ejpmr, 2020,7(7), 113-117

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

ADSORPTION OF INVERTASE ON ANIONIC RESIN

Prof. Dr. Michele Vitolo*

School of Pharmaceutical Sciences, University of São Paulo, Brazil.

***Corresponding Author: Prof. Dr. Michele Vitolo**

School of Pharmaceutical Sciences, University of São Paulo, Brazil.

Article Received on 30/04/2020 **Article Revised on 20/05/2020** **Article Accepted on 10/06/2020**

ABSTRACT

This study dealt with the adsorption of invertase on Dowex 1X8-50 (in 0.010 M acetate buffer, pH 4.6) and the kinetics between soluble invertase (SI) and the Dowex-invertase complex (DIC). Immobilization yield was over 85%. The activity and stability against pH (3.5 – 7.5) and temperature (25 ^{o}C – 60 ^{o}C), and the kinetic parameters (calculated by the Hanes-Woolf plot) for boh forms of invertase were **(a) SI:** K_M = 17.6 mM; V_{max} = 0.091 U/mg; activation energy (E_a) = 14.8 J/mol; highest stability (HS) at 3.5 ≤ pH ≤ 5.5 and 25 ^{o}C ≤ T ≤ 45 ^{o}C; and highest activity (HA) at pH 4.5 and 40 ^{o}C; **(b) DIC:** K_M = 17.6 mM; V_{max} = 0.064 U/mg; E_a = 32.8 J/mol; HS at 3.5 ≤ pH ≤ 5.5 and 25 ^{o}C ≤ T ≤ 55 ^{o}C; and HA at pH 4.0 and 50 ^{o}C.

KEYWORDS: Invertase, immobilization, adsorption, anion exchange resin.

INTRODUCTION

Invertase (β-D-fructofuranosidase; E.C.3.2.1.26) is an enzyme that catalyzes the hydrolysis of β-fructofuranosides. It has been used in analytical chemistry (biosensors and ELISA kit), in confectionery (fondants, chocolate coatings, artificial honey, chocolate-coated candies having soft centers etc.), in the production of inverted syrup, in the conservation of blackstrap molasses during storage prior to use in ethanol production, and in medicines (as anti-microbial and anti-oxidant agent).[1,2]

Invertase was adsorbed in many carriers, such as bentonite, DEAE-cellulose, and polyethylene.[3,4,5] Another possibility is to adsorb invertase on strong basic anion exchange resins, such as Dowex 1X8-50®, which derive from styrene-divinylbenzene polymer. Industrial unit operations (purification, concentration, and fractionation) have used this resin in the last 50 years. Dowex 1X8-50 is a non-toxic material, inert at the conditions used for bioconversion. Moreover, its market supply is large, with the possibility of material regeneration after use. This resin retains proteins by ionic and electrostatic bonds.

This study analyzed the catalytic performance of the Dowex-1X8-50-invertase complex against the variation of pH, temperature, and sucrose concentration.

MATERIAL AND METHODS

MATERIAL

We purchased invertase and Dowex 1X8-50 from SIGMA®, and all other P.A. grade reagents from traditional suppliers (Fluka, Aldrich, Merck etc.).

METHODS

Invertase Adsorption

We dissolved invertase (100 mg) in 100 mL of 0.010 M acetate buffer (pH 4.6) under agitation in a rotary shaker at 120 min^{-1}. Then, we added 50 mg of Dowex 1X8-50, and left the suspension under agitation of 120 min^{-1} at 25 ^{o}C for 4 h, followed by overnight resting at 4 ^{o}C. We separated the Dowex-invertase complex (DIC) by centrifugation (3,000*g*; 20 min), then rinsed it threefold with cold deionized water, and stored the final sediment at 4 ^{o}C in 10 mL 0.010 M acetate buffer (pH 4.6) until use. We measured protein content in the supernatant by using the Bradford reagent.[6] For adsorption in deionized water, we adjusted the pH to 5.0 or 4.6 by dropping 1 M HCl before the addition of resin and enzyme. The neutral pH corresponded to deionized water. We controlled pH by using a pH-meter (equipped with a combined glass electrode: Ingold-U402-K7).

We calculated the percent of protein retained in Dowex 1X8-50 as follows:

$$AI = (TAP - STP).100 \div TAP \qquad \text{(Eq. 1)}$$

Where AI = adsorption index; TAP = total amount of protein before adsorption; STP = supernatant total protein after adsorption.

To verify any detachment of the invertase, we left the DIC under agitation (120 min^{-1}) for 6 h in 0.010 M acetate buffer (4.6). After centrifugation (3,000x*g*; 20 min), we collected the supernatant and determined soluble protein by the Bradford method. After that, we added 10 g of sucrose to the remaining supernatant, and left the solution under agitation (120 min^{-1}) at 37 ^{o}C for 4

h. We took an aliquot of the solution for determining the presence of reducing sugars by the Somogyi method.[6] Using the accuracy range of the Bradford and Somogyi methods, we did not detect protein and invertase activity in the supernatant.

Measurement of Invertase Activity

A standard test for both forms of enzyme consisted of mixing 90 mL of sucrose solution (100 g/L in 0.010 M acetate buffer, pH 4.6) with 10 mL of invertase solution (2.7 mg powder/mL) or 10 mL of DIC suspension (10 mg powder/mL). We performed hydrolysis for 10 min at 37 ^{o}C under agitation (120 min^{-1}). Then, we took 0.5 mL aliquots every 2 minutes for measuring reducing sugars (RS) as previously described.[6]

We calculated the initial invertase activity (v_o) (always in triplicate) from the slopes of RS = f(t) curves. We defined one soluble or adsorbed invertase unit (U) as the amount of RS formed (mg) per minute under the conditions of the test.

Effect of pH, Temperature, and Sucrose Concentration

By varying individually the conditions of the standard test (pH 3.5 – 6.5; temperature 25 – 60 ^{o}C, and initial sucrose concentration 5.0 – 40.0 mM), we determined the following parameters: a) kinetic constants (K_M and V_{max}), by the Hanes-Woolf plot; b) effect of pH and temperature on invertase activity (soluble and insoluble forms); c) activation energy (E_a)[7]; d) stability of both forms of invertase against pH after 15 min of enzyme-buffer contact, maintaining the mixture at 37 ^{o}C; e) stability of both forms of invertase against temperature, maintaining the mixture at each temperature for 15 min, at pH 4.6.

Non-enzymatic sucrose decomposition was negligible at all temperatures and pH. Moreover, the total volume of samples withdrawn for testing was lower than 5% of the reaction volume.

RESULTS AND DISCUSSION

The adsorption of invertase on Dowex 1X8-50 was influenced by the pH of the medium, with adsorption index (AI) over 85% (Table 1). Invertase molecules in the interval 4.6 ≤ pH ≤ 7.0 charge negatively because the enzyme isoelectric point (pI) is between 3.5 and 4.4. [8] Thereby, invertase molecules can remove chloride anions from the positively charged styrene-divinylbenzene polymer, the main component of Dowex 1X8-50. However, **Cl$^-$/Invertase$^-$ exchanging** at pH 7.0 led to an AI of 21%, therefore low when compared with the AI > 85% observed at pH 5.0 or 4.6 adjusted either with 1 M HCl or 0.010 M acetate buffer (Table 1). This result highlights that the net negative charge of invertase molecules, acquired when pH > pI, is a necessary condition for **Cl$^-$/Invertase$^-$ exchanging**, but not sufficient for optimizing adsorption on the resin surface. At neutral pH, invertase molecules are likely to remain separated in aqueous solution (monomer form prevails), whereas in acidic pH the molecules aggregate forming trimmers and tetramers (Figure 1). With a higher negative charge density than monomers, molecular aggregates can efficiently remove chloride anions from the resin. Moreover, invertase activity varies as follows: monomer < dimmer < trimmer < tetramer.[8]

Although we achieved the highest AI (91%) at pH 5.0 adjusted with 1 M HCl, we chose the 0.010 M acetate buffer (pH 4.6) for invertase adsorption because this buffer was set in the standard test for invertase activity measurement. Furthermore, the acetate buffer establishes the following equilibrium in water: HAc + H_2O ⇆ Ac^- + H_3O^+, being pivotal to maintain pH at 4.6 during sucrose hydrolysis by invertase. The latter process includes the formation of HO-, which must be neutralized, otherwise the medium pH will increase and invertase catalytic activity will decrease. The hydrolysis mechanism involves the following steps: [9]

EH + G-O-FOH → EFOH + G-OH
EFOH + HOH → EH + FOH + **HO$^-$**
H_3O^+ + **HO$^-$** → 2 H_2O

Where EH = invertase; G-O-FOH = sucrose; EFOH = invertase-fructose complex; G-OH = glucose; HOH = water; FOH = fructose.

Table 1: Adsorption index (AI) of invertase on Dowex 1X8-50 at different pH.

pH	AI (%)
7.0 (deionized water)	21
5.0 (adjusted with 1 M HCl)	91
4.6 (adjusted with 1 M HCl)	86
4.6 (0.010 M acetate buffer)	88

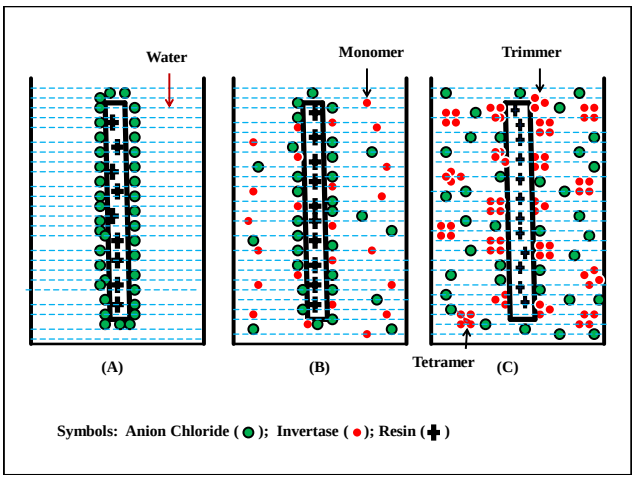

Figure 1: Sketch depicting the interaction between invertase and resin. (A) Resin in deionized water (pH 7.0); (B) Resin and invertase in deionized water (pH 7.0); (C) Resin and invertase at pH 4.6 or 5.0 (adjusted with 1 M HCl or 0.010 M acetate buffer).

We measured soluble invertase (SI) and DIC activities by the variation of reducing sugars (RS) formed against reaction time (Figure 2).

Minimum square linear regression equations for SI and DIC are, respectively:
RS = 0.0413 + 0.021.t (r = 0.9998) (Eq. 2)
*RS = 0.0429 + 0.0519.t (r = 0.9992) (Eq. 3)

Where RS = reducing sugars for SI (mg/mL); *RS = reducing sugars for DIC (mg/mL); t = reaction time (min).

We calculated SI and DIC activities, expressed as U/mg of enzyme, as follows:
$v = (a.V_r) \div (c.V)$ (Eq. 4)

Where v = enzyme activity (U/mg); a = angular coefficient (mg RS/min.mL); V_r = volume of reaction (mL); V= volume of SI solution or DIC suspension (mL).

Soluble invertase (SI) and Dowex-invertase complex (DIC) activities were 0.078 U/mg and 0.052 U/mg, respectively. It is noteworthy that DIC activity corresponded to 67% of the SI activity, differently from the 88% adsorption index in 0.010 M acetate buffer (pH 4.6) (Table 1). The immobilized enzyme activity being lower than the soluble one is a common result.[10] The following events are likely to have occurred: interactions other than ionic bonding (Van der Walls and/or dipole-dipole interactions, for instance) between resin and invertase, blockage of the active site or another sensitive domain of invertase by a chemical group of the resin, and diffusion barrier on the sucrose, fructose, and glucose transit through the resin-invertase matrix. Diffusion could be due to the resin being covered by a sticky web-like layer formed by the overcrowding of invertase aggregates (mainly trimmers and tetramers). According to the literature, invertase immobilized in DEAE-cellulose (ionic bonding), hen white egg (covalent bonding), and polyacrylamide (entrapment) yielded 100%, but showed activities of 75%, and 31%, respectively.[4]

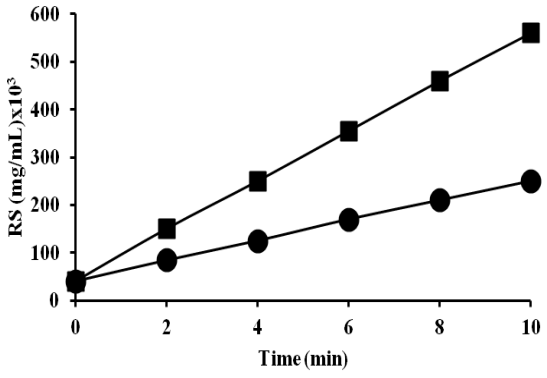

Figure 2: Sucrose hydrolysis by soluble (●) and immobilized (■) invertase.

We calculated the kinetic constants for SI and DIC by the Hanes-Woolf plot (Figure 3).

Minimum square linear regression equations were:
$(S/v)_{soluble} = 193.5 + 11.(S)$ (r = 0.99991) (Eq. 4)
$(S/v)_{immobilized} = 276.5 + 15.7.(S)$ (r = 0.99990) (Eq. 5)

Thereby, the kinetic constants were K_M = 17.6 mM and V_{max} = 0.091 U/mg for SI, and K_M = 17.6 mM and V_{max} = 0.064 U/mg for DIC.

Both forms of invertase had the same K_M, probably due to the predominance of high invertase aggregates (trimmers and tetramers), either in solution or adsorbed on the resin surface.

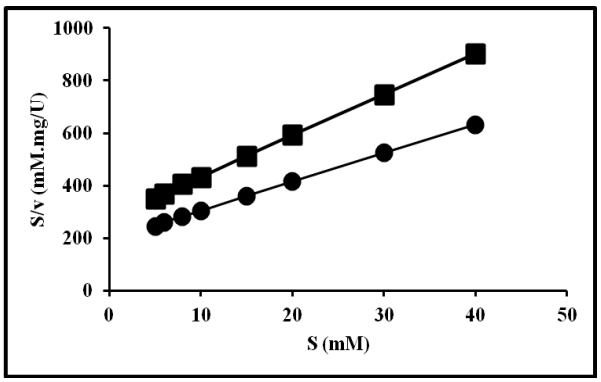

Figure 3: Hanes-Woolf plot for calculating the kinetic constants of soluble (●) and immobilized (■) invertase.

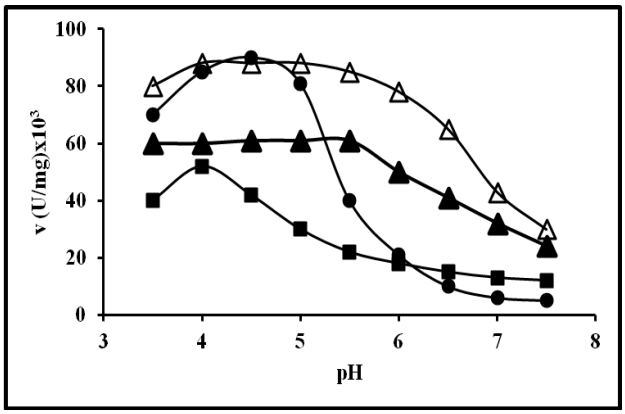

Figure 4: Effect of pH on the activity and stability of soluble (●;Δ) and immobilized invertase (■;▲).

The highest SI (0.092 U/mg) and DIC (0.052 U/mg) activities occurred at pH 4.5 and 4.0, respectively (Figure 4). Peak shiftings by 0.5 pH unit resulted from the unpaired positively charged groups on resin surface, which pull hydrogen ions (H^+) to the bulk solution, where pH is measured. In this regard, it is noteworthy that the resin used (Dowex 1X8-50) is a styrene-divinylbenzene copolymer cross-linked with quaternary ammonium salts (manufacturer information).

Soluble invertase and Dowex-complex invertase were stable in the pH interval 3.5 - 5.5, which comprised the optimum pH for both enzymes (Figure 4). Moreover, at 5.5 ≤ pH ≤ 7.5, SI and DIC activities decreased about 88% and 45%, respectively. This sharp decrease is due to the irreversible change in the molecular structure of both soluble and immobilized invertase. However, invertase molecules anchored on the resin surface had high endurance against pH changing because supramolecular aggregates (trimmers and tetramers) remain compacted, originating an extra protection of the enzyme active site. Conversely, at pH < 5.5, the molecular structure suffers a reversible change, with restoration of activity as soon as pH reaches the optimum value, i.e., 4.0 and 4.5 for DIC and SI, respectively.

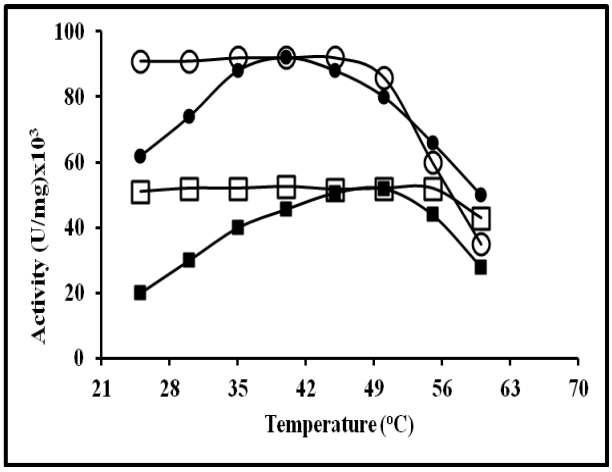

Figure 5: Effect of temperature on the activity and stability of soluble (●;o) and immobilized invertase (■;□).

The highest SI (0.092 U/mg) and DIC (0.052 U/mg) activities occurred at 40 °C and 50 °C, respectively. Furthermore, stability intervals were 25 °C – 45 °C for SI, and 25 °C – 55 °C for DIC (Figure 5). Over the stability temperature limits, 45 °C for SI and 55 °C for DIC, the activities decreased by about 62% and 17%, respectively. The higher thermostability of DIC over SI was due to the best protection (conferred by the compaction of enzyme molecules on the support surface) against the irreversible unfolding of tertiary and quaternary structures of invertase molecules.[5,11]

We calculated the activation energy (E_a) for the reactions catalyzed by SI (14.8 J/mol) and DIC (32.8 J/mol) by applying the Arrhenius plot (Figure 6). Minimum square linear regression equations were:

$\ln v_{si} = 3.27 - 1.78.(1/T)$ (r = 0.996) (Eq. 6)

$\ln v_{dic} = 9.52 - 3.95.(1/T)$ (r = 0.992) (Eq. 7)

Where v_{si} = soluble invertase activity (U/mg); v_{dic} = immobilized invertase activity (U/mg); T = absolute temperature (oK).

The high activation energy required for sucrose hydrolysis by DIC is due both to the compactness of invertase molecules on the resin surface and to the electrostactic field provoked by non-neutralized charged groups in the resin. These events lead, respectively, to diffusion and atractive/repulsive constraints for sucrose to reach the invertase active site, and for fructose and glucose to leave it. This result explains why the highest activities occurred at 50 °C for DIC and 40 °C for SI (Figure 5).

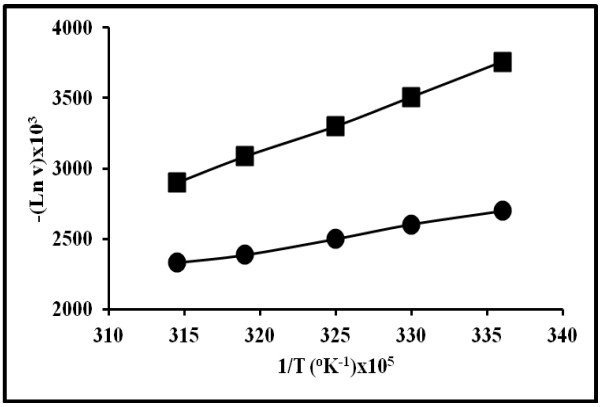

Figure 6: Arrhenius plot for calculating the activation energy of reactions catalyzed by soluble (●) and immobilized invertase (■).

CONCLUSION

From the data presented, we conclude that Dowex 1X8-50 can be used as a support to immobilize invertase in 0.010 M acetate buffer (pH 4.6) and in deionized water at pH 4.6 or 5.0 (adjusted with 1M HCl), yielding more than 85%. The Dowex-invertase complex (DIC) was more stable than soluble invertase (SI) against temperature in the range of 25 °C – 60 °C. Nevertheless, both enzymes were stable at 3.5≤ pH ≤ 5.5. Although SI and DIC had the same K_M (17.6 mm), they differed about 30% for Vmax (0.091 U/mg and 0.064 U/mg for SI and DIC, respectively). This could result from the establishment of unsuitable chemical interactions between invertase and resin; modification of the active site structure; and/or unbalanced substrate-product diffusion across the sticky layer of compacted invertase molecules on the resin surface.

REFERENCES

1. Vitolo M. Invertase activity of intact *Saccharomyces cerevisiae* cells cultured in sugarcane molasses by batch fermentation process. World Journal of Pharmacy and Pharmaceutical Sciences, 2019; 8(3): 126-137.
2. Suzuki S, Kanube I. Bioelectrochemical sensors based on immobilized enzymes, whole cells, and proteins. Applied Biochemistry and Bioengineering, 1981; 3: 145-174.
3. Monsan P, Durand G. Preparation of insolubilized invertase by adsorption on bentonite. FEBS Letters, 1971; 16: 39-42.
4. Abdellah HA, Abou B, Taisser M. Characteristics of invertase immobilized on three different types of supports. Food Chemistry, 1992; 43: 369-375.

5. De Queiroz AAA, Vitolo M, Oliveira RC, Higa ZO. Invertase immobilization onto radiation-induced graft copolymerized polyethylene pellets. Radiation Physics Chemistry, 1996; 47: 873-880.
6. Rocha-Filho JA, Vitolo M. Guide for Practical Classes on Biotechnology of Enzymes and Fermentation. São Paulo; Edgard Blücher, 2017.
7. Owusu RK, Makhzoum A, Knapp JS. Heat inactivation of lipase from psychrotrophic *P. fluorescens* P38: activation parameters and enzyme stability at low or ultra-high temperatures. Food Chemistry, 1992; 44 (4): 261-268.
8. Vitolo M. Effect of freezing on invertase activity of *Saccharomyces cerevisiae* cell. World Journal of Pharmacy Research, 2020; 9(6): 120-131.
9. Vitolo M. Invertase. In: Enzymes as Biotechnological agents. Said S, Pietro RCLR (Eds). Ribeirão Preto, Brazil; Legis Summa, 2014; p.248.
10. Vitolo M. Encapsulation of invertase in barium alginate beads. World Journal of Pharmaceutical Research, 2019; 8(10): 222-233.
11. Schellenberger A, Ulbrich R. Protein stabilization by blocking the native unfolding nucleus. Biomedical Biochemistry Acta, 1989; 48: 63-67.