KINETIC MECHANISM OF β-GLUCOSIDASE FROM *PRUNUS DULCIS*

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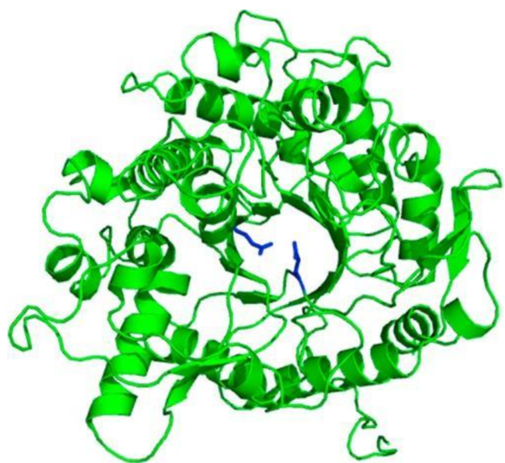
Introduction

β-glucosidase is a key enzyme in the hydrolisis of cellulose to D-glucose. This enzyme is crucial in many important physiological processes as the plant defense system, or the plant development and growth regulation. But β-glucosidases also play a critical role in xenobiotic metabolism in humans. In fact, deficiency of β-glucosidase leads to Gaucher disease. These enzymes have diverse biotechnological applications such as ethanol and biofuel production or wine and tea making. The aim of these experiments was to determinate the optimal conditions for the catalysis, the temperature effect and possible inhibitors.



3. Temperature effect was studied by measuring pNP realese at different temperatures (20, 30, 35, 50, 60 and 70º) reapiting assay described before (2).

4. Inhibition studies:

-Glucose: Assay described in section 2 was carried out with 0.25, 0.5 y and 1 M glucose. Experimental data were fitted into Michaelis-Menten equation and kinetic parameters were determined for each inhibitor concentration. Inhibition constant was calculated using secondary plot.

-Gluconoδ-lactone: Assay described in section 2 was carried out with 60, 120 y and 170 µM gluconoδ-lactone. Experimental data were fitted into Michaelis-Menten equation and kinetic parameters were determined for each inhibitor concentration. Inhibition constant was calculated using secondary plot.

Results

1. Assay standarization

About the standardization of the assay, an optimal enzyme concentration was determined to be 4 nM, an approximate KM of 3.2 mM and a trial time of 10 minutes, so the assays were made according to these results.

2. Kinetic parameters

The enzyme has a KM = 2.42 ± 0.76 mM and a Vmax = 11,053 M m/min, which was determined from the Figure 2, and its Kcat is 2875 min $^{-1}$.

Table 1: Kinetic parameters of the enzyme depending on the reaction temperature.

Temp (ºC)	KM (mM)	Vmax (µM/min)	Kcat (min-1)
20	3,15	4,636198	1324,628
30	2,62	7,713687	2203,910571
35	3,17	12,2234875	3492,425
40	2,65	10,633	3038
50	2,39	17,43476875	4981,3625
60	2,29	18,3771	5250,6
70	2,68	12,12051544	3463,004412

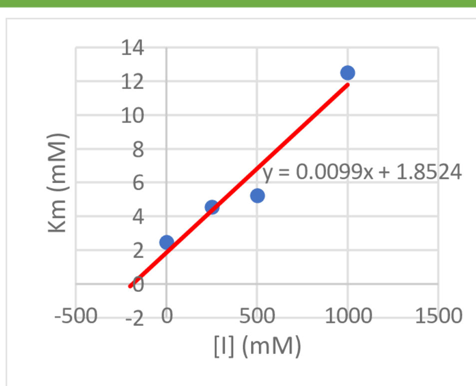


Figure 4: Graph made from the data in table 2. The cut on the x-axis is-Kis of glucose in this reaction, which is **187.1 mM.**

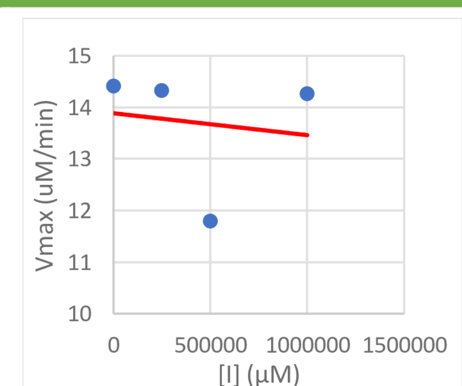


Figure 5: Representation of the Vmax according to the [I]. The cut on the x-axis would be Kii.

Materials and methods

Materials:

β-glucosidase from *Prunus Dulcis* 170 nM (FLUKA), *p*-nitrophenyl (pNP) 25mM (FLUKA), *p*-nitrophenyl-ß-D-glucopyranoside (*p*NPG) 50 mM (FLUKA), glucose 2M (FLUKA), gluconoδ-lactone 20mM (FLUKA), NaOH (PANREAC), citric acid (PANREAC).

Methods:

General procedure: β-glucosidase activity was assayd in incubations containing pNPG, enzyme adn 100mM citrate buffer (pH 5.0) in a final volumen of 1mL at 40º for 10 minutes. The realese of pNP was measured at 410 nm after addition of 1.5 mL NaOH 0.2mM.

*pNPG and β-glucosidase concentration depend on the assay

1. Assay standarization:

-**Calibration curve** for pNP was determined by measuring different concentrations among the linear range at 410 nm.

-**Optimun enzyme** concentration was calculated using saturating pNPG concentration, 7.5 mM, and enzyme concentrations between 0.5-5 nM, following the general procedure. Optimun enzyme concentration must have a A410 value of 0.7.

-**KM determination** : General procedure was carried out with three different pNPG concentrations: 0.75, 1.5 and 6,15 mM and 4nM enzyme. An approximate KM was calculated by fitting experimental data into Eisenthal and Cornish-Bowden ecuation.

-**Incubating time** was determined following the general prodecure using 3.2 mM pNPG and 4nM enzyme and incubating at different times: 1, 5, 10, 15 and 20 minutes with pNPG 3.2 mM and measuring pNP at 410nm.

2. Kinetic parameters:

Different sustrate concentrations: 0.6, 1.5, 2.5, 5, 7.5, 10 y 15 mM were incubated as described previously, using 4nM enzyme The kinetic parameters KM and Vmáx have been calculated by fitting the experimental data to the Michaelis-Menten equation using Hyperbolic Regression program.

3. Temperature effect

In the Table 1 the kinetic parameters calculated for each temperature are shown, while in the Figure 3 the test results are represented at T = 20, 30 and 40 ºC.

4. Inhibition studies

-Glucose: Figures 4 and 5 represent the values of KM and Vmax, respectively, against the inhibitor concentration.

- δ-Gluconolactone: Figures 6 and 7 represent the values of KM and Vmax, respectively, against the inhibitor concentration.

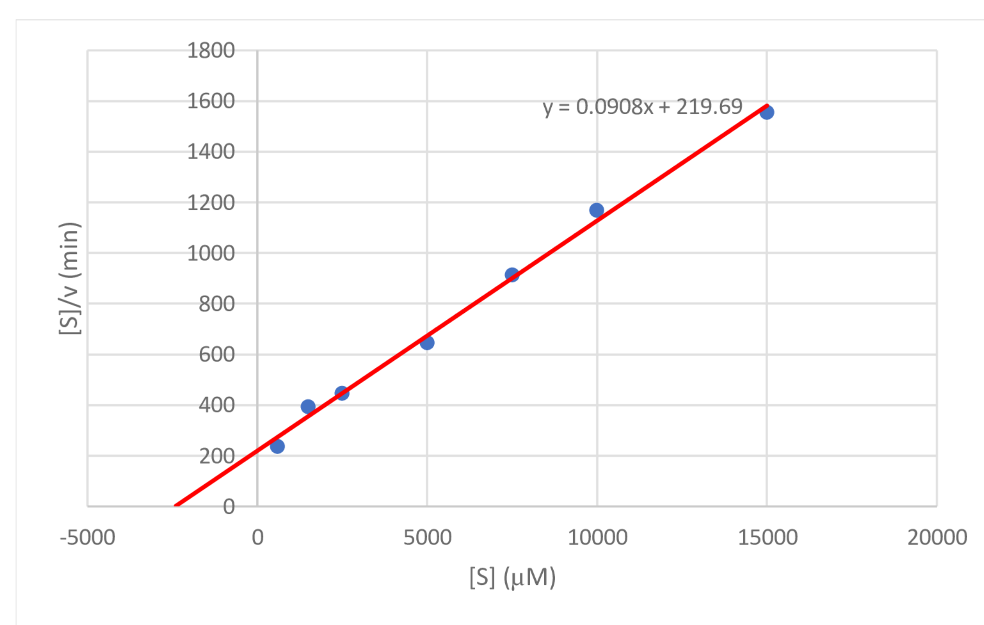


Figure 2: Representation of Hanes-Woolf ([s]/v = [s]/Vmax + KM/Vmax) of the test results. The slope is 1/Vmax and the cut on the x-axis is – KM.

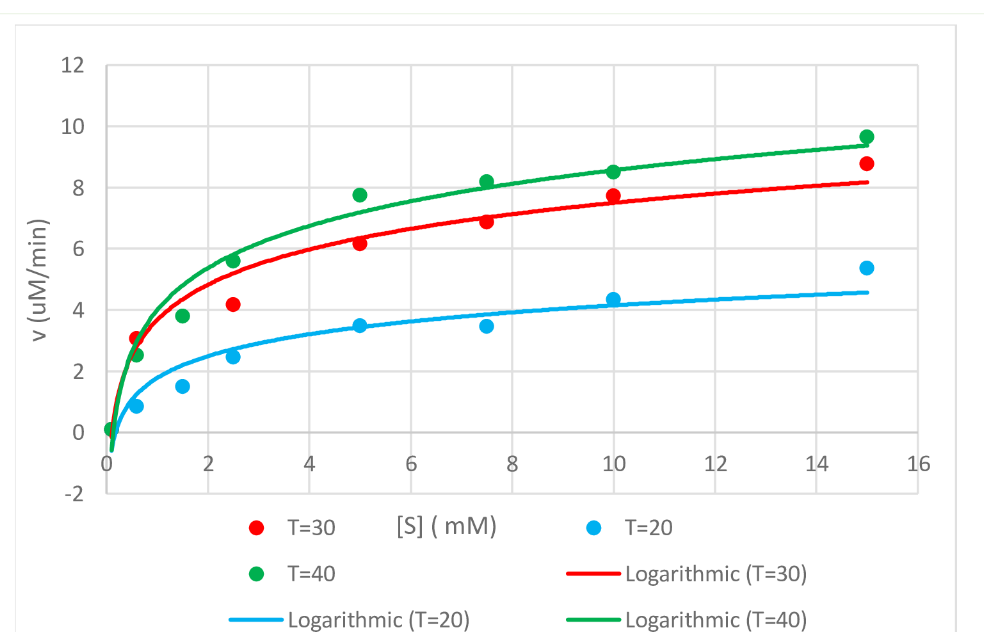


Figure 3: Representation of Michaelis-Menten for T = 20 º C, 30 º C and 40 º C.

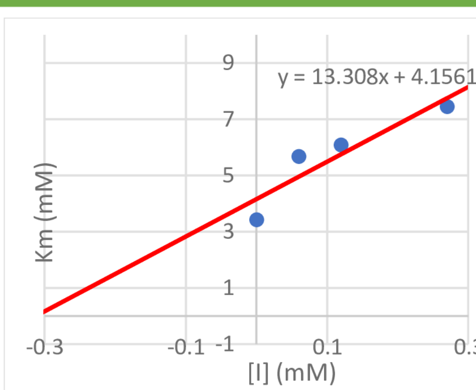


Figure 6: Representation of the results collected in the Table 2. The cut on the x-axis is-Kis of the δ-Gluconolactone for this reaction, which is **0.139 mM.**

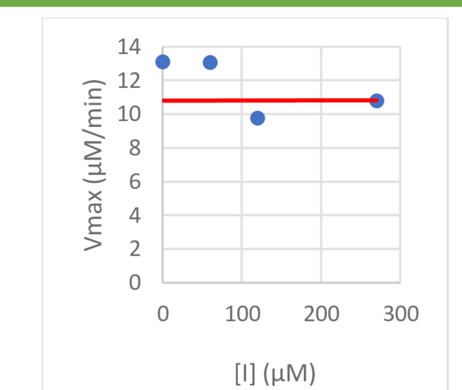


Figure 7: Representation of the Vmax according to the inhibitor concentration. The cut on the x-axis would be kii.

Discussion

The KM determined for the β-glucosidase from *Prunus Dulcis* for pNPG resembles the KM of β-glucosidase studied in other plant species **[1]**, which indicates a structural similarity between them, and probably a high similarity in the sequence of amino acids. The maximum of the Kcat is observed at 55 º C, but it would be necessary to do stability studies to determine if that temperature is the optimal for the reaction. Although both inhibitors seemed to be competitive, δ-gluconolactone is three orders of magnitude bigger than glucose (Figures 4 and 6), due to the fact that δ -gluconolactone is an analogue of the transition state, with a half-chair structure. **[2, 3]**. Inhibition studies carried out with glucose and δ-gluconolactone supported an ordered uni bi mechanism for the reaction using p-nitrophenyl β-D-glucopyranoside as substrate, in which, after the entry of the pNPG, the release of the pNP would be produced first, and then the glucose.

Bibliography

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