Determination of α -mannosidase enzymatic activity

Research article

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Abstract

α -mannosidase is a hydrolytic enzyme that cleaves α -glycosidic bonds of mannopyranoside derivatives. In this study, the enzyme activity and the specific activity of α -mannosidase have been determined using the *p*-nitrophenyl- α -D-mannopyranoside hydrolysis assay.

Keywords: enzyme, α -mannosidase, glycosidic link, enzyme activity, *p*-nitrophenyl- α -D-mannopyranoside.

1. INTRODUCTION

Alpha-mannosidase (EC 3.2.1.24), is an exoglycosidase, that cleaves α -linked mannose residues of N-linked oligosaccharides. Its systematic name is α -D-mannoside mannohydrolase. It is a hydrolytic enzyme that catalyzes the degradation of glycoproteins in lysosomes [1, 2, 3].

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If the enzyme has an insufficient activity, the oligosaccharides aren't degraded properly and therefore, they accumulate in the cell, leading to cell death. This rare disease, caused by mutations in the MAN2B1 gene located on chromosome 19 (that encodes α -mannosidase enzyme), is called mannosidosis.

Alpha-mannosidosis was firstly discovered and described in 1967, by a Swedish Physician, Okerman in 1967 [4]. It is a rare disease that affects approximately 1 of 500,000 live births. Among the main characteristic of the disease, the following have been mentioned by D. Malm and Ø. Nilssen. "immune deficiency (manifested by recurrent infections, especially in the first decade of life), skeletal abnormalities (mild-to-moderate dysostosis multiplex, scoliosis and deformation of the sternum), hearing impairment (moderate-to-severe sensorineural hearing loss), gradual impairment of mental functions and speech, and often, periods of psychosis. Associated motor function disturbances include muscular weakness, joint abnormalities and ataxia. The facial trait include large head with prominent forehead, rounded eyebrows, flattened nasal bridge, macroglossia, widely spaced teeth, and prognathism. Slight strabismus is common" [1].

Research has been devoted to the study of this enzyme, among which anticancer therapy is one of the main targets. Compounds able to modulate the enzyme activity have been investigated. Swainsonine is the natural alpha-mannosidase inhibitor [5]. It has been investigated in cervical cancer progression research [6]. Other studies target the effects of synthetic mono- and multivalent inhibitors over the enzyme [7,8,9]. The effect of plant extracts over alpha-mannosidase activity has also been evaluated [10].

Therefore, the enzyme activity determination is important. It is usually realized using the *p*-nitrophenyl-alpha-D-mannopyranoside hydrolysis assay, that will be detailed in this paper.

2. MATERIALS AND METHODS

2.1. Reagents and solutions

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

Solutions:

- *Buffer solution*: acetic acid/ sodium acetate, 200 mM (pH=5).
- α -mannosidase (*Jack bean*) was purchased in buffer solution, 5.1 mg protein/mL, 19 units/mg protein. For the enzymatic tests, the commercial solution has been diluted: 10 μ L of *Sigma* α -mannosidase (*Jack bean*) solution have been diluted with 960 μ L acetic acid/ sodium acetate buffer solution, 200 mM (pH=5).
- *p*-nitrophenyl- α -D-mannopyranoside served as enzymatic substrate. It was used in acetic acid/ sodium acetate buffer solution, 200 mM (pH=5).
- NaOH 2M was used for stopping the reaction;
- *p*-nitrophenol (PNP): A 1mM stock solution of PNP in acetic acid/ sodium acetate buffer solution, 200 mM (pH=5) was used for standards preparation.

2.2. Apparatus

The spectrophotometric measurements were performed with a Varian Cary 50 UV-Vis spectrophotometer using 1 cm pathlength plastic cuvettes. Reagents were weighted using an Adam PW124 Lab Balance. A Consort C533 pH-meter was used for pH measurements.

2.3. Method

2.3.1. *General considerations.* Enzymatic activity of Jack-bean α -mannosidase was determined using a known spectrophotometric method, using *p*-nitrophenyl- α -D-mannopyranoside as substrate [8, 9]. Briefly, the substrate is hydrolyzed into mannose and *p*-nitrophenol (pNP) (Scheme 1). At alkaline pH, pNP turns into yellow *p*-nitrophenolate, having a maximum absorption at 410 nm (A_{410}). In order

to ensure that more than 99% of the product is ionised, the final pH must be superior to 9.2.

Scheme 1. α -mannosidase assay using *p*-nitrophenyl- α -D-mannopyranoside as substrate

2.3.2. Preparation of the calibration curve. The calibration curve was prepared using 6 standards of sodium *p*-nitrophenolate, in 0-0.5 μ mole/mL concentration range, obtained from *p*-nitrophenol (pNP), buffer solution and 2M NaOH. The standards have been prepared according to Table 1. Absorbance values have been read after 5 minutes at 410 nm (A_{410}), using the buffer as blank solution. Based on A_{410} values, the calibration curve has been obtained (see Results and discussion section).

Table 1. p-NP standards preparation

No.	p-nitrophenol stock solution (mL)	Buffer solution (mL)	μ mole pNP/probe	C_{pNP} (μ mole/mL)	NaOH 2M (mL)
Std. 1	0	2	0	0	2
Std. 2	0.2	1.8	0.2	0.1	2
Std. 3	0.4	1.6	0.4	0.2	2
Std. 4	0.6	1.4	0.6	0.3	2
Std. 5	0.8	1.2	0.8	0.4	2
Std. 6	1	1	1	0.5	2

2.3.3. Determination of enzyme activity. In order to determine the enzyme activity (EA), test solutions and a control were prepared. In the control tube, the enzyme was added after the NaOH solution (according to Table 2).

Table 2. Preparation of tests and control solutions

No.	Reagent	Sample	Control
1	Substrate	$V_{\text{substrate}}^a$	0.8 mL
2	Enzyme	V_{enzyme}^b	-
3	Buffer	V_{buffer}^c	0.9 mL
4	Rest at room temperature for various periods of time (t)	t = 2 min, 5 min, 10 min, 20 min or 25 min ^e	t = 10 min.
5	NaOH	V_{NaOH}^d	1 mL
6	Enzyme	-	0.02 mL

^{a,b,c,d,e}: For the values of these volumes and reaction times, see Table 3.

Absorbance values have been read after 5 minutes at 410 nm (A_{410}), using the buffer as blank solution. Using the calibration curve, these values have been transformed into pNP quantity (μmole). Thereafter, the enzyme activity (EA) and the specific enzyme activity (usually stated simply as “specific activity” - SA) have been calculated using equations (1) and (2), respectively.

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

where:

EA = enzyme activity ($\text{U}\times\text{mL}^{-1}$)

t= time (min)

V= Volume of the enzymatic solution (mL)

$$\text{SA (U}\times\text{mg}^{-1}\text{)} = \frac{\text{EA}}{\text{conc.}} \quad (2)$$

where :

SA= specific activity ($\text{U}\times\text{mg}^{-1}$);

conc = concentration of the enzyme solution ($\text{mg}\times\text{mL}^{-1}$).

3. RESULTS AND DISCUSSION

For the six standards, A_{410} values presented in Table 3 were obtained. The calibration curve has been traced with these values (Figure 1).

Table 1. A_{410} values for standards

No.	A_{410}
Std. 1	0.0035
Std. 2	0.6868
Std. 3	1.3580
Std. 4	2.1180
Std. 5	2.7497
Std. 6	3.3803

Figure 2. Calibration curve for sodium *p*-nitrophenolate

The calibration curve equation has been obtained (equation 3):

$$A_{410} = 6.809 \text{conc} + 0.013; R^2 = 0.999 \quad (3)$$

where:

A_{410} = Absorbance value read at 410 nm;

conc = pNP concentration (μmole/mL)

For the test and control solutions, A_{410} values have been transformed into the corresponding pNP amount (μmole). For control, A_{410} had the value of 0.0338 (0.0029 μmole pNP). The enzyme and the specific activities were further calculated with equations (1) and (2), respectively. The results are gathered in Table 4. It can be observed that the maximum values for EA and SA have been obtained when the conditions from entry 4 have been applied (substrate: 0.04 mL; enzyme: 0.02 mL; buffer: 0.44 mL; 2M NaOH: 0.5 mL, reaction time: 20 minutes, at room temperature). In these experimental conditions, EA value equalled 0.5217 U/ mL and AS value was 0.1023 U/mg.

Table 2. Experimental conditions and results

No.	Reagents	Volume (mL)	Time (minutes)	A ₄₁₀	μmole pNP	EA (U/ mL enz. sol.)	SA (U/mg protein)
1	Substrate Enzyme Buffer 2M NaOH	0.08 mL 0.02 mL 0.9 mL 1 mL	2 min.	0.1534	0.0205	0.44	0.0863
2	Substrate Enzyme Buffer 2M NaOH	0.08 mL 0.02 mL 0.9 mL 1 mL	5 min.	0.2993	0.0420	0.3910	0.0767
3	Substrate Enzyme Buffer 2M NaOH	0.08 mL 0.02 mL 0.9 mL 1 mL	10 min.	0.4647	0.0662	0.3165	0.0621
4	Substrate Enzyme Buffer 2M NaOH	0.04 mL 0.02 mL 0.44 mL 0.5 mL	20 min.	1.4551	0.2116	0.5217	0.1023
5	Substrate Enzyme Buffer 2M NaOH	0.04 mL 0.06 mL 0.4 mL 0.5 mL	20 min.	2.5780	0.3650	0.3018	0.0592
6	Substrate Enzyme Buffer 2M NaOH	0.04 mL 0.06 mL 0.44 mL 0.5 mL	25 min.	2.4162	0.3529	0.2333	0.0458

4. CONCLUSION

Alpha-mannosidase is a hydrolytic enzyme involved in the cleavage of α -linked mannose residues of N-linked oligosaccharides. Its activity can be determined using the *p*-nitrophenyl- α -D-mannopyranoside assay. In our determinations, Jack bean α -mannosidase has been used and tested in

different experimental conditions. The enzyme and enzyme specific activities have been calculated and the most suitable experimental conditions for highest EA and SA values have been identified.

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