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Biocatalytic action of proteases in ionic liquids: Improvements on their enzymatic activity, thermal stability and kinetic parameters

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ABSTRACT

This study evaluated the effect of the addition of the following ionic liquids (IL): choline chloride (CC), tetramethylammonium bromide (TB) and 1 ethyl 3 methylimidazolium bromide (EM), on some biochemical properties including enzymatic activity and different kinetic parameters of commercial proteases. The enzyme-IL combinations that showed the highest increases in enzyme activities were as follows: CC (0.5 mM) and Neutrase® 0.8 L; CC (5 mM) and Flavourzyme® 500 L; TB (2000 mM) and Alcalase® 2.4 L, with relative increases of 20, 15 and 150% in protease activities, respectively, compared to the control assays. The combination TB and Alcalase® 2.4 L showed a reduction of 50% of the activation energy (E_a), an increase of the relation V_{max}/K_m of 35% and a 16-fold rise in the values of $t_{1/2}$, and D. Neutrase® 0.8 L combined with CC showed an increase of 20% in the relation V_{max}/K_m. The combination Flavourzyme® 500 L and CC presented a 20% higher value of the relation V_{max}/K_m and a 2-fold increase in the values of $t_{1/2}$ and D compared to the control assay. In summary, the most positive effects observed in this study included proteases with improved activity and stability properties and a greater affinity for the substrate.

1. Introduction

The term ionic liquid (IL) can be used to describe asymmetric organic salts composed entirely of ions that have a melting point below 100 °C or typically near room temperature. Commonly, these compounds have organic cations associated with anions whose coordination strength is relatively low. Molten salts, in turn, are a class of ionic liquid, which, though normally solid at standard temperature and pressure, are in the liquid phase due to elevated temperature [1]. In many cases, it has been observed that the addition of these salts promoted a beneficial effect on the reaction rate (and/or selectivity) when acting as entropic conductors for the system [2].

Currently, ILs are highlighted due to their application in ecologically cleaner catalytic processes when compared to traditional media [3]. Their use is described in the synthesis of biodiesel, in the substitution of organic solvents, in the electrodeposition of metals/semiconductors and as fuel cells, and in presenting promising potential for biological catalysis [4]. Several studies reported the use of ILs in lipase-catalyzed reactions, such as esterification and transesterification [5–8].

The use of enzymes in ionic liquids has presented many advantages, such as high conversion rates, high enantioselectivity, better enzyme stability, and better recoverability and recyclability [9,10]. The addition of IL also exerts an influence on the enzymatic activity: it was reported that the relative activity increases initially before subsequently decreasing, generating a bell-shaped profile with the IL concentration. This effect on the enzymatic activity can be explained by the formation of micelles by the ionic liquid which alter the enzyme-substrate interaction [11]. The high industrial and technological applicability of the enzymes is increasing the search for new forms of production and the improvement of their characteristics. In this sense, the use of enzymes derived from microorganisms is highlighted instead of those of animal and vegetable origin, due to the lower cost and larger scale of production [12].

Among the different classes of enzymes, proteases are widely studied because they play critical roles in multiple biological pathways [13]. They have a great commercial importance among enzymes, accounting for nearly 60% of the whole enzyme market, and are often used in the detergent, leather, pharmaceutical, food and biotechnology industries [14,15]. Despite the well-examined investigation of enzyme activity and stability in ILs, there are few studies regarding the use of these compounds in proteases. In this way, the aim of this work was to evaluate the effects of the addition of ionic liquids on the biochemical characteristics of different commercial preparations of proteases.

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2. Materials and methods

2.1. Enzymes and ionic liquids

The commercial preparations of proteases used in this study were as follows: Alcalase® 2.4 L from *Bacillus licheniformis*, Flavourzyme® 500 L from *Aspergillus oryzae* and Neutrase® 0.8 L from *Bacillus amyloliquefaciens*. The molten salts choline chloride (CC) and tetramethylammonium bromide (TB) and the compound 1 ethyl 3 methylimidazolium bromide (EM), were used as ionic liquids in the present study. All the mentioned materials were purchased from Sigma-Aldrich Brazil (São Paulo, Brazil).

2.2. Determination of protease activity

The enzymatic activity of commercial protease preparations was determined according to the methodology of Charney and Tomarelli [16], using azocasein as a substrate. The reaction mixture, containing 0.5 mL of the enzyme solution and 0.5 mL of 5 mg mL^{-1} azocasein, was incubated for 40 min. Subsequently, the reaction was stopped by adding 0.5 mL of 10% TCA, and the test tubes were centrifuged at 17000 ×*g* and 25 °C for 10 min. Aliquots of 1 mL of the supernatant were neutralized by the addition of 1 mL of 5 M KOH. A unit of proteolytic activity (U) was defined as the amount of enzyme that produced a difference of 0.01 in absorbance at 428 nm per minute of reaction between the blank reaction and the sample under the assay conditions, expressed as U mL^{-1}. The blank reaction was defined as the same reaction mixture, but with the addition of 10% TCA before the enzyme solution.

The pH and temperature conditions of the assays were adjusted according to the optimum values for maximal enzyme activity of each protease, provided by the manufacturer as follows: Alcalase® 2.4 L (pH 8 and 50 °C), Flavourzyme® 500 L (pH 6 and 50 °C) and Neutrase® 0.8 L (pH 7 and 50 °C).

2.3. Effect of concentration of ionic liquids on the enzymatic activity

Aliquots of the enzyme solutions, prepared from the dilution of the commercial proteases in distilled water, were added in solutions of the ionic liquids at different concentrations as follows: 0 to 500 mM for Neutrase® 0.8 L and Flavourzyme® 500 L, 0 to 3000 mM for Alcalase® 2.4 L. The control assay was prepared using the enzyme in distilled water. The final dilution for each commercial protease was 1:20000 for Neutrase® 0.8 L and Flavourzyme® 500 L with the three ionic liquids; 1:100000 for Alcalase® 2.4 L with CC and EM; and 1:200000 for Alcalase® 2.4 L with TB. The samples were gently homogenized and maintained for 30 min at room temperature. The protease activity was determined for each sample according to the previously described method.

2.4. Determination of activation energy

The activation energies (E_a) of the best enzyme-IL combinations were determined by incubating the previously described reaction mixture for 40 min at different temperatures, which varied by 5 °C increments, ranging from 35 to 60 °C as a function of the assessed protease (35–55 °C for Neutrase® 0.8 L and Flavourzyme® 500 L; 40–60 °C for Alcalase® 2.4 L). It was considered that the variation of the enzymatic activity followed the Arrhenius model, and the E_a was determined by the coefficient (slope) of the linear regression generated by the graph 1000/T vs. ln (enzymatic activity), where $E_a = -\text{slope} \times R$, R (gas constant) = 8.314 J K^{-1} mol^{-1} and T is the absolute temperature in Kelvin (K) [17].

2.5. Determination of kinetic parameters K_m and V_{max}

For the determination of the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) kinetic parameters of the most suitable enzyme-IL combinations, azocasein was used with concentrations ranging from 1 to 20 mg mL^{-1} and the assay was followed as described previously. The parameters K_m and V_{max} were obtained through the Lineweaver-Burk linearization methodology.

2.6. Determination of kinetic parameters for thermal inactivation

The assays were performed for the most suitable enzyme-IL combinations. Enzymes were incubated under the optimal pH and temperature conditions for each preparation and aliquots of the samples were collected every 30 min for 4 h to determine the residual protease activity. The residual activity values were used to estimate the kinetic parameters for thermal inactivation.

The value of the inactivation constant (k_d) for the proteases, expressed as exponential decay, was determined by plotting $\ln(A/A_0)$ as a function of time using the experimental data, as follows:

$$A = A_0 \times e^{-kdt}$$

where *t* is the time, A_0 is the initial activity of the enzyme and A is the residual enzyme activity for a given time *t*.

The protease half-life ($t_{1/2}$), defined as the time required for enzymes to reduce their initial activity by 50%, was estimated as follows:

$$t_{1/2} = \ln(0.5)/k_d$$

The decimal reduction time (D value), defined as the time required for a 90% reduction of the initial enzyme activity at a specific temperature, was calculated as follows:

$$D = 2.303/k_d.$$

3. Calculations and statistics

The results were statistically analyzed according to the Tukey test, using the software Minitab® 18 from Minitab Inc. (USA). The values were expressed as the arithmetic mean and were considered significantly different when the *p*-value ≤0.05. Additionally, the Pearson correlation coefficient (which ranges from −1 to 1) was used to evaluate the intensity of the linear dependence between two responses. A perfect positive relation between the responses is denoted by the value of 1. On the other side, a value of −1 indicates a perfect negative correlation, while the value of 0 demonstrates no linear correlation. The correlations between analyzed parameters were considered significant when the *p*-value ≤0.10.

4. Results and discussion

4.1. Enzymatic activity

The effect of the addition of IL in different concentrations on the catalytic activity of the commercial preparations of proteases is shown in Figs. 1, 2 and 3. In general, it is verified that the increase of the IL concentration caused an initial increase in relative activity of the proteases and a subsequent decrease.

For the commercial preparation Neutrase® 0.8 L (Fig. 1), it was observed that the ionic liquid CC at 0.5 mM promoted an increase of 20% in protease activity when compared to the control ($p \leq 0.05$). The enzyme activities showed a positive correlation with IL concentrations between 0 and 0.5 mM (Pearson coefficient = 0.91 and *p*-value = 0.09) and negative correlation for IL concentrations between 0.5 and