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Lectin–glycoenzyme column chromatography monitored by enzyme flow microcalorimetry

D. Mislovičová*, M. Chudinová, A. Vikartovská, P. Gemeiner

Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, SK-842 38 Bratislava, Slovak Republic

Abstract

A method based on the flow microcalorimetric determination of catalytic activity of immobilized enzyme in a so-called enzyme thermistor was used to monitor the process of lectin affinity chromatography of invertase on Concanavalin A-bead cellulose. The strong biospecific interaction between Concanavalin A and invertase was employed to determine the bound enzyme and this principle was used for the investigation of an alternative direct method for monitoring the lectin affinity chromatography of glycoenzymes.

The results obtained by flow microcalorimetry showed that the catalytic activity of invertase immobilized on Concanavalin A-bead cellulose can be compared directly with the thermometric value ΔT_{max}. The validity of the method was also confirmed by the enzyme thermistor post-column method, which is based on the determination of the product from the immobilized invertase enzymatic reaction. The adsorption and desorption in the chromatography column were examined by flow microcalorimetry in small samples withdrawn from the column. Attention has been given to the operating parameters and the storage stability of the affinity sorbent. The binding ability of the affinity matrix decreased with the number of consecutive chromatographic runs, although its storage stability was satisfactory.

1. Introduction

Numerous applications of lectins are reported in affinity chromatography where their biochemically and biologically properties to bind compounds containing saccharide chains are utilized [1–4]. Glycoenzymes, the glycosyl residues of which are not involved in the catalytic function [5,6], can be adsorbed with retention of their catalytic activity in high yields [7]. Biospecific adsorption of biologically active glycoproteins on suitable lectins combines the isolation of the glycoproteins with their oriented immobilization. The advantages of such an immobilization are the good steric accessibility of the active binding sites and the increased thermal and operational stability.

Hitherto Concanavalin A (Con A) is the best studied lectin. Its capability to bind molecules with glucosyl as well as with mannosyl residues is well known [2]. If the saccharide moiety of the glycoconjugate contains a high mannosyl chain (oligomannose-type) its interaction with Con A can be very strong [8]. Therefore, Con A has been investigated as an effective ligand not only in affinity chromatography [9,10] but also in the immobilization of some glycoenzymes [7]. Inver-

* Corresponding author.

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tase is a glycoenzyme exhibiting strong biospecific affinity to Con A immobilized on different matrices [11,12]. In our previous work we used the sorbent Concanavalin A–triazine bead cellulose (Con A–TBC) for both affinity purification [13] and immobilization [14,15] of invertase. In those studies the extraordinarily strong binding was confirmed. This stimulated us to develop a direct method for the determination of the amount of coupled invertase (as a catalytic activity), evaluating the calorimetric measurements of the thermometric response of the enzymatic reaction in the enzyme thermistor (ET) [14,15].

The aim of this work was to use the flow microcalorimetric method for monitoring the affinity chromatography of invertase on a Con A–TBC matrix. We have studied the performance of this matrix during consecutive runs. The storing stability of the Con A–TBC matrix with and without immobilized invertase was also investigated.

2. Experimental

2.1. Materials

Concanavalin A (Con A) from jack bean in lyophilized form was provided by Lectinola (Charles University, Prague, Czech Republic). Invertase (INV) (EC 3.2.1.26, β-D-fructofuranoside, grade V: practical, Sigma, St. Louis, MO, USA) from baker's yeast exhibited, after removal of the water-insoluble material, a specific activity of 100 U/mg of protein at 25°C. Perlose MT [bead cellulose (BC), particle diameter 100–250 μm, dry weight 12.5%, bed volume 1.5 ml/g of wet gel] was supplied by Lovochemie (Lovosice, Czech Republic). α-Methyl-D-mannopyranoside (α-MMP), microbiologically pure, was obtained from Fluka (Buchs, Switzerland). The affinity matrix Concanavalin A–triazine bead cellulose (Con A–TBC) was prepared in a two-step procedure through the intermediate chlorotriazine derivative of bead cellulose as described in a previous article [13].

2.2. Methods

Batch experiments

The invertase was sorbed on Con A–TBC in 50 m*M* acetate buffer (pH 4.7) containing 0.1 *M* NaCl, 0.1 m*M* $CaCl_2$ and 0.1 m*M* $MnCl_2 \cdot 4H_2O$. Samples of wet Con A–TBC (0.5 g; containing 1.03 mg Con A/ml of gel) were stirred slowly with solutions of INV in the above-mentioned buffer (2 ml; 2–32.5 mg INV/ml with an activity of 4.04–79 U/ml) at ambient temperature for 1 h. After INV immobilization the preparations were washed with 0.05 *M* acetate buffer pH 4.7, containing 1.0 *M* NaCl. The activity of the immobilized INV was determined with Spekol 11 (Carl Zeiss Jena, Germany) by the balance method using the Bio-La-Test (Oxo-chrom glucose test; Lachema, Brno, Czech Republic) [16] for the spectrophotometric determination of glucose (at 492 nm) produced by the enzymatic hydrolysis of saccharose at 25°C. A series of six preparations of INV–Con A–TBC with different activities of adsorbed INV was prepared in this way. The immobilized invertase was monitored alternatively by two methods:

(i) As the thermal change in the enzyme thermistor (ET) due to the enzymatic reaction. The procedure for the characterization of the INV kinetics in a flow microcalorimeter, the so-called enzyme thermistor (3300 Thermal Assay Probe, Thermo-Metric, Jarfälla, Sweden), was described in our previous articles [13,14]. The standard Teflon (Delrin) column (2.0 × 0.4 cm I.D.) packed with a support containing immobilized invertase (ca. 0.17 g) was used. The flow-rate of the mobile phase (0.1 *M* acetate buffer, pH 4.65, containing 0.3 *M* saccharose) was 1 ml/min and the temperature of the enzymatic reaction was 30°C. Thermograms were recorded with a TZ 4100 recorder (Laboratorní Přístroje, Prague, Czech Republic). The normalized value of ΔT_{max} (°C) [14] was defined as the maximum response to the thermal change caused by the enzymatic hydrolysis under steady-state conditions.

(ii) As the rate of saccharose hydrolysis catalyzed by immobilized INV by post-ET column analysis. The flow-through ET column was

adapted to a different reactor system with substrate recirculation [15]. The catalytic activity of the immobilized invertase was spectrophotometrically estimated in the recirculate (the volume ranging from 5 to 30 ml depending on the activity of the immobilized INV) in 3, 5, 7, 10 and 12 min time intervals as the change in glucose concentration [16].

Chromatographic experiments

Minicolumns were packed with 1.2 g of wet Con A–TBC (2.5 × 1.0 cm I.D.) (concentration of the immobilized Con A was 1.2 mg per ml of gel). The columns were equilibrated with 50 m*M* acetate buffer (pH 4.7) containing 0.1 *M* NaCl or 1 *M* NaCl and 0.1 m*M* $CaCl_2$, 0.1 m*M* $MnCl_2 \cdot 4H_2O$. Afterwards 2 ml of invertase solution in the above-mentioned buffer (33.3 mg INV per 1 ml with an activity of ca. 100 U/ml) was applied to the column. The nonbound proteins were washed out with 30 ml of the equilibration buffer (without $MnCl_2$ and $CaCl_2$), followed by elution of the bound INV with 20 ml of 0.5 *M* α-MMP in the same buffer. The flow-rate of the mobile phase was 0.2 ml/min; the first 2 ml of the eluent (α-MMP) were acting at 8°C for 20 h (after an initial 2-ml eluent influx the eluent flow was stopped and started again after 20 h). The enzyme activity was determined in 10-ml fractions. The columns were regenerated by washing with 0.05 *M* acetate buffer containing 0.1 *M* NaCl before repeating the chromatographic experiment.

After each loading and elution procedure, 0.17-g samples were taken form the column packing to determine the immobilized INV in ET. The samples were then returned to the column.

3. Results

As already mentioned in the Introduction, this paper aims at monitoring the affinity chromatography of invertase on the Con A–TBC carrier by a thermometric method that allows the direct determination of the amount of invertase bound on the column after sorption and elution. A mathematical model for the reaction catalyzed in a minicolumn in a flow microcalorimeter was derived and experimentally verified in our previous studies [14,17]. Nevertheless, the relationship between the ΔT_{max} values and the activity of the immobilized INV, as estimated by the balance method, was calibrated for practical purposes (to determine the amount of INV bound). Six samples of immobilized INV, with concentrations ranging from 10.7 to 204.7 U/ml of gel (as determined by the balance method), were prepared in batch experiments. Fig. 1 shows the dependence of the experimental ΔT_{max} values on the amount of invertase bound; its curve is linear with slope $k = 1069.1$ and regression coefficient $r = 0.992$. Alternative data ($k = 1004.1$, $r = 0.999$), confirming the linear dependence of the activity of the immobilized invertase on ΔT_{max} (Fig. 2), were obtained by the post-ET column determination of the amount of glucose formed

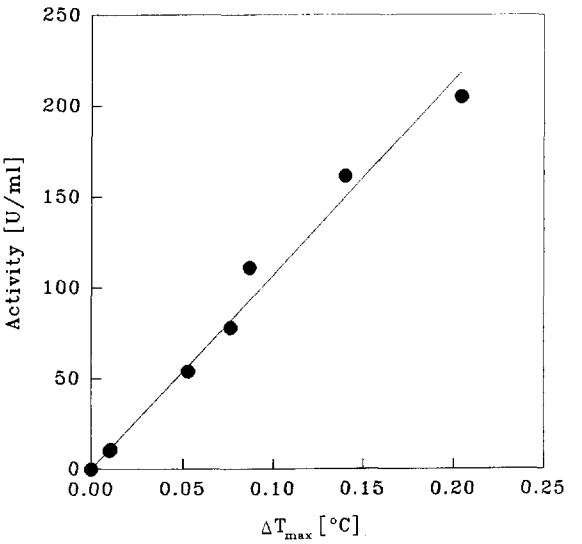

Fig. 1. Calibration plot showing the correlation between the maximum ET response and the activity of invertase bound to Con A–TBC determined by the balance method. Samples of INV–Con A–TBC (10.7–204.7 U/ml of the gel) were prepared in a batch system with adsorption of INV on Con A–TBC (1.03 mg Con A per ml of gel). Slope $k = 1069.1$, correlation coefficient $r = 0.992$.

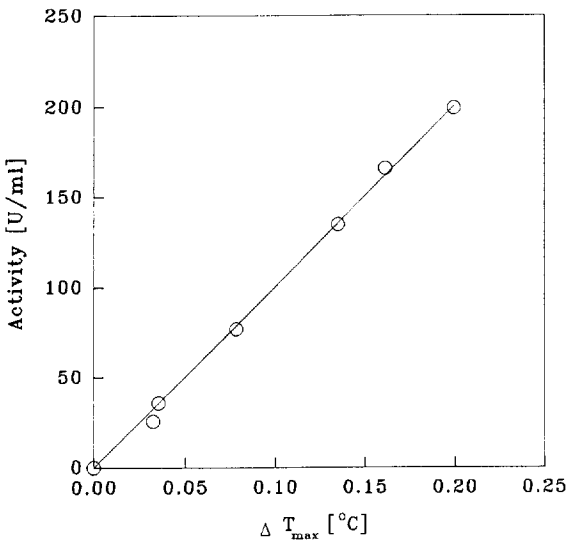

Fig. 2. Calibration plot presenting the correlation between the maximum ET response and the activity of invertase immobilized on Con A–TBC obtained by the post-ET column determination of the change in glucose concentration. Samples of INV–Con A–TBC (25–205 U/ml of the gel) were prepared in the batch system with adsorption of INV on Con A–TBC (1.03 mg Con A per ml of gel). Slope $k = 1004.1$, correlation coefficient $r = 0.999$.

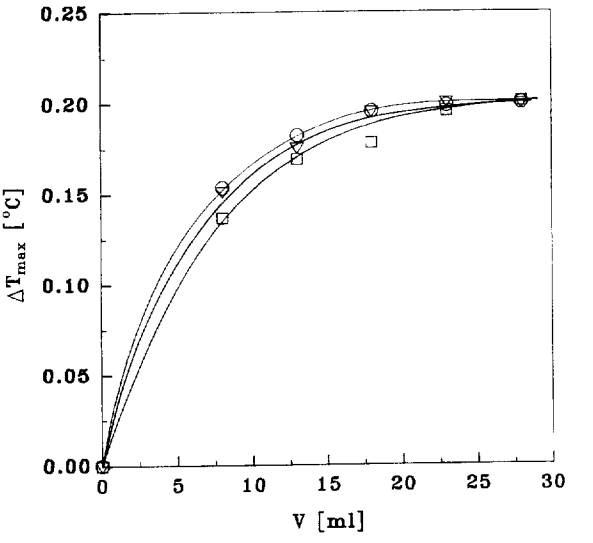

Fig. 3. pH Dependence of invertase adsorption on Con A–TBC determined using the enzyme thermistor. Invertase was bound on the Con A–TBC (1.03 mg Con A per ml of gel) in the flow ET. The ET-minicolumn was saturated with solutions of INV (5 U/ml) at a 1 ml/min flow-rate and 30°C. (○) Acetate buffer pH 4.7, (▽) acetate buffer pH 5.8, (□) phosphate buffer pH 7.0.

in the recyclate. Both mutually independent methods showed the ΔT_{max} value to be directly proportional to the amount of the enzyme bound. Comparison of the activity values for immobilized invertase obtained by both the balance method and the post-column determination of the enzymatic product showed that about 97% of the original activity was maintained after binding invertase to Con A–TBC. The ΔT_{max} values allow the direct determination of the amount of immobilized invertase as its activity; therefore, flow microcalorimetry was considered to be a reliable method for monitoring the enzyme sorption and desorption process.

The flow microcalorimeter was employed to monitor the binding curve of invertase to Con A–TBC at different pH values. Fig. 3 shows the invertase sorption at pH 4.7, 5.8 and 7.0 in relation to the amount of enzyme loaded; slight differences were observed in the sorption curves, but eventually the total amount of invertase bound was the same for all three pH values.

Chromatographic experiments were carried out in a minicolumn containing wet Con A–TBC (1.2 g); equal amounts of invertase (200 U) applied in five consecutive experiments were eluted in the standard manner with 0.5 *M* α-MMP. To attain an optimal effect, the elution reagent was left on the columns overnight [13]. The amounts of invertase bound and eluted were monitored (in %). The results obtained by the balance method were compared with those from the microcalorimetry experiments (Table 1).

Although the data obtained by the two methods were not completely identical (Table 1), this way of checking is reliable. However, iteration of the chromatographic experiments on the same packing resulted in a decrease of the binding capacity of the carrier. Moreover, the finding presented in our previous paper [13] that ca. 10–20% of the bound INV cannot be liberated even with 0.5 *M* α-MMP was confirmed. Quite unfavourable results were found with a buffer containing 1.0 *M* NaCl; only 20% of the loaded invertase was bound in the fourth iteration.

Table 1
Iterated chromatography with INV on Con A–TBC (0.1 *M* NaCl)

Step no.	Bound INV after loading activity (%)		Remaining INV after elution activity (%)	
	BM	ET	BM	ET
1	94.12	92.50	10.5	10.5
2	88.00	86.10	19.1	13.1
3	81.94	78.21	20.6	20.9
4	78.62	60.33	12.1	11.8
5	75.60	76.03	9.1	14.4

Wet Con A–TBC (1.2 g; 1.2 mg Con A/ml of gel) packed in a glass minicolumn was equilibrated with 0.05 *M* acetate buffer pH 4.7 containing 0.1 *M* NaCl, 0.1 m*M* $CaCl_2$ and 0.1 m*M* $MnCl_2 \cdot 4H_2O$. On the column were loaded 2-ml solutions of INV (activity ca. 100 U/ml). For elution of adsorbed INV 0.05 *M* α-MMP in equilibration buffer was used. Activities of the immobilized INV were determined by the balance (BM) and ET methods.

Table 2 shows data obtained by the microcalorimetric method. Also here, the first experiment gave good results, while iterations displayed a strong decrease of the binding capacity.

Estimation of the stability of the affinity carrier Con A–TBC with and without immobilized INV is very important from the practical point of view. The flow microcalorimetric method proved suitable to establish this property. It was also employed for monitoring the influence of storage of this carrier on its properties at various conditions (ambient temperature, 12°C, different periods of storage) in chromatographic experiments. Table 3 summarizes the results of iteration experiments with Con A–TBC columns kept cold or at room temperature.

The data presented in Table 3 show clearly that storage at lower temperature did not influence the binding properties of Con A–TBC. Properties of the sorbent tested deteriorated due to the repeated use, as was the case with preceding experiments. On the other hand, storage at room temperature strongly diminished the sorption properties of the carrier.

The good binding stability of Con A–TBC at the given conditions is very important for the direct determination of the activity of the immobilized invertase [ET, differential minireactor (DMR)]. Iterated activity estimation by flow microcalorimeter showed that the operational stability of invertase bound on Con A–TBC is reliable. It is, however, necessary to wash the conjugate INV–Con A–TBC thoroughly with a

Table 2
Iterated chromatography with INV on Con A–TBC (1 *M* NaCl)

Step no.	Bound INV after loading activity (%)	Remaining INV after elution activity (%)
1	92.8	7.9
2	71.2	6.5
3	21.2	5.9
4	23.3	5.9

Wet Con A–TBC (1.2 g; 1.2 mg Con A/ml of gel) packed in a glass minicolumn was equilibrated with 0.05 *M* acetate buffer pH 4.7 containing 1 *M* NaCl, 0.1 m*M* $CaCl_2$ and 0.1 m*M* $MnCl_2 \cdot 4H_2O$. On the column were loaded 2-ml solutions of INV (activity ca. 100 U/ml). For elutions of adsorbed INV 0.5 *M* α-MMP in the equilibration buffer was used. Activity of the immobilized INV was determined by the ET method.

Table 3
Iterated chromatography with INV on Con A–TBC stored under different conditions

Temperature	1st day		50th day		140th day	
	Bound INV activity (%)	Remaining INV activity (%)	Bound INV activity (%)	Remaining INV activity (%)	Bound INV activity (%)	Remaining INV activity (%)
Ambient	94.0	10.5	30.0	2.1	0	0
12°C	94.0	10.5	100	13.6	74.1	11.9

Wet Con A–TBC (1.2 mg Con A/ml of gel for one column) packed in two glass minicolumns was equilibrated with 0.05 *M* acetate buffer (pH 4.7) containing 0.1 *M* NaCl, 0.1 m*M* $CaCl_2$ and 0.1 m*M* $MnCl_2 \cdot 4H_2O$. On the column were loaded 2-ml solutions of INV (activity ca. 100 U/ml). For elution of adsorbed INV 0.5 *M* α-MMP in equilibration buffer was used. Activities of the immobilized INV were determined by the ET method. The results of these experiments are presented in the "1st day" column. After this experiment Con A–TBC columns were stored at 12°C and ambient temperature, respectively. Chromatographic experiments were repeated on the 50th and 140th day of storage by the procedure described above.

buffer containing 1 *M* NaCl, which is presumed to fully liberate the invertase not trapped biospecifically.

The stability of invertase after longer storage was also examined. Carrier with bound INV was kept wet or stored in a 0.05 *M* acetate buffer, pH 4.7, containing 0.1 *M* NaCl, and the ΔT_{max} values estimated at pre-set time intervals served to calculate the immobilized enzyme activity. Data obtained for storage up to 30 days are listed in Table 4.

The data obtained indicate that the activity of INV immobilized in this way (provided the conjugate INV–Con A–TBC was washed with a buffer containing 1 *M* NaCl and stored in a pH 4.7 buffer containing 0.1 *M* NaCl) remained constant over a 30-day period. On the other hand, the activity of INV stored in sucked form decreased markedly.

4. Discussion

Investigation of the properties of Con A–TBC as an affinity sorbent for INV with the differential method and also with direct microcalorimetric determination of the bound enzyme proved its suitability for lectin chromatography of glycoenzymes; however, its binding properties deteriorated with iteration, as was characterized by a stepwise lowered binding capacity. Noticeable deterioration of the binding properties of the Con A–TBC-packed column was found in iterative chromatographic experiments in the

Table 4
Storage stability of the immobilized INV on Con A–TBC

Storage conditions	Storage time (days)				
	1 Act. (U/ml)	2 Act. (U/ml)	3 Act. (U/ml)	10 Act. (U/ml)	30 Act. (U/ml)
In buffer pH 4.7 with 0.1 *M* NaCl	61.1	64.1	62.9	58.7	63.9
In wet form	61.1	n.d.[a]	n.d.	60.0	14.9

INV–Con A–TBC (61.1 U/ml) washed successively with 1 *M* NaCl in 0.05 *M* acetate buffer pH 4.7 was stored in a wet form or in the buffer at 4°C. Activity of the immobilized INV (U/ml of the gel) was determined by the ET method.
[a] n.d.: not determined.

presence of 1 *M* NaCl. As reported [9], the binding capacity of a carrier with immobilized Con A, employed for chromatography of hydrophobic glycoproteins, may decrease due to the unwanted loss of lectin (dissociation of Con A-tetramer). Loss of Con A during elution, especially with α-MMP, accounted for serious complications in both classic Con A chromatography and Con A high-performance affinity chromatography (HPAC).

The employed sorbent Con A–TBC gave only a minimal release of Con A during elution of the column with an acetate buffer (0.05 *M*, pH 4.7) containing 0.1 *M* NaCl. Nonetheless, elution with α-MMP could result in the liberation of Con A, this being manifested by a lower amount of invertase bound; in the fifth experiment this decrease was found to be up to 75%.

Differences were found between the differential and direct activity determinations of the amount of immobilized invertase after elution with α-MMP. According to the differential estimation, the amount of nonelutable enzyme should increase with each chromatographic cycle, but the direct determination of the amount of bound enzyme did not confirm this presumption.

Only multiple adsorption of INV to an affinity carrier and elution exclusively with a buffer without α-MMP showed a real increase in the amount of immobilized enzyme, in line with the differential determination (microcalorimetric and post-ET column determinations of D-glucose–unpublished results). As a result, only elution with α-MMP brought about liberation of Con A and also of Con A–INV; in the latter complex INV cannot be determined quantitatively after elution, because it is partly inhibited (unpublished results). To avoid this unfavourable effect, it would be necessary to crosslink the bound Con A, as has already been reported [18].

The microcalorimetric method was successfully applied to determine the storage properties of Con A–TBC and of INV–Con–TBC. Storage of the carrier for a long time (up to 150 days) in a 0.05 *M* acetate buffer pH 4.7 in a cold place neither lowered its binding capacity, nor released the immobilized invertase. This finding proved the stability of Con A–TBC and INV–Con A–TBC in the absence of α-MMP.

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