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Integration of aqueous two-phase extraction and affinity precipitation for the purification of lactate dehydrogenase

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Abstract

Integration of extraction in aqueous two-phase system and affinity precipitation was investigated as a technique for purification of lactate dehydrogenase (LDH) from porcine muscle extract. An enteric coating polymer, Eudragit S 100, which can be made reversibly soluble and insoluble by change in pH was used as the ligand carrier. The ligand used was Cibacron blue 3GA. The polymer is nearly totally partitioned to the top phase (>98%) in PEG–dextran aqueous two-phase system. The enzyme, lactate dehydrogenase, was first spontaneously partitioned to the bottom phase in a 6% (w/w) PEG 8000–8% (w/w) dextran T250 phase system. New PEG phase and Eudragit-dye were then added to the bottom phase, which helped in extraction of LDH to the top phase. After a washing step with a fresh bottom phase, Eudragit-dye–target protein affinity complex was precipitated out from the top phase by lowering the pH to 5.1. The enzyme was recovered by treatment of the complex with 0.5 *M* NaCl with a yield of 54% and a specific activity of 245 units/mg. The purification of LDH by this procedure was better than that obtained by a single step of affinity partitioning.

1. Introduction

Aqueous two-phase partitioning is an established technique for use during the initial stages of downstream processing of proteins [1]. The most attractive feature of these systems is the rapid removal of cell debris and particulate matter with minimal requirements of energy. The incorporation of affinity interactions in these systems has provided an element of specificity for purification of proteins. A number of studies have been carried out using this concept [2–4]. The most popular ligands for use in affinity partitioning have been reactive dyes, which are generally coupled to polyethylene glycol (PEG), the top phase polymer in the two-phase system.

Precipitation is another technique used often during large-scale protein purification. It is generally non-specific, the more specific form being termed affinity precipitation. One of the strategies of affinity precipitation involves the use of a reversibly soluble–insoluble polymer as a ligand carrier; the separation of the affinity complex being effected by the precipitation of the polymer [5]. The precipitation can be carried out by change in either pH, ionic strength, temperature, etc., depending on the nature of the polymer.

In an earlier report we described the integration of affinity precipitation with extraction in aqueous two-phase system for the purification of Protein A from recombinant *Escherichia coli* [6]. The affinity ligand, immunoglobulin G (IgG), was coupled to Eudragit, a polymer which precipitates under acidic conditions. Eudragit partitions to the PEG-rich phase, making it possible

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to design the affinity extraction on the same grounds as using PEG-bound ligands. This procedure combines the advantages of gentle and rapid separation obtained by aqueous two-phase extraction with the possibility of having a better control over the ligand and the easy recovery of the target protein from the phase polymers by affinity precipitation.

The present report deals with the use of the above concept for the purification of lactate dehydrogenase (EC 1.1.1.27) from porcine muscle extract using Eudragit-bound Cibacron blue.

2. Experimental

2.1. Materials

Eudragit S 100 was a gift from Röhm Pharma (Weiterstadt, Germany). Polyethylene glycol (PEG) 8000 was procured from Union Carbide Co. (New York, NY, USA). Dextran T250 (av. mol. mass 298 000), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), Cibacron blue 3GA and β-nicotinamide adenine dinucleotide, reduced form (NADH), were purchased from Sigma (St. Louis, MO, USA). 1,4-Diaminobutane was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Methods

Preparation of muscle extract

100 g porcine muscle were homogenized with 250 ml of 100 m*M* ice-cold potassium phosphate buffer pH 7.9 in a standard household mixer for 5 min. The homogenate was centrifuged at 27 300 *g* for 20 min and the supernatant was collected. About 200 ml of ice-cold water were added to the settled debris, mixed well, homogenized and then centrifuged under the same conditions. The supernatants were pooled and filtered using filter paper to remove the floating fat. Muscle extract (280 ml) thus obtained was stored at −20°C until use.

Immobilization of Cibacron blue to Eudragit S 100

Eudragit S 100 (2.0 g) was suspended in 80 ml of 0.2 *M* K_2HPO_4 solution and pH adjusted to 8.0 with 1 *M* NaOH for dissolving the polymer. Initial use of phosphate buffer facilitated subsequent solubilization of Eudragit. However, to prevent the interference of phosphate ions during activation of carboxyl groups, the Eudragit was precipitated by reducing pH to 4.5 and centrifuged (10 400 *g* for 10 min). The precipitate was washed twice with distilled water and finally solubilized at a concentration of 4% in water at pH 6.2.

The above native Eudragit solution (25 ml) was stirred together with 0.3 g carbodiimide (EDC) at 22°C for 20 min. To this were then added 10 ml of 30% diaminobutane solution at final pH 9.2. The stirring was continued for 4 h at the same temperature, after which the Eudragit was precipitated by lowering the pH to 4.5, and centrifuged at 10 400 *g* for 10 min to get rid of unreacted diaminobutane. The precipitate was redissolved in water at pH 8.0 by adding 0.5 *M* NaOH.

To the modified Eudragit solution were added 1.0 g of Na_2CO_3 and 0.5 g of Cibacron blue, and the pH of the solution was adjusted to 10 with 1 *M* NaOH. After continuous shaking at 22°C for 20 h, the polymer-bound dye (Eudragit-dye) was precipitated by lowering pH of the solution to 4.8 and centrifuged at 3000 *g* for 10 min. The supernatant with the bulk of the free dye was discarded. The remaining unbound dye in the precipitate was later removed by adsorption to pretreated DEAE-cellulose [7].

The precipitate was dissolved in 100 ml of water at pH 6.8–7.0 and mixed with the pretreated ion exchanger (2.5 g dry weight). The suspension was filtered; the filtrate contained the bulk of the Eudragit-dye. The ion exchanger was then treated with 2 *M* KCl solution to elute the adsorbed Eudragit-dye. The Eudragit-dye in the filtrate and eluate were pooled and washed twice with water by alternating precipitation at pH 4.5 and resolubilization at pH 7.0. The Eudragit-dye was finally dissolved in water at pH 7.0 with a concentration of 0.38%.

Precipitation behavior of Eudragit-dye

To 0.5 ml of 0.38% Eudragit-dye solution in a test tube was added 0.5 ml of 0.1 *M* citrate buffer of a certain pH and 1 ml of water or 20% PEG 8000 solution. After proper mixing, the tube was allowed to stand for about 10 min at room temperature. As a control, the native Eudragit solution of the same concentration was treated in a similar manner. The precipitation of the native Eudragit at a particular pH was determined by measuring turbidity at 470 nm [6], and that of Eudragit-dye by measuring the absorbance of dye in the supernatant at 612 nm after centrifugation.

Two-phase system

Stock solutions of 30% PEG 8000, 30% dextran T250 and 0.5 *M* potassium phosphate buffer, pH 7.6 were prepared. The concentrations of the polymers are given as % (w/w). Unless specifically mentioned, the aqueous two-phase systems used were composed of 6% PEG and 8% dextran and the concentration of the phosphate buffer was 50 m*M*. The volume ratio of the top to bottom phase was 1.35. For affinity partitioning, Eudragit-dye was added to the above two-phase system.

Purification procedure

30% PEG solution, 30% dextran solution, the porcine muscle extract, 0.5 *M* phosphate buffer and water were mixed to form 13 g of aqueous two-phase system having a final concentration of 6% PEG, 8% dextran, 10% muscle extract and 0.05 *M* phosphate buffer. After mixing and centrifuging at 1625 *g* for 5 min, the top and bottom phase were separated. Fresh top phase with the same composition as above but containing 0.18% Eudragit-dye was added to the bottom phase. After mixing and subsequent separation by centrifugation, the bottom phase was discarded and replaced with a fresh bottom phase. Then the top phase was removed, the Eudragit-bound affinity complex was precipitated by reducing the pH of the top phase to 5.1 with 0.5 *M* citric acid and centrifuged at 3000 *g* for 10 min. After washing once with 5 ml of 20 m*M* citrate buffer, pH 5.1, the precipitate was solubilized in 4 ml of 0.5 *M* NaCl at pH 7.0. After 30 min incubation, Eudragit-dye was precipitated at pH 5.1 and centrifuged. The supernatant containing the enzyme was collected. The treatment of Eudragit-dye for desorption of the enzyme was repeated once more. The Eudragit-dye was washed once with water and re-used for the next cycle of purification.

Analytical methods

The partition coefficients (*K*: the ratio of concentration in the top phase to that in the bottom phase) of LDH, Eudragit-dye and protein in the two-phase system were determined by measuring their concentrations in the top and bottom phase, respectively.

Lactate dehydrogenase activity was determined according to Decken [8] by reduction of pyruvate to lactate. The consumption of NADH was followed by measuring the reduction in absorbance at 340 nm at room temperature. One unit of the LDH activity was defined as the amount of enzyme which catalyzes the consumption of 1 μmol of NADH per min under the standard assay conditions.

Total protein concentration was determined by the bicinchoninic acid method as described by Smith *et al.* [9]. Bovine serum albumin was used as a standard.

The amount of Cibacron blue immobilized on Eudragit was determined by measuring the absorbance of the dye in Eudragit-dye solution at 612 nm [10]. The weight of Eudragit-dye was estimated by precipitating it at pH 4.5, washing the precipitate with water at the same pH and drying overnight at 105°C and weighing.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) with 12% acrylamide was performed to check the purity of the samples [11]. The molecular mass markers consisted of rabbit muscle phosphorylase b (M_r 97 000), bovine serum albumin (M_r 66 000), ovalbumin (M_r 45 000), bovine carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 22 000) and hen egg white lysozyme (M_r 14 400), available as a standard kit (Bio-Rad Laboratories, Richmond, CA, USA).

3. Results and discussion

3.1. Binding of Cibacron blue to Eudragit

Eudragit is an anionic copolymer based on methacrylic acid and methylmethacrylate with an average molecular mass of 135 000. The carboxyl groups on the polymer have been utilized for the coupling of enzymes, proteins and other molecules via their amino groups [6,12,13]. As Cibacron blue does not possess the required amino group, its coupling to Eudragit could be performed in the presence of a spacer group like diaminobutane [14]. The reaction between this group and the dye is favored at high pH. At 22°C, the highest amount of immobilized dye was obtained at about pH 10.0.

Coupling of Cibacron blue to Eudragit at different temperatures showed that although the initial binding of the dye was greater at higher temperature, it was reduced after a period of 1.5–2 h (data not shown). In contrast, covalent coupling of the dye was favored at lower temperature and long reaction time. About 130 mg of Cibacron blue were bound per gram Eudragit preparation after coupling at pH 10, 22°C for 20 h. This temperature dependence was in full agreement with the result reported concerning coupling of Cibacron blue to PEG [7]. The separation of the free dye from the bound dye was facilitated due to the ability of Eudragit to precipitate under acidic conditions. Small amounts of the free dye left over could then be adsorbed to DEAE-cellulose as described earlier for removal of free dye from PEG-dye [7].

3.2. Eudragit-dye characteristics

Native Eudragit has a soluble–insoluble transitional region between pH 4.6 and 5.5. The region was shifted slightly upward on the pH scale after dye was immobilized on the Eudragit (Fig. 1). A similar shift was observed for the polymer in the presence of 10% PEG. These observations are in accordance with the results reported earlier for IgG–Eudragit [6]. This shift would mean that the separation of Eudragit bound affinity complex may be carried out at relatively favorable pH values, which in turn may lower the risk of enzyme denaturation.

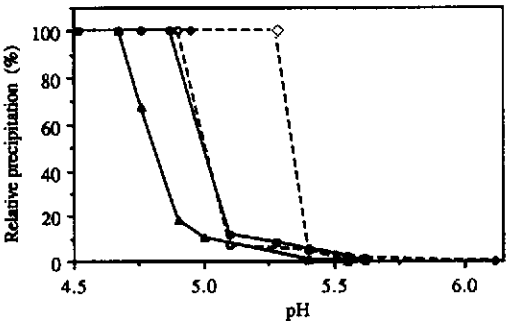

Fig. 1. Precipitation curve of Eudragit S 100 (solid lines) and Eudragit-dye (dashed lines) in response to variation of pH. Symbols: (▲) native Eudragit; (○) Eudragit-dye; (●) native Eudragit + 10% PEG and (◇) Eudragit-dye + 10% PEG.

The partitioning of Eudragit in aqueous two-phase systems is an important parameter to consider if the polymer is to be used as a ligand carrier. The earlier studies on partition of Eudragit in PEG–Reppal two phase system showed that at a concentration of 0.5%, more than 97% of the polymer partitioned into the top phase [6]. Eudragit-dye exhibited a similar partition behavior in PEG–dextran two-phase system. The effect of different salts on the partitioning was also studied (Fig. 2). Increase in potassium phosphate concentration strongly promoted the partitioning of Eudragit-dye to the top phase. Ammonium sulfate had a weaker effect while

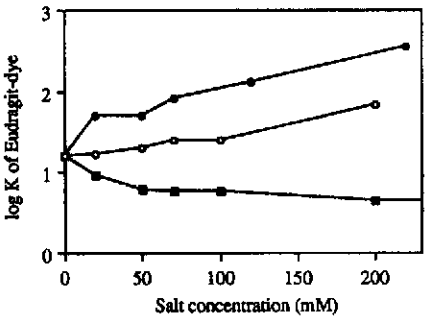

Fig. 2. Effect of salt on the partitioning of Eudragit-dye in 6% PEG 8000–8% dextran T250 system. The salts added were potassium phosphate (●), ammonium sulfate (○), and sodium chloride (■). The concentration of Eudragit-dye was 0.025%.

NaCl favored the partitioning to the bottom phase. This is also in agreement with the earlier observations [6].

3.3. Effect of Eudragit and Eudragit-dye on LDH partitioning in two-phase system

When the porcine muscle extract was added to a PEG–dextran two-phase system, most of the proteins including LDH (log $K = -1.0$) partitioned into the bottom phase. The presence of native Eudragit in the same two-phase system had little influence on protein and enzyme partitioning (Fig. 3). However, in the presence of increasing concentrations of Eudragit-dye, while most of the other proteins still remained in the bottom phase, the partitioning of LDH to the top phase was increased significantly. At an Eudragit-dye concentration of 0.05%, the increase of log K of LDH was 2.45, and more than 95% of the enzyme went into the top phase. While the partitioning of Eudragit-dye to the top phase continuously increased with the increase in phosphate concentration in the system (Fig. 2), the log K of LDH increased only up to a phosphate concentration of 70 mM (Fig. 4). With further increase in the salt concentration, the enzyme remained in the bottom phase because of the lowered affinity between the dye and the enzyme. Hence, 50 mM phosphate buffer (pH 7.6) was used during LDH purification in the two-phase system.

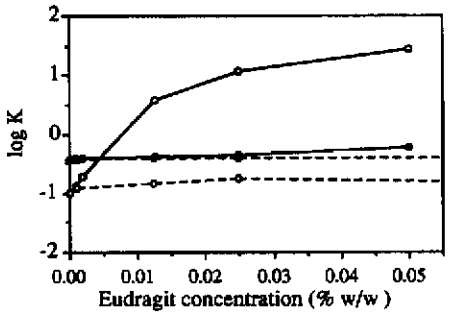

Fig. 3. Effect of native Eudragit (dashed lines) and Eudragit-dye (solid lines) concentration on the partition coefficient of the total protein (●) and LDH (○) in the 6% PEG 8000–8% dextran T 250 system at pH 7.6.

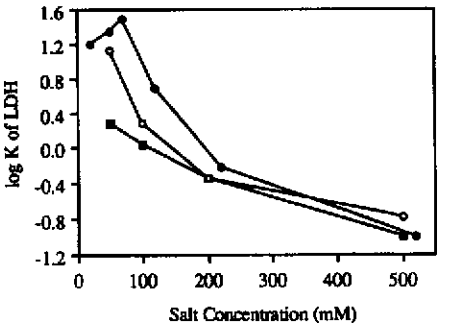

Fig. 4. Effect of salt on the partitioning of lactate dehydrogenase from porcine muscle extract in 6% PEG 8000–8% dextran T250 system containing 0.025% Eudragit-dye. The salts added were potassium phosphate (●), ammonium sulfate (○), and sodium chloride (■).

3.4. Effect of PEG and dextran concentration on LDH partitioning

Fig. 5 shows the effect of PEG and dextran concentration on the partition coefficient of LDH in a two-phase system without and with Eudragit-dye. In the absence of Eudragit-dye, enzyme partitioned predominantly into the bottom phase. The log K value of the enzyme decreased significantly with increase in PEG concentration, while a slight decrease was observed at higher dextran concentrations. The

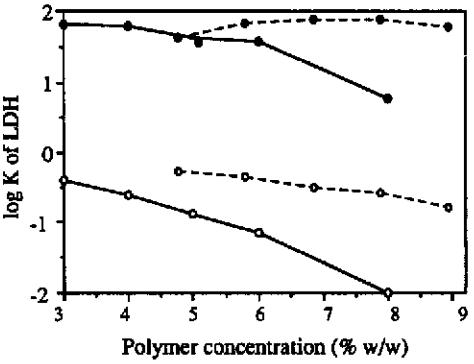

Fig. 5. Effect of PEG 8000 (solid lines) and dextran T 250 (dashed lines) concentration on partitioning of LDH from muscle extract in the two-phase system without Eudragit-dye (○) and with 0.025% Eudragit-dye (●). The dextran concentration was maintained at 7% as PEG concentration was varied, while the PEG concentration was 4% with change in dextran concentration.

extreme partitioning of proteins obtained by increasing the polymer concentrations has been documented earlier [15]. In the presence of Eudragit-dye, the enzyme partitioned efficiently into the top phase. There was a slight decrease in the partition coefficient with increase in PEG concentration, while it remained more or less constant with change in dextran concentration.

The pH of the two-phase system also influenced the enzyme partition in the presence of Eudragit-dye, the optimal pH being in the range of 7.4–8.7. This corresponds to the pH range in which LDH has maximal affinity for Cibacron blue.

3.5. Adsorption capacity of Eudragit-dye vs. enzyme concentration

The binding of LDH to Eudragit-dye in the aqueous two-phase system would be influenced by the components of the two-phase system, ion concentration, pH and protein load. Fig. 6 shows that at lower loading of the muscle extract in PEG–dextran system containing Eudragit-dye, more than 97% of LDH was taken into the top phase while the dye was still in excess with respect to LDH concentration. At the enzyme concentration of 54 U/g, the Eudragit-dye bound per mg 180 U of LDH corresponding to 89% of the total enzyme loaded in the two-phase system. Overloading of the extract in the system resulted in the excess enzyme remaining in the bottom phase. It is well known that Cibacron blue is a group-affinity ligand and that it has affinity for other dehydrogenases and kinases present in the muscle extract. Hence, the real capacity of the Eudragit-dye for pure LDH would be expected to be higher.

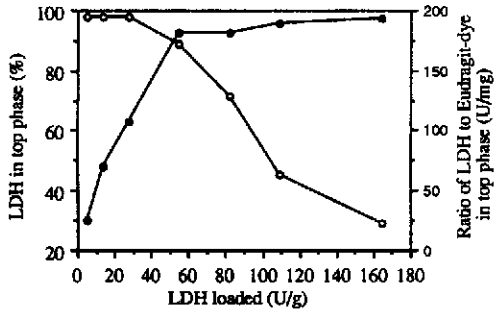

Fig. 6. Partitioning of LDH in 5% PEG 8000–7% dextran T 250 system containing 0.01% Eudragit-dye as a function of different amounts of muscle extract. Symbols: (○) % enzyme recovery in the top phase, and (●) ratio of L-LDH activity per mg Eudragit-dye.

3.6. Purification of lactate dehydrogenase from the muscle extract

Purification of LDH by affinity partitioning in aqueous two-phase systems using dye–PEG and sometimes even dye–dextran has been described earlier in several reports [2,16]. Normally 3 to 4 extraction steps are required to obtain LDH of high purity (around 300–500 U/mg protein) [4]. The effect of introducing additional "washing" steps besides that of affinity partitioning using Eudragit-dye was studied (Table 1). Affinity extraction of the enzyme directly from the crude extract resulted in only about 5-fold increase in purification. However, adding the "washing" steps before and after the affinity step resulted in almost 12 times increase in the enzyme specific activity. Fig. 7 shows the protein pattern obtained on electrophoresis of samples purified by different methods.

The number of steps required for purification would depend on the system under study. In the previous report, purification of Protein A by partitioning using IgG–Eudragit in PEG–Reppal system and subsequent elution of the protein after precipitation of the affinity complex yielded a nearly pure preparation in just one extraction step [6]. The present system is based on a ligand which is group-specific, it binds proteins present in the crude muscle extract with different affinities.

Based on the above experiments, a three-step purification protocol was adopted. Initially, the muscle extract was added to the PEG–dextran system and over 84% of LDH activity was recovered in the bottom phase (Table 2). The top phase with some of the contaminant proteins and the interface with cell debris were discarded. Fresh top phase containing Eudragit-dye (46 mg dye per gram Eudragit preparation) was equilibrated with the bottom phase, resulting in the

Table 1
Effect of different procedures on LDH purification from muscle extract

Method		Specific activity (U/mg protein)	Purification factor
1	Muscle extract and Eudragit-dye added into two-phase system. The Eudragit-dye–enzyme complex precipitated by reducing pH of PEG phase to 5.1. LDH eluted out with salt.	108.4	5.2
2	Method 1 + "washing" with fresh bottom phase before precipitation.	153.1	7.3
3	Muscle extract partitioned without Eudragit-dye before method 1.	181.0	8.6
4	Method 3 + "washing" with fresh bottom phase before precipitation.	247.7	11.8

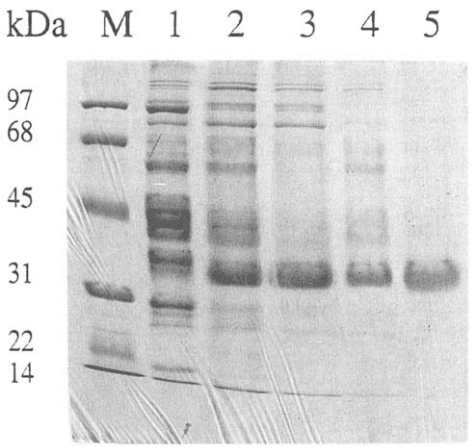

Fig. 7. SDS-PAGE pattern of LDH purification from muscle extract by different procedures. For details, see Table 1. Lane M, marker proteins; lane 1, crude muscle extract; lane 2, method 1; lane 3, method 2; lane 4; method 3; lane 5, method 4.

partitioning of LDH to the top phase. After washing, affinity precipitation and elution, the specific activity and the yield of eluted L-LDH were 245 U/mg and 54% respectively (Table 2). SDS-PAGE showed a single band having a molecular mass of 34 000 (Fig. 7). The reduced yield of LDH during the elution step may partly be due to the denaturation of the enzyme under conditions of low pH. After the whole procedure, the recovery of the Eudragit-dye was 91%. The 9% loss arose partly from the solution transfer during operation and partly from some Eudragit-dye sticking with the particulate matter from the muscle extract and partitioning into the interface. The possible dye leaching will also contribute to the loss.

As a comparison, dissociation of the enzyme from the affinity complex was performed by the addition of the salt phase (11% potassium phos-

Table 2
Purification of crude LDH by integration of two-phase extraction and affinity precipitation

Phase system no.	Stage	Total LDH (U)	Specific LDH (U/mg)	Purification factor	Yield (%)
	Muscle extract	427.1	21.0	1.0	100.0
1	Bottom phase	361.8	34.8	1.7	84.7
2	Top phase	306.9	125.0	6.0	71.9
3	Top phase	292.2	208.1	9.9	68.4
	Elution 1	196.0	245.0	11.7	45.9
	Elution 2	36.3	250.0	11.9	8.0

phate, pH 7.6) to the top phase such that the LDH was transferred to the salt rich phase. The yield of the enzyme was increased to 71%, however with a lowered specific activity of only 199 U/mg. Gel electrophoresis showed the presence of several faint protein bands along with the main band of LDH. To purify the enzyme further, the enzyme solution was dialysed against 10 m*M* phosphate buffer and then treated with DEAE-cellulose. After centrifugation at 3000 *g* for 10 min, 81% of LDH activity remained in the supernatant. The specific activity was 297 U/ml, showing a single band on the electrophoresis gel (Fig. 8).

These results show that using Eudragit as a ligand carrier in aqueous two-phase systems, one has the choice of either precipitating out the polymer bound affinity complex for subsequent enzyme desorption, or dissociating the affinity complex present in the top phase by addition of a salt phase. The former strategy avoids the introduction of salt into the PEG phase and also the removal of the phase polymers from the purified protein is simplified. This purification is also possible using the cheaper bottom phase polymer, Reppal PES, as evidenced from earlier studies [6]. Preliminary studies have also shown the possibility of reuse of Eudragit in two-phase systems.

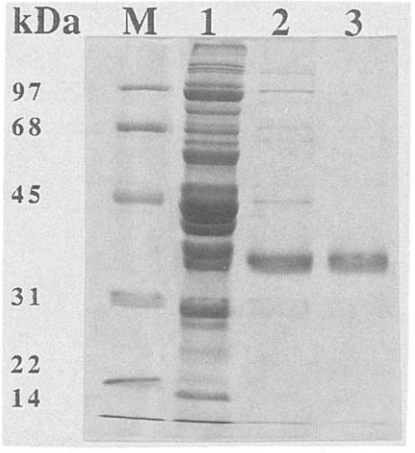

Fig. 8. SDS-PAGE pattern of muscle extract proteins at different stages of LDH purification. Lane M, marker proteins; lane 1, crude muscle extract; lane 2, the enzyme after purification by two-phase partitioning and lane 3, supernatant after DEAE-cellulose treatment.

This study has thus demonstrated that integration of aqueous two-phase extraction and affinity precipitation offers an interesting technology combining many of the strong features of the two sub-technologies.

4. Acknowledgements

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