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Liquid–Liquid Extraction of Lactate Dehydrogenase from Muscle Using Polymer-Bound Triazine Dyes

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ABSTRACT

An extract from porcine muscle containing soluble enzymes has been partitioned between the two liquid phases of an aqueous, biphasic system consisting of dextran, polyethylene glycol, and water. The influence of polymer-bound triazine dyes (Cibacron blue F3G-A and Procion yellow HE-3G) on the partition of lactate dehydrogenase and total protein was studied. The effects of pH and concentrations of polymers and buffer on this so-called affinity partitioning were also determined. The two-phase systems were used in extraction procedures for purification of lactate dehydrogenase to a specific activity of 456–494 U (7.6–8.4 μkat) per mg protein. The use of these systems for extraction of enzymes in technical scale is discussed.

Index Entries: Lactate dehydrogenase liquid–liquid extraction of; affinity partitioning; liquid–liquid extraction; two-phase systems; triazine dyes, polymer bound, influence of on partitioning of lactate dehydrogenase and total protein.

INTRODUCTION

The use of enzymes in large amounts, e.g., as catalysts in enzyme reactors, has increased the need for simple and effective isolation of enzymes on a large scale. One promising method for enzyme isolation is

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extraction between two aqueous phases (*1–3*). These phases are generated by dissolving two polymers in water or combining a solution of salt (at high concentration) with a polymer solution. The partition of proteins between the phases can be strongly influenced by covalent binding of ionic groups (*1*), hydrophobic molecules (*4,5*), or specific protein ligands (*6–8*) to one of the polymers. Since the two polymers are present in opposite phases, the polymer-bound group will be concentrated in one phase.

For technical applications, it is of advantage to use cheap but effective ligands, that can easily be bound to the polymers. One group of such ligands is the reactive triazine dyes. They can be obtained in a number of molecular structures with varying affinities for the nucleotide binding sites of dehydrogenases and kinases (*8–11*). Their effectiveness in influencing the partitioning of such enzyme in aqueous two-phase systems has been demonstrated in several cases (*3,7,8,12*). Also, preparative extractions have been described (*3,13,14*).

In the present work, the extraction of lactate dehydrogenase from an extract of porcine muscle has been studied, and optimal conditions for large-scale isolation have been determined and tested.

MATERIALS AND METHODS

Polyethylene glycol (PEG) (mol wt 6000–7500) was obtained from Union Carbide, New York. Dextran 500 (mol wt 500,000) was purchased from Pharmacia, Uppsala. Cibacron blue F3G-A was from Serva, Heidelberg, and Procion HE-3G from Swedish I.C.I., Göteborg. All biochemicals were obtained from Sigma, St. Louis.

Dye-Polymers

The triazine dyes were bound to PEG and dextran in alkaline water solution, as descibed elsewhere (*12,15,16*). The PEG-derivatives contained 140 μmol dye/g polymer, and the dextran derivative contained 8.2 μmol Procion yellow HE-3G/g polymer.

Muscle Extract

Muscle from swine, 200 g, was cut in pieces and homogenized with 360 mL ice-cold 40 m*M* sodium phosphate buffer, pH 7.9, in a standard house-hold mixer with rotating knives, for 5 min. The mixture was diluted with 300 mL of the same buffer and further treated with an Ultra-Turrax for four 5-s periods. It was centrifuged at $16{,}300g_{max}$ for 20 min in the cold. The supernatant was recovered, 550 mL, and filtered to remove traces of fat, yielding 505 mL. This extract contained 11–13 g protein/L and 190,000–266,000 U lactate dehydrogenase/L, corresponding to 3150–4450 μkat/L.

Enzyme Assays

Lactate dehydrogenase (LDH) (E.C. 1.1.1.27), malate dehydrogenase (E.C. 1.1.1.37), pyruvate kinase (E.C. 2.7.1.40), glyceraldehydephosphate dehydrogenase (E.C. 1.2.1.12), and myokinase (E.C. 2.7.4.3) were measured according to Bergmeyer (*17*), 3-phosphoglycerate kinase (E.C. 2.7.2.3) according to Scopes (*18*), and glutamate oxaloacetate transaminase (aspartate aminotransferase, E.C. 2.6.1.1.) according to Cohen (*19*), all at 340 nm and 25°C.

Protein Assay

Protein was determined according to Bradford (*20*), using Coomassie brillant blue G at 595 nm. Corrections were made for absorption from colored phases.

Two-Phase Systems

Systems were prepared from stock solutions of dextran (20 or 25% w/w), PEG (40 or 50%), dye polymers (1–4%), muscle extract, water, and, eventually, extra buffer. The polymer concentrations are given in per cent of the total system (w/w). Detailed procedures have been described elsewhere (*21,22*). The systems (4 or 8 g), in sealed centrifugation tubes, were brought to 0°C, equilibrated by careful mixing, and centrifuged at $1500g_{max}$ for 5 min to speed up the settling of the phases. Samples were withdrawn from the phases (by aid of pipets with plastic tips) for analysis.

Gel Electrophoresis

The electrophoresis was run in the buffer system of Laemmli (*23*), using slab gels containing a 12–20% polyacrylamide gradient. Samples of 50 μL vol, containing 20–80 μg of protein, were used. A current of 7.5 mA/gel slab (per six samples) was applied for 20 h at 20°C, using a Pharmacia GE-2/4 LS apparatus. The gels were stained with Coomassie brilliant blue R and photometrically scanned with an LKB 2202 Ultrascan laser densitometer.

RESULTS

The distribution of enzymes and total protein between the two liquid phases can be described by their respective partition coefficients, *K*, defined as the ratio between the concentrations of solutes in the upper and lower phases. Proteins in general show *K* values lower than one, i.e., they are concentrated in the lower phase. By introducing increasing amounts of ligand (Cibacron blue F3-GA) on PEG, the partition coef-

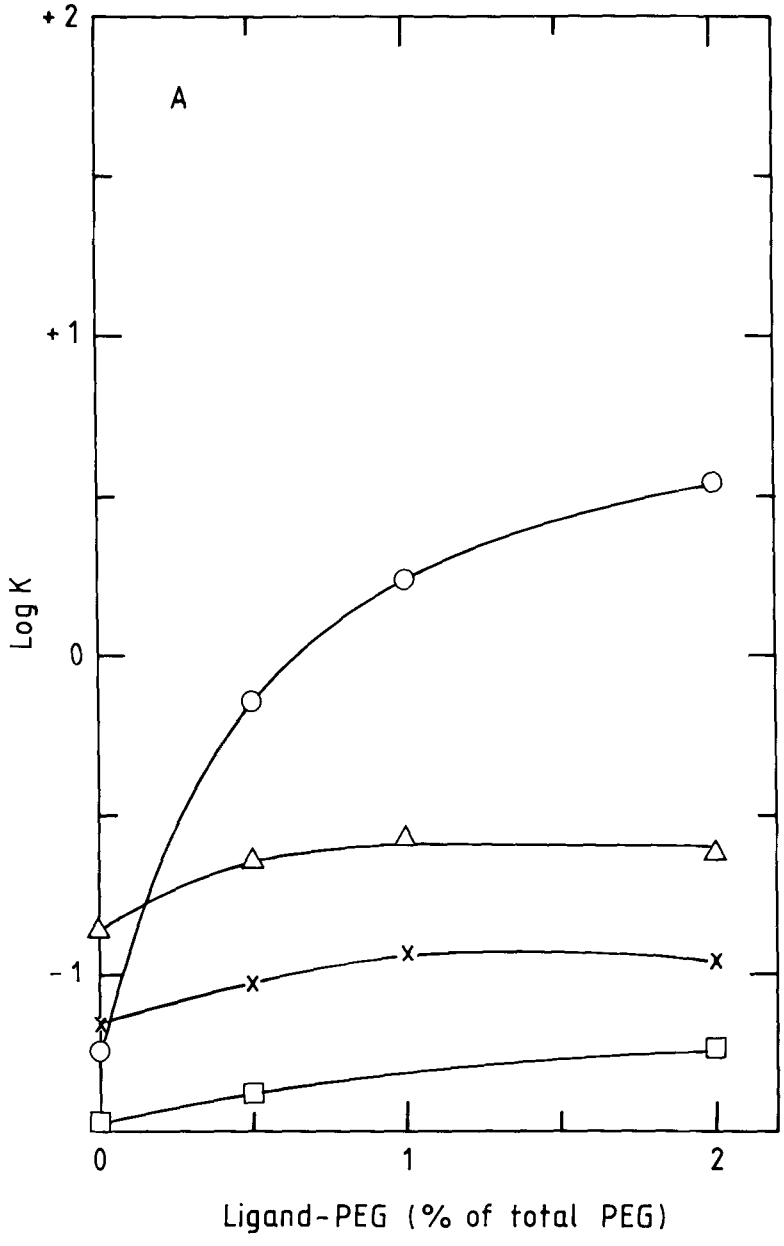

Fig. 1. Effect of the concentration of PEG-bound dye affinity ligand on the partition of muscle protein extract. A (above): with Cibracron blue F3G-A; B: with Procion yellow HE-3G. (△), Protein; (○), lactate dehydrogenase; (X), 3-phosphoglycerate kinase; and (□), glyceraldehydephosphate dehydrogenase. Two-phase system: 4% (w/w) dextran, 6% (w/w) PEG, 25 m*M* sodium phosphate buffer, pH 7.9, including 50% (w/w) muscle extract. Temperature: 0°C.

ficient of LDH increases gradually to values more than 70 times higher than without the dye (Fig. 1). The total protein present in the extract only moderately changes its partition coefficient, as do some other enzymes. The increase in the *K* values of LDH with another dye, Procion yellow

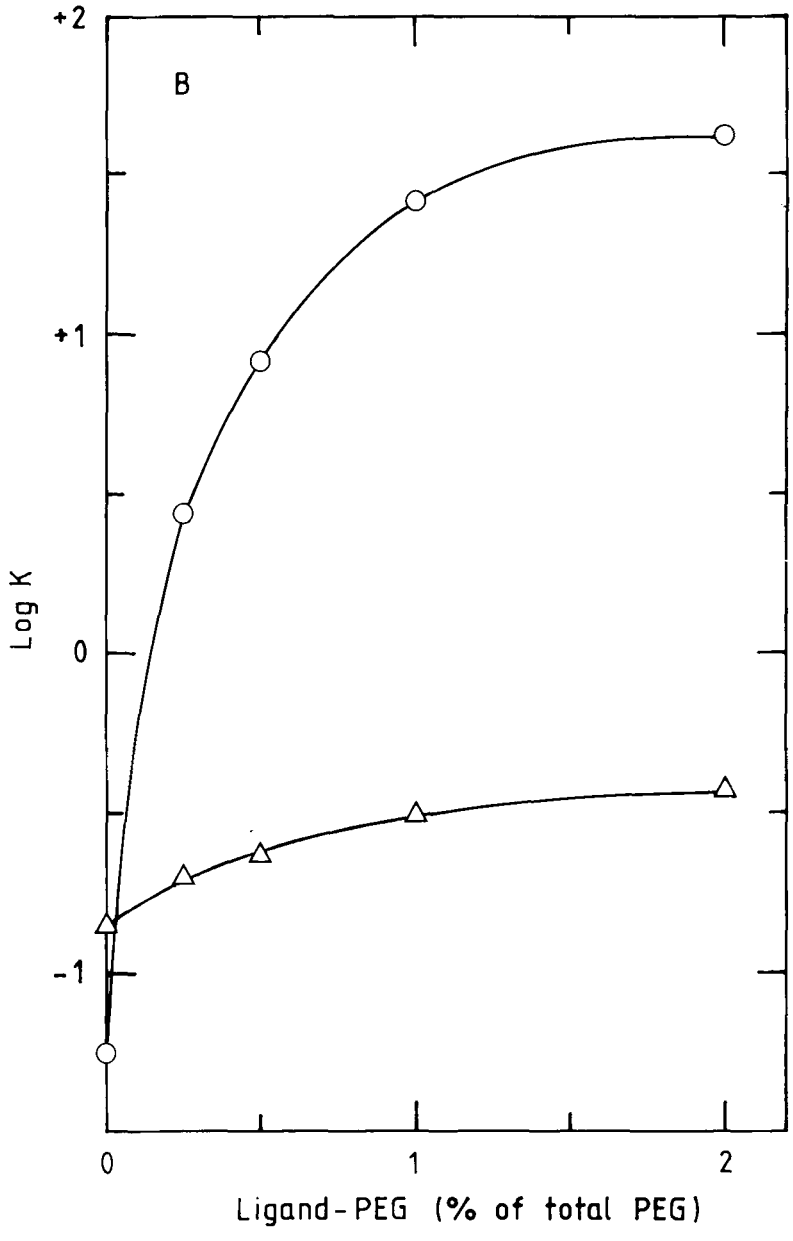

Fig. 1B.

HE-3G, is even more pronounced (760 times). This makes an extraction of the enzyme possible with a good degree of purification. To get the optimal conditions for an extraction, some important parameters were studied.

Effect of pH

The effectiveness of the extraction and the degree of purification were negatively influenced by the use of higher pH values of 7.5–9.5 (Table 1), in which PEG-bound Procion yellow HE-3G (PrY–PEG) was used.

TABLE 1
Dependence of Partition of LDH and Protein on pH[a]

pH	K_{LDH}	$K_{protein}$	Specific LDH activity in upper phase, U/mg	Recovery of LDH in upper phase, %
7.5	39	0.29	64.2	100
8.0	33	0.30	57.8	93
8.6	31	0.30	54.0	89
9.1	25	0.36	49.4	87
9.5	13	0.41	45.6	87

[a]Muscle extract (50% of system) was partitioned in a system containing 7% dextran, 4.9% PEG, 0.1% PrY-PEG, and 25 m*M* sodium phosphate buffer. Temperature 0°C. K = partition coefficient.

Effect of Concentration of Polymers

Increases in the concentrations of dextran and PEG strongly enhance the extraction effectiveness (Fig 2). Although LDH, to a large extent, could be recovered in the upper phase, the bulk proteins were effectively collected in the lower phase.

Effect of Buffer Concentration

The concentration of buffer showed moderate effects on the partition (Fig. 3). The degree of purification increased, however, with the concentration of phosphate buffer from 4.8 to 8.7 times. This salt effect was less pronounced at higher concentrations of polymers.

Multistep Extractions

To increase the degree of purification, the upper phase of the initial system (No. 0) was combined and extracted with fresh lower phases several times (Table 2). These "washing" steps removed proteins with low or no interaction with the ligand present in the upper phase. The importance of polymer and buffer concentrations, are clearly seen in Table 2. As expected, the purification factor approaches a constant value. Two or three washing steps strongly enhance the purification without major loss of the target enzyme. The better recovery obtained with Procion yellow as ligand, compared with Cibacron blue, is the result of a more effective binding of the former to the enzyme.

Two possibilities of improving the purification by a final transfer of LDH into a pure lower phase were tested. These included addition of NAD (+ sulfite) or use of dextran-bound Procion yellow HE-3G present in the lower phase. It is remarkable that the dye bound to dextran is able to extract a large part of the enzyme to the lower phase when the concen-

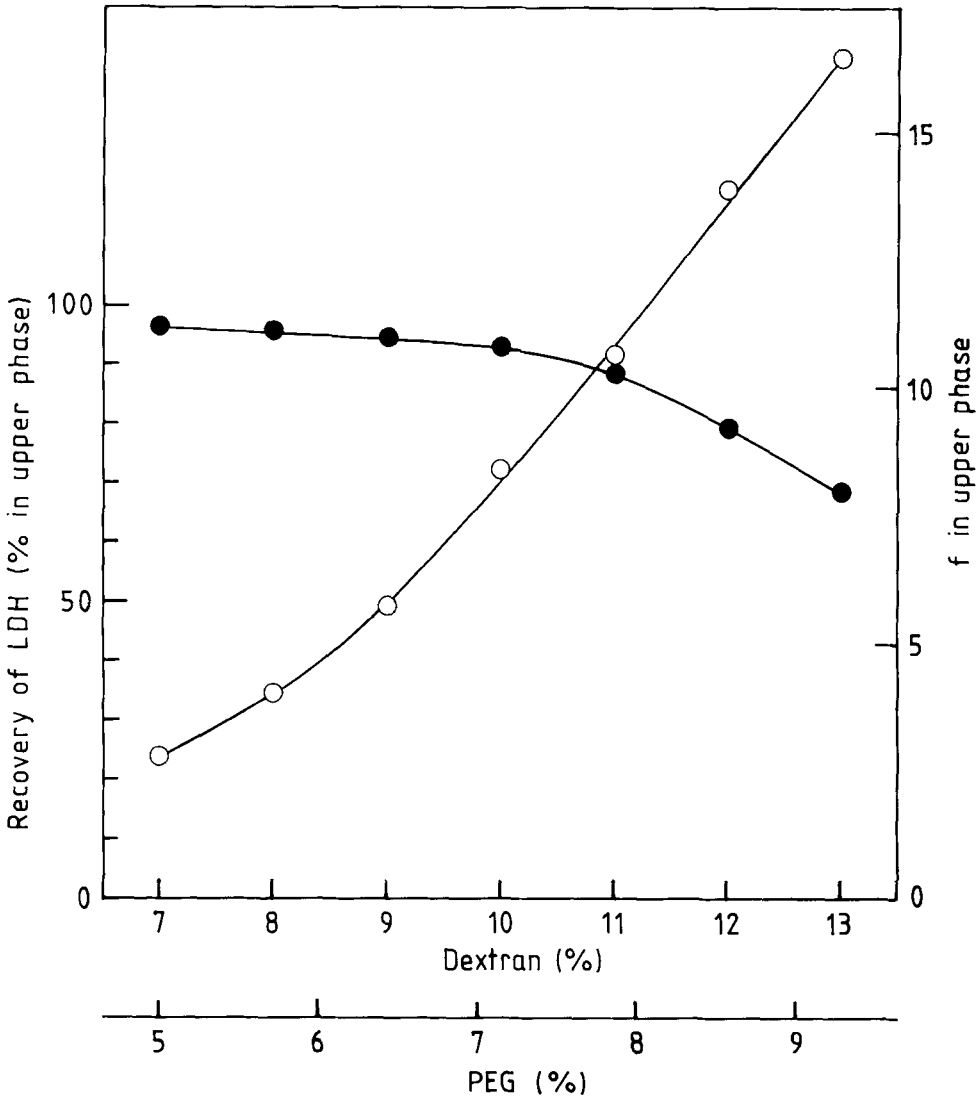

Fig. 2. (●), Recovery and (○), purification factor *f*, of LDH in the upper phase, using systems with various concentrations of dextran and PEG. Systems contained, besides the polymers, 25% (w/w) protein extract and 12.5 m*M* sodium phosphate buffer, pH 7.9. A 1% solution of PEG was carrying Procion yellow HE-3G. Temperature: 0°C.

tration of PEG-bound dye is in 10–20-fold excess (150–300 μ*M*). The competition between Cibacron blue (in upper phase) and Procion yellow (in lower phase) for LDH is shown in Fig. 4.

Large-Scale Extraction

The extraction of LDH from 100 g of muscle tissue in a 0.96-L system (10% dextran, 7.03% PEG, 0.07% PrY-PEG, 50 m*M* sodium phosphate buffer, pH 7.9, and 25% muscle extract), using two washing step, gave

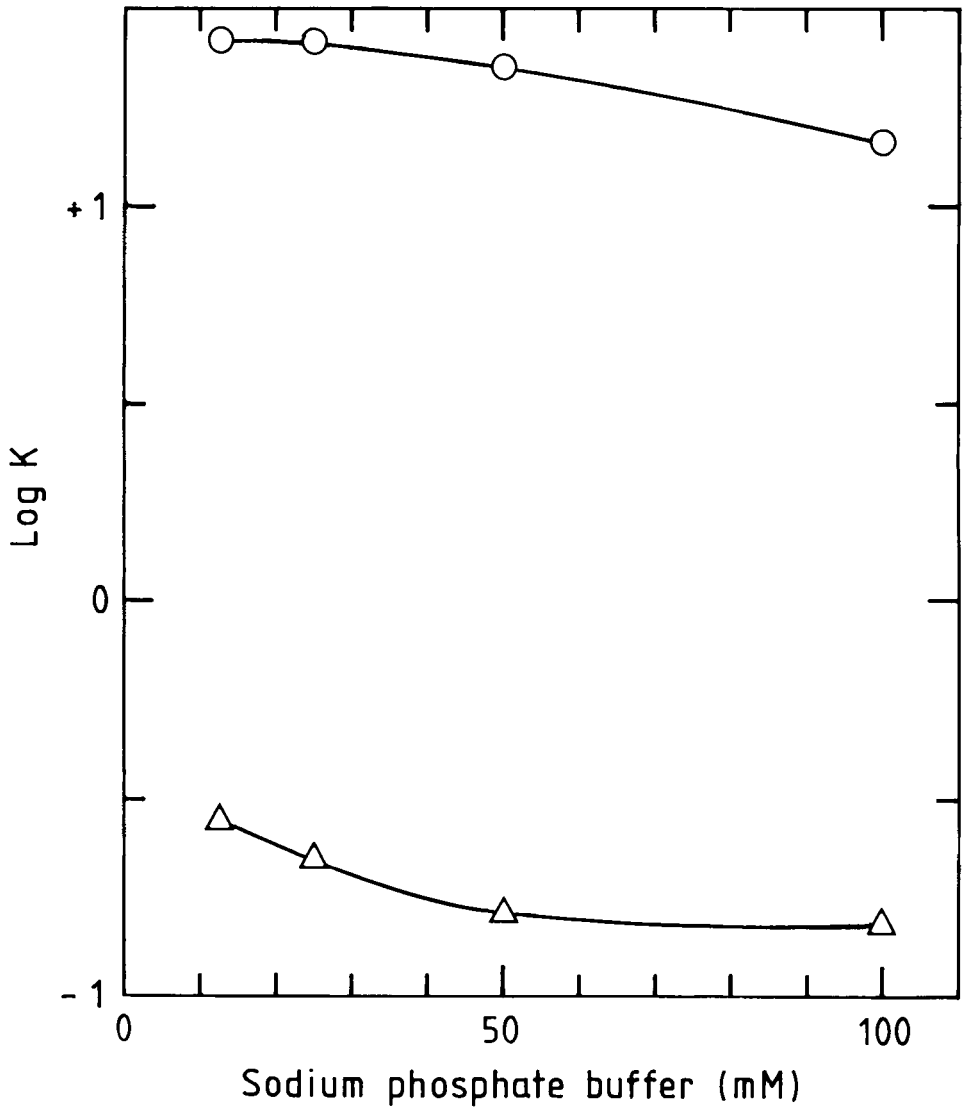

Fig. 3. (○), Effect of buffer concentration on the partition of LDH and (△), total protein. Protein extract from muscle, 25% (w/w), was partitioned in systems containing 8.0% (w/w) dextran, 5.7% (w/w) PEG, including Procion yellow PEG (1% of total PEG), and sodium phosphate buffer, pH 7.9, at various concentrations. Temperature: 0°C.

52,630 U (878 μkat) of LDH, a 79% recovery, with a purity of 486 U/mg protein or 8.09 kat/kg (corresponding to 1072 U/mg or 17.9 kat/kg measured at 37°C). This corresponds to a 25-fold purification of the enzyme relative to protein. The extraction was completed in 80 min, including three 20-s periods of mixing and three 10-min periods of centrifugation. The remaining time was used for separation of the phases and loading of the centrifuge. By addition of a 50% water solution of $NaH_2PO_4 \cdot H_2O$ and

TABLE 2
Multiple Extraction of LDH from Muscle Extract[a]

Composition of system	Phase of system No.	Specific LDH activity, U/mg	Purification factor	Recovery of LDH, %
7% Dextran	0 upper	56.6	3.5	82
4.90% PEG	1 upper	112	7.0	62
0.10% Cb-PEG	2 upper	147	9.2	56
25 m*M* Sodium phos-	3 upper	198	12.4	50
phate buffer, pH 7.1	4 upper	189	11.8	41
50% Extract	5 lower[b]	399	24.9	48
7% Dextran	0 upper	76.4	4.7	101
4.95% PEG	1 upper	144	8.8	83
0.05% PrY-PEG	2 upper	214	13.1	81
25 m*M* Sodium phos-	3 upper	268	16.4	76
phate buffer, pH 7.1		346	21.2	49
50% Extract	4 lower[b]			
10% Dextran	0 upper	163	10.6	85
7.03% PEG	1 upper	294	19.1	80
0.07% PrY-PEG	2 upper	390	25.3	86
50 m*M* Sodium phos-	3 upper	440	28.6	80
phate buffer, pH 7.8		459	29.8	75
25% Extract	4 lower[c]			

[a]After partition in system No. 0, the upper phase of this system was sequentially brought in contact with the same volumes of pure lower phases. Temperature, 0°C.
[b]Lower phase containing 14 μ*M* dextran-bound Procion yellow.
[c]System contaning 1.3 m*M* NAD and 2 m*M* Na_2SO_3.

Na_2HPO_4 in a molar ratio of 1:1, at a total of 213 g, at 50°C, to the upper phase (535 mL, room temperature) the enzyme was recovered in the appearing lower (salt-rich) phase, whereas the PEG and ligand-PEG were concentrated in a small, highly viscous, upper phase (166 mL). The recovery of this step was quantitative. The degrees of contaminating enzymes in the final lower phase (after desalting) are shown in Table 3, and the electrophoretic patterns of product and starting material are shown in Fig. 5. The upper phase could, after desalting, be reused for extraction.

DISCUSSION

The results show that LDH can effectively be extracted into the upper PEG-rich top phase when muscle homogenate is included in an aqueous, biphasic system. The extraction is performed by anchoring a ligand

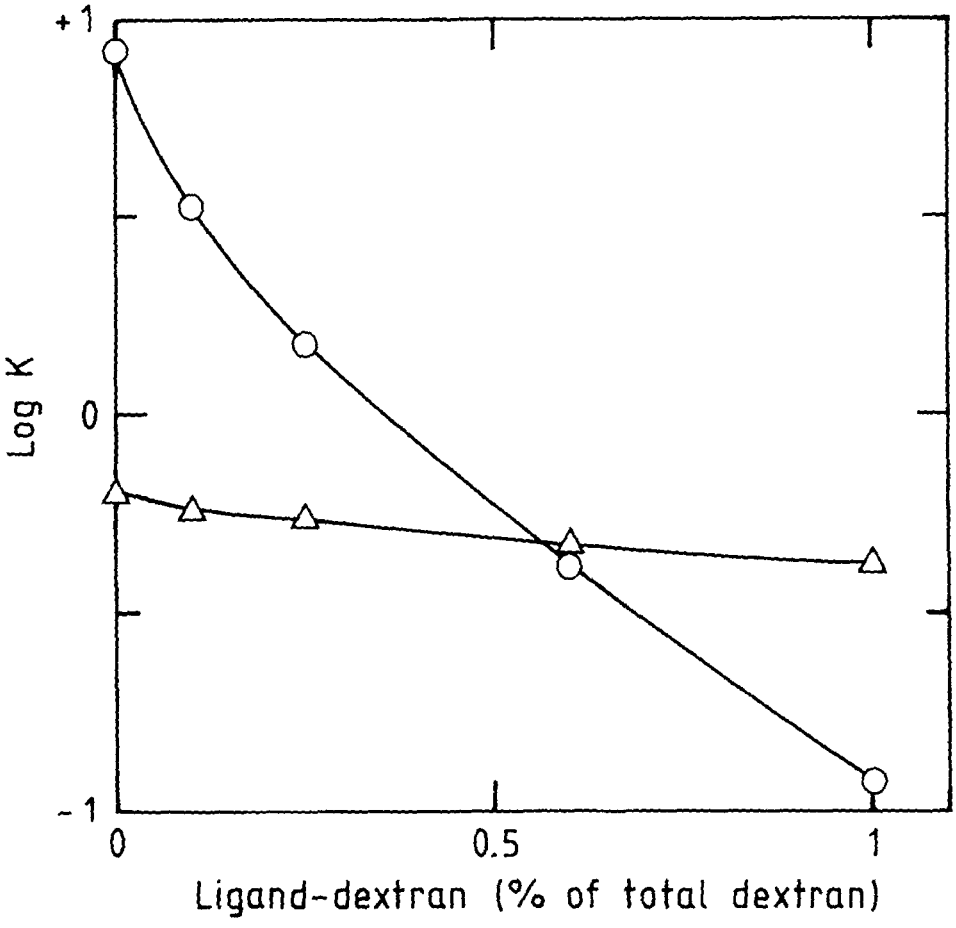

Fig. 4. (○), Effect of Procion yellow dextran on the partition of LDH and (△), total protein in a two-phase system containing Cibacron blue PEG. The upper phase of a system containing muscle protein extract and with the same composition as in Fig. 1, but with 2% of total PEG in form of Cibacron blue PEG, was combined with fresh lower phases with various concentrations of dextran-bound Procion yellow. The partition coefficients, *K*, were determined after equilibration at 0°C.

TABLE 3
Contaminating Enzymes in Muscle Extract and Final LDH Preparation[a]

Enzyme	Homogenate	LDH product
Pyruvate kinase	50	0.006
3-Phosphoglycerate kinase	19	0.06
Myokinase	14	4.7
Malate dehydrogenase	58	19
Glutamate oxaloacetate transaminase	5.6	1.2

[a]Activities are given in percentages of LDH activity.

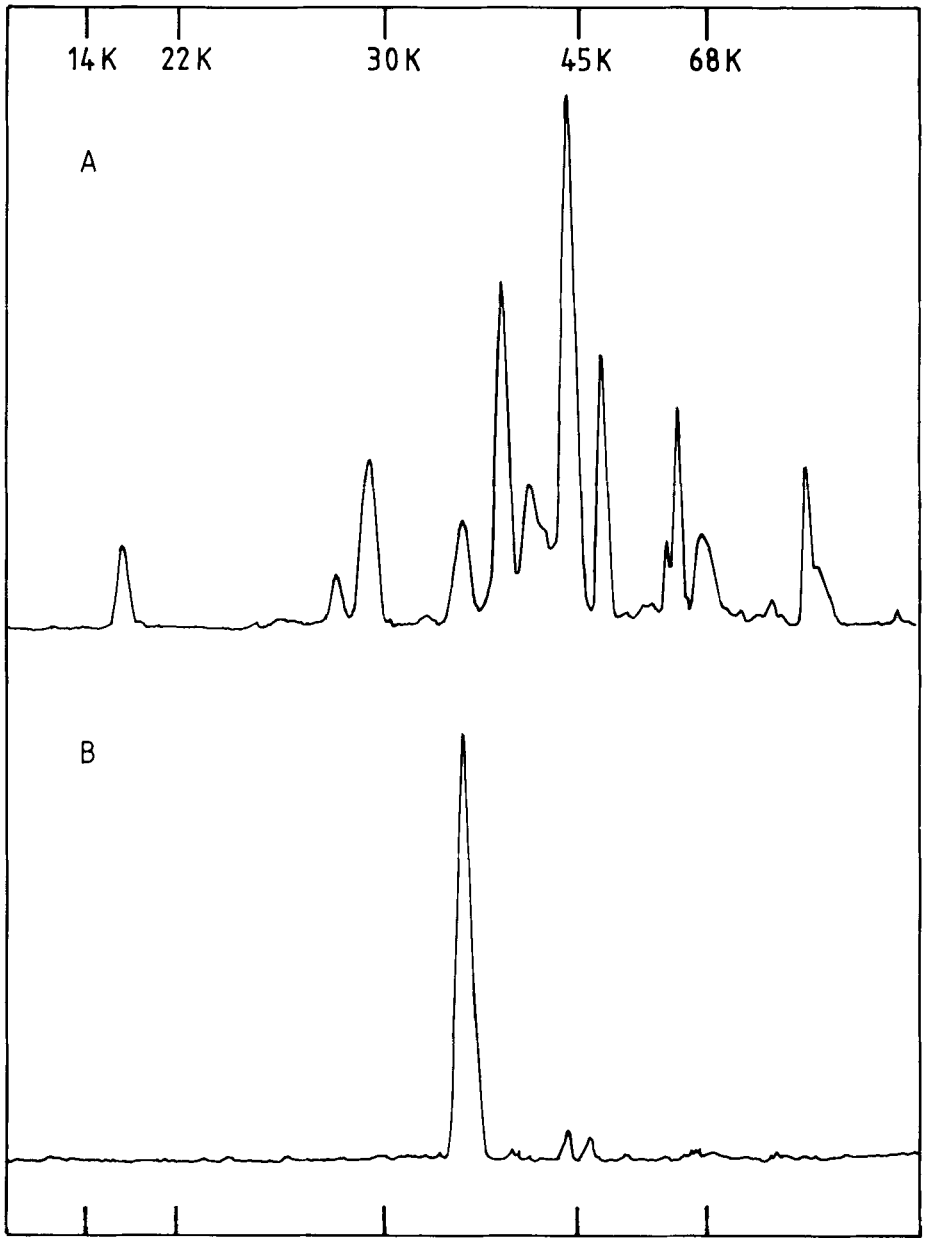

Fig. 5. Polypeptide pattern obtained by sodium dodecylsulfate gel electrophoresis and analyzed by photometric scanning. A: Original protein extract from porcine muscle; B: LDH preparation obtained by large scale affinity partitioning (*see* text for details). Molecular weights are indicated.

for the enzyme, in this case, a triazine dye, to PEG. The choice of dye is of importance (Fig. 1A,B) in both the effectiveness in extraction and the selectivity. The dye Procion yellow HE-3G was found to have favorable properties.

In contrast to some enzyme studies using yeast extract (*12*), the purification is enhanced at lower pH values. Use of higher concentrations of polymers (Fig 2) dramatically increases the purification factor, which is mainly dependent on a more effective exclusion of bulk proteins from the upper phase.

The extraction of the lower phase can be done with the same ligand. As can be seen in Fig. 4, the ligand is more effective in influencing the partition when bound to dextran. To totally reduce the effect of PEG-bound ligand (0.27 m*M* Cibacron blue in the upper phase), only 1/23 of another ligand, Procion yellow, has to be bound to dextran (0.012 m*M* concentrations in the lower phase). According to Fig. 1, the concentration of the two dyes necessary to reach saturation are nearly the same. The much higher effectiveness in using dextran as a ligand carrier can be the result of the presence of 2–3 dye molecules per dextran molecule (inducing a chelate-type of binding). Other explanations are a less-negative protein polymer interaction, better availability of ligand, and/or a more extreme partition of ligand-dextran compared with ligand-PEG.

For preparative purposes, repeated extractions are useful ways of improving the purity of the enzyme (Table 2). The aqueous two-phase systems offer some important features: (1) Extreme rapidity in enzyme extraction (10–30 min in any scale with adequate equipment); (2) high capacity, i.e., 100 g tissue/L system with only 1% of total PEG in ligandized form: (3) very good recovery of the target enzyme; and (4) the scale-up can be done with ease because of the simplicity of the process. Therefore, affinity partitioning is well suited for preparative use in large-scale production of enzymes. The preparation in larger scale (1-kg two-phase system) shows that LDH can be obtained in good purity by solely using affinity partitioning, as reflected by the gel electrophoresis pattern (Fig. 5) and excellent yield (79%). Although some contaminating enzyme activities, e.g., pyruvate kinase (Table 3), are effectively reduced, other enzymes are coextracted, e.g., malate dehydrogenase and myokinase. These activities have to be eliminated if the LDH preparation is going to be used as auxiliary enzyme for analytical purposes. It should be possible, for example, to separate myokinase from LDH by gel filtration because of the difference in their molecular weights.

In this work, inexpensive and easily attachable ligands have been used. Since ligands can be bound to both polymers and are thereby constricting to either one of the two phases, two different ligands can be used simultaneously, one in each phase (Table 2 and Fig. 4). This offers a unique way of improving selectivity of extraction.

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