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Screening of Dowex® Anion-Exchange Resins for Invertase Immobilization

Ester Junko Tomotani and Michele Vitolo*

Department of Biochemical and Pharmaceutical Technology, Pharmaceutical Sciences School, University of São Paulo, Av. Prof. Lineu Prestes, 580, B-16, 05508-900, São Paulo, SP, Brazil, E-mail: michenzi@usp.br

Abstract

Commercial yeast invertase (Bioinvert®) was immobilized by adsorption on anion-exchange resins, collectively named Dowex® (1x8:50-400, 1x4:50-400, and 1x2:100-400). Optimal binding was obtained at pH 5.5 and 32°C. Among different polystyrene beads, the complex Dowex-1x4-200/invertase showed a yield coupling and an immobilization coefficient equal to 100%. The thermodynamic and kinetic parameters for sucrose hydrolysis for both soluble and insoluble enzyme were evaluated. The complex Dowex/invertase was stable without any desorption of enzyme from the support during the reaction, and it had thermodynamic parameters equal to the soluble form. The stability against pH presented by the soluble invertase was between 4.0 and 5.0, whereas for insoluble enzyme it was between 5.0 and 6.0. In both cases, the optimal pH values were found in the range of the stability interval. The K_m and V_{max} for the immobilized invertase were 38.2 m*M* and 0.0489 U/mL, and for the soluble enzyme were 40.3 m*M* and 0.0320 U/mL.

Index Entries: Invertase; immobilization; exchange resins; Dowex®.

Introduction

Invertase (EC 3.2.1.26) catalyzes the hydrolysis of β-fructofuranosides and has been used in analytical chemistry (biosensors), in confectionary, and in the production of inverted syrup *(1)*. Invert sugar syrup, which can be obtained by acid or enzymatic hydrolysis of sucrose, is a valuable commercial product especially in countries where the main sources of sugar are beet or cane. With acid hydrolysis, the final syrup is often contaminated with colored oxidation compounds, which arise from cyclization of hexoses at low pH and high temperatures *(2–4)*. Such a problem does not occur

*Author to whom all correspondence and reprint requests should be addressed.

when using invertase as catalyst for sucrose hydrolysis owing to the mild conditions employed (low temperature and less acidic pH) *(5,6)*.

Over the last 40 yr, an increasing number of researchers studied various methods of protein immobilization and have found widespread application for these methods in many biotechnology areas such as clinical analysis, therapeutic medicine, and the production of biomaterials *(7)*. Among these techniques, adsorption of proteins is very simple, mild, and reversible, permitting reuse of both enzyme and the support *(8)*. Applications of immobilized or adsorbed enzymes as specific catalysts have gained new routes in modern applied chemistry *(9)*.

A good carrier for immobilized enzyme must be a stable material that possesses versatile chemical properties. Among a great variety of these adsorbents, polystyrene ion-exchange resins possess some very promising properties, such as high mechanical stability, good stability against chemical agents, and a matrix structure with various possibilities of altering the mode and degree of functionalization as well as physical parameters *(8,10)*. Several types of bead derivatives of styrene-divinylbenzene (such as Dowex® 1x2, 1x4, and 1x8) are strong basic anion-exchange resins that have been used for at least 50 yr in industrial unit operations such as purification, concentration, and fractionation. They are a nontoxic material and completely inert at the conditions under which the bioconversions are carried out *(11)*. Because the literature has little information about the use of Dowex as a support for immobilizing invertase, this aspect was the aim of the present work.

Materials and Methods

Invertase and Polystyrene Ion-Exchange Resins

The commercial yeast invertase (Bioinvert®) was purchased from Quest International®. Styrene-divinylbenzene bead derivatives having different granulometry (50–400 mesh) and different degrees of crosslinking (2–8%) were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Immobilization of Invertase

Immobilization of invertase on the Dowex resin was performed as follows: The anion exchanger (100 mg dry wt) was previously equilibrated in 22 mL of deionized water (pH adjusted to 5.5 by dropping 1 *M* HCl), and the suspension was left for 24 h under agitation (100 rpm) at 32°C. Then, 3 mL of original solution of Bioinvert was added, and the system was left for 4 h under the same conditions. The complex Dowex/invertase was then centrifuged (2880*g*, 30 min), and the protein content in the supernatant was measured. The separated Dowex/invertase complex was rinsed until no protein was detectable in the supernatant, and the final suspension was stored at 4°C in deionized water (pH 5.5).

The percentage of protein retention in Dowex was calculated as follows:

$$AI = \frac{(TAP - STP)}{TAP} \times 100 \quad (1)$$

in which AI is the adsorption index, TAP is the total amount of protein before immobilization, and STP is the supernatant total protein content after 4 h under stirring (100 rpm) at 32°C.

Standard Assay for Measuring Invertase Activity

A standard assay for both forms of enzyme consisted of mixing 108 mL of sucrose solution (120 g/L in 0.010 *M* acetate buffer, pH 5.5) with 12 mL of aqueous invertase solution (diluted 1:5000 [v/v]) or 12 mL of an aqueous suspension of Dowex/invertase (100 mg of powder/mL). Hydrolysis was carried out for 6 min at 37°C under agitation (100rpm), as previously described by Vitolo et al. *(1)*. One soluble or immobilized invertase unit (U) was defined as the amount of total reducing sugar (TRS) (mg) formed per minute under the conditions of the test.

The immobilization coefficient (IC) was determined using Eq. 2:

$$IC = \frac{Activity_1}{Activity_2} \times 100 \quad (2)$$

in which IC is the immobilization coefficient, $Activity_1$ is the immobilized invertase (U), and $Activity_2$ is the soluble invertase (U).

The standard deviation (SD) and the coefficient of variation (CV) related to this method were equal to 6.20×10^{-3} mg/mL and 4.35%, respectively.

Characterization of Soluble and Immobilized Invertase

The pH, temperature, substrate concentration, and storage time of the standard reaction test related to both forms of invertase were changed one by one at the intervals cited above. In each case, the activity corresponded to the slope of the straight line attained through a plot of TRS vs time.

Effect of Temperature on Activity and Stability

The activity of soluble and immobilized invertase was determined by varying the temperature of the standard test within a range of 30–60°C (30, 35, 40, 45, 50, 55, and 60°C). A blank was prepared under the same conditions for each temperature employed. The activation energy (E_a' kJ/mol) was determined by the Arrhenius method, and the thermodynamic parameters were calculated by conventional equations (Eqs. 3–5) *(12)*. The stability of both forms of invertase (aqueous invertase solution [diluted 1:5000, v/v] or an aqueous suspension of Dowex/invertase (100 mg powder/mL]) was evaluated by maintaining the enzyme solution or suspension for 12 min in acetate buffer (0.010 M, pH 5.5) at each

temperature cited. Then, the residual activity was measured as discussed under Standard Assay for Measuring Invertase Activity.

$$\Delta G = \left(\frac{R \times T}{2.303}\right) \times \log\left(\frac{v \times h}{K \times T}\right) \quad (3)$$

$$\Delta H = E_a - R \times T \quad (4)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (5)$$

in which ΔG is the Gibb's free energy (kJ/mol), ΔH is the enthalpy (kJ/mol), ΔS is the entropy (kJ/[mol·K]), R is the gas constant (8.31 J/[mol·K]), T is the temperature (K), v is the enzyme activity, h is the Planck constant (3.978×10^{-23} J/min) and K is the Boltzman constant (1.38×10^{-23} J/K).

Thermal Inactivation of Soluble and Immobilized Invertase

Aqueous invertase solutions (soluble and immobilized enzyme) were incubated in acetate buffer (0.010 *M*, pH5.5) for 20, 40, 60, 80, and 120 min at 30, 37, 40, 45, 50, and 55°C. After incubation at each specified time, both forms were assayed for residual activity according to the standard procedure (*see* Standard Assay for Measuring Invertase Activity).

Effect of pH on Activity and Stability

The effect of pH on the activity and stability of soluble and immobilized invertase was determined always at 37°C by mixing the enzyme with buffer solutions at fixed pH. The buffers were prepared according to Bacilla et al. *(13)*. For soluble invertase, the pH values employed were 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5, and for immobilized invertase they were 4.5, 5.0, 5.5, 6.0, and 6.5. The stability against pH was determined by measuring the residual activity of both forms of invertase after 12 min of enzyme-buffer contact.

Effect of Sucrose Concentration on Invertase Activity

The kinetic parameters (K_m and V_{max}) were determined through the conventional Lineweaver-Burk method, by varying the sucrose concentration between 10.0 and 292 m*M*.

Evaluation of Shelf Life of Immobilized Invertase

Concerning storage stability, four Dowex/invertase complexes were kept in deionized water (pH adjusted to 5.5) for 28 d at 4°C. After every 7 d the residual activity was measured.

Analytical Techniques

Determination of Protein

Protein was determined according to Segel *(14)*, by the difference between ultraviolet absorbance measured at 215 and 225 nm, using bovine serum albumin (Sigma) as a standard. The SD and the CV were 1.51 µg/mL and 3.48%, respectively.

Table 1
AI, Catalytic Activity, IC and Enzyme Retention After Hydrolysis for Invertase Adsorbed in Several Types of Dowex Anion-Exchange Resins

Dowex	AI (%)	Activity (soluble enzyme) (U/mL)	Activity (immobilized enzyme) (U/mL)	IC (%)	Retention after hydrolysis (%)
1x2-100	100	0.0214	0.0287	100	86.2
1x2-200	100	0.0214	0.0178	83.2	100
1x2-400	100	0.0214	0.0170	79.4	78.2
1x4-50	92.4	0.0214	0.0136	63.7	100
1x4-100	73.2	0.0214	0.0182	84.9	100
1x4-200	100	0.0214	0.0214	100	100
1x4-400	100	0.0214	0.0153	71.6	67.9
1x8-50	99.5	0.0214	5.00×10^{-4}	2.10	100
1x8-100	98.6	0.0214	0.0108	50.6	99.9
1x8-200	97.0	0.0214	0.0122	56.8	100
1x8-400	81.3	0.0214	0.0291	100	89.0

Measurement of TRS

TRS were measured by the method of Somogyi-Nelson *(15)* as described by Arruda and Vitolo *(16)*. The SD and the CV were 3.50×10^{-3} mg/mL and 3.86%, respectively.

Results and Discussion

Immobilization Efficiency

By applying the immobilization procedure described under Immobilization of Invertase, invertase was bound by all polystyrene beads tested, having an AI always higher than 70% (Table 1). Since the p*I* of invertase is equal to 4.0 *(17)*, its molecules in the immobilization medium (pH 5.5) had a net negative charge, which allowed it to interact strongly with the positively charged groups of the resins.

Table 1 shows that some Dowex/invertase complexes, in which the resins used were 1x2-200, 1x4-50, 1x4-100, 1x4-200, 1x8-50, and 1x8-200, retained 100% of protein molecules during sucrose hydrolysis. The absence of enzyme desorption from the support enhances the half-life of the immobilized complex when employed in repeated-batch or continuous processes *(18)*.

Furthermore, the IC for almost all Dowex/invertase complexes varied from 50.6 to 100% (Table 1), probably owing to the different mesh and crosslinking degree of the resin granules. Immobilization coefficient values of that magnitude (50.6–100%) are quite relevant when compared with other methods for invertase immobilization described in the literature *(5)*.

Table 2
Thermodynamic Parameters Calculated at 37°C[a]

Invertase	E_a (kJ/mol)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (kJ/[mol·K])
Soluble	37.3	–14.2	34.5	0.16
Immobilized	37.2	–14.0	34.6	0.16

[a]E_a was calculated through Arrhenius' plot.

By contrast, the Dowex-1x8-50/invertase complex had an IC of 2%, though AI and protein retention were 100%. A reasonable hypothesis for understanding this result might be related to the quite high occurrence of undesirable interactions between the active site and/or another sensitive domain of the enzyme with charged chemical groups of the resin *(11)*. Such a phenomenon, although with more or less intensity, could also have contributed to the IC variation observed for other Dowex/invertase complexes.

Undoubtedly, the best immobilized complex attained was Dowex-1X4-200/invertase, because all its immobilization efficiency parameters (AI, IC, and protein retention) were equal to 100% (Table 1). Therefore, this complex was chosen for characterization assay purposes.

Effect of Temperature

From Fig. 1 it can be seen that the highest activity for both soluble (0.0569 U/mL) and immobilized (0.0559 U/mL) invertase occurred at 55 and 50°C, respectively. As the maximal activities differed <2%, one can assume that the immobilization procedure did not significantly affect the tertiary and quarternary structures of the invertase.

By applying the conventional Arrhenius method to the data related to the activity vs temperature (Fig. 1), it was possible to establish the following equations (Fig. 2):

$$\log v = 4.61 - 1.95 \times 10^3 \left(T^{-1}\right) \quad (6)$$

$$\log v = 4.70 - 1.94 \times 10^3 \left(T^{-1}\right) \quad (7)$$

in which T is the absolute temperature (K).

The activation energy (E_a), calculated through the inclination of $\log v \times T^{-1}$ (Eqs. 6 and 7), and the thermodynamic parameters (ΔH, ΔS, and ΔG), calculated through Eqs. 3–5, for both forms of invertase are presented in Table 2. As can be seen, the values of those parameters were quite similar for both soluble and immobilized invertase. This fits very well with the previous assumption that the immobilization technique has little effect on enzyme structure. It could be speculated that the reaction catalyzed by either soluble or insoluble invertase is constituted by identical thermodynamic systems.

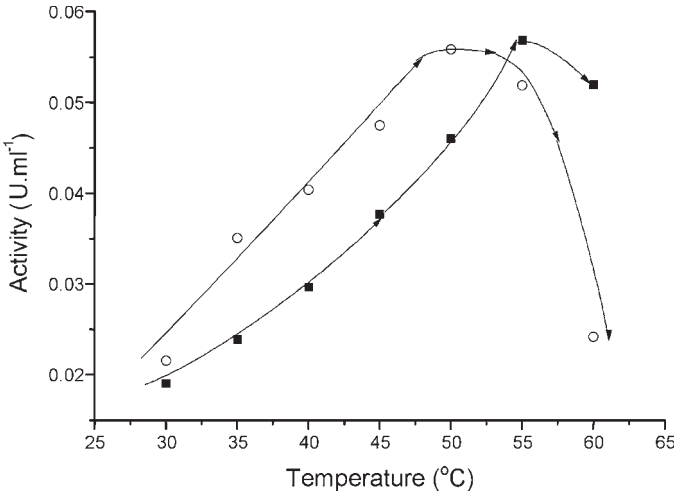

Fig. 1. Effect of temperature on activity of soluble invertase (■) and Dowex-1x4-200/invertase complex (○).

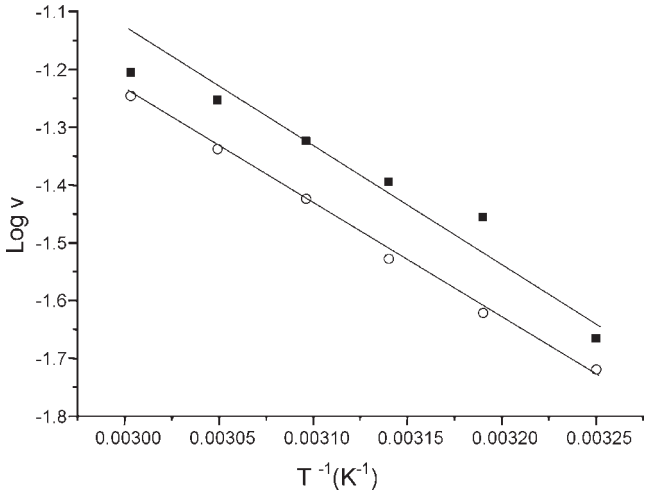

Fig. 2. Arrhenius plot for calculation of E_a for both soluble invertase (■) and Dowex-1x4-200/invertase complex (○).

The same entropy values (0.16 kJ/[mol·K]) should indicate a similar distribution of invertase molecules inside both systems. In other words, when dissolved in aqueous medium, the invertase molecules, which are natural dimers, attract each other, forming aggregates with high molecular weight (mainly hexamers and octamers) and increased hydrolytic activity *(19)*. The hexamers and octamers should be adsorbed unaltered by the pellets of Dowex-1x4-200.

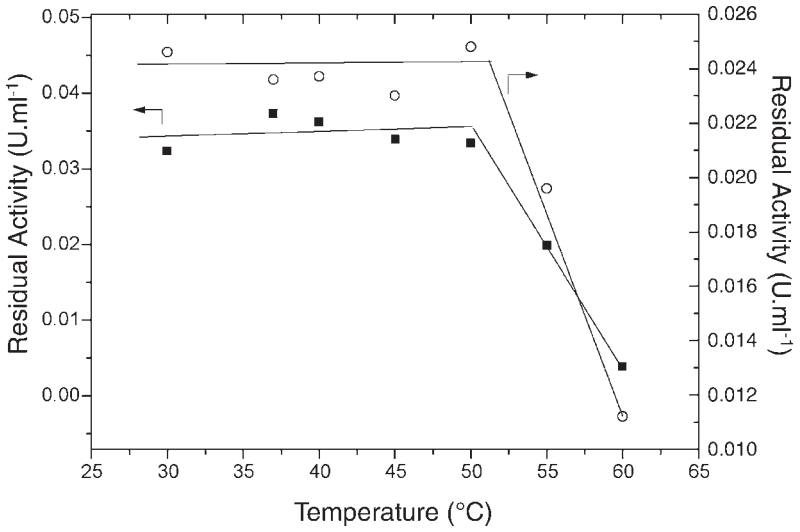

Fig. 3. Effect of temperature on stability of soluble invertase (■) and Dowex-1x4-200/invertase complex (○).

Table 3
Logarithm of Soluble Invertase Activity
Against Time at Several Temperatures

	log v				
Time (min)	(37°C)	(40°C)	(45°C)	(50°C)	(55°C)
20	–1.61	–1.62	–1.59	–1.57	–2.27
40	–1.63	–1.63	–1.62	–1.57	–2.37
60	–1.66	–1.65	–1.58	–1.61	–2.51
80	–1.66	–1.60	–1.60	–1.59	–2.62
120	–1.62	–1.62	–1.59	–1.61	–2.86

As shown in Fig. 3, both invertase forms were stable in the temperature range of 30–50°C. At 55°C the residual activity for soluble and insoluble invertase diminished about 18 and 40%, respectively, whereas at 60°C the reductions were 55 (soluble invertase) and 88% (Dowex-1x4-200/invertase).

In the range of temperatures selected for evaluation, the soluble invertase presented detectable instability only at 55°C (Table 3 and Fig. 4). Hence, the thermodynamic parameters for the heat inactivation of invertase ($\Delta G'$, $\Delta H'$, $\Delta S'$ and E_a') were not determined, because the correlation $\log k' \times T^{-1}$ might necessarily be established, as proposed by Owusu and

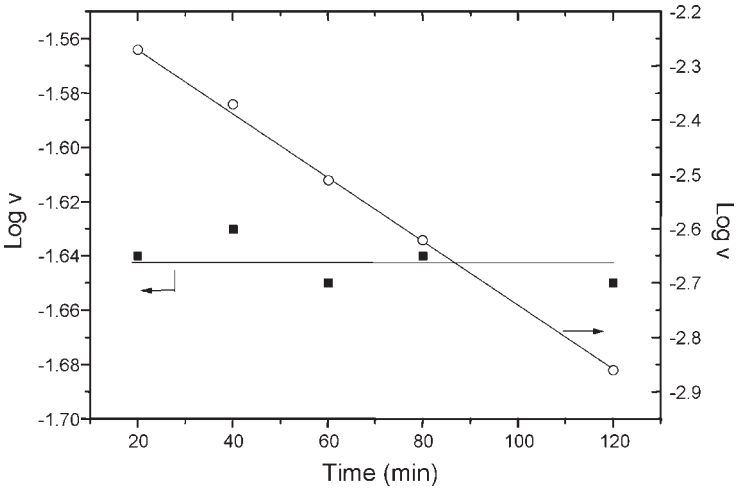

Fig. 4. Logarithm of soluble invertase activity vs time at 37°C (■) and 55°C (○).

Makhzoum *(20)*. However, the deactivation rate constant (k') at 55°C for soluble invertase was equal to 6.00×10^{-3}/min, as calculated through Eq. 8 (Fig. 4):

$$\log v = -6.00 \times 10^{-3} \cdot t - 2.14 \quad (r = 0.9998) \tag{8}$$

The relevance of the latter information lies in the fact that the activity loss (0.6%/min, in this case) can be compensated by adding an appropriate quantity of invertase, when the hydrolysis reaction is carried out at 55°C. Such a condition could apply when the reaction is carried out at a sucrose concentration higher than 150 g/L, at which viscosity should be diminished by increasing the operational temperature.

Since all ($\log v \times t$) curves were linear (Fig. 5), the following equations were established:

$$(30°C) \quad \log v = -6.00 \times 10^{-4} \cdot t - 1.57 \quad (r = 0.996) \tag{9}$$

$$(37°C) \quad \log v = -2.30 \times 10^{-3} \cdot t - 1.70 \quad (r = 0.993) \tag{10}$$

$$(45°C) \quad \log v = -1.09 \times 10^{-2} \cdot t - 2.44 \quad (r = 0.990) \tag{11}$$

$$(55°C) \quad \log v = -2.92 \times 10^{-2} \cdot t - 2.71 \quad (r = 0.997) \tag{12}$$

By plotting $\log k'$ against T^{-1}, a straight line was attained (Fig.6), the equation was as follows:

$$\log k' = 19.2 - 6.77 \times 10^{3} \cdot T^{-1} \quad (r = 0.993) \tag{13}$$

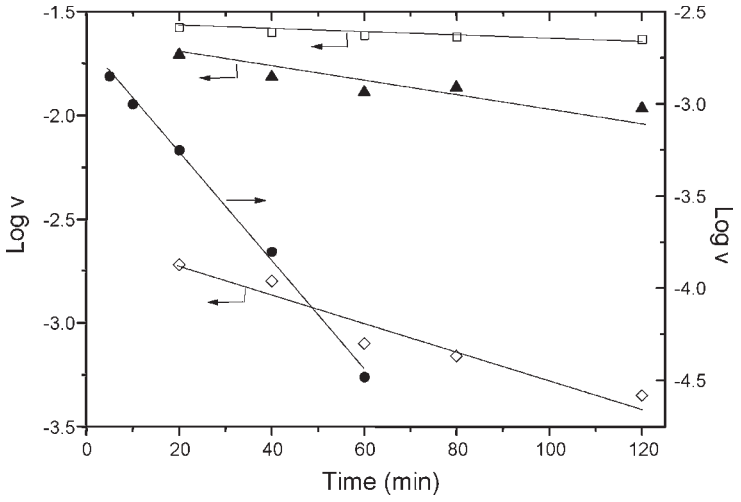

Fig. 5. Logarithm of Dowex-1x4-200/invertase complex activity vs time at 30 (□), 37 (▲), 45 (◇), and 55°C (●).

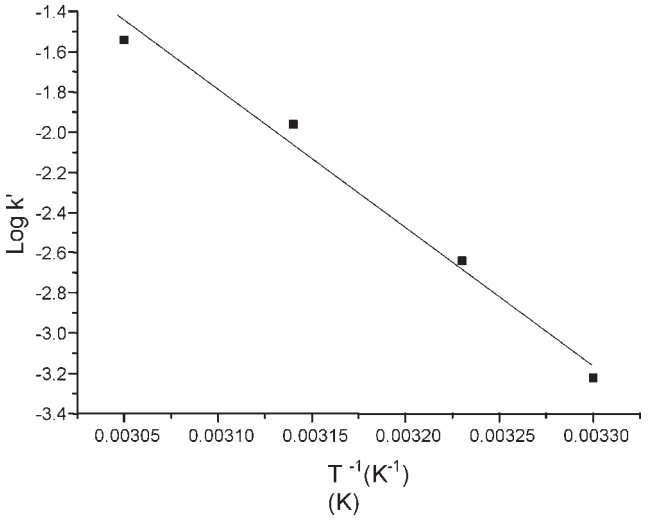

Fig. 6. Arrhenius plot for calculation of E_a related to heat inactivation of Dowex-1x4-200/invertase complex.

From Eq. 13 the activation energy (E_a') related to the heat inactivation of Dowex-1x4-200/invertase complex was calculated as 129 kJ/mol. Combining the E_a' value with the conventional thermodynamic equations *(20)*, it was possible to calculate the corresponding $\Delta G'$, $\Delta H'$ and $\Delta S'$, which were equal to –81.0 kJ/mol, 127 kJ/mol, and 0.660 kJ/(mol·K), respectively.

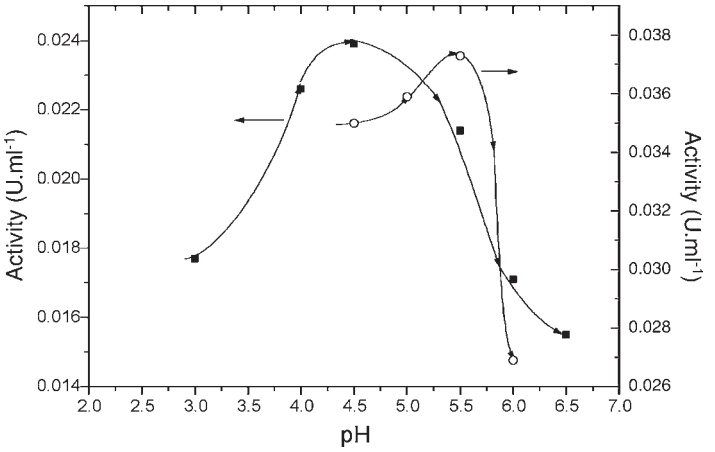

Fig. 7. Effect of pH on activity of soluble invertase (■) and Dowex-1x4-200/invertase complex (○).

According to Owusu and Makhzoum *(20)* $\Delta H'$ values of about 200–300kJ/mol would lead to unfolding of the tertiary structure of a protein. However, the Dowex-1x4-200/invertase complex had a $\Delta H'$ 37% lower than these values, indicating that the declining invertase activity vs temperature is probably owing to the breakup of the supramolecular structures of invertase rather than to the irreversible unfolding of the macromolecular tertiary structure.

At 55°C, k' was 0.006 (Eq. 8) and 0.0292 min^{-1} (Eq. 12), respectively, for soluble and insoluble invertase. As can be seen, the activity of Dowex-1x4-200/invertase complex diminished at a rate fivefold higher than the soluble enzyme, as the time increased. One possible explanation could be related to the probable different patterns of internal heat transference presented by a solution and a suspension. In a suspension, a significant portion of the thermal energy would accumulate in the resin particles in which the invertase molecules are adsorbed. As a consequence, the molecules should be submitted to a higher thermal energy than when they are homogeneously dispersed in a solution.

Effect of pH

The activity and stability of both forms of invertase against pH are presented in Figs. 7 and 8, respectively. Since the p*I* of invertase is equal to 4.0 *(17)*, the immobilized invertase was not submitted at a pH lower than 4.0 to avoid desorption of the enzyme from the support. The highest activities for soluble and immobilized invertase occurred at pH 4.5 and 5.5, respectively. Moreover, both soluble and Dowex-1x4-200/invertase were stable at a pH interval of 4.0 to 5.0 and 5.0 to 6.0, respectively. It must be pointed out that in both cases the optimum pH was within the stability interval.

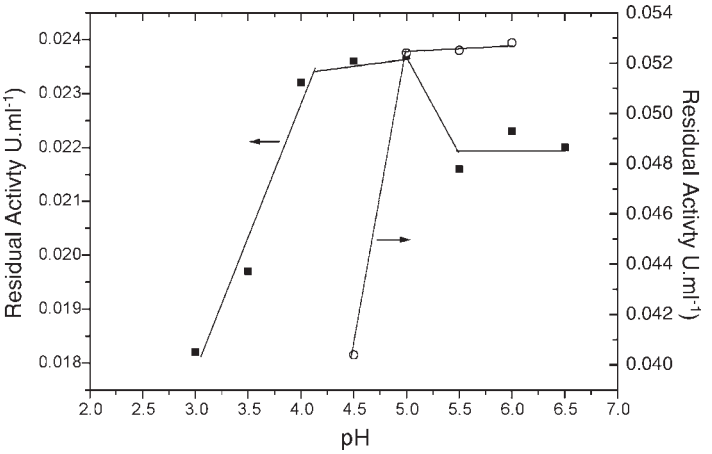

Fig. 8. Effect of pH on stability of soluble invertase (■) and Dowex-1x4-200/invertase complex (○).

By comparing the profiles of activity vs pH curves of both forms of invertase, a shifting of about one pH unit toward less acidic pH values was observed for the immobilized form (Fig. 7). According to Vitolo and Barros *(5)* such a shift could be attributed to the fact that the concentration of charged particle species (e.g., hydrogen ions) in the domain of the immobilized enzyme is different from that in the bulk solution, owing to electrostatic interactions with the fixed charges on the carrier. Unfortunately, the glass electrode effectively measures the pH in the bulk solution. Since the Dowex resins used in the present work had positively charged chemical groups, a shift toward a more acidic pH range would be expected, because the hydrogen ions should be repelled from the pellets, increasing their concentration in the bulk solution. However, exactly the opposite was observed (Fig. 7). One explanation is that the resin granules were fully covered by negatively charged invertase molecules, and as a consequence, the hydrogen ions would preferably accumulate near the particles of Dowex/invertase complex, leaving the bulk solution less acidic.

Effect of Sucrose Concentration

The activities of free and immobilized invertase for various substrate concentrations are plotted in a Lineweaver-Burk graph, from which V_{max} and K_m values were calculated (Fig. 9). The K_m of free enzyme was 40.3 m*M*, while the apparent K_m was 38.2 m*M* for the immobilized one. These similar K_m values for both forms indicate that enzyme-substract interaction is not substantially altered after immobilization. However, the V_{max} for immobilized invertase (0.0489 U/mL) was 35% higher than the V_{max} for soluble invertase (0.0320 U/mL). Such a difference could be explained by the predominance of supramolecular aggregates (hexamer, octamer forms) guaranteed under the conditions of the immobilization assay.

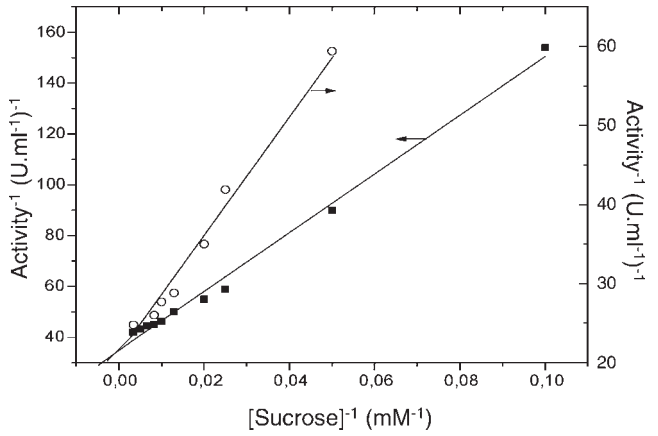

Fig. 9. Lineweaver-Burk plot for soluble invertase (■) ($V^{-1} = 1.26 \times 10^3[S] + 31.2$ [r = 0,997]) and Dowex-1x4-200/invertase complex (○) ($V^{-1} = 7.81 \times 10^2[S] + 20.5$ [r = 0,990]).

Table 4
Storage Stability of Dowex-1x4-200/Invertase
Complex Maintained in Deionized Water at 4°C for 28 d

Time (d)	AI (%)	Activity (U/mL)	IC (%)
1	100	0.0263	100
7	100	0.0325	100
14	100	0.0211	98.6
21	100	0.0246	100
28	100	0.0167	78.0

Storage Stability of Immobilized Invertase

Enzymes are not stable during storage in solution and their activities decrease gradually over time *(21)*. Immobilization technique, on the other hand, is applied considering lifetime, durability, and storage stability as important parameters. It puts the enzyme into a more stable position in comparison to free enzyme. The Dowex/invertase complexes kept at 4°C in deionized water were evaluated. Table 4 shows that after 21 d, the immobilized complexes maintained 100% of AI and retained their initial activity. These results are in accordance with the storage stability of immobilized pectinase on anionic polystyrene beads, which was stable and retained its initial activity for at least 7 wk of storage at 4°C *(22)*.

Conclusions

The data presented lead to the general conclusion that Dowex anion-exchange resins (types 1x8:50-400, 1x4:50-400, and 1x2:100-400 styrene-

divinylbenzene copolymers with different granulometry [50–400 mesh] and different degrees of crosslinking [2–8%]) are suitable for adsorbing invertase (aqueous medium at pH 5.5 and 32°C). We selected the complex Dowex-1x4-200/invertase, taking into account aspects such as high operational stability (no release of enzyme from the carrier during sucrose hydrolysis), good storage performance in deionized water (100% of the invertase activity was maintained after 21 d at 5°C), and AI and IC both equal to 100%. Since the maximal activity of soluble invertase and Dowex-1x4-200/invertase differed <2 %, it was assumed that the immobilization procedure did not significantly affect the enzyme conformation. These points make the complex Dowex-1x4-200/invertase highly suitable for use in invertase immobilization. The reutilization of Dowex/invertase complex and diminution of 5°C in the reaction temperature (less demand for energy) will lead to an overall cost reduction in the future use of the complex Dowex-1x4-200/invertase in the continuous sucrose hydrolysis process.

Acknowledgments

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