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Process Design for Purification of Muscle Lactate Dehydrogenase by Affinity Partitioning Using Free Reactive Dyes

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ABSTRACT

It was shown that by using free reactive dyes as affinity ligands, lactate dehydrogenase (LDH) can be purified with affinity partitioning directly from rabbit muscle homogenization. The free reactive dyes not bound to polyethylene glycol (PEG) showed a strong tendency toward the top PEG-rich phase in aqueous two-phase systems, and thus enhanced the affinity partitioning effect. Wide-ranged reactive dyes were screened in terms of their partitioning abilities for LDH. The effects of various parameters on affinity partitioning behavior of LDH, such as phase composition, impurities in raw material, concentration of dyes, pH of the systems, and addition of salts, were studied. The optimized affinity extraction process has been carried out for the large-scale purification of LDH from rabbit muscle homogenization. The enzyme was recovered with a yield of 81.3% and a purification factor of 7.4. Both PEG and dyes were recovered and reused directly without lowering the quality of the product.

Key Words. Affinity partitioning; Aqueous two-phase systems; Lactate dehydrogenase; Free reactive dyes

INTRODUCTION

The use of affinity ligands in the purification of proteins has led to rapid and very selective separation methods. Affinity extraction based on aqueous

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two-phase systems has advantages for large-scale bioseparation processes (1, 2). The large amount of water (70–90%) and the low interfacial tension in aqueous two-phase systems provide an excellent environment for dissolving biological molecules such as enzymes and other proteins and for preserving their structure and activities (3). Moreover, the introduction of affinity ligands has a profound and selective influence on the partitioning of the biological materials in the systems. During recent years, reactive textile dyes have been used in several cases as cheap and relatively specific ligands usually attached to polyethylene glycol (PEG) in systems containing this polymer as one phase-forming component (4, 5). However, this procedure still has some limitations in the recovery and reuse of the ligands and polymers (6).

There are three forms for using affinity ligands: polymer-bound ligand, carrier-bound ligand, and free ligand. Previously, it was considered that free ligands partition in both phases in polymer–polymer aqueous two-phase systems and that they have to bind to one of the phase-forming polymers or some carriers to enhance the distribution of ligand. However, covalent binding of dye to the polymer reduces the number of available binding sites to the target enzyme (7). This might be due to unfavorable interaction between the attached polymer chain and that part of the surface of the protein molecule which surrounds one of the binding sites. Another possible explanation could be that some parts of the dye molecule interact with the polymer chains and contact points on the dye molecule for the binding protein may be masked. Therefore, free ligands have a tendency to bind more strongly than ligands bound to polymer as the long tails of polymer-bound ligands or carrier-bound ligands. Wang et al. (8) reported that the free dyes show a one-sided partition to the top phase in PEG/salt systems and thus enhance the affinity partitioning effect in the systems. Bhide et al. (9) suggested that free reactive dyes could be used in PEG/phosphate systems for the separation of some enzymes from pressed bakers' yeast. However, neither group provided a feasible separation process. In an earlier report (10) we confirmed the possibility of using free reactive dye as an affinity ligand for the separation of lactate dehydrogenase (LDH) from rabbit muscle homogenization. A two-step continuous extraction process was described and employed tentatively, but the purification factor and total yield of LDH were only 4.4 and 68.8%, respectively. Since affinity extraction using free dye ligands offers new potential advantages, a detailed technical study is needed in order to fully utilize this type of separation process.

In the present work, particular emphasis has been placed on the strategies of optimization of the separation process using free dye ligands. A wide range of reactive dyes was screened in terms of their partitioning ability for LDH. A systematic study of the effects of various parameters, such as phase composition, impurities in raw material, concentration of ligands, pH of the systems,

and addition of salts, was carried out to explore an efficient and economic large-scale separation process.

MATERIALS AND METHODS

Materials

Polyethylene glycol (PEG), purchased from Shanghai Chemical Reagent Factory (People's Republic of China), had number-average molecular weights (M_n) of 2000 (1900–2200) and 6000 (5500–7500). Hydroxypropyl starch (PES), Reppal PES100 (MW = 100,000) and Reppal PES 200 (MW = 200,000), was a kind gift from Carbamyl AB (Sweden). Cibacron Blue F3GA and Procion Red HE-3B were obtained from Dalian University of Technology (People's Republic of China). The other reactive dyes, Blue M-BR, Blue K-3R, Blue K-GR, Blue X-R, Red M-2B, Red K-2BP, Red X-3B, Red KE-3B, Yellow X-7G, Yellow K-RN, and Orange KE-2G, were purchased from Shanghai 8th Dyes Chemical Co. (People's Republic of China). Lactate dehydrogenase (E.C.1.1.1.27) was purchased from Dongfen Biochemical Reagent Factory (People's Republic of China). Pyruvic acid and NADH were from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Experimental Techniques

The aqueous two-phase systems were prepared from stock solutions: 30% PES, 30–50% PEG, and 40% $(NH_4)_2SO_4$. All concentrations are given in weight per weight (w/w) percentage. A detailed description of the preparation of aqueous two-phase systems has been given elsewhere (3, 11).

The leg and back muscles from rabbit were cut into pieces and then homogenized for 5 minutes at 4°C in 0.03 M NaOH solution (1–1.5 L/kg tissue). The homogenization was carried out with a high-speed tissue blade crasher.

Protein concentration was measured using Bradford's method (12), with bovine serum albumin used for calibration. The reactive dyes showed a strong and nonlinear effect on absorbance measurement at 595 nm, so corrections were made for the influence of reactive dyes. Lactate dehydrogenase was determined according to Bergmeyer (13) at 340 nm and 25°C. When the concentration of dyes was less than 10 μM in the assay solution, they did not interfere with the enzyme determination. Orange KE-2G was determined photometrically at 480 nm; Yellow K-RN at 395 nm.

The partition coefficient was defined as the ratio of activity of the enzyme in the top phase to that in the bottom phase.

RESULTS AND DISCUSSION

Partitioning Behavior without Affinity Ligands

In the first stage of enzyme extraction using aqueous two-phase systems, the desired enzyme is expected to partition into the top phase while the debris and other mixed proteins (relative to the target enzyme in the homogenization) remain in the bottom phase. However, in PEG/PES or PEG/salt systems, LDH and most proteins concentrated in the bottom PES-rich or salt-rich phases (data not shown). The partition coefficient of LDH in PEG/PES systems, K_e, ranged from 0.05 to 0.8 and decreased with an increasing tie-line length of the systems. K_e in PEG/salt systems was less than 0.01. An increase in PEG molecular weight brought about a reduction of K_e. Moreover, the bottom phases of systems containing higher molecular weight PES were too viscous to be used. Therefore, PEG2000 and PES100 were considered to be favorable for the follow-up research. Since LDH could not be separated from mixed proteins in basic aqueous two-phase systems, affinity ligands were introduced to efficiently separate LDH from muscle homogenization.

Affinity Partitioning of Pure LDH

Partitioning of Free Reactive Dyes

The partition coefficients of free reactive dyes in some PEG/salt and PEG/PES systems are listed in Table 1. In the PEG/salt systems, free reactive dyes tend strongly to the top PEG-rich phase. The partition coefficients in PEG/PES systems are lower than those in PEG/salt systems, but the values are enough to indicate a tendentious partition behavior toward the top PEG-rich phase. This may be caused by the hydrophobic difference between the two phases of the systems. The one-sided partitioning behavior of free dyes may enhance the affinity partitioning effect in aqueous two-phase systems.

Influence of Free Dyes on the Partition of LDH in Polymer–Polymer Systems

The affinity partitioning behavior of pure LDH was studied in the presence of various free reactive dyes. An increase in $\Delta \log(K_e)$ was determined by increasing the dye concentration in the two-phase systems as shown by the hyperbolic curves in Fig. 1. The maximum extraction power ($\Delta \log K_{max}$) and the relative affinity of the dye ligand ($C_{1/2}$, the concentration of dye yielding 0.5 $\Delta \log K_{max}$) are listed in Table 2. The highest values of $\Delta \log K_{max}$ were obtained with Yellow K-RN and Orange KE-2G. The results indicate that the structural differences of various dyes significantly influence the affinity partitioning effect. Because of the similarity in structure of Cibacron

TABLE 1
Partition Coefficients of the Reactive Dyes in PEG/$(NH_4)SO_4$ and PEG/Hydroxypropyl Starch Systems, 25 mM Sodium Phosphate Buffer (pH 7.4), 0.5 mg Dye/g System, 25°C

		K_d		
Reactive dyes	M	PEG2000 12.8%, $(NH_4)_2SO_4$ 11.5%	PEG2000 11%, PES100 16.6%	PEG6000 7.0%, PES100 16.6%
Cibacron Blue F3-GA	774.2	⩾100	3.72	5.65
Procion Red HE-3B	1338.0	~100	9.38	12.05
Blue M-BR	948.2	⩾100	8.34	
Blue K-3R	733.1	>100	5.60	
Blue K-GR	662.0	⩾100	4.98	
Blue X-R	822.0	~100	4.04	6.22
Yellow X-7G	655.3	94.4	2.55	6.19
Yellow K-RN	815.1	>100	7.58	
Orange KE-2G	1776.0	⩾100	12.45	16.73
Red M-2R	948.2	~100	7.08	
Red K-2BP	808.5	~100	10.62	
Red X-3B	615.3	~100	5.69	
Red KE-3B	1470.0	~100	11.72	

Blue F3-GA, Blue M-BR, Blue K-3R, and Blue K-GR, they have a similar affinity. The two 2-amino-8-naphthol-6-sulfonic acid rings within Orange KE-2G seem to enhance its affinity for LDH; Red M-2B and Red K-2BP, which have only one 1-amino-8-naphthol-3,6(or 3)-sulfonic acid ring, show a low affinity for the enzyme. Yellow K-RN, which shows a relative high Δ log K_{max} value and a relative low $C_{1/2}$ value in comparison with all the other dyes, seems to be a new and effective class of dye to bind LDH.

Affinity Partition in PEG/Salt Systems

The effect of the free dye ligands on the partition of pure LDH in the PEG/$(NH_4)_2SO_4$ systems was observed. The influence of free dyes in PEG/$(NH_4)_2SO_4$ systems was very little (Fig. 2). The free dyes concentrated significantly in the top PEG-rich phase ($K_d > 100$) while LDH remained in the bottom salt-rich phase ($K_e < 0.01$).

Preliminary Idea on Separation Process

The reactive dyes tended to concentrate in the top phase of PEG/PES systems, and two kinds of dyes, Yellow K-RN and Orange KE-2G, increased K_e more effectively than other dyes as shown in Fig. 1. Therefore, Yellow K-RN and Orange KE-2G were considered to be favorable for the affinity

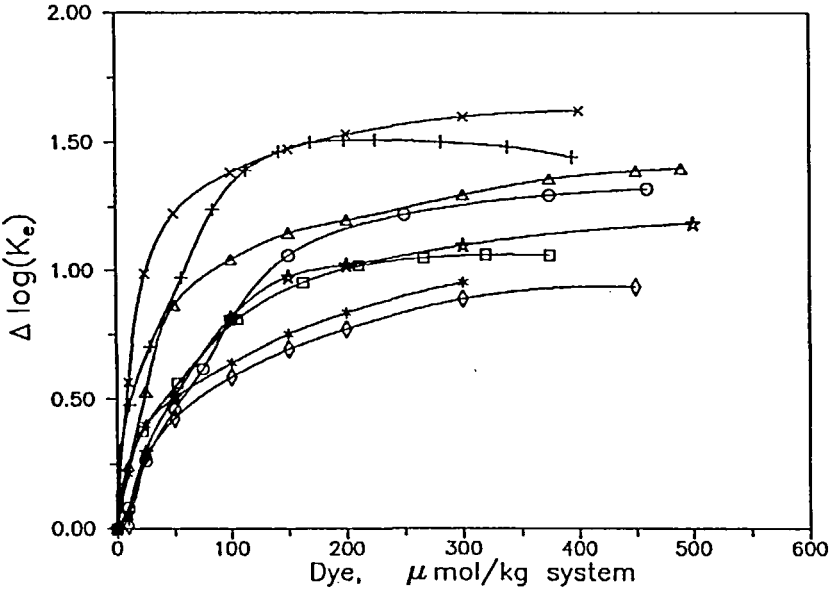


FIG. 1 Influence of the concentration of free dyes on the partition coefficient of pure LDH. The system (5 g) contained 11% PEG2000, 16.6% PES100, 25mM sodium phosphate buffer (pH 7.4), and 10 units of the enzyme. The systems were equilibrated at 25°C. Test dyes: (+) Orange KE-2G; (×) Yellow K-RN; (○) Cibacron Blue F3-GA; (□) Blue M-BR; (△) Blue K-3R; (☆) Blue K-GR; (*) Red M-2B; (◇) Red K-2BP.

TABLE 2
Affinity Partitioning of LDH Expressed by Δ log K_{max} and $C_{1/2}$

Reactive dye	Δ log K_{max}	$C_{1/2}$ (μM)
Yellow K-RN	1.63	17
Orange KE-2G	1.51	33
Blue K-3R	1.43	37
Cibacron Blue F3-GA	1.33	73
Blue K-GR	1.18	60
Blue M-BR	1.07	48
Red M-2B	0.98	43
Red K-2BP	0.94	58

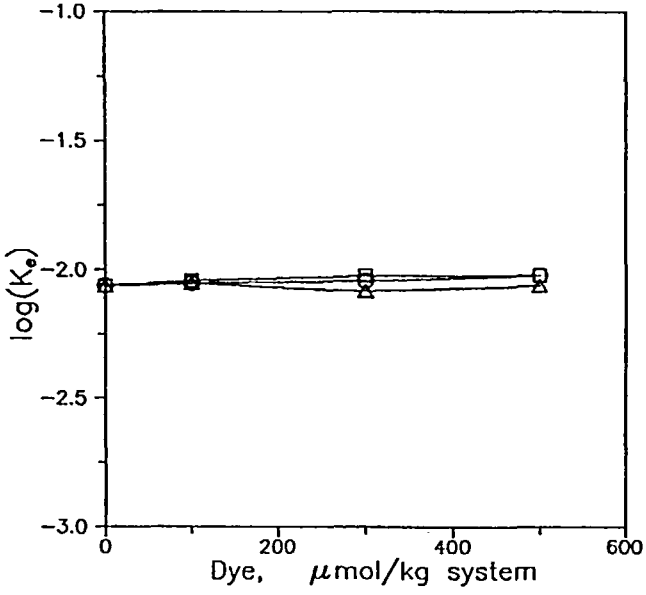


FIG. 2 Influence of free dyes on the partition behavior of pure LDH in PEG/$(NH_4)_2SO_4$ systems. The system (5 g) contained 12% PEG2000, 11.5% $(NH_4)_2SO_4$, and 10 units of the enzyme. The systems were equilibrated at 25°C. Test dyes: (△) Orange KE-2G; (□) Yellow K-RN; (○) Cibacron Blue F3-GA.

extraction. Furthermore, the partitioning behavior in PEG/salt systems may be used to separate LDH from a dye–LDH complex and recover the free dyes and PEG. To purify LDH from muscle homogenization and explore a feasible large-scale separation process, more details of the influence factors should be investigated.

Optimization of Affinity Extraction Conditions for Practical Use

Selection of Ligand Dyes for Raw Materials

Using Yellow K-RN and Orange KE-2G, the partitioning behavior of LDH of muscle homogenization in PEG/PES systems was studied (Fig. 3). The affinity behavior of homogenization systems had some differences from that of pure LDH. For rabbit muscle homogenization, Orange KE-2G was more effective than Yellow K-RN, indicating that the mixed proteins of muscle extract significantly influenced the affinity partitioning behavior of LDH.

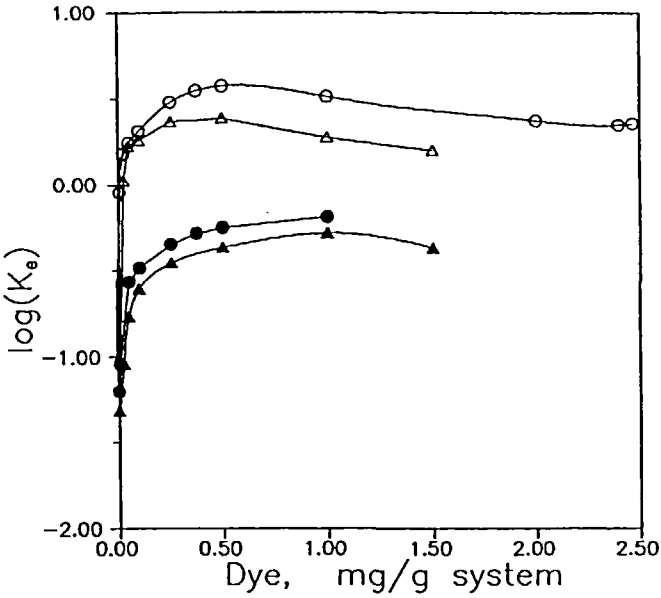


FIG. 3 Influence of the concentration of free dyes on the partition coefficient of LDH of muscle homogenization. System 1 (○, ●): 11% PEG2000, 16.7% PES100. System 2 (△, ▲): 13% PEG2000, 16.7% PES100. 25 mM sodium phosphate buffer (pH 7.4). In all cases the homogenization concentration was 10% (w/w). T = 25°C. Dyes: (○, △) Orange KE-2G; (●, ▲) Yellow K-RN.

Figure 4 shows that the partition coefficient of the target enzyme and free dyes increased significantly with an increasing concentration of dye in the systems. This indicates that the partition coefficients of free dye do not keep a constant value, which may be an important factor on affinity partitioning using free ligands. Various proteins mixed with the target enzyme may significantly influence the partitioning of dyes and thus change the affinity partitioning behavior. Therefore, Orange KE-2G had a stronger selectivity for the separation of LDH from muscle homogenization than did Yellow K-RN, and so it was chosen as the best dye for affinity extraction.

Selection of Extraction Systems, Phase Ratio, and pH

For a given tie line the effect of phase ratio on the partition coefficient and top-phase yield of LDH was observed (see Fig. 5). The values showed a concave-down curve with increasing phase ratio from 0.7 to 5. The minimum value reached when the phase ratio was 1.5. Customarily K_e should remain

constant while the phase ratio is changed for a given tie line. However, in our experiments, as the total concentration of dye in the systems maintained constant, the top-phase concentration of free dye obviously decreased with increasing phase ratio. The changes of free dye in the top phase certainly cause K_e to vary. Thus, the influence of the phase ratio is important for affinity partitioning. The increase in phase ratio brought about a steady increase of yield from 71.4 to 91.4%, but a reduction of the purification factor. On the other hand, the effectiveness of affinity partitioning of LDH decreased with an increasing tie line length of the systems (result not shown). An appropriate system we chose was PEG2000 13.0% and PES100 11.8%.

A maximum in the partition coefficient of LDH was observed at neutral pH (pH 7.4), and a decrease was encountered at acid solution (Fig. 6). The dependence of the enzyme activity on pH is consistent with that of thermal stability on pH (14). A neutral pH was suitable to maximize the stability and thus enhance the affinity effect of the target enzyme in partition experiments.

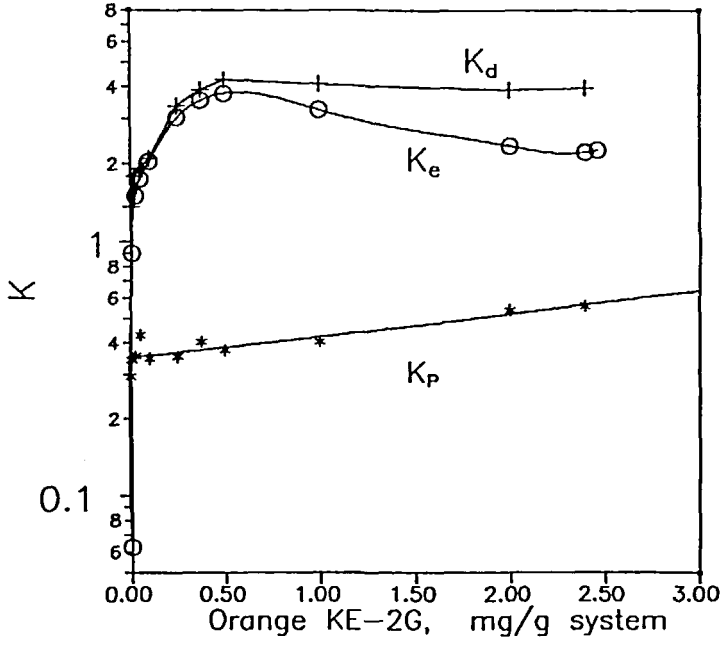


FIG. 4 Affinity partition behavior of LDH (○), dye (+), and protein (*) in PEG/PES systems. System: 11% PEG2000, 16.7% PES100, 25 mM sodium phosphate buffer (pH 7.4), 10% (w/w) homogenization. T = 25°C.

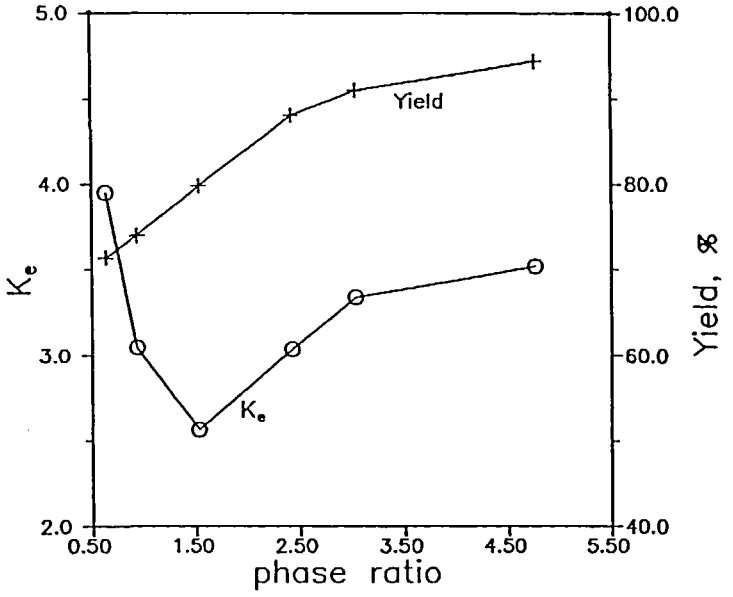


FIG. 5 Influence of phase ratio on the partition coefficient (O) and top-phase yield (+) in PEG2000/PES100 systems. In all cases the homogenization concentration was 10% (w/w), including the Orange KE-2G 0.5 mg/g system and 25 mM sodium phosphate buffer (pH 7.4). $T = 25°C$.

On the other hand, the reactive dyes are believed to mimic the natural ligands NAD^+ and ATP, and could be bound specifically to the nucleotide binding domain of dehydrogenase (15). Therefore, the optimum pH (7.4) for LDH is suitable to enhance the dye–LDH interaction.

Influence of Salt Additions in PEG/PES Systems

Different salts showed diverse effects on the affinity partitioning of LDH (Fig. 7). For $(NH_4)_2SO_4$ and NaCl, the partitioning was almost independent of the concentration of the addition salts. When $MgCl_2$ was added to the systems, a remarkable dependence was observed. The partition coefficient of LDH decreased from 5.6 to 0.38 when the $MgCl_2$ concentration was increased from 0 to 50 mM. Addition of salts to aqueous two-phase systems is known to influence the partitioning of charged molecules between the phases. The addition of $MgCl_2$ probably influences the partitioning of free reactive dyes or destroys the dye–enzyme interaction, thus forcing LDH into the bottom phase.

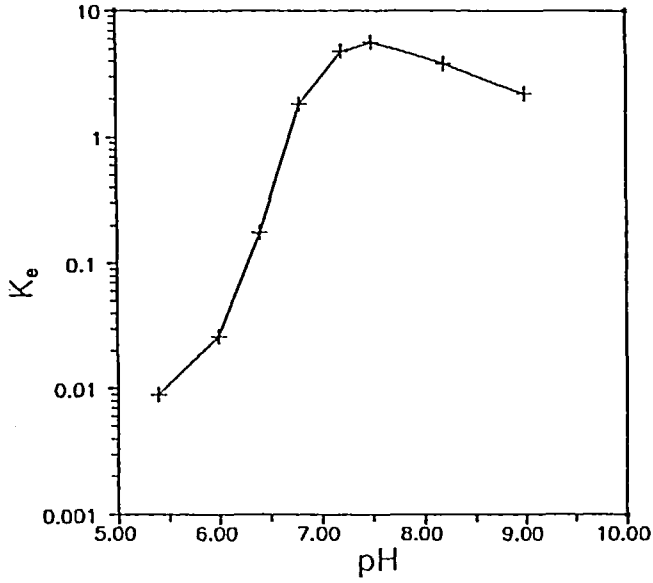


FIG. 6 Influence of pH on affinity partitioning of LDH of muscle homogenization. System: 13% PEG2000, 11.8% PES100, Orange KE-2G 0.5 mg/g system, and 25 mM sodium phosphate buffer (pH 7.4). In all cases the homogenization concentration was 10% (w/w). T = 25°C.

Separation Process for the Continuous Extraction of LDH from Rabbit Muscle Homogenization

Scheme for Separation Process

Based on the above results, Orange KE-2G was chosen as the best dye ligand, and the appropriate system for affinity partitioning was PEG2000/PES100 (PEG2000 13.0%, PES100 11.8%, pH 7.4). A process scheme for the continuous extraction of LDH from rabbit muscle homogenization is given in Fig. 8. In this scheme the homogenization is added directly in the first-step phase system (PEG/PES aqueous two-phase system). The debris is distributed to the bottom phase, and the target enzyme is extracted into the top phase by the use of free reactive dyes. After separation in a centrifugal separator, the top phase can be treated in two way: 1) directly combined with a solution of ammonium sulfate (40%) to yield a PEG/salt two-phase system, which simplifies the process but has a low purification factor; (2) after being washed with the pure bottom PES-rich phase, a PEG/salt

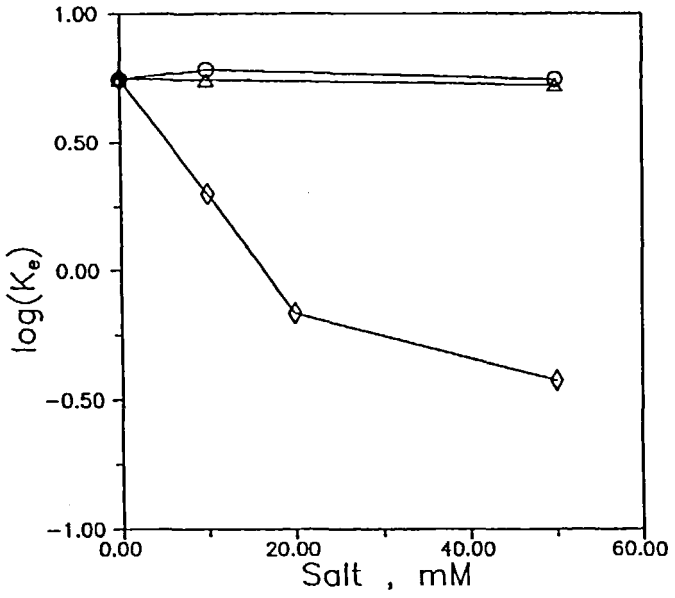


FIG. 7 Influence of addition of salts on affinity partitioning of LDH of muscle homogenization. System: 13% PEG2000, 11.8% PES100, Orange KE-2G 0.5 mg/g system, and 25 mM sodium phosphate buffer (pH 7.4). T = 25°C. In all cases the homogenization concentration was 10% (w/w). Addition salts: (○) NaCl; (△) $(NH_4)_2SO_4$; (◇) $MgCl_2$.

system is the yield, which has a highly efficient extraction but includes a time-consuming extra step. In the PEG/salt two-phase system the enzyme is recovered in the bottom salt-rich phase, and both PEG and free dye concentrating in the top PEG-rich phase are recycled.

Optimization of Operation Conditions

Various operation conditions, such as concentration of homogenization, washing process, phase ratio, pH control, and recycling of PEG and dye, were studied to optimize the extraction process. Different conditions and results of extraction experiments are listed in Table 3. From Experiments 1, 2, 3, and 4 it was found that: 1) the washing process significantly enhances the purification factor but the total yield is reduced a little; and 2) the addition of 10% homogenization is more suitable for the affinity extraction process. The objective of Experiments 5 and 6 was to improved

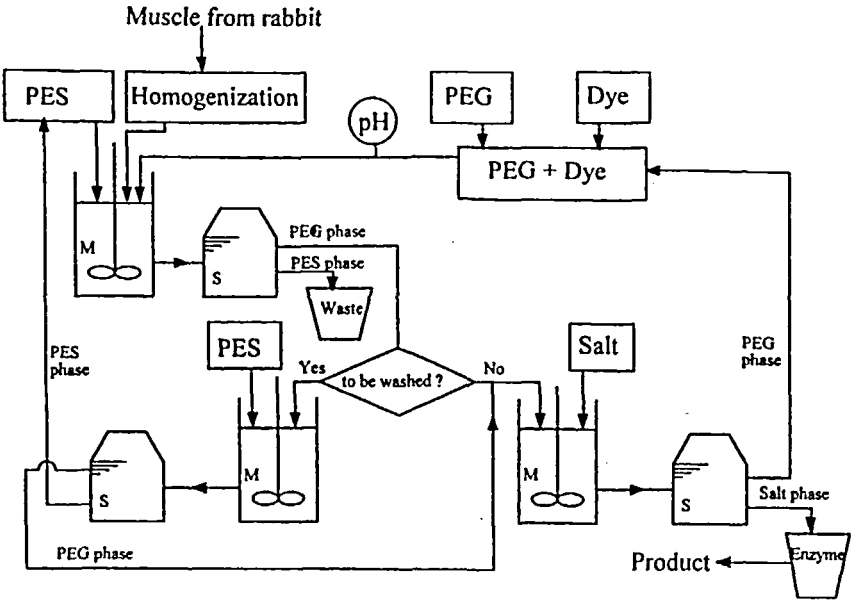


FIG. 8 A process scheme for the continuous extraction of LDH from rabbit muscle homogenization (S = separator, M = mixer).

TABLE 3
Different Operation Conditions and Results of Separation Process Using Orange KE-2G as Free Ligand (system: 100 g)

	Extraction conditions							Result	
	First-step extraction[a]				Second-step extraction				
Exp.	Type	Homogenization (%)	Dye (%)	Washing	Type	Phase ratio	Other	Purification factor	Yield (%)
1	PEG/PES	20	0.15	—	PEG/$(NH_4)_2SO_4$	0.9		4.07	85.1
2	PEG/PES	20	0.15	yes	PEG/$(NH_4)_2SO_4$	0.9		5.27	72.1
3	PEG/PES	10	0.05	—	PEG/$(NH_4)_2SO_4$	0.9		3.97	86.6
4	PEG/PES	10	0.05	yes	PEG/$(NH_4)_2SO_4$	0.9		5.75	80.8
5	PEG/PES	10	0.05	yes	PEG/$(NH_4)_2SO_4$	3.8		7.38	81.3
6	PEG/PES	10	0.05	yes	PEG/PES[b]	3.1		12.48	60.9
7	PEG/PES[c]	10	0.05	yes	PEG/$(NH_4)_2SO_4$	2.7	No pH control	6.67	58.8
8	PEG/PES[c]	10	0.05	yes	PEG/$(NH_4)_2SO_4$	3.2	pH control	7.21	81.6

[a] First-step extraction: 13% PEG2000, 11.8% PES100, 25 mM sodium phosphate buffer (pH 7.4).
[b] After washed with the pure bottom PES-rich phase, the top PEG-rich phase was added with pure bottom PES-rich phase and 0.1 M $MgCl_2$.
[c] Recycling process: The PEG and dye used in the first-step extraction came from recycling the PEG-rich phase of second-step PEG/salt extraction.

the purification factor. Experiment 5 changed the phase ratio of the second-step system (PEG/salt) to 3.8, which increased the purification factor to 7.4 and maintained a total yield at 81.3%. The specific activity could reach 110 U/mg protein. Experiment 6 added 0.1 M $MgCl_2$ to the PEG/PES system to force LDH into the bottom PES-rich phase, which significantly increased the purification factor to 12.5 but the total yield was only 60.9%. Based on both the purification factor and recovery, the extraction conditions of Experiment 5 were more efficient and suitable for the separation of LDH from muscle homogenization. In order to study the recycling process (Experiments 7 and 8), PEG and dye recovered from the top phase of PEG/salt systems were used again without any purification. For Experiment 7 the top phase of the PEG/salt system was used directly to generate a new PEG/PES system. It was found that the yield of the first-step extraction was only 68.8% and the pH value of the system decreased to 5.5. Since the pH strongly influences affinity partitioning behavior (as shown in Fig. 6), the total yield of Experiment 7 decreased to 58.8%. In Experiment 8 the pH value was adjusted to 7.4 by adding the appropriate NaOH solution. The purification factor and the total yield reached 7.2 and 81.6%, respectively. The values were similar to those of Experiment 5. This demonstrates that the recycling process is feasible and efficient.

Economic and Industrial Aspects for Large Scale Use

The attractiveness of liquid–liquid extraction is due to the ease of scaling up the process. The partition behavior can be determined on a very small scale in the laboratory and then applied directly to a large scale (16). The above separation process was carried out on a laboratory scale (100 g system) to determine the optimal conditions (such as a suitable ligand, a suitable way to handle affinity partitioning, and the effective recovery of the ligand and the phase-forming polymers) in order to achieve an economical process for industrial application. The reactive dye and PEG recoveries of 96 and 88%, respectively, made the process reasonably economical. The results proved that the continuous extraction process described above is feasible for the large-scale purification of LDH from rabbit muscle. Preliminary analysis of the cost indicates that the main cost is due to the bottom-phase polymer (in the first extraction step). The bottom phase contains high concentrations of proteins, membranes, and other cell debris, and it is therefore not feasible to recover the polymer in this step. The use of PES instead of dextran as the phase-forming polymer obviously reduces the cost of the separation process. Moreover, hydroxypropyl starch

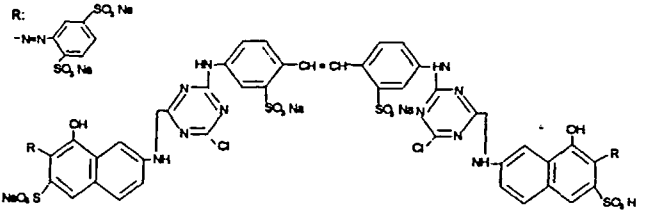
is biodegradable and is classified as an acceptable food additive (1). In the case of animal tissue, the bottom phase could be used as cattle food after drying.

CONCLUSIONS

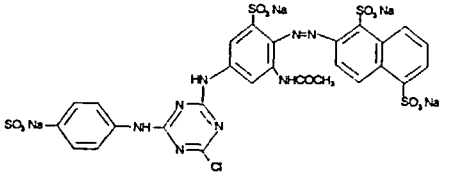
Free reactive dyes not bound to PEG show one-sided partition to the top PEG-rich phase in PEG/salt and PEG/PES aqueous two-phase systems, and can be used directly for affinity partitioning. Therefore, no chemical modification to PEG is needed for some cases. A continuous affinity extraction process has been carried out for the large-scale purification of LDH from rabbit muscle homogenization. The enzyme was recovered with a yield of 81.3% and a purification factor of 7.4. The relative low price of dye ligand and the effective recycling of PEG and dye made the separation process reasonable economically. Affinity partition offers a rapid and selective extraction of enzymes, well-suited for large-scale application. By using a cheap phase-forming polymer and a high degree of recovery of ligands, the process may have considerable economic advantages in many cases.

APPENDIX

Orange KE-2G



Yellow K-RN



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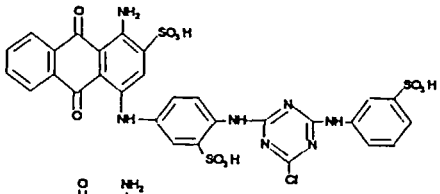
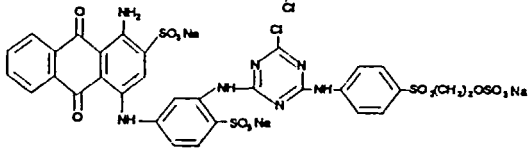
Cibacron Blue F3-GA



Blue M-BR



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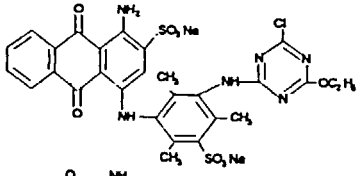

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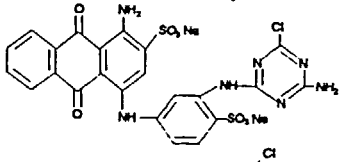

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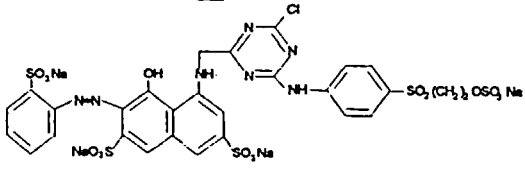

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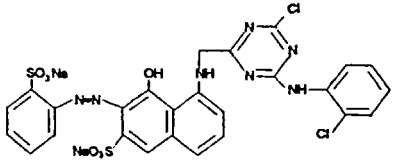

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