KINETIC CHARACTERIZATION OF ALMOND BETA-GLUCOSIDASE

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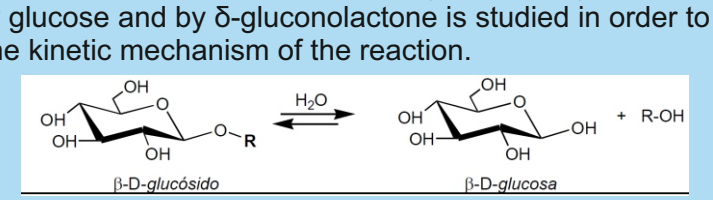
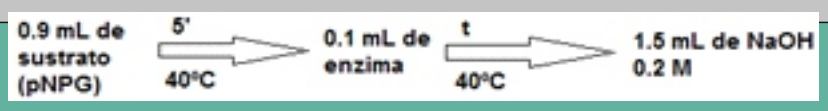
1·Introduction

β-glucosidases (EC 3.2.1.21) are a group of enzymes that acts on bond β(1->4). This group of enzymes catalyzes the hydrolysis of O-β-glycosidic bond located at the non reducing end of oligosaccharides and of aril- or alqui-β-D-glycosides produces β-D-glucose.
In fungi and bacteria are part of multi-enzyme complex called cellulases(1) that are responsible of the degradation of cellulose. In plants(2) are involved in several mechanisms such as lignification, that is a process of cell growth, in defense mechanisms against pathogens by releasing various compounds or in the releasing of aglycones responsible for the aroma and flavor of the fruit. In mammals there are cytosolic and lysosomal β-glucosidases, whose natural substrate is a ceramide attached to a glucose. In the case of humans, the deficiency of this enzyme causes Gaucher's disease (3).
Nowadays, glycoside hydrolases are grouped by their sequence and folding similarities in over 100 different families(4). The main applications of these enzymes are in the food and textil industries, in the manufacture of beverages or in the synthesis of molecules with pharmaceutical interest. (5)
The objective of this experiment is the kinetic characterization of almond β-glucosidase and for this the first step is the standardization of the assay conditions. Then, macroscopic kinetic parameters of the almond β-glucosidase for the substrate p-nitrophenil-β-D-glucoside are determined and the effect of temperature in the kinetic parameters is analyzed. Finally, inhibition of the catalysis by glucose and by δ-gluconolactone is studied in order to propose a model for the kinetic mechanism of the reaction.



2· Methods



2.1. Standardization of assay conditions

- Standardize the assay conditions of almond β-glucosidase using pNPG as substrate by choosing: optimal concentration of enzyme and substrate concentration (approximate Km) from the theoretical Km.
- Check the linearity of appearance of product with the time
- Check the percentage of transformed substrate (%S=[pNP]10' /[S]0') and the ratio substrate/enzyme in the chosen assay conditions (S/E=[pNPG]0'/[E])

2.2. Determination of kinetic parameters

To determine the kinetic parameters, the dependence of the initial velocity of catalysis with substrate concentration has been analyzed. Assays have been performed with assay conditions previously established and with different substrate concentrations. From the values of initial velocities, the macroscopic kinetic parameters have been calculated.

2.3. Temperature effect in the catalysis

To study the effect of temperature in the catalysis of ß-glucosidase: the kinetic parameters of the enzyme for the pNPG incubating at different temperaturas have been determined. To estimate the activation energy, ln kcat against the inverse of temperature has been plotted. It also can be calculated Q10 coefficient: Q10=Vmáx(T)ºC/Vmáx(T-10)ºC

2.4. Reversible inhibition studies

The inhibitory behavior of the glucose, one of the products of the hydrolysis of pNPG, and of the δ-gluconolactone, a transition estate analogue, will be determined by analyzing the kinetic parameters of the enzyme for the pNPG in the presence of different concentrations of inhibitor.

3·Results

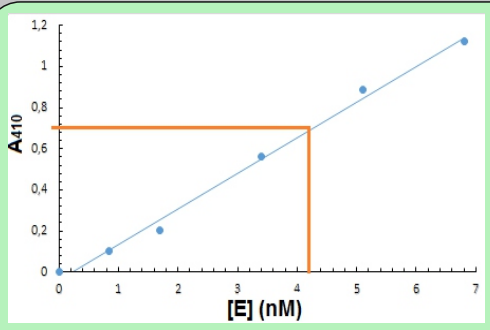


Figure 1: Plot to determine the optimal concentration of enzyme ([E]=4.25nM)

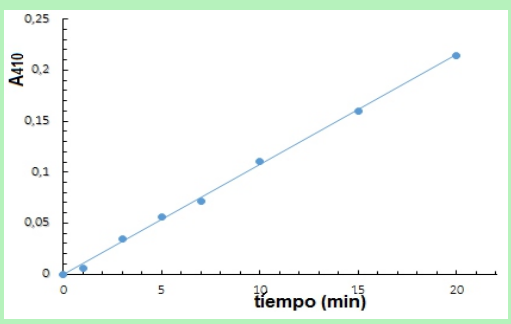


Figure 2: Plot to check the linearity of appearance of product with the time ([E]=4.25nM [S]=0.4mM)

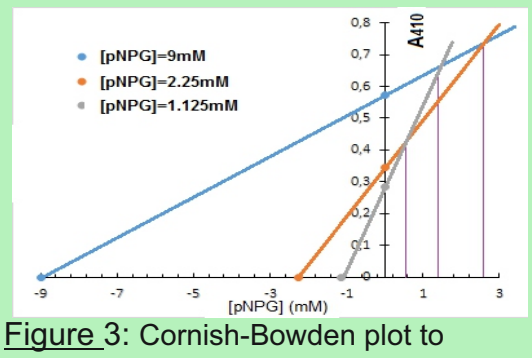


Figure 3: Cornish-Bowden plot to determine the approximate Km (Km*=1.7mM). Percentage of transformed substrate=4% and ratio S/E=94117.65

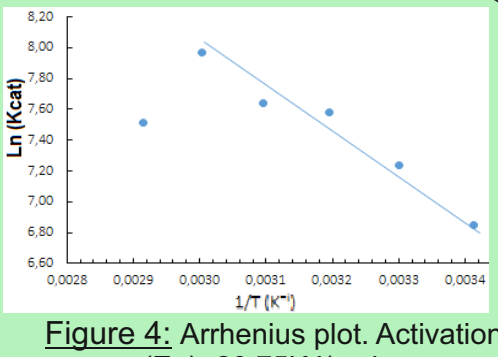


Figure 4: Arrhenius plot. Activation energy (Ea)=20.75KJ/mol
Q10(50ºC-40ºC)=1.06
Q10(40ºC-30ºC)=1.417

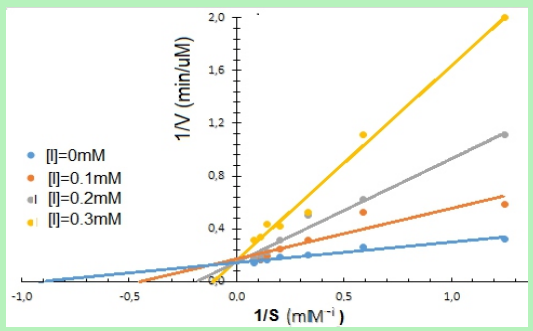


Figure 5:Lineweaver-Burk plot for various δ-gluconolactone concentrations. Different slope and same intercept: Competitive inhibitor

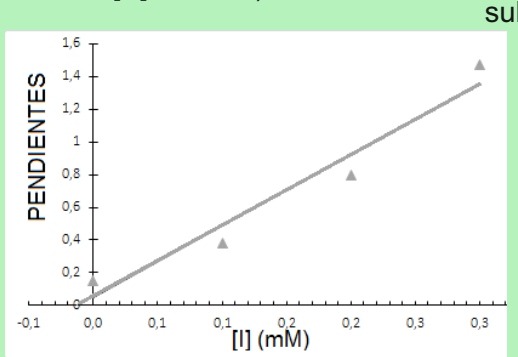


Figure 6: Dixon plot for a competitive inhibitor (slope vs inhibitor concentration). Gluconolactone Ki=0.01mM

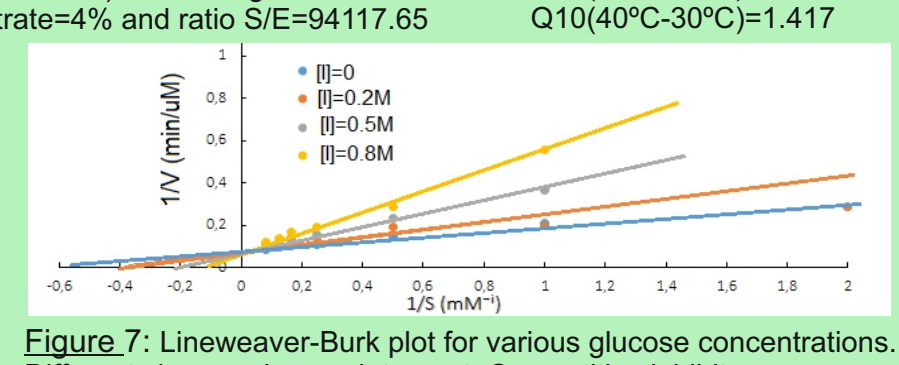


Figure 7: Lineweaver-Burk plot for various glucose concentrations. Different slope and same intercept: Competitive inhibitor

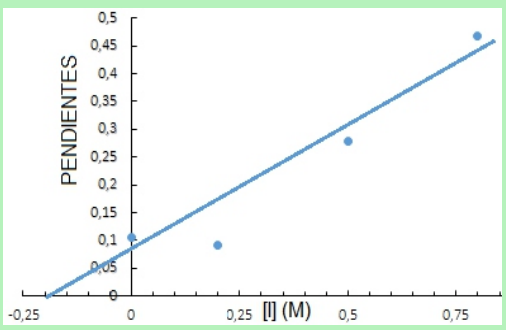


Figure 8: Dixon plor for a competitive inhibitor (slope vs inhibitor concentration). Glucose Ki=250mM

Table 1: Macroscopic kinetic parameters for beta-glucosidase.

Representation	Lineweaver-Burk	Eadie-Hofstee	Hanes-Woolf	Eisenthal	Hyperbolic Regression
Vmáx (uM/min)	10,7	10,8	11,3	11,2	11,1
Km (mM)	1,7	1,8	2,0	1,8	2,0
Kcat (min^-1)	2510,6	2545,9	2654,1	2630,6	2618,8
Kcat/Km (mM^-1min^-1)	1449,5	1427,9	1321,1	1443,0	1337,5

4·Conclusion

Taking into account all the previous assays, it is possible to conclude that the kinetic mechanism of the reaction catalyzed by beta-glucosidase is a non sequential crypto-ping-pong. The results of these assays agree with an orderly mechanism of double displacement and, consequently, with the formation of a covalent intermediate state. To confirm that the enzyme follows this mechanism, different experiments can be made, for example: pH studies, studies to isolate chemically the altered form of the enzyme or studies to demonstrate the existence of partial reactions.
With the studies of the effect of temperature in the catalysis it is possible to conclude that the enzyme can be used until 60ºC in these conditions.
Finally, with the reversible inhibition assays it is possible to conclude that the gluconolactone is better inhibitor than glucose, although both are competitive inhibitors.

5· References

1· Bhat, MK.; Bhat, S. (1997) "Cellulose degrading enzymes and their potential industrial applications" Biotechnology Advances, 15, 583-620
2· Morant, AV. Et al (2008) "β-glucosidases as detonators of plant chemical defense" Phytochemistry, 69, 1795-1813
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4· Henrissat, B. (1991) "A classification of glycosyl hidrolases base on amino-acid-sequence similarities" Biochemical Journal, 280, 309-316
5· Bhatia, Y. et al (2002) "Microbial beta-glucosidases: Cloning, properties, and applications" Critical Reviews in Biotechnology, 22, 375-407