KINETIC CHARACTERIZATION OF β-GLUCOSIDASE FROM SWEET ALMOND (*PRUNUS DULCIS*)

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INTRODUCTION

β-glucosidases (β-D-glucopyranoside glucohydrolases E.C. 3.2.1.21) are enzymes that catalyze the hydrolysis of O-glycosidic bonds to release nonreducing terminal β-D-glucose residues from oligosaccharides and glycosides [1]. They are found in different organism where they have different functions: glycolipid metabolism in animals, defense in plants and biomass conversion in microorganisms [2]. These enzymes have many potential applications in the pharmaceutical and detergent industries, in the production of fuel ethanol or in the release of aromatic compounds in the food industry [3]. The purpose of this project is the kinetic characterization of β-glucosidase from sweet almond to propose a catalytic mechanism of the enzyme. For this, we have standardized the conditions to perform the assays that have allowed us to estimate the kinetic parameters of the enzyme and determine the stability against temperature and the inhibition behavior of glucose and δ-gluconolactone.

MATERIALS AND METHODS

β-glucosidase from sweet almond; *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), used as a substrate; glucose and δ-gluconolactone, used as inhibitors and *p*-nitrophenol (*p*NP) were supplied by FLUKA.

After adding *p*NPG (from 0.5 to 15 mM) and 100 mM citrate buffer (pH 5.0) into an assay tube, the mix was incubated for 5 minutes. Then the enzyme was added and the reaction was stopped after 10 minutes by adding 1.5 mL 0.2 M NaOH and putting the assay tubes into a beaker filled with ice. The activity of β-glucosidase was determined by release of *p*NP, whose absorbance was measured at 410 nm and corrected by subtracting the absorbance of substrate controls. Kinetic and inhibition assays were performed at 40 ºC while the effect of temperature was determine at six different temperatures (20, 30, 40, 50, 60 and 70 ºC).

STANDARIZATION OF ASSAY

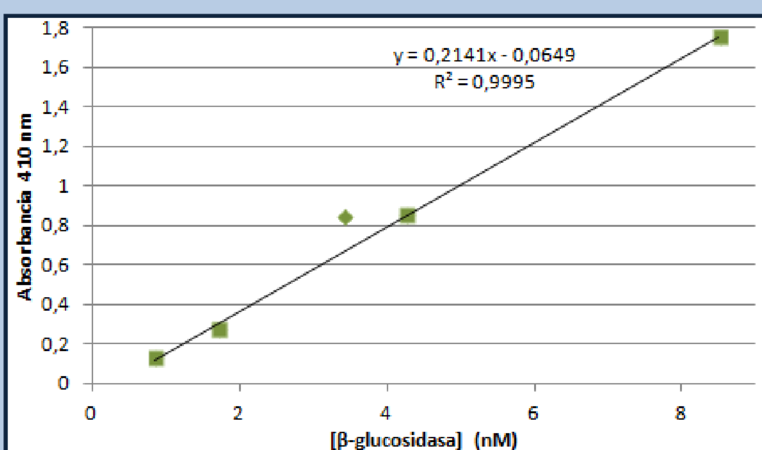

Figure 1. Absorbance at different enzyme concentrations. We estimated that the optimum enzyme concentration, 3.5 nM, corresponds to an absorbance of 0.7.

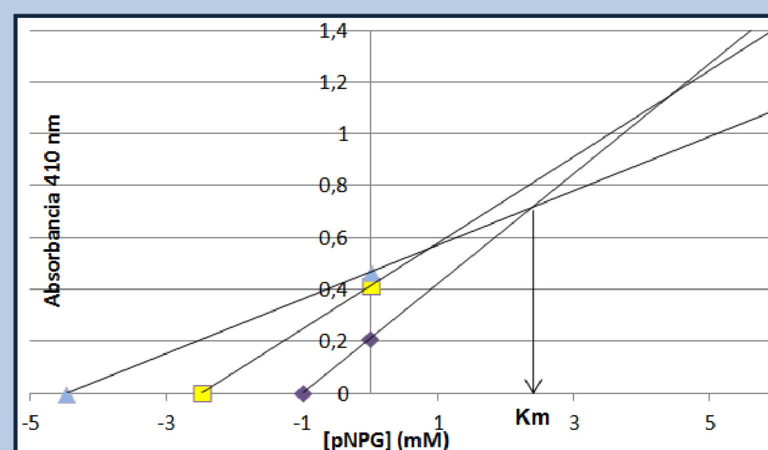

Figure 2. Representation of Eisenthal and Cornish-Bowden. The median of the three cut points allowed us to calculate the approximate Km, 2.5 mM.

EFFECT OF TEMPERATURE

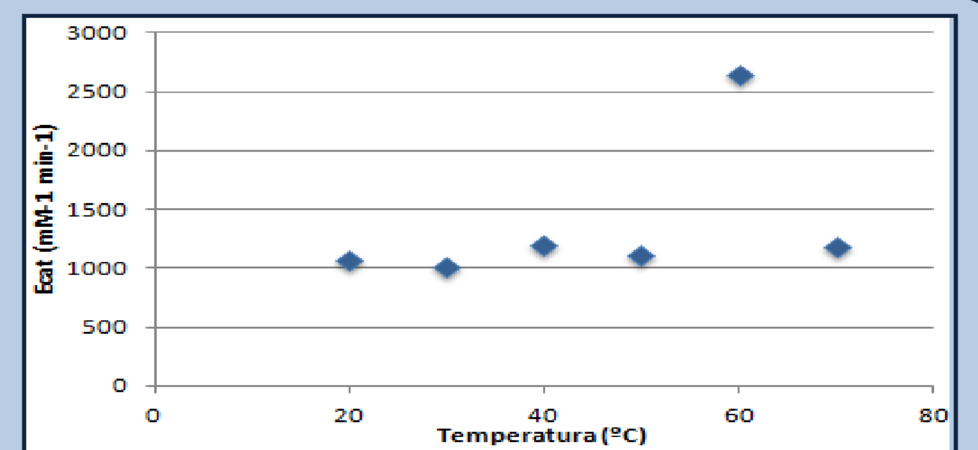

Figure 3. Enzymatic assays at different temperatures. It shows a maximum catalytic efficiency at 60 ºC. The activation energy, 25.21 KJ/mol, was calculated with the Arrhenius plot (figure not shown).

KINETIC PARAMETERS

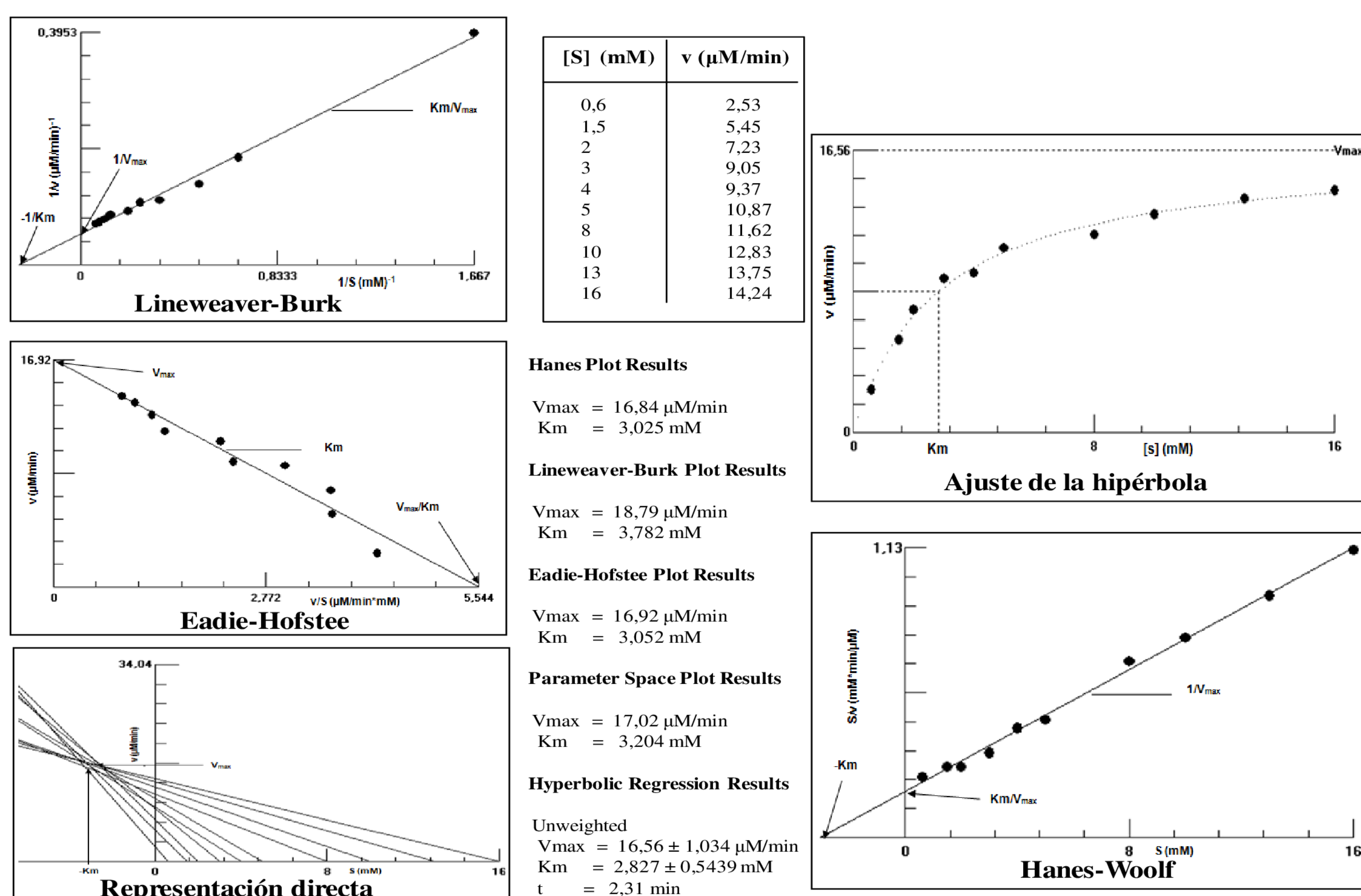

Figure 4. Representations for the determination of the kinetic parameters of β-glucosidase using the program "Hyperbolic Regression". Assays were performed as described in materials and methods using an enzyme concentration of 3.5 nM and different substrate concentrations (shown in the figure). Each plot provided estimated values of kinetic constants, which appear in the middle of the figure.

INHIBITION ASSAYS

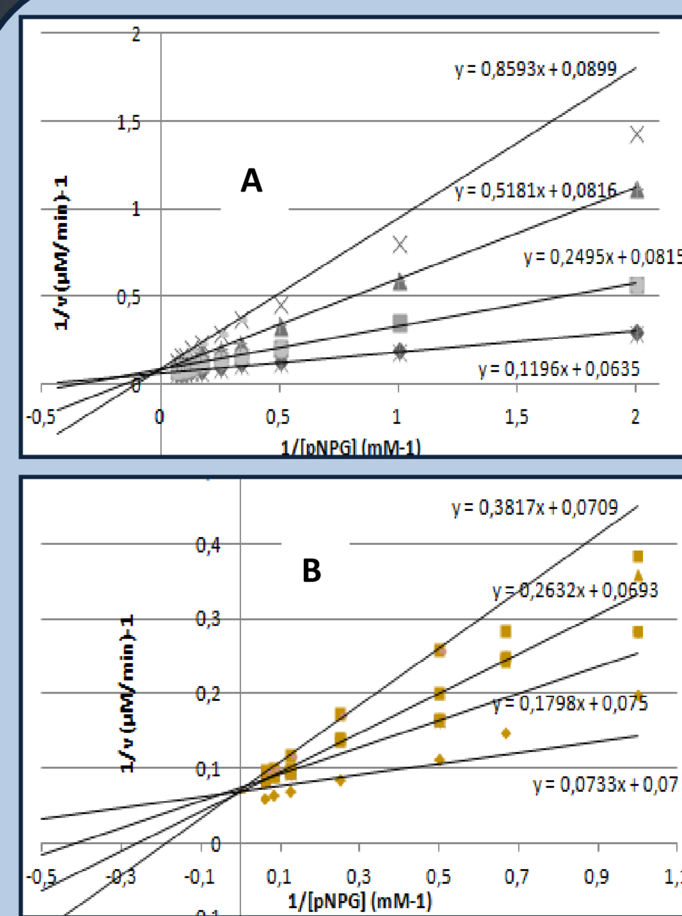

Figure 5. A) Lineweaver-Burk plot for enzyme assays using glucose as inhibitor. Lines cut on the Y-axis, indicating a competitive mechanism. **B) Lineweaver-Burk plot for enzyme assays using δ-gluconolactone as inhibitor.** The interception shows a competitive behavior.

Their Kis values, which were calculated using secondary plots (not shown), are 128.75 mM for glucose and 61.5 µM for δ-gluconolactone.

CONCLUSIONS

- The kinetic parameters determined by Michaelis-Menten equation are as follows: Vmax = 16.56 µM/min; Km = 2.83 mM; Kcat = 4731.43 min^{-1} y Ecat = 1673.66 $mM^{-1}min^{-1}$.
- Although the enzyme shows its maximum activity at 60 ºC, assays have not been performed at this temperature because enzyme stability has not been tested.
- Both glucose and δ-gluconolactone are competitive inhibitors. Inhibition assays with *p*NP indicate it is a mixed inhibitor [4]. The proposed catalytic mechanism to the hydrolysis of *p*NPG by β-glucosidase is an ordered sequential Uni-Bi mechanism in which *p*NPG is hydrolyzed into *p*NP (first product to leave the active site) and glucose (which is released when water enters).

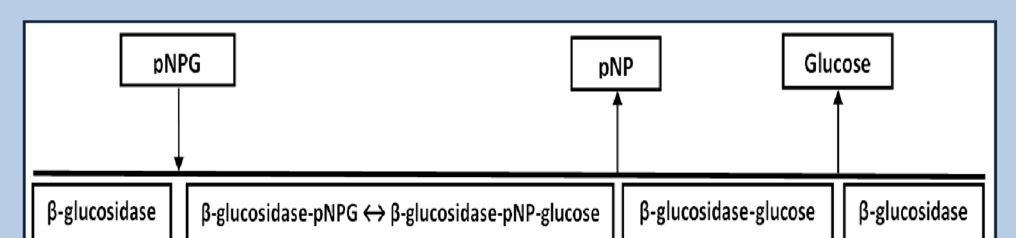

Figure 6. Cleland plot

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