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Effect of zinc ions and of EDTA on α-mannosidase activity

Research article

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Abstract
The modulation of α-mannosidase enzyme activity has been investigated. Two studies have been conducted: (a) the effect of zinc ions over enzyme activity has been evaluated; if stored for 20h in acetic acid/acetate buffer, pH 5, supplemented with 1mM $ZnSO_4$, the enzyme displayed higher catalytic capacity than the enzyme stored in the same buffer without zinc ions; (b) the effect of EDTA on enzyme activity has been investigated, in presence of 10 mM EDTA in acetic acid/acetate buffer, pH=5; EDTA inhibited the enzyme, even without pre-incubation.

Keywords: α-mannosidase, enzyme activity, EDTA, zinc ions

1. INTRODUCTION

Alpha-mannosidase (EC 3.2.1.24) is a hydrolytic enzyme with lysosomal localization involved in the degradation of glycoproteins [1]-[3].

The modulation of α-mannosidase activity has received attention, a number of inhibitors being reported, with swainsonine the best known among them [4]. The enzyme is also inhibited by

ethylenediaminetetraacetic acid (EDTA) [10]-[7] and metal ions, like $Cu^{2+,}$ $Fe^{2+,}$ Cd^{2+}, $Co^{2+,}$ Hg^{2+} and Ag^{+} [10].

α-Mannosidase is a metalloenzyme, having one zinc ion in the active site [10]-[7]. It is known that at pH=8, enzyme preparations are stable [6]. At a more acidic pH, upon storage, especially in diluted solutions, the activity of enzyme preparations is known to decrease. Addition of Zn^{2+} stabilized the enzyme at pH=5 and slowly restored the activity of decayed preparations, with a rate that was dependent on Zn^{2+} concentration [10].

In this paper, we report the experimental results obtained when the catalytic activity of α-mannosidase was evaluated in the presence and in the absence of: (a) zinc ions; (b) ethylenediaminetetraacetic acid (EDTA).

2. MATERIALS AND METHODS

2.1. Materials

The reagents were of analytical purity. Alpha-mannosidase and p-nitrophenyl-alpha-D-mannopyranoside were Sigma products.
Solutions:

- *α-mannosidase (Canavalia ensiformis, Jack bean)* was purchased in buffer solution, 5.1 mg protein/mL, 19 units/mg protein. For the enzymatic tests, 10 μL of commercial solution has been diluted with 960 μL acetic acid/ sodium acetate buffer solution, 200 mM (pH=4.5).
- *p-nitrophenyl-α-D-mannopyranoside*, 5 mM solution in acetic acid/ sodium acetate buffer solution, 200 mM (pH=4.5), was used as enzymatic substrate.
- *Na_2CO_3 0.4M* was used for stopping the reaction.
- *p-nitrophenol (pNP) 1mM* - stock solution was used for the preparation of standards for the calibration curve.

2.2. Apparatus

The spectrophotometric measurements were performed with a Varian Cary 50 UV-Vis spectrophotometer using plastic cuvettes for

spectrophotometer, having 1 cm pathlength. Reagents were weighted using an Adam PW124 Lab Balance.

2.3. Method

2.3.1. General considerations

A method previously reported has been used [8], with a slight modification, which consisted in using a Na_2CO_3 0.4M to stop the enzymatic reaction.

2.3.1. Preparation of the calibration curve

1 mL pNP solutions in acetic acid/sodium acetate buffer, 200 mM (pH=4.5) have been obtained in the concentration range 0-0.2 μmol/mL and 1 mL of Na_2CO_3 0.4 M has been added to each standard. The absorbance has been read at 405 nm (A_{405}) and the equation (1) has been obtained, having R^2=0.9996.

A_{405}=9.8223x conc (μmol/mL) + 0.0092 (1)
where: A_{405}= Absorbance value read at 405 nm; Conc= pNP concentration (μmole/mL).

2.3.2. Determination of enzyme activity

The enzyme assays consisted in preparing:

- test solutions (with $ZnSO_4$ or EDTA, buffer acetic acid/sodium acetate buffer, enzyme and substrate). After specific incubation times, aliquots have been taken away and the reaction has been stopped with Na_2CO_3 0.4 M.
- control samples, prepared in a similar manner to the tests, with the exception of $ZnSO_4$ or EDTA solutions, which have been replaced with buffer.
- witness samples, prepared in a similar manner to the tests, with the exception of the enzyme, that has been added in the end, after the Na_2CO_3 solution.

Effect of zinc ions on enzyme activity. The enzyme used in this assay has been stored in commercial form for 4 years at 4 ℃ prior to dilution (see *Materials* section) and testing. 0.60 mL of 5 mM $ZnSO_4$ solution and 2.16 mL of acetic acid/sodium acetate buffer, 200 mM (pH=4.5) have been incubated with 0.12 mL of diluted α-mannosidase at room temperature (20 ℃) for 20 hours. 0.12 mL of p-nitrophenyl-α-D-mannopyranoside solution have been added. $ZnSO_4$ concentration in assay mixture was 1mM. The reaction was incubated at room temperature (20 ℃) and at specific moments (after 5 min, 15min, 25 min, 35 min, 45 min), 0.5 mL of aliquots have been taken away and 0.5 mL of Na_2CO_3 0.4M solution have been added. In control samples, no $ZnSO_4$ solution has been added and the volume of the buffer was 2.76 mL. In witness samples, 0.02 mL of enzyme solution have been added in each aliquot, after the addition of the Na_2CO_3 solution.

Effect of EDTA on enzyme activity. The enzyme used in this assay has been stored in commercial form for 3 years at 4 ℃ prior to dilution (see *Materials* section) and testing. 0.70 mL of 50 mM EDTA solution and 2.59 mL of acetic acid/sodium acetate buffer, 200 mM (pH=5) have been incubated with 0.14 mL of p-nitrophenyl-α-D-mannopyranoside solution and 0.07 mL of diluted α-mannosidase at room temperature (20 C). EDTA concentration in assay mixture was 10mM. At specific moments (after 30 min, 1h, 1h 30 min, 1h 45 min, 2h, 2h 15 min), 0.5 mL of aliquots have been taken away and 0.5 mL of Na_2CO_3 0.4M solution have been added. In control samples, no EDTA solution has been added and the volume of the buffer was 3.29 mL. In witness samples, 0.01 mL of enzyme solution have been added in each aliquot, after the addition of the Na_2CO_3 solution.

Calculations. Absorbance values have been read at 405 nm (A_{405}) within 5 minutes at the end of the assay, with the buffer as blank solution. Using the calibration curve equation (1), A_{405} values have been transformed into pNP concentration (μmole/mL). The concentration has been converted into quantity (μmole) with equation (2). The quantity of pNP liberated for witnesses has been extracted from the quantity of pNP

liberated for control and test samples and the results have been plotted versus reaction times.

pNP quantity (μmole) = pNP concentration/2 (2)

EAAP (enzyme activity augmentation percentage) and EIP (enzyme inhibition percentage) have been calculated using equations (3) and (4):

$$EAAP = (Q_{E, Zn} - Q_E)/Q_E * 100 \quad (3)$$

$$EIP = (Q_E - Q_{E, EDTA}/Q_E * 100 \quad (4)$$

where: Q_E = Quantity of pNP liberated by enzyme (μmole); $Q_{E, Zn}$ = Quantity of pNP liberated by enzyme, in presence of zinc ions (μmole); $Q_{E, EDTA}$= Quantity of pNP liberated by enzyme, in presence of EDTA (μmole).

3. RESULTS AND DISCUSSION

3.2. Effect of zinc ions on enzyme activity

For this assay, the enzyme has been stored for 20 h in acetic acid/sodium acetate buffer, 200 mM, pH 4.5, in the presence and in the absence of $ZnSO_4$ and was thereafter submitted to assay. In Figure 1, the quantities of reaction product liberated by enzyme are plotted versus time. The pNP quantity varies linearly with time, in the measured interval (0-45 minutes), both for the enzyme alone and for enzyme + Zn^{2+}. The reaction product quantity liberated when zinc ions are added is more elevated, when compared to the quantity of reaction product liberated when Zn^{2+} is not added to storage buffer and, subsequently, to the reaction mixture. Therefore, zinc ions protect the enzyme during storage. EAAP calculated at 45 minutes of reaction was 41.6%.

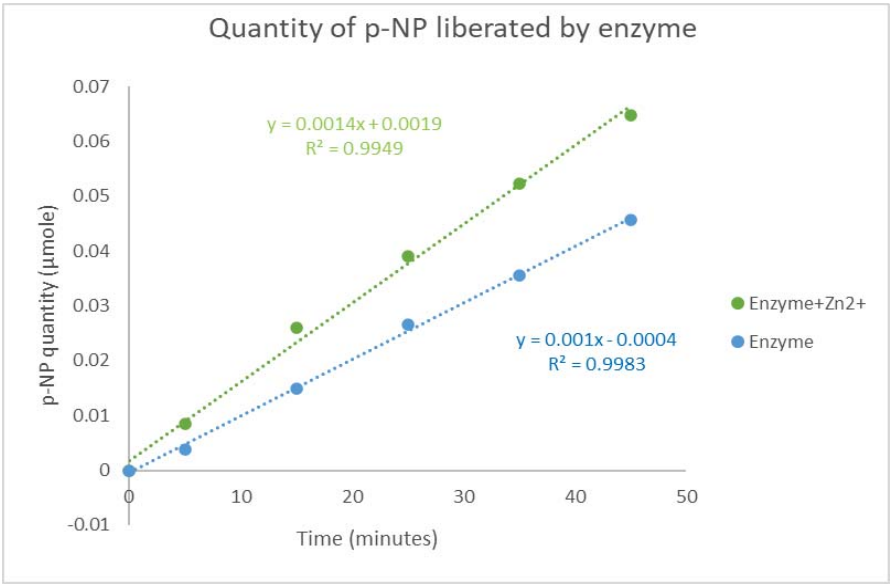

Figure 1. Quantity (μmole) of enzymatically liberated pNP, after storage in presence (green) and absence (blue) of 1 mM Zn^{2+} ions.

Reversible inactivation of the enzyme has been reported, α-mannosidase activity falling slowly at pH 5 upon storage at 0 ℃ and more rapidly upon incubation at 37 ℃, especially in diluted preparations. Zn^{2+} ions (0.1-1 mM) stabilized the enzyme and restored activity in decayed preparations, in a manner dependent to zinc ions concentrations. Zinc was the only ion found to possess this property [10]. This can be explained by the fact that α-mannosidase is a zinc metalloenzyme; it has an active site composed of two unidentical subunits linked in a non-covalent manner [10]. Another substance known to possess a protective effect upon α-mannosidase storage is albumin [10].

3.1. Effect of EDTA on enzyme activity

In Figure 2, the quantities of reaction product liberated by enzyme, in the presence and absence of EDTA, are plotted versus time. The pNP quantity varies linearly with time, in the measured interval (0-135 minutes), both for the enzyme alone and for enzyme + EDTA. The reaction product quantity liberated when EDTA is added is smaller,

when compared to the quantity of reaction product liberated when EDTA is not added to the reaction mixture, therefore EDTA inhibits the enzyme. EIP calculated at 135 minutes of reaction was 42.1%.

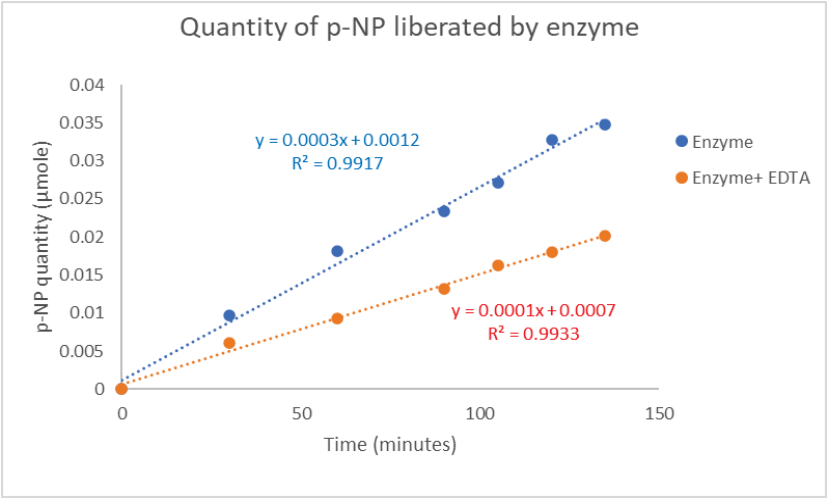

Figure 2. Quantity (µmoles) of enzymatically liberated pNP, in presence (red) and absence (blue) of 10 mM EDTA.

At low concentrations (micromolar range), EDTA has been reported to protect the enzyme against spontaneous inactivation, but at the tested concentrations values, in millimolar range, EDTA exhibits an effect opposed to zinc ions, inhibiting the enzyme [10]-[7]. This can be understood taking into account the known chelation properties of EDTA [9], used as chelation agent in a variety of diseases [10], [11]. EDTA chelation therapy is also applied in the treatment of neurotoxicity [12]. EDTA is known to inhibit an important number of metalloenzymes, by removing or by replacing the metal ions [13].

4. CONCLUSION

Zinc ions and EDTA had opposed effects over α-mannosidase activity.

Zinc ions are known to protect the enzyme over spontaneous reversible inactivation, known to take place at acidic pH. In our experiment, when incubated in the presence of zinc ions, at pH=5, the

enzyme showed higher catalytic activity when compared to the enzyme stored in a buffer without zinc ions. Zinc ions play therefore a protective role; the percentage of augmentation of enzyme activity achieved in presence of 1 mM zinc ions compared to the non-treated enzyme, after 45 minutes of reaction time, was 41.6%.

The effect of EDTA on enzyme activity has also been investigated, in presence of 10 mM EDTA in acetic acid/acetate buffer, 200 mM, pH=5. Contrary to zinc ions, EDTA inhibited the enzyme when added in the reaction mixture, even without prior pre-incubation. The inhibition percentage achieved after 135 minutes of reaction time in presence of 10 mM EDTA was 42.1%.

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