionic liquids (ILs) have been studied. The ILs selected were 1-ethyl-3-methylimidazolium 2-(2-methoxyethoxy) ethylsulfate, [emim][MDEGSO$_4$], and 1-ethyl-3-methylimidazolium ethylsulfate, [emim][EtSO$_4$]. Experiments were performed at room temperature using concentrations of ILs between 5 and 50% (v/v) and pH values in the range from 5 to 9. The initial activity of the enzyme in these ILs at optimized conditions (pH 7 and 5−10% IL) was similar or higher than that achieved with buffer solution. Kinetic studies showed that maximum reaction velocity (V_{max}) decreased with increasing concentration of IL. The effect of [emim][EtSO$_4$] concentration on the decrease of V_{max} was higher than that of [emim][MDEGSO$_4$]. It was found that [emim][EtSO$_4$] was a more potent inhibitor on peroxidase activity. The peroxidase studied was active in the ILs investigated, and the enzyme exhibited a higher stability in ILs at the optimized conditions. Kinetic studies showed that inhibition was a noncompetitive type in both ILs.

1. Introduction

Research on new processes involving peroxidase has been intensified and presents new applications in the analytical, environmental, clinical, and industrial fields.[1] Recently, the use of peroxidase to remove a variety of phenolic compounds from polluted wastewater has also been the topic of several works.[2] Phenols are oxidized by peroxidase in the presence of hydrogen peroxide to form oligomeric and polymeric products.[3] Heme-containing peroxidases catalyze the two-electron oxidation of a wide range of structurally diverse aromatic compounds generating phenoxy radicals using hydrogen peroxide, which forms polymeric products when coupled with other substrates.[4–6] For different reasons, the yield of some peroxidase-catalyzed processes decreased in the presence of organic solvents. The addition of organic solvents to an aqueous enzyme solution can diminish substrate binding and the catalytic effect, thus reducing the reaction rates. The molecules of solvent may interact directly with the active center of the enzymes, thus changing their structure and causing inactivation (reversible or irreversible). But, the effect of these organic solvents on the stability and catalytic activity of peroxidases also depends on the source of the enzyme, its cultivation conditions, purification procedure, and organic solvent concentration.[7] Studies about the effect of organic solvents on peroxidase structure and kinetics suggest that the catalytic activity for phenols decreases with the increase of solvent hidrophobicity. However, a partial denaturation of the enzyme also occurs and the loss in catalytic efficiency was associated to several factors: penetration of organic solvent into the enzyme active site and stabilization of the phenolic ground state in organic media.[8] The use of organic solvents in bioprocesses presents additional problems due to the toxicity of these solvents for both the process operators and for the environment (ecotoxicity). The volatile and flammable nature of these solvents also raises process safety issues.[9,10]

The use of ionic liquids (ILs) as nonaqueous solvents or cosolvents for biocatalysis has been attracting a great interest because of the range of properties that they can offer. These chemicals are organic salts composed only by ions which are liquids at or near room temperature, with melting points below 100 °C. They are often considered as environmentally friendly solvents when compared to organic solvents. ILs are distinguished from classical molten salts by their lower melting points. Their lower melting points are commonly obtained from combinations of highly asymmetric organic cations (namely derivatives of *N*,*N*′-substituted imidazolium, *N*-substituted pyridinium, tetraalkyl-ammonium, and tetraalkyl-phosphonium) and either organic or inorganic anions. They are often called "designer solvents" due to the easiness in changing cations and anions, or even functionalizing them, in order to modify the solvent properties, which allows ILs to be designed for specific reaction systems. Properties such as density, viscosity, melting point, or solvation ability can be finely tuned.[11] Recent research studies with ILs as alternative solvents for enzymatic catalysis demonstrated high enzymatic activity and stability in these solvents, which makes them a promising replacement for organic solvents in biocatalysis at both laboratory and industrial scale.[12–14] Nowadays, prices will mainly depend on the quantity in which specific ILs are produced. Most of the ILs today are manufactured only in kilogram quantities and therefore are offered at high prices. Nevertheless, it is expected that prices are lowered as standards ILs. The growing demand of ILs will make some of then standard products, with prices for ton quantities below 30 euro/kg.[15] As the recycling of imidazole ILs is easy (can be distilled), their application in the enzymatic process becomes viable and sustainable.

The objective of this work is to evaluate the activity and the stability of peroxidase in the presence of two different ILs, 1-ethyl-3-methylimidazolium 2-(2-methoxyethoxy) ethylsulfate, [emim][MDEGSO$_4$], and 1-ethyl-3-methylimidazolium ethylsulfate, [emim][EtSO$_4$], at different concentrations and pH values. The peroxidase inhibition mechanism of these ILs and the kinetic parameters are also determined.

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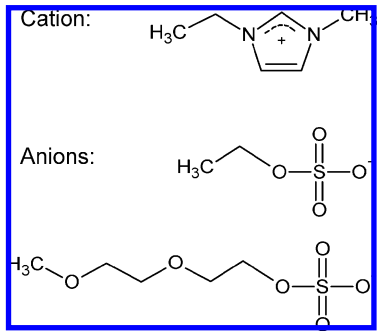

Figure 1. Cation and anions of the two ionic liquids used in this study.

2. Materials and Methods

2.1. Chemicals. The ILs 1-ethyl-3-methylimidazolium 2-(2-methoxyethoxy) ethylsulfate, [emim][$MDEGSO_4$], and 1-ethyl-3-methylimidazolium ethylsulfate, [emim][$EtSO_4$], were kindly provided by Solvent Innovation GmbH (Köln, Germany) with nominal purities 99% (puriss.) and 98% (purum.), respectively, and used without further purification. Their chemical structures are shown in Figure 1. The water content of ILs was measured prior to use by Karl Fisher titration (Metrohm 756 Karl Fisher Coulometer). The results obtained were 0.113% wt for [emim][$MDEGSO_4$] and 0.178% wt for [emim][$EtSO_4$]. 2,2′-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) (98%) was obtained from Sigma (Spain). Hydrogen peroxide 30% was obtained from Merck (Germany). Commercial peroxidase (E.C. 1.11.1.7) (concentrated solution) was kindly provided by Novozymes (Denmark). The only information provided by Novozymes is that the peroxidase is from a fungus source.

2.2. Determination of Enzyme Activity and Stability. The enzyme activity is a measure of the ability of an enzyme to catalyze a specific reaction. The most commonly used quantity is the unit (U). One activity unit is defined as the amount of enzyme that oxidizes 1 μmol of ABTS per minute, and the activity is expressed in units per liter.[16] The residual peroxidase activity (the enzyme that remains in solution) was assayed spectrophotometrically (Thermo Electron, model UV1 spectrophotometer) by a standard method.[17] The ABTS substrate solution was prepared mixing 0.4 mL of ABTS (0.4 mM) with 1.9 mL of 0.05 mM citrate/0.1 mM phosphate buffer pH 4.5 and 0.1 mL of H_2O_2 (100 μM) and was the same for all experiments. To measure the peroxidase activity, 0.1 mL of the enzyme solution (containing ILs, buffer, or a mixture of both) were added to 1.9 mL of the ABTS solution at 40 °C.[17] The change in absorbance at 420 nm ($\varepsilon = 36 \times 10^3$ $M^{-1}\cdot cm^{-1}$) was recorded for 30 s, and the catalytic activity was determined by measuring the slope of the initial linear portion of the kinetic curve. The experiences were performed in duplicate.

The enzyme stability is the extent to which the peroxidase retains its activity when subjected to storage. Peroxidase stability at different pH values and for different concentrations of ILs was investigated by incubating (maintenance of the enzyme at the controlled temperature, 25 °C) the enzyme solution in a (IL + buffer) solution. The concentration of the ILs was set to 5, 10, 25, and 50% (v/v). The water content of the pure ILs is neglected (see section 2.1 above) and does not affect this essay. The effect of pH was studied using buffer solutions of pH 5.0, 7.0, or 9.0. The pH of buffer solution was adjusted adding small amounts of phosphoric acid and checking the value with a pH-meter. A sample incubated with phosphate buffer only (50 mM) at the corresponding pH (5.0, 7.0, or 9.0) was used as the reference for each case (called the control in tables and figures). The peroxidase initial activity was measured after the addition of the enzyme solution (final concentration of 2000 U/L) into the incubation media. Residual activities were determined from small samples of the incubated enzyme solution withdrawn at regular time intervals, up to 7 days, using the standard procedure described above. The activity loss (%) is the difference between the final activity (A_f) and initial activity (A_i): $(A_i - A_f)/A_i \times 100$. All activity measurements were performed in duplicate, and the average standard deviation found was 4.2% and 4.1% for the [emim][$EtSO_4$] and the [emim][$MDEGSO_4$] series of experiments, respectively.

2.3. Enzyme Kinetics and Inhibition. The kinetics of the reaction with the ABTS substrate and the effect of IL inhibition on peroxidase activity were evaluated. The enzyme was incubated in a solution with 50 mM phosphate buffer (pH 7.0) and 5, 10, or 25% (v/v) IL (corresponding to 0.263, 0.525, and 1.313 M for [emim][$EtSO_4$]; 0.195, 0.390, and 0.975 M for [emim][$MDEGSO_4$]). As explained above, the rate of ABTS oxidation was studied spectrophotometrically at 420 nm ($\varepsilon = 36 \times 10^3$ $M^{-1}\cdot cm^{-1}$). The substrate (ABTS) concentration was varied within the range 0.005−0.1 mM. The experimental data obtained were analyzed by nonlinear regression analysis using the Michaelis−Menten and inhibition equations. The experiences were performed in duplicate.

3. Results and Discussion

3.1. Peroxidase Activity and Stability. The initial activity of peroxidase with ABTS substrate was measured using two different water-miscible ILs as cosolvents, [emim][EtSO4] and [emim][$MDEGSO_4$], in order to assess the effect of the IL on enzyme activity. Three pH values (5, 7, and 9) and IL contents varying from 5 to 50% v/v were evaluated. The results obtained are presented in Table 1. Initial activities in [emim][$EtSO_4$] at moderate concentrations (5−25% IL) show a slight decrease with increasing IL concentrations for all pH values. Compared to the aqueous buffer (control), a decrease in initial activity was observed for pH 5 and 9. However, promising results were obtained at pH 7 for concentrations of 5 and 10% (v/v), with the initial activity approximately 8% higher. [emim][$MDEGSO_4$] showed a similar behavior to [emim][$EtSO_4$], but its initial peroxidase activities were slightly higher than those with [emim][$EtSO_4$]. Compared to the buffer (control), a decrease in enzyme activity around 1.4 times was observed in the presence of a high [emim][$MDEGSO_4$] concentration (50%) for the three pH values studied. Nevertheless, the differences in enzymatic activity found for both ILs are small as can be observed in Table 1. A similar tendency for horseradish peroxidase in organic solvents has been reported, and the denaturing effect of the organic cosolvent was observed for DMSO contents higher than 20%.[7]

The stability of peroxidase was studied at the same conditions as those for the initial activity tests over 7 days. The enzyme activity loss with respect to its initial activity (%) is shown in Table 1, and the deactivation profiles are presented in Figure 2. The residual activities of peroxidase were well-maintained, with final values around 86% for [emim][$EtSO_4$] and 75% for [emim][$MDEGSO_4$] for a IL content of 5% (v/v) and pH = 7 after 7 days of incubation (see Table 1). Figure 2 shows that for this concentration of IL the stability of peroxidase at pH 7 in both ILs was higher than or approximately the same as in the control sample. When the IL content was increased from 25 to 50% (v/v), a decrease in activity was observed, complete inactivation with [emim][$EtSO_4$] and 1.5 times for [emim]-

Table 1. Initial Activity, Residual Activity, and Activity Loss of Peroxidase in Ionic Liquids (ILs) at Different pH Values and IL Concentrations

		enzymatic activity, U/L (activity loss, %)			
pH	[IL] % v/v	initial	1 day	3 days	7 days
		[emim][$EtSO_4$]			
5	control[a]	1316	978 (26%)	1008 (23%)	937 (29%)
	5	1022	577 (44%)	578 (43%)	647 (37%)
	10	952	458 (52%)	483 (49%)	532 (44%)
	25	769	276 (64%)	233 (70%)	229 (70%)
	50	667	247 (63%)	25 (96%)	2 (100%)
7	control	1269	1176 (7%)	1243 (2%)	1178 (7%)
	5	1368	1058 (23%)	1172 (14%)	1176 (14%)
	10	1345	1017 (24%)	1069 (20%)	1040 (23%)
	25	1178	709 (40%)	701 (41%)	630 (47%)
	50	1045	265 (75%)	48 (95%)	0 (100%)
9	control	1501	1311 (13%)	1327 (12%)	1300 (13%)
	5	1399	927 (34%)	998 (29%)	930 (34%)
	10	1271	971 (24%)	980 (23%)	900 (29%)
	25	1247	309 (75%)	181 (85%)	145 (88%)
	50	1059	70 (93%)	8 (99%)	7 (99%)
		[emim][$DMEGSO_4$]			
5	control	1129	1003 (11%)	666 (41%)	323 (71%)
	5	1208	1112 (8%)	754 (38%)	179 (85%)
	10	1047	1038 (1%)	825 (21%)	774 (26%)
	25	1075	783 (27%)	489 (55%)	466 (57%)
	50	869	77 (91%)	1 (100%)	2 (100%)
7	control	1506	1312 (13%)	1081 (28%)	965 (36%)
	5	1447	1408 (3%)	996 (31%)	1081 (25%)
	10	1476	1209 (18%)	1083 (27%)	1070 (28%)
	25	1461	1326 (9%)	1091 (25%)	927 (37%)
	50	979	913 (7%)	712 (27%)	624 (36%)
9	control	1231	1039 (16%)	943 (23%)	936 (24%)
	5	1284	1069 (17%)	963 (25%)	886 (31%)
	10	1313	1005 (23%)	948 (28%)	813 (38%)
	25	1277	826 (35%)	807 (37%)	771 (40%)
	50	1077	837 (22%)	573 (47%)	340 (68%)

[a] Buffer solution without IL.

[$MDEGSO_4$]. Comparable results were recently reported for laccase, in a comparison with the same ILs and organic solvents.[14]

It has been previously reported that enzymes may express higher stability in certain ionic liquids under certain conditions: Lipases generally present good activity in ILs and higher stability than in organic solvents.[18–20] The stability of laccases and peroxidases in ILs has also been previously studied and confirmed the previous statement: Liu and co-workers[21] reported significant catalytic activity of peroxidase in the presence of 25% (v/v) of the water-miscible ionic liquid 4-methyl-butylpyridinium tetrafluoroborate.

Continuous and faster enzyme deactivation was observed in [emim][$EtSO_4$] at 50% (v/v) in all pH values after 3 days of incubation, while for [emim][$MDEGSO_4$] a complete deactivation of peroxidase was determined only at pH 5 and an IL concentration of 50% (v/v). From these results, a good initial activity and stability could be obtained for peroxidase in both ILs for concentrations up to 25% (v/v). At higher concentrations of both ILs, structural changes associated with the incubation of the enzyme may be attributed to conformational changes of the enzyme active site. It is known that the water content and solvent nature are important for the enzymatic reactions in nonaqueous medium due to the influence on the flexibility of the protein which is responsible for enzyme activity, selectivity, and stability.[22] These conformational changes may be due to interactions between the ions of the ILs and charged groups of the enzyme. Researchers have suggested that electrostatic interactions between ILs and enzymes should result in a higher kinetic barrier and a more rigid enzyme.[12] Similar studies of stability in ILs with oxidative enzymes showed the same tendency in enzyme deactivation with increasing IL content. Sgalla et al.[23] studied the activity of horseradish peroxidase in 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF_4])/water mixtures. No activity was observed for contents above 25% and 60% at pH 7.0 and 8.0, respectively. However, for pH 9.0, the enzyme retained some catalytic activity up to 90% of IL. A similar study of oxidative enzyme stability (laccase and peroxidases) shows that these enzymes present catalytic activity at moderate concentrations (up to 25% v/v) of water-miscible ionic liquid 4-methyl-*N*-butylpyridinium tetrafluoroborate [4-MBP][BF_4], while in water-immiscible ionic liquid 1-butyl-3-methylimdizaolium hexafluorophosphate (BMIM)PF_6, the laccase did not show a measurable activity.[24]

3.2. Enzyme Inhibition. In order to study the type of inhibition produced by the ILs used, the Michaelis−Menten parameters for the enzymatic reaction in the presence and absence of [emim][$EtSO_4$] and [emim][$MDEGSO_4$] were determined. The Michaelis−Menten model was successfully used in the past enzymatic reactions with peroxidase.[25,26] The kinetic experiments were carried out varying both the IL and the ABTS concentrations, as explained above. The calculation of the Michaelis−Menten parameters was done by nonlinear regression, based on the Michaelis−Menten equation:

$$v_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

where v_0 is the initial reaction rate, V_{max} is the maximum reaction velocity, K_m is the Michaelis−Menten constant, and [S] the substrate concentration. The K_m and V_{max} parameters were obtained by least-squares nonlinear regression using the SigmaPlot (SPSS, Inc.) software, and the results are presented in Table 2 together with the corresponding correlation coefficient, R^2.

The K_m values in the control sample and in the presence of [emim][$MDEGSO_4$] (for any composition) present close values (in the range 0.009−0.012 mM) while in the presence of [emim][$EtSO_4$] (for any composition) the result was a bit lower (0.0064−0.0079 mM). The effect of the presence of IL was much clearer in V_{max}. As the IL concentration was increased, the V_{max} obtained decreased. The effect of [emim][$EtSO_4$] concentration was higher than that of [emim][$MDEGSO_4$], and so V_{max} was reduced from 3.10 mM/min in pure buffer (control) to 2.19 mM/min for the first IL (a reduction of 29%) and 2.35 mM/min for the latter (a reduction of 24%). K_m is the value of substrate concentration at which the reaction rate reaches half of its maximum value ($V_{max}/2$) and is understood as the affinity of the enzyme by the substrate. Thus, low K_m values indicate that the enzyme attains its maximum catalytic efficiency at a lower substrate concentration. The V_{max} values obtained decrease as the IL concentration increases. According to these results, the K_m value can be considered approximately constant for all IL concentrations, while V_{max} gradually decreased with the increase in IL. This type of behavior would correspond to a noncompetitive inhibition mechanism (Table 2), so the ILs do not affect the apparent substrate binding to the enzyme but indeed affect the rate of the reaction (lower V_{max}). The same conclusion is obtained when the results are plotted in Hanes−Woolf diagrams, as presented in Figure 3. The diagram is a linearization of the Michaelis−Menten equation,

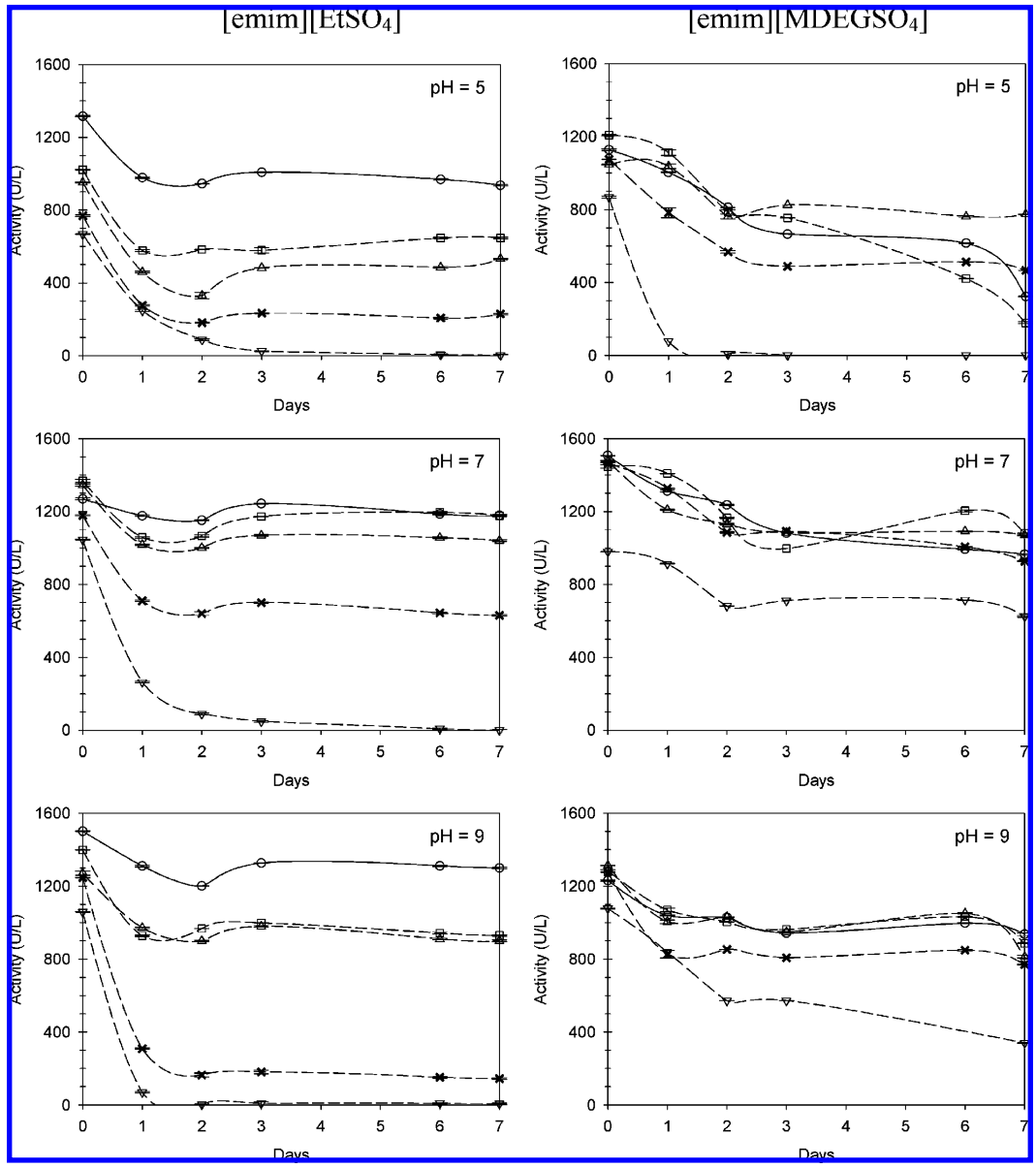

Figure 2. Effect of ionic liquid concentration ([emim][$EtSO_4$] and [emim][$MDEGSO_4$]) on peroxidase stability at different pH values. -•- control, -□- 5%, -Δ- 10%, -×- 25%, -▽- 50%.

Table 2. Michaelis−Menten Parameters (Equation 1) for Peroxidase in the Presence of [emim][$EtSO_4$] and [emim][$MDEGSO_4$] ILs

IL concentration				
% v/v	M	V_{max}, mM/min	K_m, mM	R^2
		control sample		
0	0	3.10 ± 0.31	0.0118 ± 0.0042	0.906
		[emim][$EtSO_4$]		
5	0.263	2.69 ± 0.21	0.0079 ± 0.0025	0.896
10	0.525	2.54 ± 0.25	0.0069 ±0.0029	0.831
25	1.313	2.19 ± 0.16	0.0064 ±0.0021	0.880
		[emim][$MDEGSO_4$]		
5	0.195	2.87 ± 0.17	0.0090 ± 0.0020	0.950
10	0.390	2.90 ± 0.24	0.0094 ±0.0030	0.911
25	0.975	2.35 ± 0.14	0.0091 ±0.0021	0.947

obtained when the ratio of substrate concentration to reaction rate is plotted against the substrate concentration itself:

$$\frac{[S]}{v_0} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}}[S] \qquad (2)$$

This type of diagram allows a quick identification of the noncompetitive inhibition: Data for different concentrations of the inhibitory component will show different slopes, but the same intercepts with the *x*-axis (different V_{max}, the same K_m). The plots in Figure 3 present approximately that situation, specially in the case of [emim][$MDEGSO_4$]. Moreover, the same type of inhibition mechanism was reported for a different IL, 1-butyl-3-methylimidazolium tetrafluoroborate, on the kinetics of horseradish peroxidase for guaiacol oxidation.[27] This highlights the fact that the Hanes−Woolf diagram[28,29] is used for ease of visualization only and that all kinetic parameters were obtained by nonlinear regression of the experimental data.

In pure noncompetitive inhibition, the inhibitor decreases V_{max} but has no effect on K_m. The noncompetitive inhibitor does not compete with substrate, and thus, substrate concentration has no influence on the degree of inhibition of the enzyme. The inhibitor binding does not affect enzyme−substrate affinity; both the substrate (S) and the inhibitor (I) can simultaneously bind to the enzyme, but the resultant E−S−I complex is catalytically inactive. As a result, the inhibitor (*I*) can hider the adequate

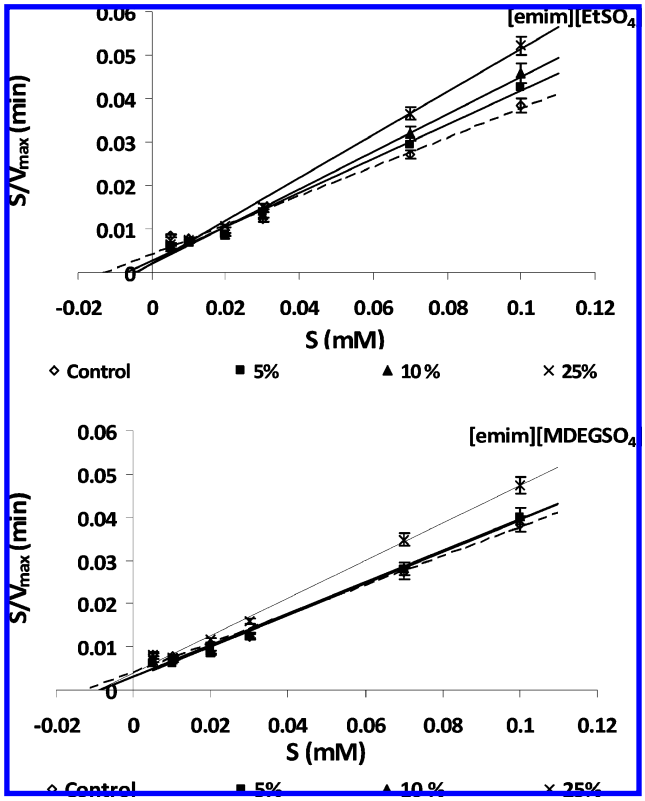

Figure 3. Hanes−Woolf plots (eq 2) for inhibition of peroxidase by [emim][MDEGSO$_4$] and [emim][EtSO$_4$] ILs at pH 7.0 and 25 °C using ABTS as substrate (S).

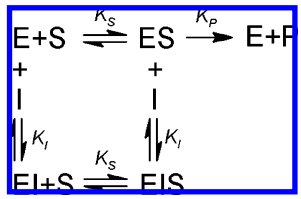

Figure 4. Reaction mechanism for noncompetitive inhibition, with enzyme (E), substrate (S), inhibitor (I), and products (P).

positioning of the catalytic center. In that case, the reaction of the noncompetitive inhibitor is irreversible and the substrate cannot overcome the inhibitor's impact on the enzyme. The mechanism of noncompetitive inhibitor is shown in Figure 4. The reaction rate for that mechanism is given by the following equation:[30]

$$v_0 = \frac{V_{max}[S]}{([S] + K_m)\left(1 + \frac{[I]}{K_i}\right)} \quad (3)$$

where [I] is the concentration of the inhibitor (here, the IL) and K_i is the inhibitory constant. The K_m and V_{max} values used are those for the pure buffer (control sample) which correspond to absence of IL. The K_i values were obtained by fitting of the experimental data to eq 3. This fitting was again carried out by least-squares nonlinear regression using the SigmaPlot (SPSS, Inc.) software. To do so, the concentration of ionic liquid was transformed to molarity using the densities of the corresponding ionic liquid at 20 °C: 1.241 g·cm^{-3} for [emim][EtSO$_4$][31] and 1.21 g·cm^{-3} for [emim][MDEGSO$_4$].[32]

The inhibition constant of each IL, K_i, and its correlation coefficient, R^2, are presented in Table 3 together with the K_m and V_{max} values used. Figure 5 shows the experimental and calculated initial reaction rates for the different concentrations of IL. It is important to highlight that the inhibitory effect of the ILs can be modeled with fair agreement using just one adjustable parameter (K_i). The Michaelis−Menten parameters (K_m and V_{max}) were obtained in the pure buffer solution (control) without any IL (see Table 2). It can be seen that both ILs produce a similar effect on peroxidase, but [emim][EtSO$_4$] gave a slightly larger reduction on the reaction rate (higher inhibition) due to a smaller K_i value (see Table 3).

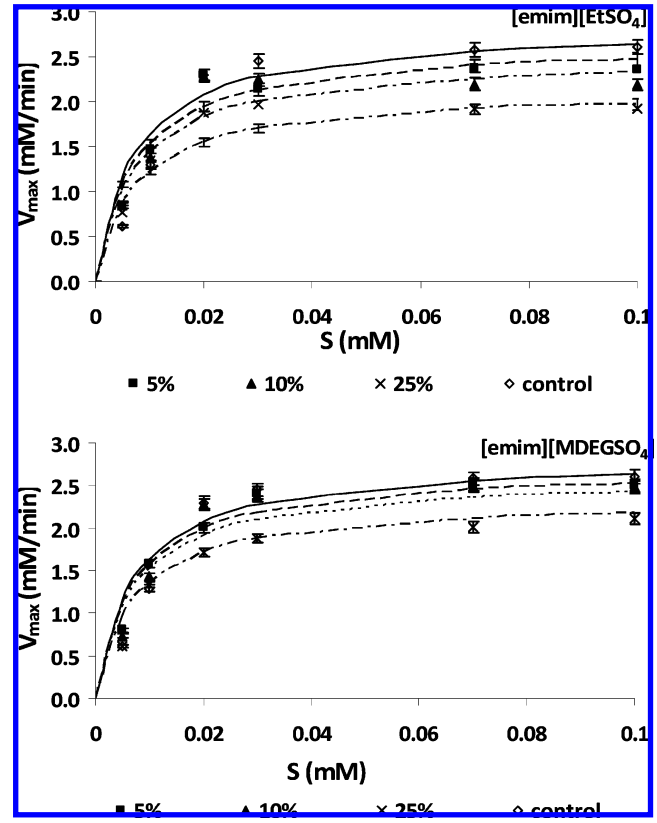

Figure 5. Effect of ILs on ABTS oxidation rate by peroxidase: points (experimental) and lines (inhibition model, eq 3).

Table 3. Inhibition Parameter (Equation 3) Values for the Oxidation of ABTS by Peroxidase in [emim][EtSO$_4$] and [emim][MDEGSO$_4$] ILs

IL	K_i (M)	R^2	V_{max} (mM/min)	K_m (mM)
[emim][EtSO$_4$]	0.982 ± 0.259	0.790	3.10	0.0118
[emim][MDEGSO$_4$]	1.263 ± 0.308	0.904		

Conclusions

The peroxidase studied was active in the two ionic liquids investigated ([emim][EtSO$_4$] and [emim][MDEGSO$_4$]) and exhibited similar initial activity for both ILs. Furthermore, the enzyme showed a higher stability in the ILs at the optimized conditions of pH and IL concentration (pH 7 and IL 5−10%). From the two ILs studied, [emim][EtSO$_4$] was the most promising IL for peroxidase stability with an activity loss of about 14% after 7 days of incubation. However, the initial activities of the IL [emim][MDEGSO$_4$] were higher. The Michaelis−Menten model described well the experimental results. Both ILs were found to inhibit the oxidation of ABTS by peroxidase, although this effect can be more perceived with [emim][EtSO$_4$], which is in agreement with the initial activities results once the kinetic data were obtained from the initial

activities. The noncompetitive inhibition effects of the ILs on the peroxidase reaction could be modeled adequately with an extension of the Michaelis−Menten kinetics.

Acknowledgment

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