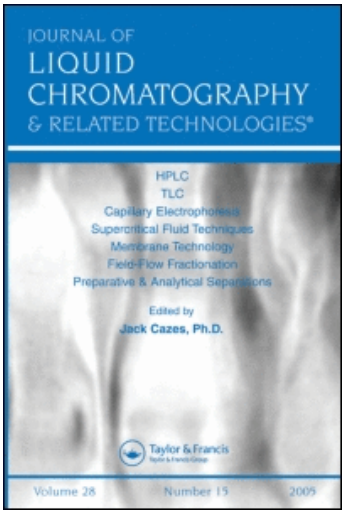


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# Effect of Dextran Filling in Macroporous HEMA Sorbent on Its Behavior in Dye-Affinity Chromatography

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# EFFECT OF DEXTRAN FILLING IN MACROPOROUS HEMA SORBENT ON ITS BEHAVIOR IN DYE-AFFINITY CHROMATOGRAPHY

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## ABSTRACT

Macroporous and microparticulate poly(hydroxyethyl methacrylate) gel (HEMA) and HEMA containing pores filled with a dextran network were used as supports for immobilization of reactive dye Cibacron Blue 3G-A. The performance of both dextran-filled and non-filled HEMA sorbents, with various ligand densities, in dye-ligand affinity

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chromatography of several enzymes and of bovine albumin were studied. The obtained results show the positive effect of dextran filling on enzyme recovery and on binding capacity of the separation media.

## INTRODUCTION

The poly(hydroxyethyl methacrylate)-based gel Separon HEMA (HEMA) belongs to the organic polymer supports suitable for HPLC of proteins [1-4]. It involves a large number of hydrophilic groups on its surface, what is condition for a good protein recoveries, and for introduction of various selective groups by a number of common methods. The application of HEMA for immobilization of different ligands, and subsequently for affinity chromatography, were presented by Taylor and Marenchic [5,6]. However, hydrocarbon polymer backbone chains of HEMA cause certain hydrophobicity of the material. It results in undesirable hydrophobic interactions of the HEMA sorbents with proteins which often prevent the direct application of HEMA in affinity-based separations. The hydrophility of HEMA surface can be increased by various low-molecular-weight, or better, polymeric modifiers.

In the present paper the use of macroporous and microparticulate HEMA particles with pores filled with crosslinked dextran (HEMA-D) as a support for high-performance affinity chromatograpfy (HPLAC) is described. The preparation, the permeation properties and the preliminary study of elution behaviour in dye-affinity chromatography of various dextran-filled HEMA sorbents were objects of study in our previous papers [7,13]. The dextran-HEMA composite sorbent showed suppressed nonspecific interaction with lactate dehydrogenase in dye-affinity chromatography, as compared with non-filled HEMA support. The aim of present work was to find out how the dextran filling effects the binding capacity of chromatographic media and the recovery of chromatographed enzymes, when different amount of reactive dye Cibacron Blue was bound to the supports. The positive effect of dextran filling on all chromatographic properties studied was found.

## EXPERIMENTAL

### Materials

Separon HEMA 1000, particle size 10 m was purchased from Tessek (Prague, Czech Republic). Dextran D-40, $M_W$=40 000 (Biotika, Slovenská Lupča, Slovak Republic) and 1,4-butanediol diglycidyl ether (Aldrich, Milwaukee, USA) were used for modification of sorbent. Cibacron Blue 3G-A (CB) was purchased from Ciba-Geigy (Basle, Switzerland). Lactate dehydrogenase (LDH; E.C.1.1.1.27) from beef flank muscle was prepared as crude lyophilizate (4 U/mg of lyophilizate) [8]. Other enzymes, such as LDH from rabbit muscle, malate dehydrogenase from pig heart (MDH; EC 1.1.1.37), glucose-6-phosphate dehydrogenase from yeast (G-6-PDH; EC 1.1.1.49) and glycerol kinase from yeast (GK; EC 2.7.1.30) were from Fluka (Buchs, Switzerland). Hexokinase from yeast (HK; EC 2.7.1.1.) and pyruvate kinase from rabbit muscle (PK; 2.7.1.40) were from Serva (Heidelberg, Germany). Bovine serum albumin was from SEVAC (Prague, Czech Republic), NADH from Reanal (Budapest, Hungary).



### Apparatus

Equipment for gradient HPAC included LKB 2152 HPLC gradient programmer; LKB 2150 HPLC high pressure pump; LKB 2151 detector (all LKB, Bromma, Sweden), automatic injector and collector (Gilson typ 4, Viliers-Le-Bel, France). Date were collected and evaluated with Data Apex integrator software ( Datapex, Prague, Czech Republic) programed for HPLC analysis . Columns were filled on Pneumatic HPLC pump (Knauer, Berlin, Germany). Spekol 11 and Specord M 400 (both from Carl Zeiss, Jena, Germany) were used for spectrophotometric determination of dyes, enzymes and proteins.

### Methods

Dextran was incorporated into macropores of HEMA and subsequently cross-linked with 1,4-butanediol diglycidyl ether according

the procedure described previously [7,13]. The mass ratio of incorporated dextran vs. HEMA was 0.32 g/g. The amount of cross-linking agent added was 3.10-4 mol per gram of dextran.

Preparation of sorbents for scanning electron microscopy was performed using procedure of fixation with cacodylate buffer and glutaraldehyde as described by Casson and Emery [9]. The samples were examined in scanning BS 301 electron microscope (Tesla, Brno, Czech Republic).

The described technique of modifying of HEMA and HEMA-D with CB was used [7], where the mass ratio of sorbent : CB were 1 : 0.016-0.070. The degree of substitution was determined spectrophotometrically at 610 nm from the difference between the initial amount of CB in the reaction mixture and the residual amount of CB in the washing solution.

The activities of LDH, MDH, G-6-PDH, GK, HK and PK were determined spectrophotometrically at 340 nm (10) and the protein content was determined according to the method of Bradford at 595 nm [11].



## Chromatographic experiments

### *Elution experiments*

The described procedure [7] was applied in this case. The glass columns were filled with 0.3 g of dyed sorbents (1.5 x 1 cm I.D.) and equilibrated with 50 mmol/l phosphate buffer pH 7. The solutions of enzymes (ca 15 U of LDH/0.2 ml, 10 U of MDH/0.2 ml) or bovine serum albumin (ca 2 mg/0.5 ml) were loaded. The bound proteins were eluted with water solution of 1.3 mol/l KCl (LDH, MDH) or 0.5 mol/l KSCN (BSA). The flow-rate was 0.2 ml/min. The activity of enzymes and protein content were determined in 1 ml fractions.

### *Loading experiments*

The same dyed columns as described above were used for loading experiments. The columns were saturated with solution of LDH from bovine muscle (18 U/ml). The saturation of LDH was controlled by

determination of activity of unbound LDH in 3 ml fractions. The bound LDH was eluted after the saturation with 1.3 mol/l solution of KCl. The recoveries of LDH activities were determined in all fractions.

*HPAC experiments*

Mixtures of enzymes were separated on a CB-HEMA-D column (12.5 x 0.6 I.D.) with 16.03 μmol of immobilized CB per g of sorbent. 20 ml of enzymes from rabbit muscle (LDH and PK) or yeast enzymes solution ( HK, GK and G-6-PDH) were loaded. The flow rate was 1 ml/min, pressure 2 - 6 MPa. The bound enzymes were eluted with a KCl gradient (0-1.5 mol/l). UV absorbance at 280 nm was monitored and the enzyme activities in 1.2 ml fractions were determined in the effluent.



## RESULTS and DISCUSSION

Dextran gel was created within macropores of rigid microparticulate poly(hydroxyethyl methacrylate-co-ethylene dimethacrylate) resin (HEMA). The preparation and properties of dextran-filled HEMA supports (HEMA-D) were described more in detail previously [7,13]. The non-filled and dextran-filled HEMA supports were studied by scanning electron microscopy (SEM). The swollen dextran network usually can not withstand the conditions of SEM sample preparation. Therefore, the porous structure of incorporated dextran was fixed by the method of Casson and Emery [9] before electron microscopy study. Figure 1 shows the SEM electron micrographs of the particles of HEMA and HEMA-D. It is possible to see that filled HEMA has smoother surface with rare presence of macropores in comparison with non-filled one. No differences were seen if the fixing procedure was not applied to HEMA- D.

The incorporated dextran gel has its own porous structure with smaller pore size than those of original HEMA. The change in porous structure of support due to filling with dextran was seen on size-exclusion chromatographic calibration curves. It was found previously

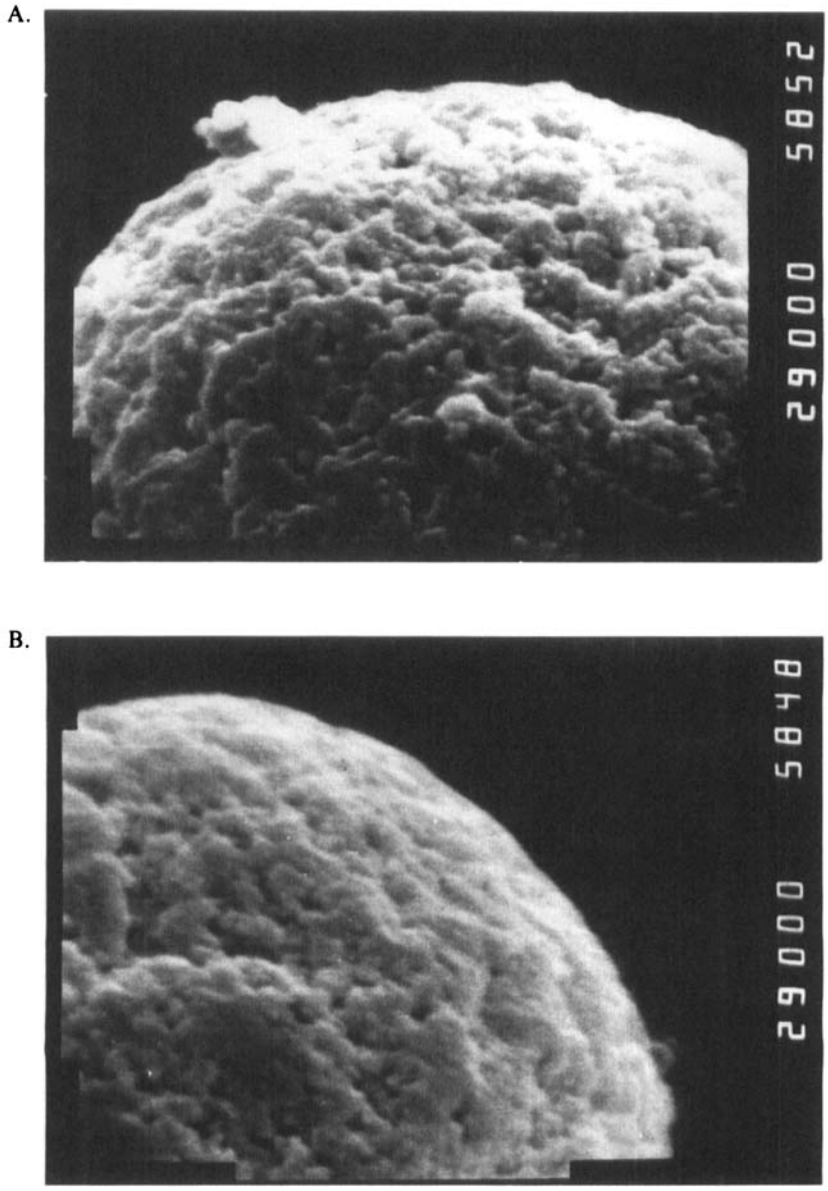


FIGURE 1. Scanning electron micrograph of HEMA (A) and HEMA-D (B). Original magnification: 29,000X, Present magniification 18,850X.

that permeation properties of dextran-filled HEMA depend on amounts of both incorporated dextran and crosslinking agent as well as on the porous structure of the starting HEMA [13]. The exclusion limits of HEMA and HEMA-D supports used in the study, as determined by SEC calibration for dextrans, were 1,000 and 400 kDa, respectively. SEC calibration showed that proteins with molecular weights under 700 kDa were still able to penetrate the incorporated dextran network.

Reactive dye Cibacron Blue (CB) was immobilized on both the original Separon HEMA 1000 (HEMA), and dextran-filled HEMA (HEMA-D), by the procedure described previously [7]. In the first series of experiments with dyed sorbents, we focused on the study of recoveries of selected proteins from both supports. Lactate dehydrogenase (LDH), malat dehydrogenase (MDH) and bovine serum albumin (BSA) were used for this purpose. The proteins were loaded at constant conditions and then eluted with equilibration and eluting buffers. The activities determined in effluents are summarized in Table 1. The differences in elution allows us to compare binding abilities of the sorbents studied. All proteins used in the study showed improved specific binding to the affinity ligands immobilized on dextran-filled HEMA, in comparison with non-filled support. This is probably due to suppression of non-specific interactions by dextran filling and improved accessibility of CB-ligands which are spread on the incorporated dextran network.



The loading characteristics of LDH expressed as breakthrough curves are shown in Figure 2. It shows that dextran- filled HEMA with immobilized CB can bind larger amount of enzyme than non-filled HEMA of about the same total ligand concentration.

Since amount of immobilized ligand seems to be a factor which significantly influences the effectivity of separation, the effect of this one on enzyme recovery was studied more in detail. Two different approaches were used in the recovery study. First, a constant amount of LDH from bovine muscle (15 U, ca 2.5 mg) was loaded (small loading - elution experiments on page 4)on all sorbents and eluted with both equilibration and eluting buffers. In another set of experiments, the sorbents were fully loaded (loading experiments on page 4) with LDH and recovered activities were determined. Recoveries, purification

TABLE 1

The recoveries of enzymes and BSA from CB-HEMA and CB-HEMA-D sorbents

| Support | Dye content (μmol CB/g) | Recovery (%) Elution with | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Equilibration buff. | | | | 1.3 M KCl | | | 0.5 M KSCN |
| | | LDH RM | LDH BM | MDH | BSA | LDH RM | LDH BM | MDH | BSA |
| CB-HEMA | 9.6 | 0 | 3.4 | 2.4 | 44.0 | 68.0 | 68.2 | 62.4 | 20.1 |
| CB-HEMA-D | 7.6 | 1.7 | 1.2 | 0 | 60.6 | 68.6 | 94.3 | 87.0 | 36.1 |



factors and binding capacities of bovine muscle LDH for CB-HEMA and CB-HEMA-D sorbents of various ligand densities, as determined in these experiments, are summarized in Table 2. All columns were thoroughly washed with equilibration buffer after enzyme loading. Only a small part of enzyme activity (0.5-2.9%) was released in this step (at small loading).

The data in the Table 2 are obtained from the activities determined in effluent by using 1.3 M solution of KCl as eluting buffer. All of the important parameters of affinity sorbents such as recoveries, binding capacities, and purification factors are improved if dextran-filled support is used instead of bare HEMA. As expected, the binding capacities of both kinds of sorbents increased with increasing amount of ligand bound, however, the dextran-filled sorbents showed significantly higher binding capacities then the non-filled ones at the same dye content. The binding capacity related to dye content was about 26 U/ μmol CB in the case of CB-HEMA and about 42 U/ μmol CB for CB-HEMA-D

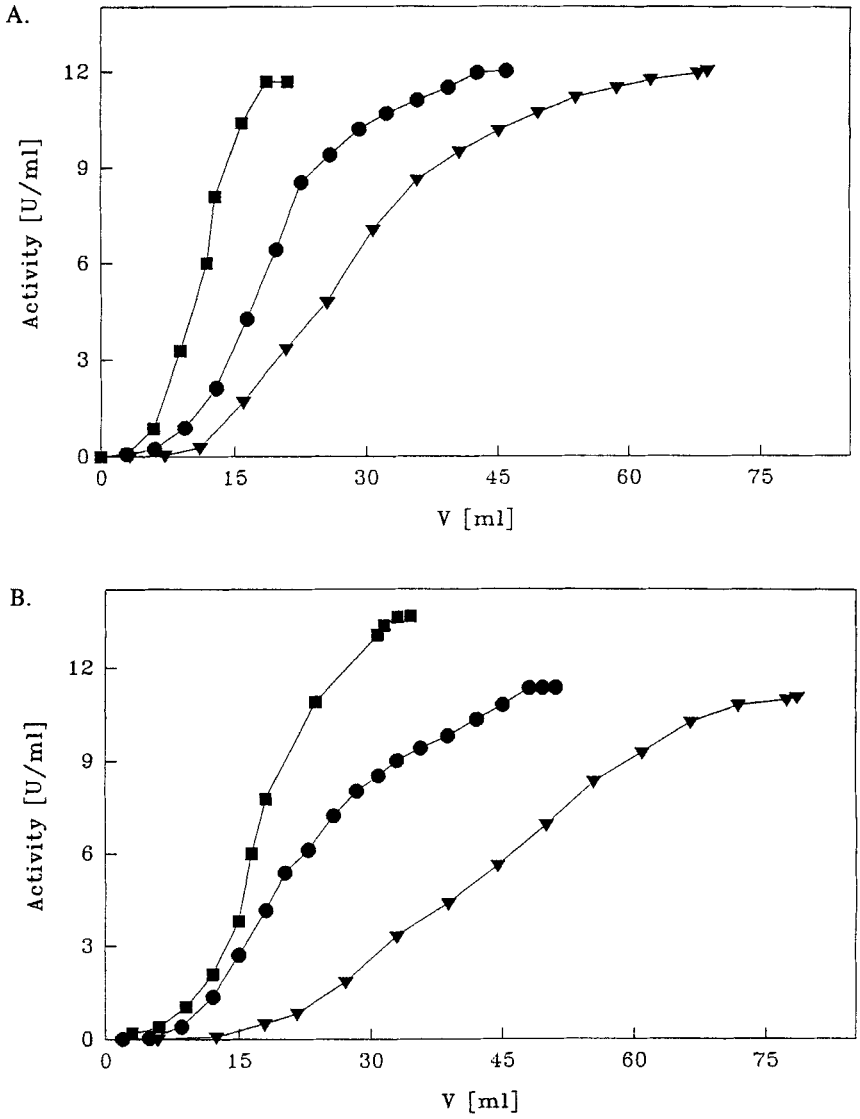




*FIGURE* 2. Breakthrough curves of LDH from rabbit muscle obtained on :

A - CB-HEMA
- ■ - 9.6 μmol CB/g
- ● - 14.4 μmol CB/g
- ▼ - 26.3 μmol CB/g

B - CB-HEMA-D
- ■ - 7.6 μmol CB/g
- ● - 15.9 μmol CB/g
- ▼ - 26.5 μmol CB/g

TABLE 2

Results from experiments with loading and elution of LDH-bovine muscle from CB-HEMA and CB-HEMA-D sorbents

| | | Small loading | | Full loading | |
|---|---|---|---|---|---|
| Support | Dye content (μmol CB/g) | Recovery (%) | Purification factor | Recovery (%) | Binding cap. (U/mg) |
| CB1-HEMA | 9.6 | 68.2 | - | 40.7 | 253.6 |
| CB2-HEMA | 14.4 | 86.0 | 9.3 | 60.4 | 390.0 |
| CB3-HEMA | 26.3 | 63.0 | 8.2 | 49.9 | 659.0 |
| CB1-HEMA-D | 7.6 | 94.3 | 21.4 | 82.3 | 342.6 |
| CB2-HEMA-D | 15.9 | 95.0 | 19.7 | 73.3 | 700.6 |
| CB3-HEMA-D | 26.5 | 78.7 | 14.1 | 44.7 | 942.3 |



sorbents. It means that the same number of affinity ligands can bind about 1.6 times higher amount of enzyme if the ligands are spread on the incorporated dextran network, in comparison with ligands bonded only on the surface of starting HEMA support. The higher capacity of CB-HEMA-D sorbents could be caused also by effect of stronger interaction of LDH with CB-dextran conjugate than CB.(14)

Figure 3 compares the recoveries and the binding capacities for the both small and full loadings of LDH. Even if the three-point-plots do not allow us to speak about dependency between the recovery and the binding capacity or dye content, respectively, some tendencies can be seen from such a graph. The interval between lower (full loading) and upper (small loading) curves corresponds to the recoveries which are possible to obtain for whole range of possible loadings for the both CB-HEMA and CB-HEMA-D sorbents. The recoveries are higher for dextran-filled sorbents, but the tendency of decreasing recovery at high concentration of immobilized dye can be seen for the both kinds of

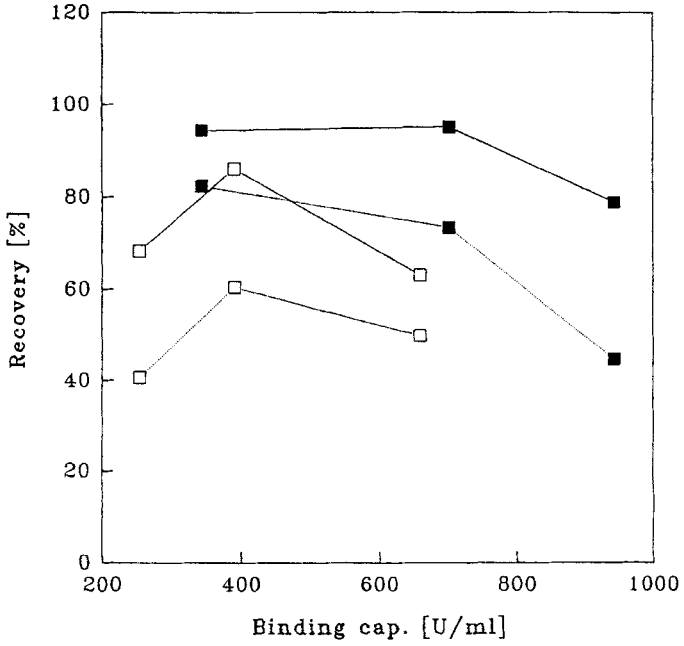


FIGURE 3. Graphical expression of the relation between the binding capacity of affinity sorbent and the enzymatic recovery of LDH. Upper curves corresponds to small loading of LDH, lower curves corresponds to full loading of LDH.

□ - CB-HEMA

■ - CB-HEMA-D



sorbents. It is probably due to multivalent interaction between enzyme molecules and affinity ligands that the binding capacity still increases with increasing amount of dye bound, however, the recovery begin to decrease at high ligand densities. Decrease in recovery of LDH from CB-HEMA sorbents with lower binding capacities may be caused by non-specific interaction of enzyme with support. It did not occur in the case of CB-HEMA-D sorbents which corresponds with our previous knowledge [7,13] that dextran filling suppress the hydrophobic interactions with HEMA surface.

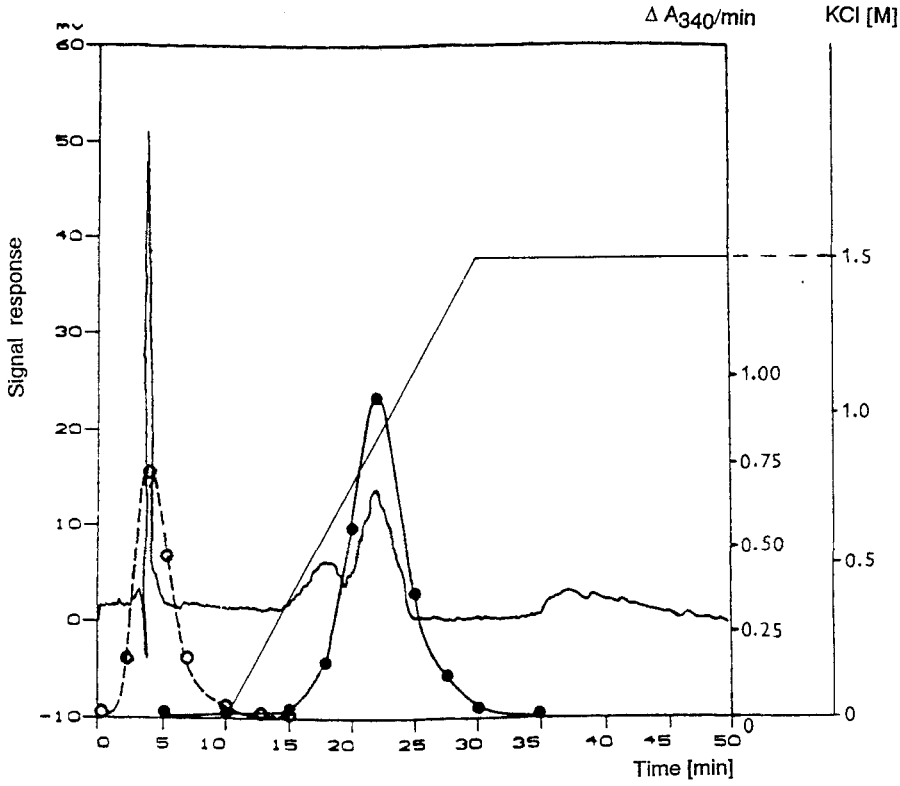


FIGURE 4. HPAC chromatogram of mixture of LDH and PK enzymes from rabbit muscle on CB-HEMA-D (16.03 mol CB/g) with a linear gradient of KCl concentration.

● - activity of LDH

○ - activity of PK

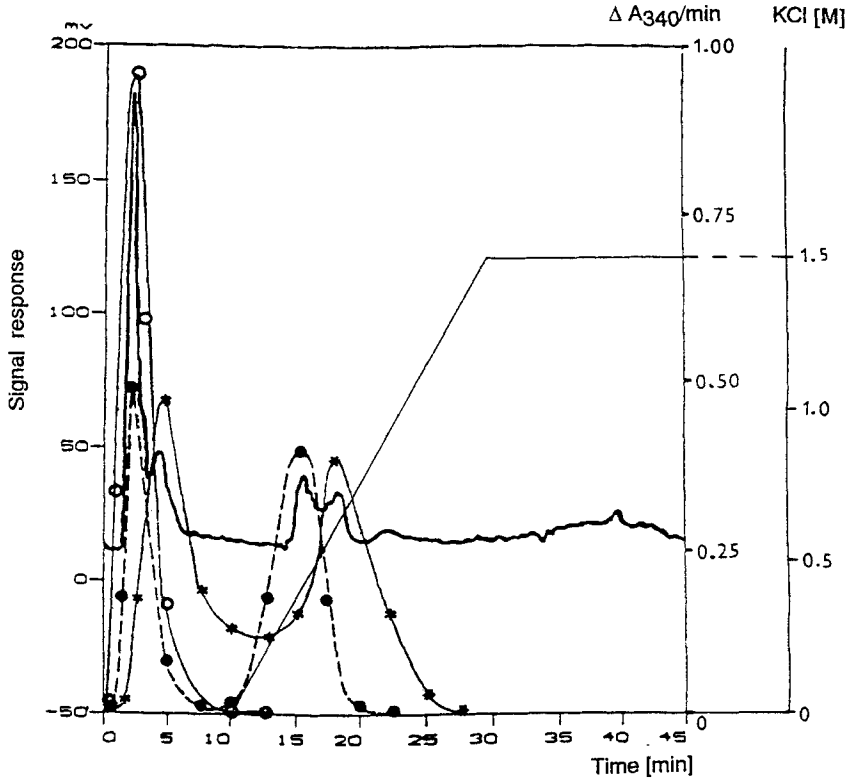


FIGURE 5. HPAC chromatogram of mixture of yeast enzymes GK, HK and G-6-PDH on CB-HEMA-D (16.03 mol CB/g) with a linear gradient of KCl concentration.

○ - activity of GK

● - activity of HK

✱ - activity of G-6-PDH

Columns packed with CB-HEMA-D sorbent containing 16.03 μmol CB/g were used for HPAC. Figure 4 shows separation of LDH and PK from rabbit muscle and Figure 5 shows HPAC of mixture yeast enzymes GK,HK and G–6–PDH. Both the UV- traces, and the activity assays, were used for detection of chromatographed enzymes. Several enzymes differing in the strength of affinity interaction with sorbent can be separated with high recoveries in a single injection.

## CONCLUSION

The crosslinked dextran in macropores of HEMA-sorbent not only suppresses nonspecific sorption in dye-ligand chromatography, supports the LDH-CB interaction but also allows speading of the immobilized dye in the whole volume of the macropores. In such case, the ligands are better accessible for both molecules of chromatographed proteins and also a elution agent. As a result, an improved recovery of separated enzyme even at low ligand densities was obtained .



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