Revista de Ciências Farmacêuticas Básica e Aplicada
Journal of Basic and Applied Pharmaceutical Sciences

Rev. Ciênc. Farm. Básica Apl., v. 26, n.3, p. 175-179, 2005
ISSN 1808-4532

Anion exchange resin as support for invertase immobilization

Ribeiro, R.R.[1]; Vitolo, M.[2]*

[1]*Presbyterian Mackenzie University, Faculty of Biological, Exact and Experimental Sciences, São Paulo-SP, Brazil.*
[2]*University of São Paulo, School of Pharmaceutical Sciences, Dept. of Biochemical and Pharmaceutical Technology, São Paulo-SP, Brazil.*

Recebido 16/01/06 / Aceito 09/05/06

ABSTRACT

The invertase (EC 3.2.1.26) from *Saccharomyces cerevisiae* was employed as a model enzyme in the evaluation of the adsorption capacity of DOWEX-1X8-50®, a basic anion exchange resin, when used as support in enzyme immobilization. By mixing 100mg of resin with 27mg of invertase (pI = 4.0) in buffer solution (pH 4.6, 25°C), stirred at 100rpm, an adsorption of 93% was achieved. The activities (1U = amount of enzyme forming 1mg reducing sugars/min) of soluble and insoluble invertase were 0.084 U/mg$_E$ and 0.075 U/mg$_E$, respectively, giving an immobilization coefficient of 90.4%. The immobilized invertase had a higher thermal stability than the soluble form. The highest activity was observed at pH 4.5 in both forms of the enzyme, whereas the pH stability ranges for soluble and insoluble invertase were 3.5-5.0 and 4.5-5.5, respectively. The kinetic constants for soluble invertase were K_M = 18.3 mM and V_{max} = 0.084 U/mg$_E$, and for the insoluble form, K_M = 29.1 mM and V_{max} = 0.075 U/mg$_E$. The resin tested adsorbed the invertase very well, provided the enzyme molecule had a net negative charge, i.e., the immobilization and reaction procedures had to be carried out at pH > pI.

Keywords: Invertase, immobilization, adsorption, anion-exchange resin.

INTRODUCTION

Adsorption, when used to immobilize enzymes, involves an electrostatic and/or ionic interaction between a charged protein and the oppositely charged groups on the surface of the support. Supports for adsorbing enzymes, such as bentonite, porous ceramic, alumina and ion-exchange resins, are cheap, in plentiful supply, mechanically resistant, chemically stable, nontoxic, non-polluting and easily regenerated after use. They also have a suitable density for compaction (in a packed-bed column) or suspension in solution (batch, fed-batch, fluidized-bed or continuous stirred tank reactor) (Godfrey & West, 1996).

Among all types of adsorbing materials used for enzyme immobilization, the ion-exchange resins (e.g. DEAE-cellulose, amberlite IRA-94 and amberlite IRC-50) are widely used (Vitolo, 2004). In particular, the DOWEX® ion-exchange resins deserve special attention for their high ion-exchange capacity and long use in both industry and the laboratory - at least 60 years in purification, concentration and fractionation unit operations, as well as in chromatography columns - besides the common characteristics already cited for adsorbing supports in general (Li et al., 2001). In spite of their suitable qualities, until now DOWEX® resins have been used to immobilize only a few enzymes (xylanase, β-xylosidase, dextranase, protease, α-amilase, pectinase and lipase) (Abdel-naby et al., 1999; Ivanova et al., 1999; Demir et al., 2001; De Oliveira et al., 2000).

Undoubtedly, one of the greatest industrial successes achieved with an immobilized enzyme was the isomerization of glucose into fructose, catalyzed by glucose isomerase adsorbed on DEAE-cellulose (Godfrey & West, 1996). In this case, the glucose is generally obtained from starch hydrolysis, but a similar product called invert syrup, an equimolar solution of glucose and fructose, is produced by the hydrolysis of sucrose present in sugarcane or beet. The hydrolysis can be carried out either by hydrochloric acid at 75-80°C or by invertase (EC 3.2.1.26) at 35-45°C. The advantages of enzymatic over acid hydrolysis are well established, mainly in terms of energy savings and low by-product formation (Vitolo, 2004). However, to enhance those advantages the invertase must be used in the immobilized form, as the catalyst has to be recovered and reused several times and the hydrolysis should preferably be carried out in a continuous reactor. Invertase has also been used in analytical chemistry (biosensors) and in confectionery (Barlikova & Miertus, 1991; Walsh & Headon, 1994). In spite of invertase having been immobilized over the last 90 years on innumerable types of support (charcoal, clay, bentonite, DEAE-cellulose, polyethylene, chitosan, polyacrylamide, polyethylenimine, for example), using all kinds of immobilization techniques (entrapment, adsorption and covalent-binding), only a few initial studies have been published on using DOWEX® resins as carriers for this enzyme (Barros & Vitolo, 1992; Torres et al., 2002; Cirpan et al., 2003; Danisman et al., 2004).

This paper reports a study of the catalytic performance of the invertase-DOWEX-1X8-50® complex (I-D1X8-50) at various pH values, temperatures and sucrose concentrations.

**Corresponding author*: Prof. Dr. Michele Vitolo - Dept. of Biochemical and Pharmaceutical Technology - University of São Paulo, School of Pharmaceutical Sciences - Av. Prof. Lineu Prestes, 580, B.16 - 05508-900, São Paulo, SP, Brazil.
e-mail: michenzi@usp.br - Phone: 0055-11-30912382

MATERIALS AND METHODS

Materials

Invertase (β-D-fructofuranosidase, EC 3.2.1.26) and DOWEX-1X8-50® (anionic resin) were purchased from SIGMA. All the other chemicals used were of analytical grade.

Methods

Immobilization Procedure: The DOWEX-1X8-50® (100mg dry weight) and the invertase (27mg of powder) were mixed in 100mL of 0.010 M acetate buffer (pH 4.6) stirred at 100rpm for 30 min at 25ºC. Next, the suspension was left to stand at 4ºC for 12 h. The I-D1X8-50 complex was centrifuged (3000×g; 20 min), rinsed twice with buffer and the final sediment stored at 4ºC in 10mL of 0.010 M acetate buffer (pH 4.6). The protein content in the supernatant was measured, so as to evaluate the percentage of protein adsorbed on the resin, which was 93%.

Measurement of Invertase Activity*:* A standard assay for both forms of invertase consisted of mixing 90mL of sucrose solution (100g/L in 0.010 M acetate buffer, pH 4.6) with 10mL of aqueous invertase solution – in which 27mg of invertase powder were dissolved – or 10mL of I-D1X8-50 suspension, containing 25.1mg of invertase adsorbed on 100mg of resin powder. The hydrolysis was carried out for 10 min at 37°C with stirring (100 rpm). At intervals of two minutes, 0.5mL aliquots were withdrawn for measurement of reducing sugars (RS). One soluble or immobilized invertase activity unit (U) was defined as the amount of enzyme forming 1mg of RS per minute under the conditions of the test. The initial invertase activity (v) was calculated from the slope of the straight line (in mg RS/min or U) drawn through a plot of RS versus time (Figure 1). All assays were performed in triplicate. The specific activity for soluble invertase $[(v_p)_S]$ or I-D1X8-50 $[(v_p)_I]$ was defined as v per mg of invertase (expressed as total protein) dissolved in the medium or adsorbed on I-D1X8-50.

Measurement of Soluble Protein: Protein determination was based on the difference between UV absorbance measured at 215nm and 225nm, using bovine serum albumin (BSA, from SIGMA) as a standard (Segel, 1979).

Measurement of Reducing Sugars (RS): In a Folin-Wu test tube, 0.5mL of the sample and 1.0mL of alkaline cupric sulfate solution (Somogyi, 1952) were mixed. The test tube was then immersed in a boiling water bath for 10 min. After cooling, 2.0mL of an arsenic-molybdenum solution (Somogyi, 1952) were added and the color of the solution, read in a spectrophotometer at 540nm, was calibrated against a standard curve obtained with 0.2mg/mL glucose solution.

Characterization of Soluble and Immobilized Invertase: The pH (3.5-7.5), temperature (25°C – 60°C) and substrate concentration (5.0 mM – 40 mM) of the standard assay reaction were varied, one at a time, for both forms of invertase. The range of pH values was attained by using suitable buffer solutions, *viz.* 0.010 M acetate buffer (pH 3.5 to 5.6) and 0.010 M phosphate buffer (pH 6.0 to 7.5). The thermal and pH stability of each form of invertase was evaluated by maintaining the enzyme for 12 min in 0.010 M acetate buffer (pH 4.6) at a selected temperature in the interval 25°C–60°C, or at 37°C in 0.010 M buffer solution at a selected pH in the intervals 3.5-6.5 (for soluble invertase) and 4.5-7.5 (for I-D1X8-50). Protein desorption was checked after each assay by measuring the protein present in the supernatant. A blank was prepared under the same conditions for each temperature or pH.

RESULTS

The data obtained are presented in Figure 1 and Tables 2 and 3.

The linear plots of concentration of reducing sugars versus time shown in Figure 1 can be represented by the equations:

(Soluble Invertase): $RS = 2.24.t + 3.87$ ($r = 0.9998$) (Eq. 1)

(Insoluble Invertase): $RS = 1.88.t + 2.92$ ($r = 0.9996$) (Eq. 2)

where RS = reducing sugar concentration and t = reaction time.

From Equations 1 and 2 the specific activities of soluble and insoluble invertase under standard assay conditions (pH 4.6, 37°C) were calculated as follows: 1mg of dissolved Sigma invertase had an activity of 0.083U (the slope 2.24 divided by 27mg of enzyme), whereas 1mg of insoluble invertase (I-D1X8-50) had an activity of 0.075U (the slope 1.88 divided by 25.1 mg of enzyme).

The immobilization coefficient (Y) was calculated from the equation:

$$Y\ (\%) = [(v_p)_I/(v_p)_S].100 \qquad \text{(Eq. 3)}$$

where $(v_p)_I$ and $(v_p)_S$ are, respectively, the specific activity of insoluble and soluble invertase. At this pH and temperature, Y = 90.4 %.

The data in Tables 2 and 3 show, respectively, the effect of temperature and pH on the activity and stability of both forms of invertase.

From the conventional Arrhenius' plot (the Napierian logarithm of enzyme activity versus the reciprocal

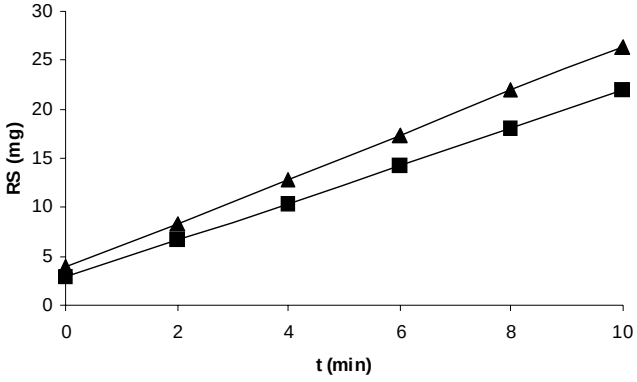

Figure 1 - Formation of reducing sugars during sucrose hydrolysis catalyzed by soluble invertase (▲) and I-D1X8-50 (■). The equations for the straight lines shown are: a) soluble invertase: $RS = 2.24t + 3.87$ ($r = 0.9998$); b) I-D1X8-50: $RS = 1.88t + 2.92$ ($r = 0.9996$).

of absolute temperature, T) of the values of invertase activity at various temperatures (Table 2), the following equations were obtained:

(Soluble Invertase) $\ln (v_p)_S = 19.7 - 6858.(1/T)$ (r=- 0.998) (Eq. 4)

(Insoluble Invertase) $\ln (v_p)_I = 19.3 - 6858.(1/T)$ (r = - 0.998) (Eq. 5)

DISCUSSION

The immobilization coefficient (Y) calculated from Equation 3 (90.4%) is quite high compared to those found in the literature (Table 1). Given that polystyrene polymers,

Table 1 - Some values of immobilization coefficients for invertase immobilized on various supports.

Support	Y (%)	Immobilization	Reference
Calcium alginate	50	Entrapment	Arruda & Vitolo, 1999.
Bentonite	93	Adsorption	Monsan & Durand, 1971
Chitin	50	Covalent bond	Barros & Vitolo, 1992
Polyethylene copolymerized with acrylamide	17	Covalent bond	De Queiroz et al., 1996
Polyacrylamide	75	Adsorption	Abdellah, et al., 1992
Acrylic-ethylenediamine	93	Ionic binding	Maxim et al., 1987
Hen white egg	42	Covalent bond	Abdellah et al., 1992
DEAE-cellulose	50	Covalent bond	Suzuki & Ozawa, 1966
Modified chitosan	85	Covalent bond	Cirpan et al., 2003
Polyacrylamide-gelatine	79	Covalent bond	Danisman et al., 2004
Polyethylenimine-grafted poly(GMA-MMA)*	66	Adsorption	Torres et al., 2002

*Poly(glycidyl methacrylate-co-methylmethacrylate) or poly(GMA-MMA) for short.

Table 2 - Effect of temperature on the activity and stability of soluble invertase [$(v_p)_S$] and I-D1X8-50 [$(v_p)_I$]. The thermal stability was evaluated by determining the residual enzyme activity after leaving each form of the invertase for 12 min at the specified temperature. The pH was set at 4.5.

T	$(v_p)_S$ (U/mg_E)		$(v_p)_I$ (U/mg_E)	
(^oC)	Activity*	Stability**	Activity*	Stability**
25	0.0360	0.0800	0.0227	0.0700
30	0.0530	0.0810	0.0335	0.0715
35	0.0800	0.0800	0.0730	0.0722
40	0.110	0.0830	0.0793	0.0730
45	0.156	0.0810	0.0983	0.0725
50	0.219	0.075	0.130	0.0720
55	0.304	0.0680	0.192	0.0712
60	0.280	0.0546	0.176	0.0687
65	0.185	0.0400	0.117	0.0610

*Activity at each temperature

*Residual enzyme activity (stability)

Table 3 - Effect of pH on the activity and stability of soluble invertase [$(v_p)_S$] and I-D1X8-50 [$(v_p)_I$]. The pH stability was evaluated by determining the residual enzyme activity after leaving each form of the invertase for 12 min at the specified pH. The temperature was set at 37^{o}C.

pH	$(v_p)_S$ (U/mg_E)		$(v_p)_I$ (U/mg_E)	
	Activity*	Stability**	Activity*	Stability**
3.5	0.0500	0.0820	0.0500	0.0710
4.0	0.0630	0.0835	0.0585	0.0715
4.5	0.0840	0.0840	0.0740	0.0720
5.0	0.0820	0.0830	0.0728	0.0730
5.5	0.0795	0.0770	0.0700	0.0725
6.0	0.0730	0.0700	0.0685	0.0680
6.5	0.0632	0.0655	0.0645	0.0645
7.0	0.0570	0.0600	0.0600	0.0600
7.5	0.0495	0.0550	0.0547	0.0575

*Activity at the given pH

**Residual enzyme activity (stability)

such as DOWEX-1X8-50®, can have their adsorption capacities enhanced by altering their inner structure with the cross-linking agent divinylbenzene (Li, et al., 2001), the invertase immobilization coefficient could still be improved. Indeed, a great variety of DOWEX® resins are available, which will be tested as adsorbents for invertase in forthcoming trials.

The highest initial activity per unit mass, for both I-D1X8-50 (0.192 U/mg_E) and soluble invertase (0.304 U/mg_E), occurred at 55°C (Table 2). Moreover, the soluble invertase and I-D1X8-50 were stable over the temperature ranges 25°C – 45°C and 25°C – 55°C, respectively (Table 2). Knowing that the invertase molecules (the enzyme is originally a dimer) can aggregate to form larger structures, such as tetramers, hexamers and octamers, with increasing catalytic activity (Reddy et al., 1990), the overall improvement in enzyme thermostability may be a consequence of the stabilization of those structures by adsorption on DOWEX-1X8-50® resin. According to De Queiroz et al. (2002), after immobilization the invertase aggregate is destabilized by the direct unfolding of the tertiary/quaternary structure rather than the previous separation of the aggregate followed by the unfolding, as observed in soluble invertase. Indeed, by working with temperatures of 50°C – 55°C, the hydrolysis of concentrated sucrose solution (over 150g/L) with I-D1X8-50 becomes manageable on the industrial scale, due to the diminished viscosity of substrate solution.

The activation energy (E_a) calculated from equations 4 and 5 was the same for both forms of invertase, about 57 kJ/mol. This result may corroborate, if indirectly, the explanation suggested above for the stabilization of supra-molecular invertase structures through the adsorption on DOWEX-1X8-50®.

The highest specific activity for soluble invertase (0.084 U/mg_E) and I-D1X8-50 (0.074 U/mg_E) occurred at pH 4.5, while the soluble form and I-D1X8-50 were stable in the pH ranges 3.5-5.0 and 3.5-5.5, respectively (Table 3). It must be pointed out that in both cases the optimum pH is located within the pH range for stability, enabling both forms of invertase to be used at the same pH – for instance 4.5 – without loss of catalytic activity.

From the conventional Lineweaver-Burk plot (reciprocal initial reaction rate versus reciprocal substrate concentration), the kinetic constants for soluble (K_M = 18.3 mM and V_{max} = 0.084 U/mg_E) and insoluble (K_M = 29.1 mM and V_{max} =0.075 U/mg_E) invertase were calculated, using the equations:

Soluble Invertase: $1/v_p = 218.1.(1/S) + 11.9$ ($r = 0.9990$) (Eq. 6)

I-D1X8-50: $1/v_p = 388.(1/S) + 13.3$ ($r = 0.9996$) (Eq. 7)

The K_M for I-D1X8-50 was 37% higher than for soluble invertase, possibly due to the diffusional limitation imposed on the flow of substrate and product molecules to and from of the catalytic site. The diffusion restriction could be explained by the fact that each particle of I-D1X8-50 was covered with dozens of oligomeric forms of invertase (hexamers and octamers, mainly), resulting a sticky web-like coating over the bead surface through which the molecules would have to pass.

The data presented reinforce the potential use of the anion-exchange resin DOWEX-1x8-50® as a support for invertase immobilization. As both forms of invertase act at the same pH and require the same amount of activation energy (57 kJ/mol) for sucrose hydrolysis, the immobilization procedure does not interfere in the catalytic mechanism of the invertase. The increased thermostability exhibited by I-D1X8-50 is advantageous because concentrated sucrose solution, whose viscosity diminishes as the temperature increases, can be manipulated in very large volumes, as required in industrial plants.

ACKNOWLEDGMENTS

This work was supported by a grant provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

RESUMO

Resina trocadora de ânion como suporte para imobilizar a invertase

A invertase (EC 3.2.1.26) de *Saccharomyces cerevisiae* foi usada como enzima modelo na avaliação da capacidade de adsorção da DOWEX-1X8-50®, uma resina trocadora de ânions, para emprego como suporte na técnica de imobilização. Obteve-se 93% de adsorção ao misturar-se 100mg da resina com 27mg de invertase (pI = 4,0) sob condições definidas de imobilização (pH 4,6, agitação de 100rpm e 25ºC). As atividades (1U = mg de açúcares redutores formados/min) determinadas para a invertase solúvel e imobilizada foram iguais a 0,084 U/mg_E e 0,075 U/mg_E, respectivamente. O coeficiente de imobilização obtido foi de 90.4%. A termoestabilidade da invertase imobilizada foi maior do que a da enzima solúvel. O pH de maior atividade para ambas as formas da enzima foi 4,5, e as faixas de pH de maior estabilidade foram 3,5-5,0 e 4,5-5,5, respectivamente, para a invertase solúvel e imobilizada. As constantes cinéticas para a invertase solúvel foram K_M = 18,3 mM e V_{max} = 0,084 U/mg_E, enquanto que para a forma imobilizada foram K_M = 29,1 mM e V_{max} = 0,075 U/mg_E. A resina testada mostrou ter alta capacidade adsorvente, quando a molécula da invertase está carregada negativamente, ou seja, a imobilização e a reação de hidrólise conduzidas em pH > pI.

Palavras-chave: Invertase, imobilização, adsorção, resina trocadora de ânions.

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