Annals of the University of Craiova
The Chemistry Series
Volume XLIX, No. 1 (2023) 35-43
homepage: chimie.ucv.ro/annals/
10.52846/AUCCHEM.2023.1.04

Influence of heating on the activity of α-mannosidase

Research article

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Received: 20. 05. 2023 / Accepted: 30. 06. 2023 / Published: 01.09.2023

Abstract
The influence of heating on the activity of Jack bean α-mannosidase has been studied. The aged enzyme (3 years in concentrated commercial solution) has been stored at 4 ˚ C for 5 days in acetic acid/sodium acetate buffer (pH=5) with no added ions or albumin. The enzyme has been heated for various amounts of time (5 min, 10 min, 20 min, 30 min) at different temperatures (50 ˚C, 60 ˚C, 70 ˚C, 75 ˚C), then it was cooled and assayed at 20˚C. The residual enzyme activity has been calculated. After 5 minutes of heating at 50 ˚ C, the enzyme retained 65 % of activity but is almost completely inactivated when heated for 5 minutes at 70˚C.

Keywords: α-mannosidase, inactivation, heating

1. INTRODUCTION

Alpha-mannosidase (EC 3.2.1.24) is a hydrolytic enzyme involved in the processing of N-linked oligosaccharides in lysosomes [1]-[3].

Enzyme activity might be influenced by a series of inhibitors, among which the most potent is swainsonine, a natural compound with

anti-cancer properties [4]-[6]. Synthetic analogues also proved to be effective inhibitors of the enzyme [7], [8]. α-Mannosidase is also inhibited by EDTA, a known chelator agent [10]-[7] and by a series of metal ions, with the exception of Zn^{2+} which protects the enzyme from spontaneous inactivation and is able to restore enzyme activity in decayed preparations [10]. Another molecule able to protect the enzyme from spontaneous inactivation is albumin [10].

Enzyme activity is also influenced by pH. With *p*-nitrophenyl-*α*-D-mannopyranoside as substrate, the maximal enzyme activity was found to be between 4 and 5 [11]. Enzyme activity is influenced by storage time, temperature and pH value of the storage buffer [12].

In this paper, we investigated the influence of temperature on Jack bean α-mannosidase activity, upon enzyme storage at 4˚C in concentrated commercial solution for 3 years and in diluted solution in acetic acid/sodium acetate buffer, 200 mM, pH=5, for 5 days, in absence of zinc ions or albumin.

2. MATERIALS AND METHODS

2.1. Materials

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

- *Buffer solutions:* acetic acid/ sodium acetate, 200 mM (pH=5) and citric acid/sodium citrate buffer, 100 mM (pH=5)
- *α-mannosidase (Canavalia ensiformis, Jack bean)* was purchased in buffer solution, 5.1 mg protein/mL, 19 units/mg protein. After storage at 4˚C in concentrated commercial solution for 3 years, 30 μL of commercial solution has been diluted with 2.88 mL acetic acid/ sodium acetate buffer solution and stored at 4˚C for 5 days prior to testing. This solution has a theoretical enzyme activity of 1mU/μl.
- *p-nitrophenyl-α-D-mannopyranoside*, 5 mM solution in acetic acid/ sodium acetate buffer, 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. pH was measured with a Consort C533 pH-meter.

2.3. Methods. Enzyme has been assayed using a method previously reported [8], with a slight modification, consisting in using a Na_2CO_3 0.4M to stop the enzymatic reaction.

2.3.1. Preliminary enzyme assay

The substrate (p-nitrophenyl-α-D-mannopyranoside) was equilibrated in citric acid/sodium citrate buffer (pH=5) to a substrate concentration of 0.1 μmol/mL at room temperature (t=20° C), then the enzyme was added, with a final concentration of 1.05 μg/ml in solution.

Aliquots (0.5 mL) were taken at regular intervals, as it follows:

-every 3 minutes during the first 20 minutes;

-then every 5 minutes up to 30 minutes;

-every 10 minutes up to 90 minutes;

-finally, every 20 minutes up to 130 minutes.

-after 24 hours of enzyme reaction.

The reaction was stopped with 0.5 mL of 0.4M Na_2CO_3. A_{400} was determined within 10 minutes at the end of the assay and its value was then converted to concentration using the calibration curve.

2.3.2. Thermal processing of the enzyme

Both the enzyme and the substrate have been diluted 10 times in citric acid/sodium citrate buffer, pH=5, just before use. The enzyme was heated in citric acid/sodium citrate buffer (pH=5) at different

temperatures (50 ˚ C, 60 ˚ C, 70 ˚ C, 75 ˚ C) for various periods of time (5 min, 10 min, 20 min, 30 min). The enzyme solution was then cooled and enzyme activity was determined at room temperature (t= 20 ˚C).

2.3.3. *Enzyme assay after heating*

The assay consisted in analyses performed for test samples, control and witness. Briefly, diluted enzyme solution (at a final concentration of 2.65 μg/mL in reaction mixture) is mixed with diluted substrate (at a final concentration of 0.25 μmol/ml in reaction mixture) and the resulting solution (with a volume of 0.5 mL) is incubated for 70 minutes at 20 ˚ C. The biochemical reaction is stopped with 0.5 mL of 0.4M Na_2CO_3. In the witness, the enzyme is added after Na_2CO_3. Enzyme activity at room temperature (without heating) is considered 100% (control). The absorbance at 400 nm is determined for test samples, control and witness within 10 minutes at the end of the assay.

2.4. *Calculations.*

A_{400} values are converted to reaction product concentration (p-nitrophenol) using the calibration curve.

Calibration curve. 1 mL pNP solutions in citric acid/sodium citrate buffer, 100 mM (pH=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 400 nm (A_{400}, yellow color) and the equation (1) has been obtained, having R^2=0.9986.

$$A_{400}=11.491x \text{ conc } (\mu mol/mL) + 0.0284 \quad (1)$$

where A_{405}= Absorbance value read at 405 nm and Conc= pNP concentration (μmole/mL)

The quantity (μmole) of product in each sample has been calulated using equation (2); the quantity of pNP liberated for witnesses has been extracted from the quantity of pNP liberated for control and test samples.

$$\text{pNP quantity } (\mu mole) = \text{pNP concentration}/2 \quad (2)$$

The enzyme activities (EA) have been calculated using equation (3):

$$EA(U\times mL^{-1})=\frac{\mu mol\ pNPtest-\mu mol\ pNP\ control}{txV} \quad (3)$$

where: EA = enzyme activity ($U\times mL^{-1}$); t= time (min); V= Volume of the enzymatic solution (mL)

The percentages of residual enzyme activity have been calculated as follows (equation 4):

$$\%\ Enzyme\ activity\ (residual) = (EAtest/EAcontrol)\ x100 \quad (4)$$

where: EAtest=Enzyme activity for heated enzyme (or test samples); EAcontrol=Enzyme activity for unheated enzyme (control).

3. RESULTS AND DISCUSSION

Preliminary enzyme assay. 24 Hours before the thermal processing of the enzyme, an enzymatic assay has been conducted, in order to establish the best assay conditions. In **Figure 1**, the absorbance spectra of the reaction mixture after alkalinisation with Na_2CO_3 are displayed. It can be observed that with reaction time increase, there is also an increase of absorbance around 400 nm and therefore, an increase of reaction product or in the quantity of hydrolysed substrate. If this quantity is plotted versus time, the graph from **Figure 2** is obtained. After 130 minutes of reaction, 51.4% of the substrate was hydrolysed. After 70 minutes of reaction time, A_{400}=0.45. This test indicated that higher quantities of enzyme and substrate might be better suited for the experiment used to assay the enzyme, in order to have higher absorbance values, for increased sensitivity of determinations. Concentrations of 2.5 fold increase have been decided to be appropriate for the enzyme assay used after thermal processing of α-mannosidase.

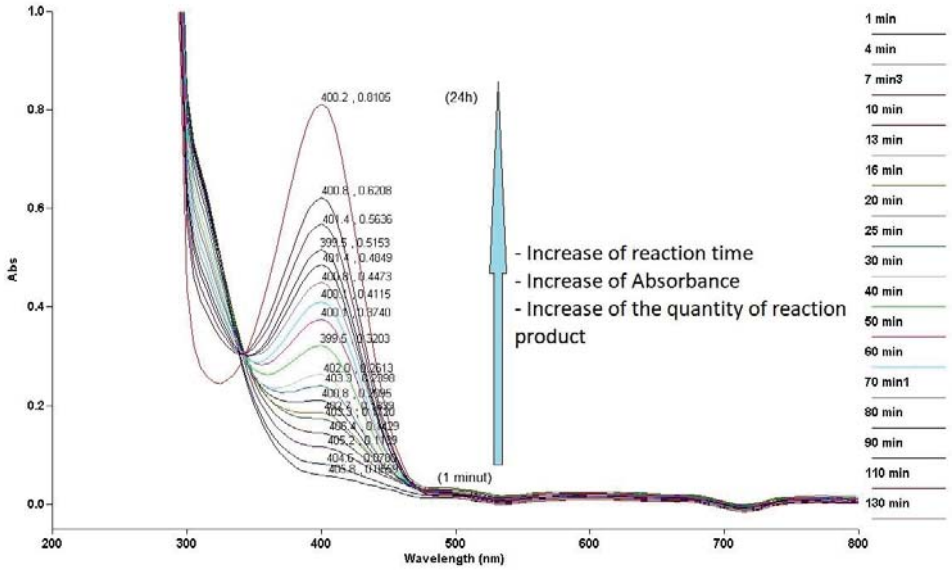

Figure 1. Time evolution of absorption spectra (220-800 nm) of enzymatic hydrolysis reaction mixture of p-nitrophenyl-α-D-mannopyranoside. obtained after alkalinisation with Na_2CO_3

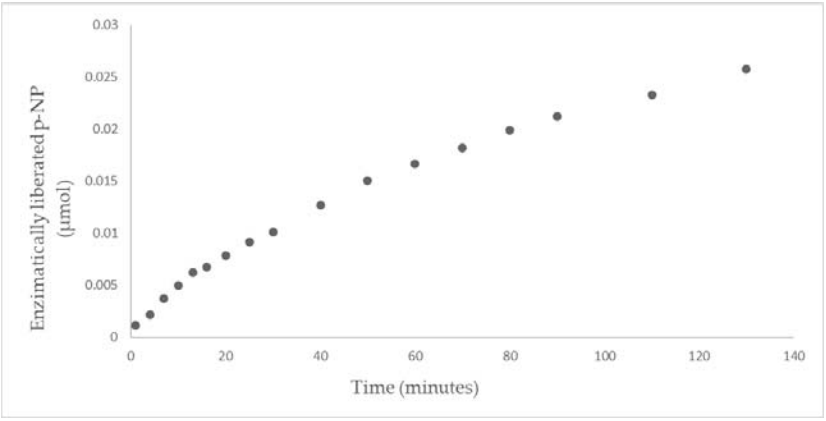

Figure 2. Evolution in time of enzymatic hydrolysis of p-nitrophenyl-α-D-mannopyranoside. The quantity of enzymatically released p-nitrophenol is plotted versus time.

Enzyme assay after heating. Enzyme solutions have been heated, cooled and assayed in the established experimental conditions. The percentages of residual enzyme activity have been plotted as a function of temperature and heating time (**Figure 3**).

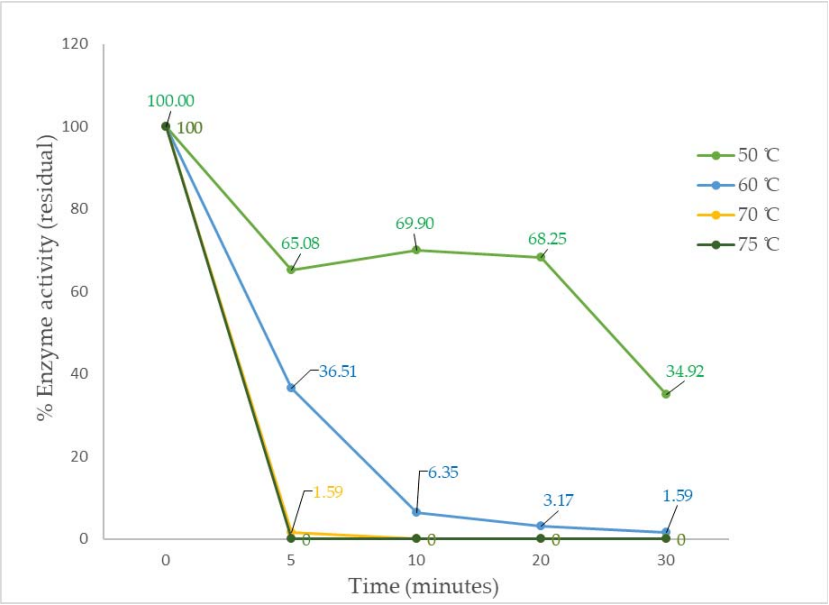

Figure 3. Influence of heating on α-mannosidase activity

Figure 3 shows that:

- after 5 minutes of heating at 50˚C, the enzyme retains about 65% of its activity. EA remains almost constant if heating is maintained for 10 and 20 minutes, then the enzyme activity decreases to 35% after 30 minutes of heating.
- if the enzyme is heated to 60 ℃, the enzyme activity decreases to 37% after the first 5 minutes of heating. It drops sharply to 6% after other 5 minutes of heating, and after 30 minutes it becomes almost zero.
- At 70 ℃, it has about 2% residual activity after 5 minutes of heating and 0% enzyme activity if heated longer.
- At 75 ℃ heated it loses all enzyme activity even after the first 5 min of heating.

In literature, there are various available reports on the activity of α-mannosidase, when heated and subsequently assayed at ambient temperature:

- α-mannosidase retained less than 50% of its activity after 5 minutes of heating at 70 ˚C; the enzyme was stable at 60 ˚C for 5 minutes [11];
- it retained full activity after 5 minutes at 70 ˚C, but was completely inactivated after 5 minutes at 80 ˚C [13];
- it retained 100%, 50% and <10% activity after 2 hours at 40, 55 and 70 ˚C, respectively [12].

In our findings, the enzyme seems to be more susceptible to thermal inactivation, when compared to other reported results. The differences observed between our values and the values from literature might be attributed to enzyme aging and storage, therefore, to spontaneous inactivation. The used enzyme has been stored in commercial buffer at 4 ˚C for 3 years, it was diluted with acetic acid/sodium acetate buffer, 200 mM (pH=5) and has been stored for 5 days at 4˚ C prior to testing, in the absence of zinc ions or albumin. Jack-bean α-mannosidase is known to be stable at pH=5 for 1 hour, but it undergoes spontaneous inactivation and loses up to 55% of activity if stored at 28˚C for 6 hours [12]. Zinc ions and albumin have a protective effect over spontaneous reversible inactivation of the enzyme, but in our assay, the enzyme has been stored and assayed in buffer only [10].

4. CONCLUSION

α-Mannosidase has been heated at temperatures between 50 and 75 ˚C for 5, 10, 20 and 30 minutes and thereafter subjected to enzymatic assay. The enzyme retains 65% of activity after heating for 5 minutes at 50 ℃ and 35 % of activity after 30 minutes at the same temperature. At 70 and 75˚C, the obtained results are almost identical, the enzyme being inactivated after 5 minutes of heating.

When compared to literature data, it might be concluded that enzyme aging and/or storage in absence of protective agents (zinc ions or albumin) makes it more susceptible to thermal inactivation.

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