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Investigation of affinity partition chromatography using formate dehydrogenase as a model[a]

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ABSTRACT

The enzyme formate dehydrogenase (FDH) was purified from the crude extract of *Candida boidinii* by affinity partition chromatography. The partition coefficient, K, of the enzyme was selectively increased by adding polyethylene glycol–Procion Red HE3b as an affinity ligand to the mobile phase in the chromatographic column. The increased K value led to early elution of the enzyme–ligand complex and separated the target protein from the main peak of the contaminants.

INTRODUCTION

Partitioning in aqueous two-phase systems was introduced by Albertsson [1] as a mild method for separating cell organelles and macromolecules. The selectivity of extraction may be increased by adding biospecific ligands covalently bound to one of the phase-forming polymers. This approach has been successfully employed for the purification of enzymes [2–9] and nucleic acids and oligonucleotides [10,11]. Commonly used general ligands for enzyme extraction are the triazine dyes [12,13], which are cheaply available commercially. Application of polyethylene glycol (PEG)–Procion Red HE3b has been investigated for the large-scale extraction of formate dehydrogenase from *Candida boidinii* [14,15].

Newly developed matrices for liquid–liquid partition chromatography (LLPC) with aqueous two-phase systems [16] combine the favourable properties of partitioning with the advantages of column chromatography. It also offers the possibility of a convenient purification method: affinity partition chromatography. Incorporation

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of ligands into the PEG–dextran system will lead to the formation of protein–ligand complexes depending on the ligand concentration and ionic strength. Fig. 1 shows schematically the simplest form of a 1:1 complex. A detailed analysis of the linked equilibria involved has been published [15]. The complex formation will selectively increase the partition coefficient and therefore change the elution volume of the target protein.

The fractionation of DNA restriction fragments has been accomplished, including base-specific DNA ligands, covalently bound to PEG in the mobile phase of a partition column [11,17], making use of the concept of affinity partition chromatography for the separation of very similar macromolecules.

In this work we investigated the application of affinity partition chromatography for the purification of formate dehydrogenase (FDH) from the crude extract of *Candida boidinii* as a model using Procion Red HE3b as the ligand. This enzyme and ligand were chosen as the system is well documented in the literature [14,18]. The method is appealing because in principle it allows the selective and early elution of the desired product from a partition column. Three methods to enhance the partition coefficient of the FDH in the partition column were investigated: first, the PEG–Procion Red HE3b was added to the sample only; second, the PEG–Procion Red HE3b was present in the sample and also at a constant, low concentration in the mobile phase, so that non-equilibrium conditions prevail and the elution volume will depend on the local concentration, dissociation rates and column length; and third, the influence of the ligand concentration in the mobile phase was tested.

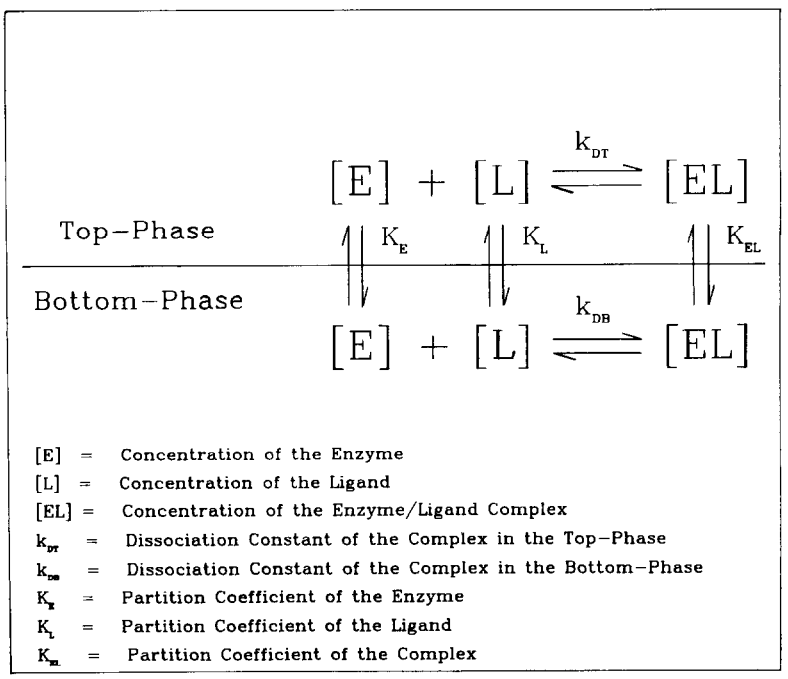

Fig. 1. Schematic representation of the linked equilibria of partition and enzyme–ligand association for the simplest case of a 1:1 complex.

EXPERIMENTAL

Materials

The support material LiParGel 750 was a gift from E. Merck (Darmstadt, F.R.G.). The triazine dye Procion Red HE3b was a gift from ICI Germany (F.R.G.). PEG 20000 was obtained from Merck Schuchard (München/Hohenbrunn, F.R.G.). Dextran Pl 500 (nominal molecular weight, MW = 500 000 dalton) and Vc 40 (MW = 40 000 dalton) were bought from Pfeifer und Langen (Dormagen, F.R.G.). The proteins were purchased from the following sources: albumin and peroxidase from E. Merck, ferritin from Pharmacia (Uppsala, Sweden), transferrin and myoglobin from Serva (Heidelberg, F.R.G.), α-chymotrypsinogen A Type II from Sigma (St. Louis, MO, U.S.A.) and formate dehydrogenase from Boehringer (Mannheim, F.R.G.).

All other chemicals were of analytical-reagent grade.

Preparation of the affinity ligand

The triazine dye Procion Red HE3b was coupled to PEG 20 000 according to Cordes and Kula [14].

Phase preparation

The concentrations of PEG and dextran in the various phase systems used for partition chromatography were taken from phase diagrams established by Albertsson [1], corresponding to a phase ratio of 1:1. The PEG- and dextran-rich phases were prepared with stirring, mixing the polymers with water and salts in the appropriate amounts for 1 h. The pH was adjusted by adding concentrated phosphoric acid or potassium hydroxide solutions. The phases were allowed to settle under gravity in a separating funnel; after 1–2 h the phases were separated. The PEG-rich phase was allowed to stand in order to obtain a totally clear top phase, removing the small amount of bottom phase after settling for several days.

The mobile phase containing the affinity ligand Procion Red HE3b was obtained by dissolving solid PEG Procion Red HE3b in the top phase of an appropriate system. The concentration of ligand was determined spectrophotometrically at 542 nm, using a laboratory-determined molar absorption coefficient of 36 200 l $mmol^{-1}$ cm^{-1}. In order to prevent oxidative degradation of the PEG, the mobile phase was stored under nitrogen.

Preparation of columns

The LiParGel 750 support was equilibrated with the phase system and packed into the chromatographic columns (Superformance system; E. Merck) according to Müller [16].

Column chromatography

The chromatographic columns were integrated into a Pharmacia FPLC system. Fractions of 1 or 0.5 ml were collected using a Frac 100 fraction collector (Pharmacia).

Sample preparation

Exactly weighed amounts of the analyte substances were dissolved in the mobile

phase and centrifuged (3000 *g*, 5 min), removing undissolved solid material. The samples were applied to the column using an MV 7 valve (Pharmacia) and the programme was started.

Determination of partition coefficient

The partition coefficients of pure proteins were determined by mixing the sample with equal volumes of top and bottom phase, separating the phases by low-speed centrifugation (1800 *g*, 10 min) and measuring the absorbance at 280 nm (Shimadzu UV 160) in each phase. The activity of formate dehydrogenase was determined according to Schütte *et al.* [19] by an enzymatic assay. The partition coefficient, K, was calculated as the ratio of the absorbance or enzyme activity in the top to that in the bottom phase according to

$$\frac{C_T}{C_B} = \frac{f_T}{f_B} = K = \text{constant} \tag{1}$$

where C_T and C_B are the concentrations of the component in the top and bottom phase and f_T and f_B are the activities of the component in the top and bottom phases.

Determination of protein concentration

The protein concentration was determined according to Bradford [20] using bovine serum albumin for calibration.

Determination of the column parameters V_s and V_m

The physical parameters of the column were obtained as described previously [21] using a special set of calibration standards. Their elution volumes from the column and the K values measured by independent batch experiments were fitted to eqn. 2 [21] derived by Martin and Synge [22]. This fit, correlating elution volumes and K values, yields sufficiently accurate values for V_s and V_m:

$$V_e = V_s/K + V_m \tag{2}$$

where V_e = elution volume, V_s = volume of the stationary phase, V_m = volume of the mobile phase and K = partition coefficient.

The results shown in Fig. 2 were obtained for the column equilibrated with a phase system of PEG 20 000 and dextran 500. V_s was found to be 5.75 ml and V_m 10.0 ml. The broken line in Fig. 2 illustrates the relationship between the partition coefficient and elution volume in the given column.

Stabilization of eluents and column against microbial degradation

Sodium azide is commonly used to protect phase components against microbial contamination. Formate dehydrogenase is completely inhibited by sodium azide [19]. Therefore, we searched for a substitute and eventually selected chloroacetamide. The addition of 5 g/l of chloroacetamide has prevented contamination of the column and the mobile phases for more than 2 years already, although storage and operation were carried out at ambient temperature.

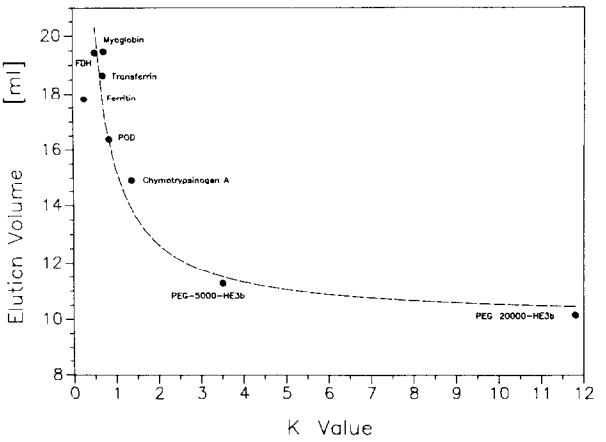

Fig. 2. Elution volume of a set of standard proteins, PEG 5000 and PEG 20 000 triazine dye derivatives in a LiParGel 750 column (30 × 1 cm I.D.). System composition, 2.7% PEG 20 000, 4.5% dextran 500, 75 m*M* potassium bromide, 10 m*M* phosphate buffer (pH 7); temperature, 30°C; flow-rate 1 ml/min. The graph illustrates the relationship between the partition coefficient and the elution volume in the given column. V_s = 5.75 ml; V_m = 10.0 ml.

Cell cultivation and harvesting

The cultivation of *Candida boidinii* on methanol as carbon and energy source was carried out according to Sahm and Wagner [23] in a 10-l Biostat E bioreactor (Braun Melsungen, Melsungen, F.R.G.) under maximum aeration. The cells were harvested using a KA2-06-075 chamber centrifuge (Westfalia Separator, Oelde, F.R.G.).

Preparation of crude extracts

The cells were suspended in 0.05 *M* potassium phosphate buffer (pH 7.5) to give a 40% (w/v) suspension and disrupted by wet milling in a Netsch LME 0.5 mill (Netsch Feinmahltechnik, Selb, F.R.G.) for 5 min at 2500 rpm using 80% (v/v) glass beads of 0.75 mm diameter. After addition of 0.5% (v/v) of a polyethyleneimine (PEI) solution [10% (w/v), pH 7.0, adjusted with HCl], the homogenate was centrifuged 10 min at 2000 *g* at 4°C and the sediment discarded. By treatment with PEI, nucleic acids are precipitated and the removal of cell debris is improved. The result was a crude extract free from solid material, which could be passed without problems through a microporous filter of 0.45-μm pore size. The crude extract was stored in aliquots at −20°C for the experiments described below.

RESULTS AND DISCUSSION

First series: addition of ligand to the sample

Addition of ligand only to the sample loaded on the column would be a cost-effective way of bringing about affinity partition chromatography and simple to operate with changing products. The ligand concentration in the sample should saturate the enzyme at the start of the chromatographic run. Multi-stage partitioning in the column will lead to dissociation and separation of the complex on the column, depending on K_L and K_{EL} (Fig. 1) and the kinetics of dissociation.

First we used a column equilibrated with a phase system of 5.45% PEG 6000 and 9.75% dextran 40 (MW = 40 000 dalton), 4 m*M* potassium bromide and 6.3 m*M* phosphate buffer (pH 7.5). The low salt concentration was chosen in order to obtain the highest efficiency of the affinity partitioning while keeping the dissociation constant of the enzyme–ligand complex small. The temperature of the column was adjusted to 23°C. To examine the general properties of the enzyme–ligand interaction in the column a partially purified formate dehydrogenase (4.8 U/mg; 12 U/ml) was used. In the absence of ligand, FDH activity was detected in the second peak in the chromatogram (Fig. 3a) at an elution volume of 25.4 ml, corresponding to a partition coefficient of 0.33. On adding increasing amounts of the affinity ligand PEG 20 000–Procion Red HE3b (PEG–Red) to the enzyme solution applied to the column,

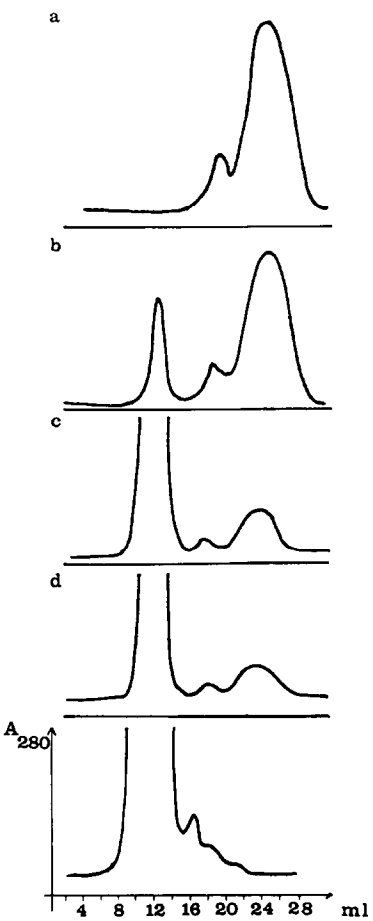

Fig. 3. Elution patterns of prepurified FDH with 12 U/ml enzymatic activity. Sample size, 100 μl. System composition, 5.45% PEG 6000, 9.75% dextran 40, 4 m*M* potassium bromide, 6.3 m*M* phosphate (pH 7.5); temperature, 23°C; flow-rate, 0.3 ml/min. The decrease in the FDH peak with increasing amounts of PEG–Red added to the sample is illustrated: PEG–Red concentration, (a) 0, (b) $0.146 \cdot 10^{-3}$, (c) $0.511 \cdot 10^{-3}$, (d) $1.022 \cdot 10^{-3}$, (e) $3.651 \cdot 10^{-3}$ *M*.

TABLE I

YIELD OF FDH IN THE ENZYME–LIGAND PEAK AND THE FDH PEAK ON INCREASING THE CONCENTRATION OF PEG–RED IN THE SAMPLE

System composition: 5.45% PEG 6000, 9.75% dextran 40, 4 m*M* potassium bromide, 6.3 m*M* phosphate buffer (pH 7.5), 23°C, flow-rate 0.3 ml/min, column size 30 × 1 cm I.D., sample size 100 µl; sample, prepurified FDH, 12 U/ml.

Concentration of PEG–Red (10^{-3} *M*)	Yield of FDH in the PEG–Red peak (%)	Yield of FDH in the FDH peak (%)	Total recovery (%)
0.146	7.8	90.2	98.0
0.511	29.2	68.7	97.9
1.022	47.4	46.6	94.0
3.651	77.0	13.9	90.9

the elution volume of the enzyme changed as illustrated in Fig. 3 b–e and quantified in Table I. Owing to the shift of the enzyme activity into the PEG-Red peak the FDH main peak decreased. However, with increasing concentration of the ligand in the sample the peak corresponding to PEG–Red becomes wider and wider and overlaps with the peak of the contaminants. Even with the highest amount of PEG–Red in the sample it was not possible to shift the FDH activity completely into the PEG–Red peak. A further increase in the concentration was not desirable owing to the increasing viscosity of the sample.

Because under the conditions employed the enzymatic activity could not be shifted completely into the PEG–Red peak and the ligand peak was not totally separated from the contaminants, we changed the system composition in order to increase the *K* value of the ligand. The higher *K* value should lead to earlier elution of the enzyme–ligand peak. On the basis of previous work we selected a phase system of PEG 20 000 and dextran 500 [24].

To find the optimum concentration of the polymers with respect to the *K* value of the PEG–Red we determined the partition coefficient of the affinity ligand as a function of the polymer concentration. The results are shown in Fig. 4. The ratio of the PEG concentration to dextran concentration was 1.0:1.5. At PEG concentrations above 5% the upper phase was difficult to clarify completely owing to the very small droplets of dextran retained in the top phase. Employing a UV monitor at 280 nm a totally clear eluate was needed in order to analyse the elution profile reproducibly. For the following experiments a system containing 4.5% PEG 20 000, 6% dextran 500, 5.5 m*M* potassium bromide and 6.3 m*M* phosphate buffer (pH 7.5) was selected. The *K* value of the PEG–Red was increased to 35 and the elution volume in the freshly prepared column was 7.5 ml, very close to the void volume.

On adding $1 \cdot 10^{-4}$ *M* PEG–Red to the enzyme sample, 38% of the FDH emerged in the enzyme–ligand peak and 62% in the main FDH peak. The yield was 100%. This is a significant improvement compared to the result given in Table I; nevertheless, the enzyme is eluted in two positions and the dissociation of the enzyme–ligand complex during the chromatographic run apparently cannot be suppressed sufficiently.

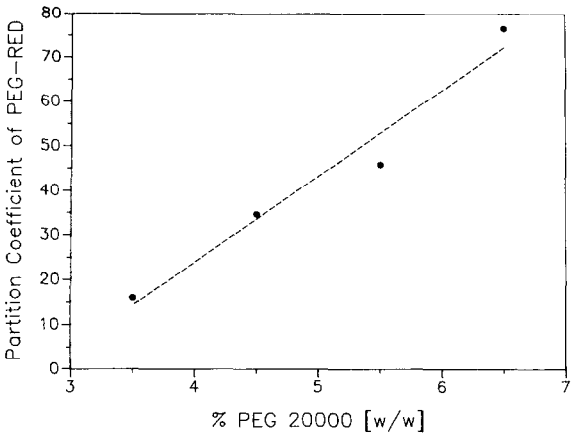

Fig. 4. Partition coefficient of the affinity ligand PEG 20 000–Procion Red HE3b as a function of the concentration of phase-forming polymers. The ratio of PEG to dextran concentration was 1:1.5.

Second series: addition of ligand to the sample and mobile phase

For the next series of experiments a crude extract of *C. boidinii* with 2.3 U/ml activity of FDH was employed. We added PEG–Red in various amounts to the sample and equilibrated and maintained PEG–Red at a low concentration of $2 \cdot 10^{-5}$ *M* in the mobile phase in the column. In this way, dissociation of the enzyme–ligand complex due to the changing concentrations of the free ligand should be minimized during the chromatography. Quantitative data are summarized in Table II.

The elution profiles are shown in Fig. 5a–c. At a PEG–Red concentration of $50 \cdot 10^{-5}$ *M* in the sample and $2 \cdot 10^{-5}$ *M* in the mobile phase the enzyme activity is shifted completely to the enzyme–ligand peak. The experiment was repeated twice to determine the reproducibility and the purification of the enzyme from the crude extract. The data are presented in Table III.

If the crude extract is heated for 10 min at 55°C in a water-bath to denature other

TABLE II

YIELD OF FDH IN THE ENZYME–LIGAND PEAK AND THE FDH PEAK USING DIFFERENT PEG–RED CONCENTRATIONS IN THE SAMPLE AND A CONSTANT CONCENTRATION OF $2 \cdot 10^{-5}$ *M* IN THE MOBILE PHASE

System composition: 4.5% PEG 20 000, 6% dextran 500, 5.5 m*M* potassium bromide, 6.3 m*M* phosphate buffer (pH 7.5), 23°C, column size 30 × 1 cm I.D., sample size 100 µl; sample, crude extract of *Candida boidinii*, 2.3 U/ml.

Concentration of PEG–Red in the sample (10^{-5} *M*)	Yield of FDH in the enzyme–ligand peak (%)	Yield of FDH in in the FDH main peak (%)	Total recovery (%)
2	40	60	100
10	63	37	100
50	100	–	93

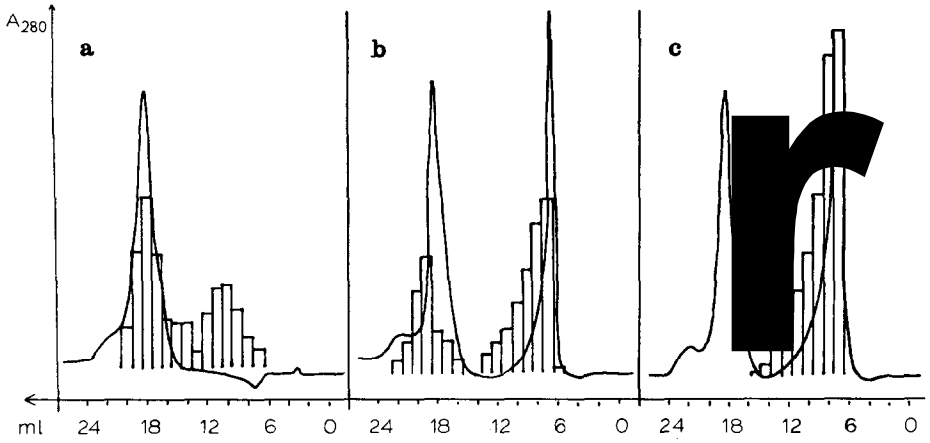

Fig. 5. Elution of FDH with increasing PEG–Red concentration in the sample (crude extract of *Candida boidinii*). The enzymatic activity in the peaks is indicated by histograms of the activity in the collected fractions. System composition, 4.5% PEG 20 000, 6% dextran 500, $2 \cdot 10^{-5}$ *M* PEG–Red, 5.5 m*M* potassium bromide, 6.3 m*M* phosphate buffer (pH 7.5); temperature, 23°C; column size, 30 × 1 cm I.D. PEG–Red ligand concentration in the sample: (a) $2 \cdot 10^{-5}$; (b) $1 \cdot 10^{-4}$; (c) $5 \cdot 10^{-4}$ *M*.

dehydrogenases and kinases competing for the ligand Procion Red HE3b [18,24], a higher specific activity is reached after the chromatography, 3.6 U/mg compared with 2.7 U/mg in the unheated sample. The yield decreased slightly to 78% after heat conditioning.

Third series: addition of ligand to the mobile phase

In a third series of experiments the ligand concentration in the mobile phase was varied while the sample was introduced without added ligand. The elution of the FDH was determined by an enzymatic test of the fractions collected. Fig. 6 shows the results. Because of the nature of partition chromatography, the elution volume is very sensitive to small changes in the *K* value in the range 0.2–3 (Fig. 2). Owing to the ligand binding

TABLE III

PURIFICATION OF FORMATE DEHYDROGENASE FROM THE CRUDE EXTRACT OF *CANDIDA BOIDINII* IN LIQUID–LIQUID PARTITION CHROMATOGRAPHY

System composition: 4.5% PEG 20 000, 6% dextran 500, 5.5 m*M* potassium bromide, 6.3 m*M* phosphate buffer (pH 7.5), 23°C, column size 30 × 1 cm I.D.; sample, 500 µl of crude extract of *C. boidinii* adjusted to a ligand concentration of $5 \cdot 10^{-4}$ *M* PEG–Red, the concentration of ligand in the mobile phase was $2 \cdot 10^{-5}$ *M*.

Specific activity of FDH in the crude extract (U/mg)	Specific activity of FDH in the enzyme–ligand peak (U/mg)	Purification factor	Yield (%)
0.58	2.62	4.5	100
0.64	2.94	4.6	84
0.54	2.48	4.6	92

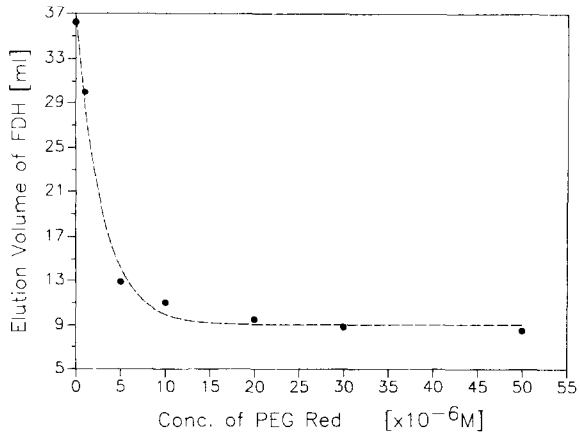

Fig. 6. Influence of the concentration of PEG 20 000–Procion Red HE3b in the mobile phase on the elution volume of FDH. Sample, 500 µl crude extract of *Candida boidinii*. System composition, 4.5% PEG 20 000, 6% dextran 500, 5.5 m*M* potassium bromide, 6.3 m*M* phosphate buffer (pH 7.5); temperature, 23°C; column size, 30 × 1 cm I.D. The elution volume of the FDH was determined by an enzymatic assay of the collected fractions.

and the resulting changes in the partition coefficient, the elution volume of the FDH decreases very rapidly until a ligand concentration of $5 \cdot 10^{-6}$ *M* and then more slowly, approaching the void volume of the column at $50 \cdot 10^{-6}$ *M* PEG–Red. The FDH activity obtained after the chromatographic separation is summarized in Table IV.

The low recovery is related to the decreasing stability of diluted FDH with time. The lowest yield is obtained in the most diluted fraction eluted from the column after 75 min. With decreasing dilution and a shorter time between injection and assay, the recovered amount of FDH increased. The fraction with 83% recovery was only 17 min in the column and three times more concentrated. The influence of dilution and time on the stability of FDH is also demonstrated with independent experiments in Fig. 7.

TABLE IV

ELUTION VOLUME AND ACTIVITY OF FDH WITH INCREASING PEG–RED CONCENTRATION IN THE MOBILE PHASE

System composition: 4.5% PEG 20 000, 6% dextran 500, 5.5 m*M* potassium bromide, 6.3 m*M* phosphate buffer (pH 7.5), 23°C, column size 30 × 1 cm I.D.; sample: 500 µl of crude extract of *C. boidinii*, 3 U/ml.

Concentration of PEG–Red (10^6 *M*)	Elution volume of the FDH (ml)	Volume of the FDH peak (ml)	Yield (%)
0	36	18	23
1	30	15	n.d.
5	13	16	43
10	11	9	n.d.
20	9.5	7	50
30	8.8	7	68
50	8.5	5	77

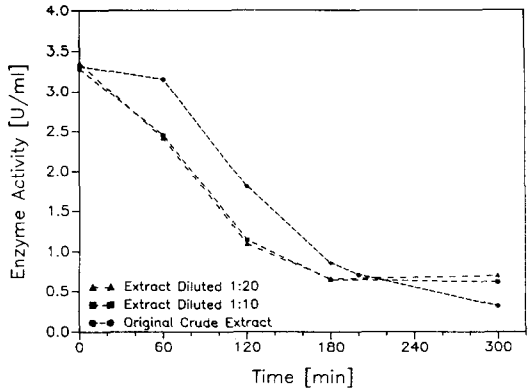

Fig. 7. Stability of FDH in the crude extract of *Candida boidinii* as a function of time at 23°C, pH 7.5 and three concentrations.

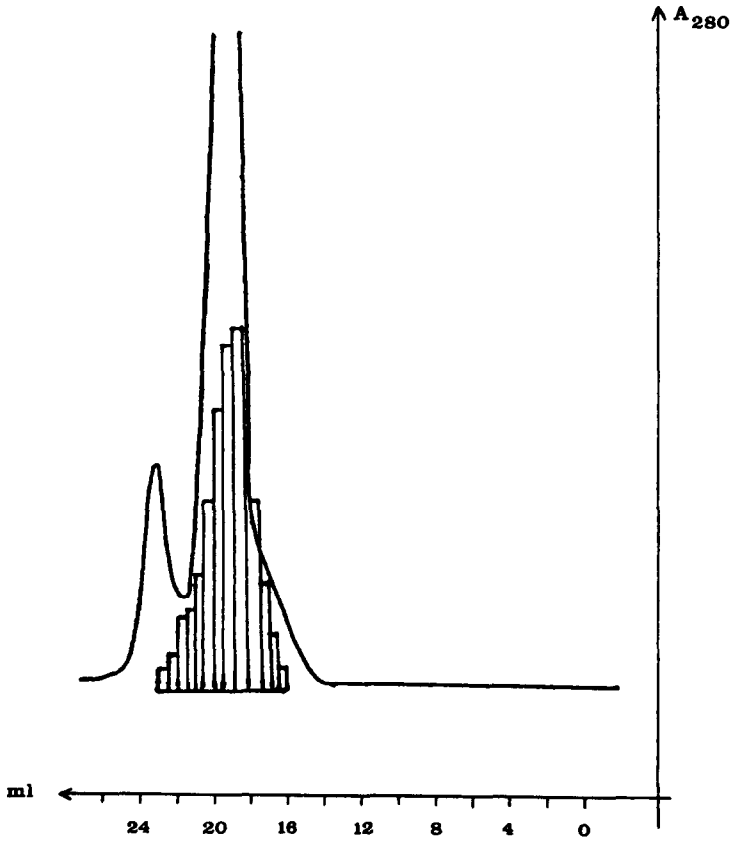

Fig. 8. Elution pattern of the crude extract of *Candida boidinii*. The elution volume of FDH indicated by the histogram of enzymatic activity in the collected fractions is 19.0 ml, which corresponds to a K value of 0.58. System composition, 2.7% PEG 20 000, 4.5% dextran 500, 75 mM potassium bromide, 12 mM phosphate buffer (pH 7.0); temperature, 30°C; column size, 30 × 1 cm I.D.; sample size, 100 μl; flow-rate, 0.6 ml/min.

In order to shorten the chromatographic separation, the viscosity of the phases was reduced by changing the concentration of the phase forming polymers and the temperature of the column was increased to 30°C. According to Fig. 5, 2.7% PEG 20 000 and 4.5% dextran 500 were selected in order to maintain a sufficiently high K value for the ligand. Further, the ionic strength was increased by adding 75 mM potassium bromide to the phase system to minimize the influence of the negatively charged groups of the support material [21]. Potassium bromide was chosen because it has a partition coefficient close to 1.0 and does not influence the elution of FDH and the ligand PEG–Red (see Fig. 10).

The elution pattern of the crude extract of *C. boidinii* under these conditions is shown in Fig. 8. The elution volume of FDH was 19.0 ml, which corresponds in this column (V_s = 5.25 ml, V_m = 10.0 ml) to a K value of 0.58. The ligand PEG–Red eluted at 10.13 ml, corresponding to a K value of 11.8. With $5 \cdot 10^{-5}$ M PEG–Red in the mobile phase, the elution volume of the FDH was reduced to 10.76 ml, corresponding to a K value of 6.9.

In order to find the maximum sample load, 100, 500 and 1000 μl of the heat-denatured crude extract were injected. The results are shown in Fig. 9. The elution volume of the enzyme–ligand complex increases with increasing sample size and eventually overlaps with the second peak. Therefore, the sample size was reduced to 500 μl in order to obtain a good separation for the purification of FDH from the crude extract. Table V gives the results.

To separate the PEG–Red from the FDH after the chromatographic run, solid potassium phosphate was added to the fractions containing FDH [14] to form a second PEG–salt phase system. A mixture of K_2HPO_4 and KH_2PO_4 was formulated to adjust the pH of the system to 7. To obtain a high yield of FDH, the influence of the

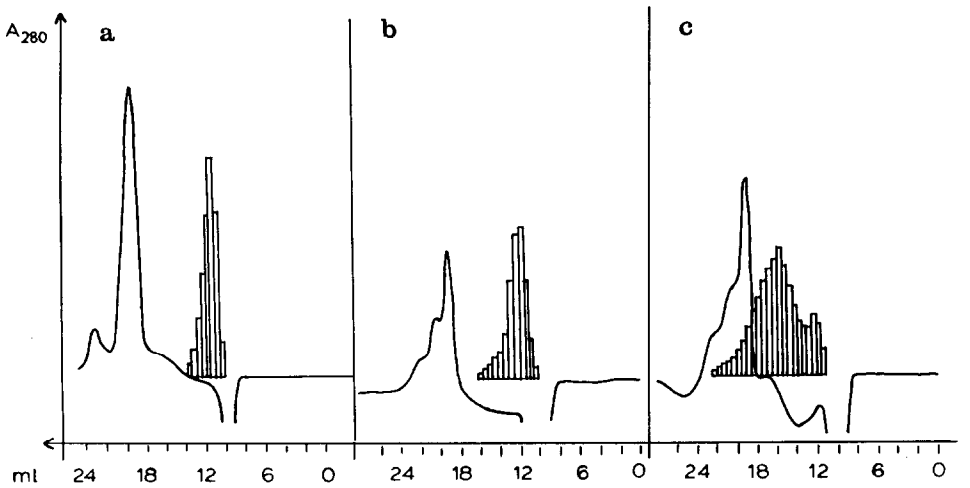

Fig. 9. Elution pattern of the crude extract of *Candida boidinii* under the conditions as in Fig. 8 but with $5 \cdot 10^{-5}$ M PEG–Red in the mobile phase. The elution volume of FDH increases with increasing sample size: (a) sample size = 100 μl, V_e (FDH) = 10.75 ml, K_{FDH} = 6.9; (b) sample size = 500 μl, V_e (FDH) = 11.97 ml, K_{FDH} = 2.7; (c) sample size = 1000 μl, V_e (FDH) = 15.8 ml, K_{FDH} = 0.9.

TABLE V

PURIFICATION OF FDH WITH PEG–RED IN THE MOBILE PHASE

System composition: 2.7% PEG 20 000, 4.5% dextran 500, 75 m*M* potassium bromide, 12 m*M* phosphate buffer (pH 7.5), 30°C, column size 30 × 1 cm I.D.; sample, 500 μl of crude extract of *C. boidinii*, heated for 10 min at 55°C, and with a concentration of $5 \cdot 10^{-5}$ *M* PEG–Red in the mobile phase.

Specific activity of FDH after heat conditioning (U/mg)	Specific activity of FDH after chromatography (U/mg)	Purification factor	Yield (%)
0.72	4.2	5.8	83
0.77	4.7	6.1	70

concentration of the phosphate on the volume ratio of the phases and the *K* value of the FDH were determined. The results are given in Table VI.

To avoid overlapping peaks with increasing sample size and to improve the separation, $5 \cdot 10^{-5}$ *M* PEG–Red was added to the sample. As in former experiments, the elution volume of the complex was reduced, but the maximum of the FDH activity emerged slightly later than the PEG–Red peak. This might be a consequence of the higher ionic strength of the phase system compared with the results reported in Figs. 2–6. To test the influence of the ionic strength on the dissociation constant of the enzyme–ligand complex, first the *K* values of FDH and PEG–Red were determined at various salt concentrations as shown in Fig. 10. An increasing concentration of potassium bromide did not change the *K* value of the PEG–Red and the *K*-value of FDH was slightly increased (elution volume decreased).

The elution volume of the enzyme–ligand complex was then investigated as a function of the salt concentration. The results are presented in Fig. 11. Using the scheme shown in Fig. 1, the following conclusion can be drawn: as the value of K_L was not affected by an increase in potassium bromide concentration (Fig. 10) and K_F varied

TABLE VI

VOLUME RATIO OF TOP AND BOTTOM PHASES AND PARTITION COEFFICIENT OF FDH AS A FUNCTION OF PHOSPHATE CONCENTRATION

Solid potassium phosphate was added to the eluate containing the FDH–ligand complex and a phase system (PEG–salt) formed. The volume ratio was determined after centrifugation (10 min, 2000 *g*) and the *K* value of FDH was determined by an enzymatic assay. Temperature, 25°C.

Concentration of potassium phosphate (%, w/w)	Volume of upper phase (ml)	Volume of lower phase (ml)	Volume ratio (top/bottom)	*K* value of FDH	Yield in the lower phase (%)
8[a]	–	–	–	–	–
9	1.1	3.5	1:3.18	0.0051	98
10	0.9	3.8	1:4.22	0.0028	99
12	0.7	4.0	1:5.71	[b]	n.d.
15	0.5	3.9	1:7.80	[b]	n.d.

[a] System remained homogeneous at this phosphate concentration.
[b] Precipitates formed at interphase.

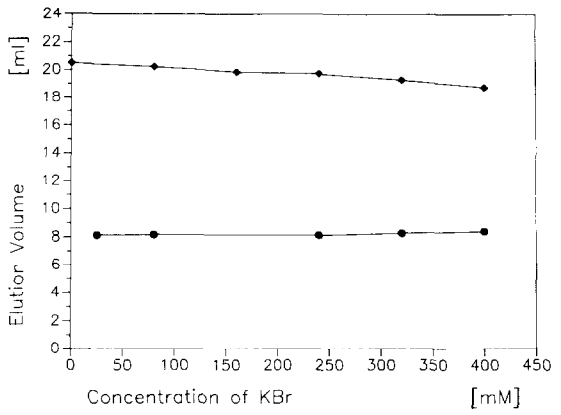

Fig. 10. Elution volume of PEG 20 000–Procion Red HE3b (●) and FDH (◆) as a function of potassium bromide concentration. System composition, 2.7% PEG 20 000, 4.5% dextran 500, 12 m*M* phosphate buffer (pH 7.0); temperature 30°C; column size, 30 × 1 cm I.D.

only slightly, the only way to explain the decrease in the partition coefficient of the enzyme–ligand complex, K_{EL}, at a given ligand concentration (corresponding to an increase in the elution volume) is a change in the dissociation constant of the complex.

It is obvious from the results presented that liquid–liquid partition chromatography incorporating affinity ligands is able to purify selectively the target protein even from fairly crude mixtures. Compared with affinity partitioning of FDH in batch systems [14], the main advantage of the partition column is the two orders of magnitude lower concentration of ligand required to achieve the desired result. This is a consequence of the nature of partition chromatography, because the elution volume is very sensitive to small changes in K values below 3 (Fig. 2). This sensitivity can be

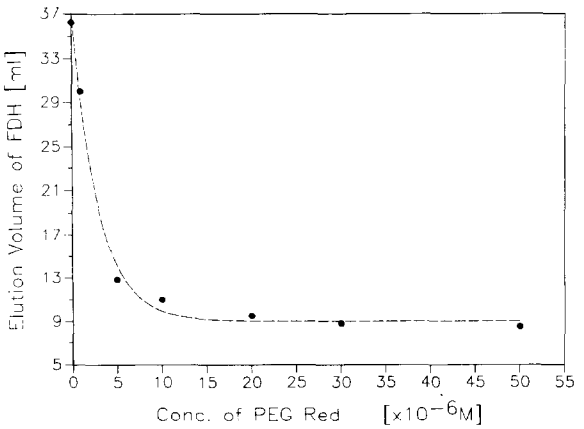

Fig. 11. Elution volume of FDH as a function of PEG–Red and potassium bromide concentration in the mobile phase. System composition, 2.7% PEG 20 000, 4.5% dextran 500, 12 m*M* phosphate buffer (pH 7.0); temperature, 30°C; column size, 30 × 1 cm I.D.

used in two directions. To separate the main peak of the contaminants of the crude extract from the enzyme–ligand peak, it is possible not only to shift the enzyme–ligand peak to the void volume of the column, but also to increase the elution volume of the contaminants by decreasing their partition coefficient through appropriate changes in the phase composition. A second advantage is that the amount of dextran required is much lower, as the column can be operated with no apparent loss of dextran over long periods of time. Uncontaminated PEG phase can be recycled directly. It also appears possible to recycle the affinity ligand after the separation from the enzyme–ligand complex [14]. As the ligand concentration is very low, this may not be worthwhile. The scale-up of liquid–liquid partition chromatography may be accomplished by increasing the diameter of the column.

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