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# Method for immobilizing invertase by adsorption on Dowex® anionic exchange resin

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*This communication describes a method for adsorbing the invertase (EC.3.2.1.26) on DOWEX® anion exchange resin. Among the types of DOWEX® resins studied (1x8:50-400; 1x4:50-400 and 1x2:100-400), 1X4-200 was the most suitable, because it adsorbed the invertase molecules completely and the complex 1X4-200/invertase retained 100% of the catalytic activity. Moreover, no leakage of enzyme from the support was noted at the end of the sucrose hydrolysis.*

**Uniterms**
- Invertase
- Immobilization
- Anionic exchange resins
- Adsorption

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## INTRODUCTION

Enzyme immobilization can be categorized according to whether the protein becomes immobilized on the inert support by chemical binding (involving covalent or non covalent bonds) or by physical retention (entrapment inside artificial membranes or compartmentalization through an ultra filtration (UF) -membrane). Immobilized catalysts have largely been used in industry, analytical and medical applications (Vitolo, 2001a).

Adsorption, a kind of chemical binding immobilization, involves an electrostatic and/or ionic interaction between a charged protein and the oppositely charged carrier. Such interaction can be achieved easily and under soft conditions (mainly gentle agitation, low temperature and ionic strength). Of course, this technique requires a tight control of variables as pH, temperature, ionic strength, the enzyme and adsorbent concentrations. Nowadays in industry, there are quite successful processes employing resin-adsorbed enzymes (as for example: aminoacylase in amino acid synthesis and glucose isomerase in glucose isomerization) (Vitolo, 2001a).

Among all types of adsorbing materials applied for enzyme immobilization (for instance, DEAE-cellulose, DEAE-Sephadex, CM-Sephadex, Amberlite IRA-94 and Amberlite IRC-50) the divinylbenzene-polystyrene derivatives (such as DOWEX® type resins) deserve special attention for characteristics as high ionic exchange capability, mechanical resistance, chemical stability, ease regeneration and no toxicity. In spite of the qualities related to DOWEX® as well as its use in industry for more than 60 years, only few enzymes (xylanase, β-xylosidase, dextranase, protease, α-amilase, pectinase and lipase) have been immobilized on it until now (Tomotani, 2002).

The sucrose hydrolysis for the production of inverted syrup (an equimolecular mixture of glucose and fructose, largely used as a sweetener) is a significant industrial activity for several countries. 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 with a notorious advantage for an enzymatic over acid process in terms of energy economy, environmental safety and low formation of by-products. However, to match the low cost of the acid

process, the invertase must be used in an immobilized form. Although the invertase has been immobilized over the last 90 years in uncountable types of supports through all kinds of immobilization techniques, there are little information on using DOWEX® resins as carriers for this enzyme (Tomotani, 2002).

Thus, the present work aims at setting the adequate conditions for adsorbing invertase on DOWEX® - type resins.

## MATERIAL AND METHODS

Invertase (β-D-fructofuranosidase, E.C.3.2.1.26) was purchased from Fluka. The derivatives of styrene-divinylbenzene (DOWEX® resins) constituted by 50-400-mesh granulated beads having different degrees of cross-linking (2-8%) were purchased from Sigma Chemical Co. All the other chemicals used were of analytical grade.

### Standard Procedure of Immobilization

The anion exchanger (100 mg dry weight) was previously equilibrated in 25 mL of desionized water (pH adjusted to 5.5 by dropping 1M HCl) leaving the suspension for 24 h under agitation (100 rpm) at 32 °C. Then, the enzyme (0.4 mg) was added and the system was left for 4 h under the same agitation and temperature. After that, the complex DOWEX-resin/invertase (DWR-I) was centrifuged (2880 g; 30 min) and the protein content in the supernatant was measured. The DWR-I was rinsed once with desionized water and no protein was detected in the supernatant. The final suspension was stored at 4 °C in desionized water (pH 5.5).

The efficiency of protein adsorption (PA) and invertase activity retention (IAR) were calculated through equations 1 and 2.

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

$$IAR = 100.(A_1/A_2) \qquad \text{(Eq. 2)}$$

Where TAP = Total Amount of Protein before immobilization, STP = Supernatant Total Protein, $A_1$ = activity of resin/invertase complex and $A_2$ = activity of soluble invertase.

Several types of DOWEX resins (1X2:100-400, 1X4:50-400 and 1X8:100-400) were tested for adsorbing invertase. Once the best DWR-I was selected, some procedure conditions were varied as follows: amount of resin (50 and 100 mg), volume of the suspension (25, 50 and 100 mL), enzyme-resin contact-time (4, 7, 24 and 48 h), pH (4.6, 5.0, 5.5 and 6.0), temperature (32, 37, 45 and 50 °C) and amount of invertase (0.2 and 0.4 mg). It was also tested the substitution of desionized water (pH adjusted to 5.5 with 1 M HCl) by 0.010 M acetate/acetic acid buffer (pH 5.5). The magnitude of PA (expressed as percentage) was used as the criterion for evaluating the effect of each modification introduced into the immobilization procedure. Each immobilization test was made in triplicate.

### Measurement of Soluble and Immobilized Invertase Activity

A standard assay for both forms of enzyme consisted of mixing 108 mL of sucrose solution (120g.$L^{-1}$ in desionized water with pH set at 5.5) with 12 mL of aqueous invertase solution (diluted 3:1000 - w/v) or 12 mL of an aqueous suspension of DOWEX-resin/invertase (100 mg powder.$mL^{-1}$). The hydrolysis was carried out for 6 min at 37 °C under agitation (100 rpm), as previously described (Arruda, Vitolo, 1996).

One soluble or immobilized invertase unit (U) was defined as milligram of total reducing sugars formed per min under the conditions of the test. It was determined that 1 mg of invertase Fluka® had an activity of 87 U.

The invertase activity considered throughout the work is the mean value of five determinations. The standard deviation and the variation coefficient related to this method were equal to 3.78 U/mg and 4.35%, respectively.

### Analytical Techniques

*Protein Determination*

Protein was determined by the difference between UV absorbance measured at 215 nm and 225 nm, using bovine serum albumin (BSA, from Sigma) as a standard (Arruda, Vitolo, 1996).

By using a 0.1 mg/mL BSA solution, the linear correlation between ΔABS ($ABS_{215nm} - ABS_{225nm}$) and protein concentration (P) (varied from 10 μg/mL) was:

$$\Delta ABS = 5.90x10^{-3}.P - 7.0x10^{-4} \qquad (r = 0.9997)$$

The standard deviation and the variation coefficient related to this technique were equal to 1.51 mg.$mL^{-1}$ and 3.48%, respectively. The protein content in 1mg of invertase Fluka® powder was equal to 0.19 mg. Thus its specific activity was equal to 458 U/mg of protein.

*Measurement of Total Reducing Sugars*

The Total Reducing Sugars (TRS) were measured as published elsewhere (Arruda, Vitolo, 1996).

The standard deviation and the variation coefficient

related to this technique were equal to $3.50x10^{-3}$ mg.mL$^{-1}$ and 3.86%, respectively.

## RESULTS AND DISCUSSION

From Table I, it can be noted that invertase was bound by all polystyrene beads tested, since the efficiency of protein adsorption (PA) was always higher than 80%. Moreover, no leakage of invertase from resins 1X2-200, 1X4-50, 1X4-100, 1X4-200, 1X8-50, 1X8-100 and 1X8-200 was detected in the reaction medium at the end of the tests (Table I). This result allows to predict an increasing on half-life of DWR-I, when it is employed in a repeated batch, fed-batch or continuous processes (Vitolo, 2001b).

Furthermore, the protein adsorption (PA) ranged from 87% (1X8-50) to 100% (1X4-200); meanwhile the invertase activity retention (IAR) varied from 50.6% (1X8-100) to 100% (1X8-400, 1X4-200 and 1X2-100), except for the DOWEX-1X8-50 (IAR = 2.1%) (Table1). These distinct adsorptive capabilities, undoubtedly linked to the superficial area available for the enzyme molecules, are due to the different mesh and cross-linking degree of the resin granules. For instance, DOWEX-1X2-400 (constituted by 2% of divinylbenzene, 98% of styrene and beads of 400 mesh) has a net wider than DOWEX-1X8-400 (constituted by 8% of divinylbenzene, 92% of styrene and beads of 400 mesh), leading to a high superficial area (Li *et al.*, 2001). IAR values of that magnitude (50.6% to 100%) are quite relevant when compared with other methods for invertase immobilization described in the literature (Tomotani, 2002). In contrast, the DOWEX-1X8-50-invertase complex had the lowest IAR, though PA = 87% and no desorption from the carrier occurred (Table I). For explaining this result, an attractive hypothesis should be a high compaction of the invertase molecules on the DOWEX-1X8-50 beads. So, the insertion of sucrose molecules into the invertase active site during catalysis would be hindered. However, conformational modification suffered by a sensitive domain, other than the active site, could also promote undesirable interactions between the enzyme and the charged chemical groups of the resin. Such a phenomenon, although with less intensity, could also have contributed to the IAR variation observed in other DWR-I complexes.

**TABLE I -** Screening of DOWEX® anionic exchange resin for immobilizing invertase, taking into account the efficiency of protein adsorption (PA), invertase activity retention (IAR), immobilized invertase activity (IIA) and protein detection in the medium on completion of the tests (PR). The total activity of soluble invertase before immobilization was 35U in all tests

| RESIN | PA (%) | IIA (U) | IAR (%) | PR (%) |
|---|---|---|---|---|
| 1X2-100 | 90 | 35 | 100 | ( + )* |
| 1X2-200 | 88 | 29 | 83 | ( - )** |
| 1X2-400 | 98 | 28 | 79 | ( + ) |
| 1X4-50 | 92 | 22 | 64 | ( - ) |
| 1X4-100 | 94 | 30 | 85 | ( - ) |
| 1X4-200 | 100 | 35 | 100 | ( - ) |
| 1X4-400 | 91 | 25 | 72 | ( + ) |
| 1X8-50 | 87 | 0.7 | 2 | ( - ) |
| 1X8-100 | 92 | 18 | 51 | ( - ) |
| 1X8-200 | 89 | 20 | 57 | ( - ) |
| 1X8-400 | 94 | 35 | 100 | ( + ) |

*( + ) means leakage of enzyme from the support; **( - ) means no leakage of enzyme from the support

Undoubtedly, the best immobilized complex attained was DOWEX-1X4-200-invertase, because no leakage occurred, PA = 100% and IAR = 100% (Table I).

Taking DOWEX-1X4-200 as the standard resin, its protein adsorption capability was checked under procedure conditions which varied as follows: (temperature and pH will be considered apart): amount of resin (50 mg and 100 mg), volume of the suspension (25 mL, 50 mL and 100 mL), enzyme-resin contact-time (4 h, 7 h, 24 h and 48 h) and amount of invertase (0.2 mg and 0.4 mg). In any case different from 100mg resin, 0.4 mg invertase, 4 h contact-time and 25 mL suspension, PA was found to be lower than 80% (data not shown).

Due to the electrostatic nature of the invertase-carrier interaction, the effects of pH and temperature on the adsorption capability of each resin employed as carrier were also evaluated.

Table II shows that all the resins adsorbed more than 60% of the protein present in the solution. Values of PA over 80% occurred with 1X4:100 and 1X2:400 resins regardless of the pH of the suspension. Moreover, at pH 5.5, all resins had an PA higher than 85%. The intense invertase-carrier interaction at this pH was due mainly to the enzyme optimized ionization (as invertase pI ranges from 4.0 to 4.4, its molecules acquire a net negative charge at pH 5.5) and strong molecular aggregation (invertase, a natural dimmer, aggregates forming structures of six and eight molecules) (Dautzemberg *et al.,* 1991; Reddy *et al.,* 1990). Accordingly, at pH 4.6, it would be expected that the resin granules had an effective positive charge

**TABLE II -** Effect of pH and temperature on the resin-enzyme interaction, which was expressed as the efficiency of protein adsorption (PA)

| Resin | pH | | | | Temperature (°C) | | | |
|---|---|---|---|---|---|---|---|---|
| | 4.6 | 5.0 | 5.5 | 6.0 | 32 | 37 | 45 | 50 |
| **1X8-50** | 68** | 68 | 87 | 68 | 87 | 73 | 70 | 69 |
| **1X8-100** | 70 | 69 | 92 | 70 | 92 | 73 | 72 | 71 |
| **1X8-200** | 72 | 76 | 89 | 76 | 89 | 71 | 68 | 64 |
| **1X8-400** | 76 | 75 | 94 | 83 | 94 | 74 | 72 | 69 |
| **1X4-50** | 69 | 69 | 92 | 79 | 92 | 73 | 72 | 71 |
| **1X4-100** | 87 | 84 | 94 | 84 | 94 | 82 | 72 | 71 |
| **1X4-200** | 75 | 77 | 100 | 78 | 100 | 76 | 72 | 68 |
| **1X4-400** | 71 | 73 | 91 | 82 | 91 | 82 | 76 | 71 |
| **1X2-100** | 71 | 71 | 90 | 83 | 90 | 78 | 74 | 72 |
| **1X2-200** | 72 | 69 | 88 | 69 | 88 | 80 | 75 | 73 |
| **1X2-400** | 90 | 95 | 98 | 82 | 98 | 92 | 79 | 71 |
| **1X4-200*** | - | - | 69 | - | 69 | - | - | - |

*In this test, the desionized water (pH adjusted to 5.5 by dropping 1 M HCl) was replaced by 0.010 M acetate/acetic acid buffer (pH 5.5); **PA expressed in percent and calculated according to Eq. 1.

whereas the invertase molecules were not completely negative charged. The contrary would occur at pH 6.0.

The substitution of desionized water (pH adjusted to 5.5 with 1 M HCl) by 0.010M acetate/acetic acid buffer (pH 5.5) led to a PA of 69% (Table II). Probably, the anion acetate present in the buffer solution competed with the negative charged invertase molecules for the cationic groups of the resin, leading to a diminution on the overall protein adsorption.

From Table II it is clear that the PA decreased when the temperature was increased from 32 to 50 °C. Certainly in the range of the temperature studied, the polymeric structure of the resin is not perturbed, because the material resists temperature as high as 120 °C (Li *et al.,* 2001). Thus, the PA decreasing could essentially be related to the inefficiency of the encounter between invertase molecules and resin beads throughout the aqueous suspension. Perhaps, this should be due to the increment of the kinetic energy of invertase molecules and/or the disruption of invertase aggregates (Reddy, *et al.,* 1990), as both events have the tendency to create a chaos inside the system. Under an increased entropy, the components of the system should preferably remain apart.

## CONCLUSIONS

The data presented led to the general conclusion on the suitability of DOWEX® anionic exchange resins for adsorbing invertase (aqueous medium with pH adjust to 5.5 with 1M HCl and temperature of 32 °C). The chosen complex was DOWEX-1x4-200/invertase, taking into account the high operational stability (no release of enzyme from the carrier during sucrose hydrolysis), PA and IAR both equal to 100%.


## RESUMO

**Método para a imobilização da invertase por adsorção em resinas trocadoras de ânions (DOWEX)**

*O presente trabalho descreve um método de adsorção da invertase (EC. 3.2.1.26) na resina de troca aniônica do tipo Dowex®. Entre os tipos de resinas Dowex® estudados (1x8:50-400; 1x4:50-400 e 1x2:100-400), 1x4-200 foi a mais apropriada devido à completa adsorção das moléculas de invertase e a sua retenção de atividade catalítica de 100% do complexo 1x4-200/invertase. Salienta-se ainda a ausência do desprendimento da enzima do suporte após o término da hidrólise da sacarose.*


***Unitermos:*** *Invertase. Imobilização. Resina de Troca Aniônica. Adsorção.*

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