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Journal of Chromatography B, 664 (1995) 145–153

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Affinity chromatography of invertase on Concanavalin A–bead cellulose matrix: the case of an extraordinary strong binding glycoenzyme

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Abstract

This work presents confirmation of the biospecific character of the interaction of Concanavalin A (Con A) immobilized on bead cellulose with invertase. In spite of the extraordinary strong binding of invertase to this Con A conjugate ($K_D = 5 \cdot 10^{-9}$ *M*), conditions have been found to use Con A–bead cellulose as an affinity chromatography medium. The effective factor in the release of the bound invertase by the counter ligand (α-methyl-D-mannopyranoside) is the time of incubation. This phenomenon was demonstrated in both batch and flow-through experiments. A concentration of 1.5 mg Con A per ml of gel was found to be suitable with regard to the maximal invertase/Con A binding ratio and the optimal invertase recovery (94%). As a result of the strong biospecific interaction the purification of invertase was very effective (above ten-fold). Verification by FPLC and PAGE of the product purity revealed only one significant protein band.

1. Introduction

Beaded cellulose (BC) showed good properties as a support for medium-pressure chromatography [1–3] in many cases and for immobilization of enzymes and proteins [3–5]. Due to its mechanical properties, porosity and chemical composition, it is a suitable material for a wide range of applications in bioaffinity chromatography, e.g. as Sepharose and Sephadex supports [3,6–10]. BC bearing immobilized Con A was successfully used as sorbent in the lectin affinity chromatography of ovalbumin [6]. It thus seems suitable as a support for the purification of glycopeptides and glycoproteins containing oligosaccharides with glucose and mannose moieties [11].

Moreover, many glycoenzymes bound to Con A matrices usually appear to be highly stable [12]. Con A can be employed, therefore, as a universal and effective ligand for immobilization of the glycoenzymes by biospecific adsorption. Many examples applying Con A matrices for the immobilization of invertase have been described [13–17]. Due to the extraordinarily strong binding, stability of the adsorbed enzyme was enhanced.

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0378-4347/95/$09.50
SSDI 0378-4347(94)00447-1

The kinetics of several invertase preparations immobilized on BC derivatives have been investigated in our previous publications [18,19]. It was confirmed that also invertase adsorbed biospecifically onto Con A–BC conjugates [19] exhibited a high stability: the stability was equal or even higher than that of invertase preparations linked covalently to different derivatives of BC [18]. The biospecific binding of invertase to Con A–BC being that strong, we doubted the suitability of such a conjugate as an affinity medium (reversible immobilization).

In the present work a conjugate of Con A with macroporous bead cellulose was investigated with respect to its interaction with invertase. Invertase as a representative of the broad family of glycoenzymes having carbohydrate moieties recognizing Con A was used. The aim was to force Con A–BC conjugate to operate as an affinity medium. Thus, the key factors mediating the required competitive effect of the counterligand (α-methyl-D-mannopyranoside) had to be found.

2. Experimental

2.1. Materials

Concanavalin A from jack bean (Con A) in lyophilized form was provided by Lectinola (Charles University, Prague, Czech Republic). Invertase (EC 3.2.1.26, β-D-fructofuranosidase) grade V: practical (Sigma, St. Louis, MO, USA) from baker's yeast after removal of the water-insoluble material, exhibited a specific activity 100 U mg^{-1} of protein at 25°C. Perlose MT 100 [bead cellulose (BC), particle diameter 100–250 μm, dry weight 12,5%] was supplied by Lovochemie (Lovosice, Czech Republic). 2,4,6-Trichloro-1,3,5-triazine was obtained from Sigma, α-methyl-D-mannopyranoside (α-mMP, microbiologically pure) was obtained from Fluka (Buchs, Switzerland). Oxochrom Glukose test, saccharose and other chemicals (analytical purity) were from Lachema (Brno, Czech Republic).

2.2. Methods

Preparation of chlorotriazine beaded cellulose (CHTBC)

CHTBC was prepared by reaction of Perlose MT 100 with 2,4,6-trichloro-1,3,5-triazine [18]. Wet bead cellulose (BC) (12 g, cellulose content 1.5 g) was suspended in 70 ml of water. Then 30 ml of 3 *M* NaOH were added dropwise with stirring during 15 min. After 30 min the BC was soaked and resuspended in a solution of 1.5 g of 2,4,6-trichloro-1,3,5-triazine in 30 ml of acetone. The reaction was continued with slight stirring at 4°C for 30 min. CHTBC was washed twice with acetone–water (50:50, v/v). CHTBC was stored in wet state in a 0.02% aqueous solution of sodium azide. Chlorine (3.7%) and nitrogen (4.1%) were determined.

Immobilization of Con A on CHTBC

The Con A was bound to the CHTBC by the method used for immobilization of proteins [20,21]. CHTBC (1 g) was washed with 50 m*M* acetate buffer (pH 5.8) containing 0.1 m*M* $MgCl_2$ and 5% (w/w) of mannose and soaked. Wet beads were suspended in 4 ml of a solution containing 0.82–32.0 mg Con A in the above-mentioned buffer. After 2 h of stirring at ambient temperature the product was filtered off and washed 3 times with 30 ml of 50 m*M* acetate buffer (pH 5.8) to remove the unbound Con A. The residual reactive groups were blocked with a solution of 150 m*M* glycine in 50 m*M* acetate buffer (pH 4.7) overnight. The amount of Con A bound was obtained from the difference in the measurement of the proteins at 280 nm and occasionally also by Lowry method [22]. Spekol 11 and Specord M400 (both from Carl Zeiss Jena, Germany) were used for spectrophotometric determinations. In the Con A-triazine derivative of bead cellulose (Con A–TBC) no chlorine could be found.

Batch experiments

The sorption of invertase on Con A–TBC was performed in 50 m*M* acetate buffer (pH 4.7) or 50 m*M* phosphate buffer (pH 7), with 0.1 *M*

NaCl or 1 *M* NaCl containing 0.1 m*M* $CaCl_2$, 0.1 m*M* $MnCl_2 \cdot 2H_2O$ and/or 30% ethylene glycol. Suspensions of Con A–TBC prepared from 0.1 g of sorbents (with different concentrations of Con A in the range of 0.92–5.17 mg Con A per ml of gel) and a 1-ml solution of invertase in the above-mentioned buffer (6–70 mg invertase was dissolved in 1 ml and centrifuged) were shaken slowly at 25°C for 2 h. The amounts of invertase bound were calculated from the spectrophotometric determination of the activity by the Bio-Lachema-Test with the reagent Oxochrom Glucose [23] at 492 nm. The dissociation constants and binding capacities were calculated by regression of the adsorption isotherm equation [24] $C/Q = C/Q_m + K_D/Q_m$ where Q is the solid-phase concentration of adsorbed material at equilibrium, Q_m is the maximum adsorption capacity of the adsorbent, C is the concentration of adsorbate in solution in equilibrium, and K_D is the dissociation constant.

Specific elution of bound invertase

Elution was carried out with the specific agent α-methyl-D-mannopyranoside (α-mMP). A 0.5-g amount of sorbent Con A–TBC (0.5–5.5 mg Con A per ml of gel) with bound invertase (157–316 U per ml of gel) was suspended in a solution of 5 ml (0.1 *M* or 0.5 *M*) of α-mMP in 50 m*M* acetate buffer (pH 4.7) with 0.1 *M* NaCl. Elution was performed by slight stirring at ambient temperature. The invertase activity in the supernatant was measured after 1, 2, 4, 6 and 20 h.

Chromatographic experiments

Minicolumns of 1 g of Con A-TBC (2 × 1 cm I.D.) with a concentration of immobilized Con A of 0.92–5.5 mg per ml of gel were used. The columns were equilibrated with 50 m*M* acetate buffer (pH 4.7) with 0.1 *M* NaCl and 0.1 m*M* $MnCl_2 \cdot 2H_2O$ and 0.1 m*M* $CaCl_2$. Then the invertase solution (2 ml, ca. 200 U, specific activity in the range 55–129 U/mg) was applied to the columns. Nonbound proteins were washed out with equilibration buffer (without $MnCl_2$ and $CaCl_2$) followed by elution with 0.5 *M* α-mMP in equilibration buffer. The flow-rate of the mobile phase was 0.3 ml/min (the first 2 ml of elution agent acted at 8°C for 20 h). Both the total enzyme activity and the protein content were determined in 3-ml fractions.

Fast protein liquid chromatography (FPLC)

The purity of the invertase was checked by size-exclusion chromatography. Pharmacia FPLC equipment (Pharmacia, Uppsala, Sweden) with a standard prepacked column (30 × 1 cm I.D.) of Superose 6 (calibrated with Dextran Blue, thyreoglobulin, ferritin, catalase and aldolase) was used. The elution buffer was 50 m*M* phosphate (pH 7) with 0.15 *M* NaCl, flow-rate 18 ml/h. The UV absorbance was monitored at 280 nm and ambient temperature. Fractions of 0.3 ml were collected for measuring the catalytic activity of invertase.

SDS electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed according to the method of Laemmli [25] using Phast system (Pharmacia-LKB, Uppsala, Sweden). Proteins were stained with Coomassie Brilliant Blue G-250. The calibration set Sigma SDS 6-H was used.

Monitoring of invertase kinetics with an enzyme thermistor

The procedure for the characterization of the invertase kinetics in a flow microcalorimeter, a so-called enzyme thermistor (3300 Thermal Assay Probe, Thermo-Metric Co., Jarfalla, Sweden), was described in our previous articles [18,19]. The standard PTFE column (2 × 0.7 cm I.D.) was packed with a support containing the immobilized Con A (0.91 mg per ml of gel). The flow-rate of the mobile phase (1 m*M*–1 *M* solution of sucrose in 0.1 *M* acetate buffer with 1 m*M* each of Ca^{2+} and Mn^{2+}, pH 4.65) was 1 ml/min and the temperature of the enzymatic reaction was 30°C. Thermograms were recorded with a TZ 4100 recorder (Laboratorni pristroje, Prague, Czech Republic). The thermal response ΔT (°C) due to the enzymatic hydrolytic reaction

was measured under steady-state condition [19]. The experimentally measured values of ΔT as a function of the substrate concentration were described by the previously tested equation [19]. The normalized value, ΔT_{max} (°C) as the maximum response value corresponding to each individual kinetic curve, was calculated from the mathematical derivation of the curves using calculated kinetic parameters V_m, K_m and K_i (not shown) [19].

3. Results

A specific sorbent for invertase was prepared from bead cellulose with covalently bound Con A. Con A was bound covalently to the chlorotriazine activated cellulose (CHTBC) in a weakly acid medium (pH 5.8) at room temperature. The concentration dependence of Con A binding to CHTBC after a 2-h reaction period (the equilibribium) was found to be linear up to a concentration of 15 mg of immobilized Con A per ml of gel, when the lectin was bound almost by 100% (Fig. 1).

The degree and character of the interaction of invertase with Con A–bead cellulose conjugate were investigated. Several parameters influencing the amount of invertase adsorbed on Con A–TBC, such as pH, ionic strength and hydrophobic substance, were examined. Fig. 2 shows the adsorption isotherms obtained under the above-mentioned conditions. The negative effect of addition of ethylene glycol on the invertase adsorption is rather evident. The sorption curve was only slightly influenced by pH; however, the binding of invertase was higher at pH 4.7 than at pH 7.0. The optimal sorption conditions (50 m*M* acetate buffer pH 4.7 containing 0.1 *M* NaCl) were investigated for the adsorption of invertase to the carrier Con A–TBC at four different concentrations of the immobilized ligand. Fig. 3 shows the adsorption isotherms obtained; the amount of the invertase adsorbed increased with the ligand concentration within the range 0.92–5.17 mg Con A per ml of gel. The dissociation constants and binding capacities were calculated by linear regression of the individual adsorption relationships and are listed in Table 1.

The K_D values indicate a very strong interaction between the invertase and Con A–TBC. A high-affinity binding of invertase onto Con A

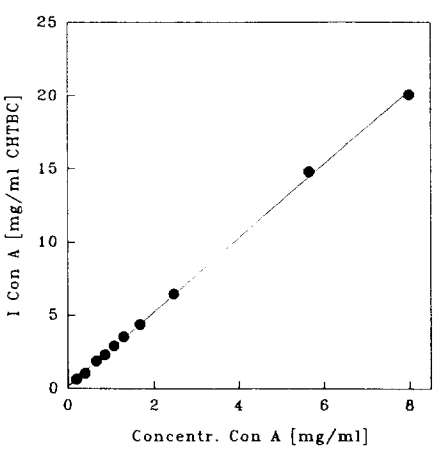

Fig. 1. Effect of Con A concentration on its immobilization yield (I). The reaction was performed in suspensions of CHTBC with solutions of Con A within the concentration range 0.21–8.00 mg/ml, at ambient temperature, at pH 5.8 for 2 h.

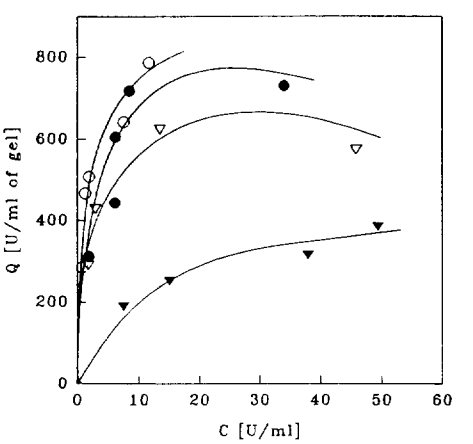

Fig. 2. Effect of pH, ionic strength and ethylene glycol on adsorption isotherms of invertase on Con A–TBC. The suspensions of Con A–TBC (4 mg Con A per ml of gel) with solution of invertase (15–175 U invertase/ml) were shaken slowly at 25°C for 2 h at following conditions: (▽) 50 m*M* phosphate buffer (pH 7) with 0.1 *M* NaCl, (○) 50 m*M* phosphate buffer (pH 7) with 1.0 *M* NaCl, (●) 50 m*M* acetate buffer (pH 4.7) with 0.1 *M* NaCl, (▼) 50 m*M* phosphate buffer (pH 7) with 0.1 *M* NaCl and 30% ethylene glycol. C is the concentration of invertase in solution at equilibrium, Q is the solid phase concentration of invertase in equilibrium.

Table 1
Quantitative parameters of adsorption

Sorbent I (mg Con A/ml gel)	Binding capacity Q_m (μmol/ml gel) · 10^3	Ratio Q_m/I	Dissociation constant K_D (μmol/l) · 10^3
0.92	0.83	0.094	8.60
1.14	0.96	0.088	5.93
3.50	1.03	0.030	4.60
5.17	1.20	0.024	5.01

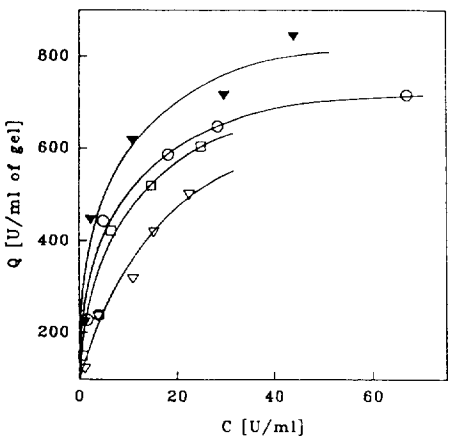

Fig. 3. Effect of the concentration of immobilized Con A on adsorption isotherms of invertase on Con A–TBC. The sorption was performed in batch systems containing Con A–TBC (0.92–5.17 mg Con A per ml of gel) and invertase solution (6–70 mg invertase/ml) in 50 mM acetate buffer (pH 4.7) with 0.1 M NaCl, at 25°C for 2 h at the following concentration of Con A: (▽) 0.92 mg Con A per ml of gel, (□) 1.14 mg Con A per ml of gel, (○) 3.50 mg Con A per ml of gel, (▼) 5.14 mg Con A per ml of gel. C is the concentration of invertase in solution at equilibrium, Q is the solid phase concentration of invertase at equilibrium.

was also evidenced by elution experiments with the specific eluent (counter ligand) α-mMP. The retention time (incubation time) of the elution agent was found to significantly influence the release of invertase (Fig. 4). The elution characteristics of the minicolumn experiments showed that minimal release of bound invertase was reached by continuous elution with a solution of α-mMP (of high concentration 0.5 M). However, the prolongation of the effect of elution agent has resulted in substantial increase of elution. In view of this point, the incubation time appeared to be a decisive parameter. The time dependence of the invertase displacement was also confirmed in batch experiments. Fig. 5 shows the time course of the elution with α-mMP solution and Table 2 lists the invertase recovery data after a 6-h desorption period in equilibrated batch systems with 0.1 M and 0.5 M concentrations of the eluent. Recoveries of invertase from Con A–TBC sorbed with a low Con A concentration (0.5 mg per ml of gel) were 85.6 and 94%, respectively. On the other hand, for a

Table 2
Recovery of invertase in batch system

Sorbent (mg Con A/ml gel)	Invertase bound (U/ml)	Invertase activity (%) eluted with	
		0.1 M α-mMP	0.5 M α-mMP
0.50	157.0	85.6	94.0
1.48	263.2	59.0	87.0
3.94	316.3	23.0	49.0
5.48	310.1	19.2	47.1

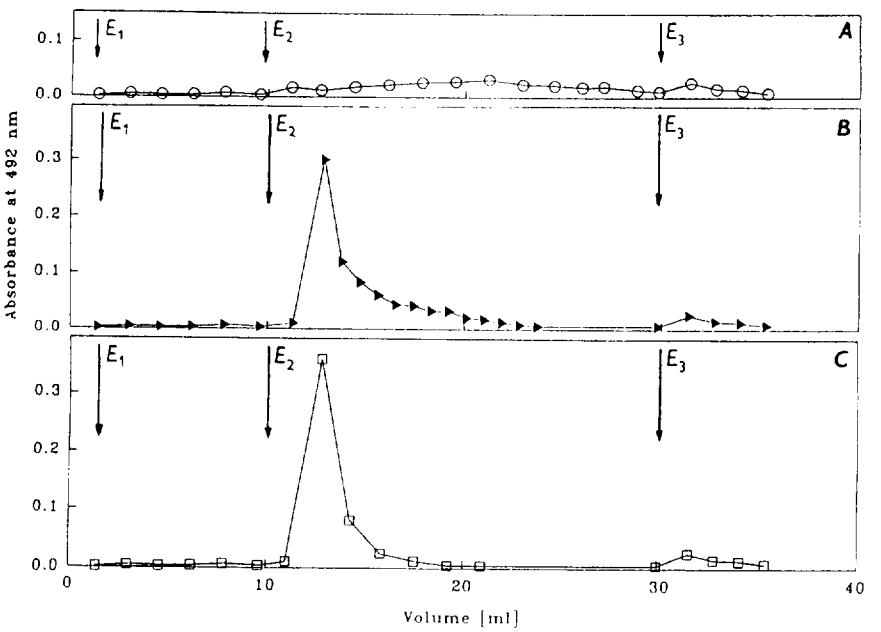

Fig. 4. Effect of incubation time on elution characteristics of invertase. The invertase 150 U bound on minicolumn (1 g Con A TBC with concentration of Con A 4 mg per ml of gel) was eluted with equilibration buffer pH 4.7 with 0.1 *M* NaCl (E_1), with specific eluent 0.5 *M* α-mMP in equilibration buffer (E_2) and with hydrophobic eluent with 30% ethylene glycol (v/v) (E_3). The incubation time was (A) none, (B) 1 h, (C) 2 h.

higher Con A concentration (5.48 mg per ml of gel) the respective recoveries were 19.2 and 47.1%. With these results in mind, further chromatographic experiments were carried out with the eluent 0.5 *M* α-mMP acting for a 20-h period. Table 3 evaluates the experiments on small columns. No enzyme recovery higher than 80% could be achieved, not even after a 20-h incubation period with the elution agent; nor could the yield be increased with the hydrophobic agent ethylene glycol.

The highest enzyme purity was achieved at an immobilized Con A concentration of ca. 1 mg per g of gel. The significant invertase purification was confirmed by FPLC (Fig. 6) and SDS-PAGE (not shown). The position of the peak in FPLC

Table 3
Results from minicolumn chromatographic experiments with invertase

Support Con A (mg/ml)	Invertase activity (%) eluted with		Specific activity of purified invertase (U/mg)
	Equilibration buffer	0.5 *M* α-mMP	
0.92	16.4	63.8	1171.5
1.47	6.4	63.7	788.8
3.41	3.4	49.9	899.0
5.48	1.9	38.9	836.6

Specific activity of invertase loaded was within the range 55–129 U/mg.

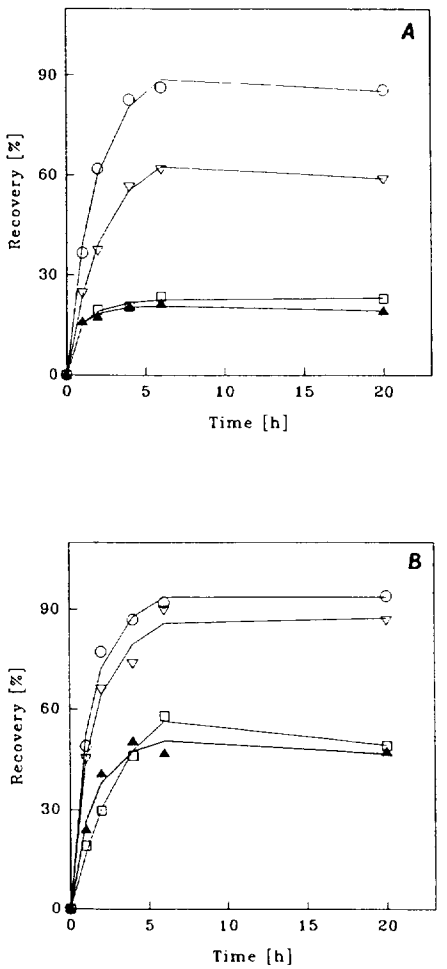

Fig. 5. Effect of the concentration of immobilized Con A on the time dependence of specific invertase elution. The invertase bound to Con A–TBC was released with α-mMP in batch system at pH 4.7 and 25°C (A) with 0.1 *M* α-mMP, (B) with 0.5 *M* α-mMP. Concentrations of immobilized Con A were (○) 0.5 mg per ml of gel, (▽) 1.48 mg per ml of gel, (□) 3.94 mg per ml of gel, (▲) 5.48 mg per ml of gel.

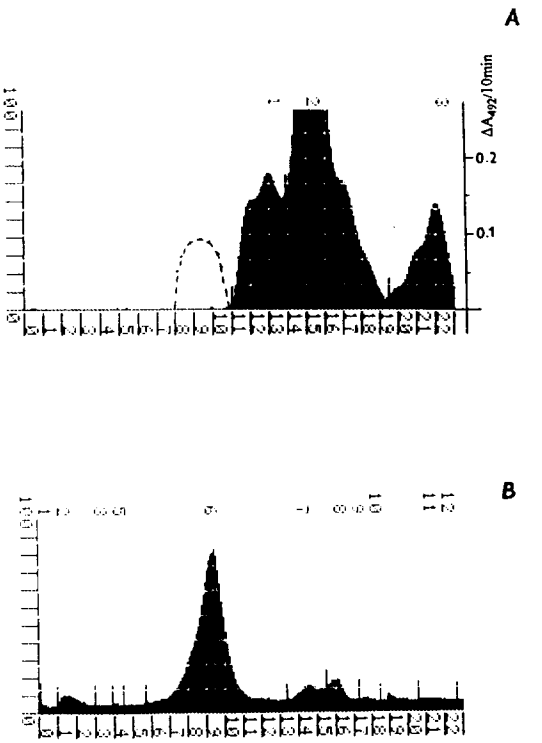

Fig. 6. Elution profiles of (A) crude and (B) purified invertase recorded with FPLC. The absorbance of the effluent was monitored at 280 nm (ordinate) where the sensitivity used in panel B is 10× higher; the numbers on the abscissa signify volume in ml. The activity of invertase ($\Delta A_{492}/10$ min) was determined with Bio-Lachema Test (dashed line) (A).

(Fig. 6B) corresponded to a molecular mass of $(26–27) \cdot 10^4$. It is worth noting that the invertase was not identified as protein in FPLC of the crude sample (Fig. 6A), but instead the activity in the fractions was measured. SDS-PAGE displayed a band of purified invertase of m_r (relative mobility) = 0.15 located between the calibration reference substances β-galactosidase (M_r = 116 000) and myosine (M_r = 205 000). This corresponded to an M_r of ca. 140 000 (dissociated invertase).

Recent papers [18,19] mentioned the possibility to determine the amount of invertase immobilized on the carrier by flow microcalorimetry. As seen (Fig. 7) from the linear relationship between the enzyme bound (determined by the balance method) and ΔT_{max} (maximal thermometric response of the immobilized enzyme), this direct method represents an alternative to the indirect balance method and offers a complementary procedure to monitor the lifetime of the respective Con A affinity chromatographic medium.

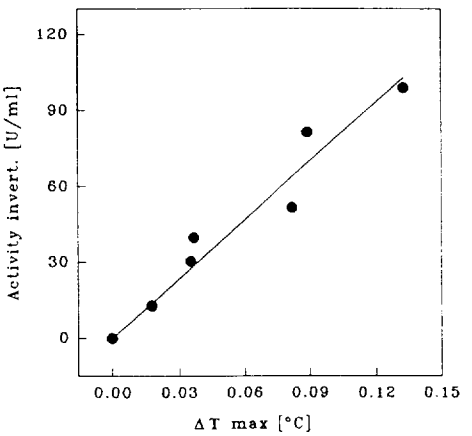

Fig. 7. Calibration plot representing the correlation between the maximum enzyme thermistor (ET) response observed and activity of invertase bound to Con A as determined by the balance method at the concentration 0.92 mg Con A per ml of gel.

4. Discussion

The practical utilization of Con A–TBC in lectin-affinity chromatography required determination of the optimal conditions for both the effective adsorption of invertase and the highest possible yield of eluted purified enzyme. Consequently, proper concentrations of the immobilized ligand and the eluent are important. An increase in the concentration of Con A linked to BC has not only a positive influence (increase of binding capacity) on the properties of this carrier; an increase in the value of I (Table 1) resulted in an increase of the amount of enzyme adsorbed, but at the same time the molar ratio of invertase to Con A decreased substantially. This ratio was found to be 0.094 and 0.024 at Con A concentrations of 0.9 and 5.17 mg per ml of gel, respectively. Decrease of the invertase/immobilized Con A molar ratio is caused by the binding of one enzyme molecule to a greater number of lectin molecules, which results in a more difficult release of the adsorbed invertase (Fig. 5, Table 3). Elution of enzyme from the carrier with a low Con A content is evidently much more effective than from that with a higher concentration. As a result, a concentration less than 1.5 mg Con A per ml of gel is advantageous since the recovery can be enhanced to 94%. This was also proved by the experiments with small columns; nevertheless, recoveries on this scale reached only 80% (0.92 mg Con A per ml of gel). A significant factor in these experiments was the time-dependence of the eluent (α-mMP) effect, because a 6-h period of action was necessary to equilibrate the batch system. In column experiments, the elution reagent should be allowed to react for at least 2 h in order to obtain a substantial improvement in the elution characteristics. As mentioned above, a longer time of interaction of the elution agent with the bound invertase has a positive effect on cleavage of the strong biospecific binding. It is worth noting that the amount of invertase immobilized on the carrier before and after elution with eluent can be directly monitored with the flow microcalorimetric method.

Acknowledgement

We thank Dr. E. Stratilová for the FPLC experiments.

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