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PROCESS DESIGN FOR LARGE-SCALE PURIFICATION OF FORMATE DEHYDROGENASE FROM *CANDIDA BOIDINII* BY AFFINITY PARTITION

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SUMMARY

It was shown that formate dehydrogenase (FDH) can be purified with affinity partitioning directly from the cell homogenate of *Candida boidinii*. The procedure is faster than the other methods known and scale-up is possible without apparent difficulties. The specific activity of the purified FDH was between 3.5 and 6 U/mg, depending on the disintegration method. The top phase containing the affinity ligand Procion Red HE3b coupled to PEG-6000 can be recycled directly at least four times without lowering significantly the quality of the product. The costs for the purification of FDH by affinity partition are comparable to other methods.

INTRODUCTION

The use of aqueous two-phase systems for the purification of proteins and the separation of cell organelles and cell debris is an established method in biotechnology [1—4]. A special field of this technique is the affinity partitioning of enzymes. It is based on the specific interaction of an enzyme with an affinity ligand that is covalently bound to a phase-forming polymer such as polyethylene glycol (PEG). The partitioning of the ligand will be governed by the PEG moiety, concentrating it in the PEG-rich phase. So, in PEG—dextran systems, a protein that binds to the ligand can be preferentially taken up into the top phase. Examples of such ligands are reduced nicotinamide-adenine dinucleotide (NADH) or triazine dyes [5, 6].

During recent years, an increasing number of papers on affinity partitioning have been published [5, 7—9]. However, a process whereby an enzyme is extracted directly from the cell homogenate by an affinity ligand has not yet been described. Theoretical considerations have shown that the binding constant between enzyme and ligand determines the selectivity, whereas the number of ligands bound to the enzyme determines the yield [10].

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Investigations of the partition coefficient of formate dehydrogenase (FDH) in the presence of PEG-Procion Red HE3b (PEG-Red), PEG-NADH and PEG-Cibacron Blue 3GA (PEG-Blue) indicated a much weaker binding of FDH to PEG-Blue than to PEG-Red and PEG-NADH. This was confirmed by the dissociation constants between FDH and the ligands (FDH—PEG-Blue: 97 μmol/l, FDH—PEG-Red: 1.7 μmol/l, FDH—PEG-NADH: 0.7 μmol/l) [10].

The present paper describes a process for the purification of FDH using PEG-Red as an affinity ligand, taking into account a possible scale-up, the recycling of the ligand and an estimation of the economics in comparison to other methods.

EXPERIMENTAL

Cells

The cultivation of *Candida boidinii* on methanol was carried out according to Sahm and Wagner [11].

Polymers

PEG-6000 was purchased from Serva (Heidelberg, F.R.G.), Dextran T 500 from Pharmacia (Uppsala, Sweden) and crude dextran was a gift from Pfeiffer and Langen (Dormagen, F.R.G.).

Affinity ligands

The triazine dye Procion Red HE3b (Deutsche ICI, Frankfurt, F.R.G.) was coupled to PEG-6000 (Serva, Heidelberg) according to the method of Morr [12]:

Chlorination. PEG-6000 was liquefied overnight at 70°C in an incubator, and then made water-free by means of a vacuum pump. In a rotary evaporator, a molar excess of 50% thionyl chloride was added. Dry nitrogen was passed through the reaction vessel to avoid water contamination. After 8 h at 70°C the reaction was terminated, and the remaining thionyl chloride was removed under vacuum.

Amination. PEG—Cl was dissolved in a glass autoclave (1.5l; Schott, F.R.G.) with a large excess of concentrated ammonia so that the vessel was filled to ca. 75%. The amination was carried out at 110°C for 30 h under pressure. Excess ammonia was removed with a rotary evaporator.

Coupling. Procion Red HE3b was mixed with amino-PEG-6000 in a molar ratio of 2.0, working in aqueous solutions as concentrated as possible. The reaction mixture was stirred at pH 11.0 and 60°C for ca. 24 h. The product was chromatographed on Sephadex G-50 using 0.01% potassium chloride as an eluent to separate the unbound dye. Dialysis in water followed to remove the salts. Working on a larger scale the procedure was changed slightly, lowering the ratio of Procion Red HE3b to amino-PEG-6000 to 1.8 so that nearly all the dye was bound to PEG. In this way, the gel filtration step could be omitted.

Cell disintegration

Unless otherwise specified, the cells were broken up as a 40% suspension

in 0.05 *M* potassium phosphate buffer (pH 8.0) by ultrasonic treatment.

Heat denaturation

After addition of 10% (w/v) ammonium formate, the disintegrated cells were heated in a water bath set at 75°C and held for 10 min at 60°C.

Partition experiments on a large scale

Phase systems for the affinity step of up to 10 kg were mixed with a magnetic stirrer and separated in a batch centrifuge (ZK 630, Hermle, Gösheim, F.R.G.). Systems above 10 kg were mixed with a blade stirrer and separated in a nozzle separator (YEB 1330 A, Alfa Laval, Glinde, F.R.G.). The PEG—salt system was separated in an open disc stack separator (Gyrotester B, Alfa Laval).

Ultrafiltration

The bottom phase of the PEG—salt system was freed from salts and PEG in a Hollow Fi PM 50 plant from Amicon (Witten, F.R.G.). The flow-rate of the filtrate was 20 $l/h/m^2$).

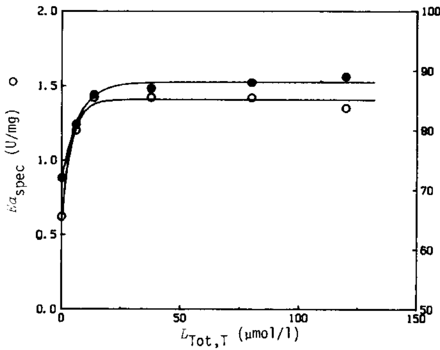
Analysis

Enzymatic activity was determined photometrically by the increase in NADH concentration using formate as the substrate [13]. It was corrected for inhibitory effects of the dye, if necessary. Protein concentration was estimated by the method of Bradford [14]. The concentration of PEG-Red was determined by absorption at 542 nm in a spectrophotometer using an experimentally determined extinction coefficient of 33 900 1 $mmol^{-1}$ cm^{-1}. Partition coefficients were calculated by dividing the activity or concentration in the top phase by the activity or concentration in the bottom phase.

RESULTS AND DISCUSSION

Parameter of the phase system

Initial experiments in systems composed of PEG-6000, dextran T500, 0.05 mol/kg potassium phosphate and 4% cells showed that by varying the PEG-Red concentration the enzyme yield in the top phase was increased to 90%, but the maximal specific activity was only 1.4 U/mg (Fig. 1). The reason for this observation was the high apparent partition coefficient for protein in the absence of the affinity ligand. Therefore, we looked for a system in which the partition coefficient of the protein falls in the range 0.04—0.08. The influences of different salts, PEGs with differing chain lengths and various pH values were examined [15]. PEG-10000 was found to lower the partition coefficients to the required value if high concentrations (16%) were used (Fig. 2). On the other hand, affinity partitioning experiments at this concentration, using different buffers, showed that by partitioning the proteins predominantly into the lower phase rather high concentrations of ligand were necessary to bring FDH into the upper phase. Moreover, the yield of FDH was low and the specific activity was unsatisfactory. This is demonstrated in Table I.



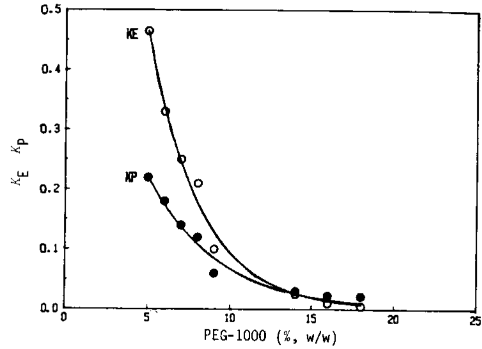


Fig. 1. Affinity extraction of formate dehydrogenase from cell debris of *Candida boidinii* with PEG-Procion Red HE3b.

Fig. 2. Dependence of partitioning of formate dehydrogenase and protein on the PEG concentration. K_P = Partition coefficient of protein; K_E = partition coefficient of FDH.

TABLE I

AFFINITY PARTITIONING OF FDH IN PHASE SYSTEMS CONTAINING SEVERAL BUFFERS AND 16% PEG-10000

$L_{Tot,T}$ = ligand concentration in the upper phase; Ea_{spec} = specific activity: K_{FDH} = partition coefficient of FDH; K_P = partition coefficient of protein. System a: 16% PEG-10000 — 1% crude dextran—20% cells—0.05 mol/kg Tris—acetate; system b: as system a, but 0.05 mol/kg Tris—Cl instead of Tris—acetate; system c: as system b, but with the addition of 0.07 mol/kg potassium chloride.

System	$L_{Tot,T}$ (mmol/l)	Ea_{spec} (U/mg)	Yield (%)	K_{FDH}	K_P
a	1.89	2.19	48.4	0.18	0.031
b	1.87	2.23	55.4	0.31	0.028
c	3.63	2.43	45.9	0.19	0.029

Effect of heat denaturation

Heat denaturation was included in the process, as it is known to improve the specific activity of FDH by coagulation of contaminating proteins [2, 13]. At the same time, it was expected that other dehydrogenase and kinases competing for the ligand would be deactivated. In fact, a specific activity of 3.7 U/mg and a yield of between 70 and 80% were obtained even though the PEG-10000 concentration was only 9% in a PEG—crude dextran system including 0.05 mol/kg potassium phosphate buffer (pH 8.0). Table II summarizes the conditions of the process and the results obtained.

Variation of cell disintegration

Further improvement in the purity of the isolated enzyme was accomplished by changing the disintegration step. It has been previously observed that proteins leak out when suspending frozen cells of *Candida boidinii* in buffer [16]. This effect depends on the incubation time. Furthermore, it was found that FDH was among those proteins released first (Fig. 3). In this way, it was possible to increase the specific activity of the end product to 6 U/mg. Higher

TABLE II

PROCESS FOR THE PURIFICATION OF FDH

Method	Conditions	Yield (%)	Specific activity (U/mg)
Cell disintegration	30 min ultrasonic	100	0.57
Heat denaturation	10 min, 60°C, 10% ammonium formate	99.2	0.81
Affinity partition	$L_{Tot,T}$ = 0.96 mmol/l	68.4	—
Separation of FDH—ligand	Top phase of the affinity partition step + 9% potassium phosphate	72.2	3.71

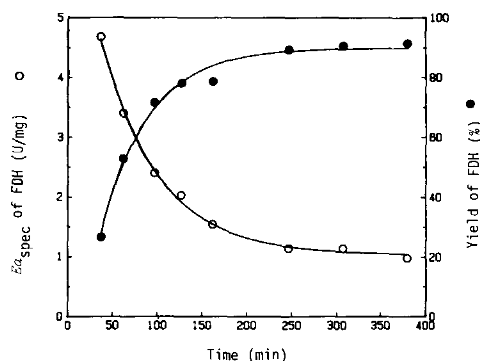


Fig. 3. Release of formate dehydrogenase and proteins from cells of *Candida boidinii* as a function of time.

values would be possible if cuts in the yield were to be accepted (for comparison: the pure enzyme has a specific activity of ca. 8 U/mg; [15]). It should be noted that specific activities based on protein determination according to Lowry et al. [17] give lower values.

Recovery of the polymer-bound ligand

To separate enzyme and ligand, 9% potassium phosphate was added to the top phase of the first extraction. In the resulting PEG—salt system, FDH was shifted nearly 100% to the salt-rich lower phase, whereas PEG-Red was completely enriched in the top phase. One problem in the design of an economic process of enzyme purification is the recycling of the expensive affinity ligand. Kopperschläger and Johansson [9] suggested the extraction of PEG and the ligand bound to PEG with chloroform. This technique has some disadvantages: (a) it is not generally applicable for triazine dyes, because dyes with a higher number of ionic groups are not very soluble in the organic phase. For example, for PEG-Red carrying six sulphonate groups the yield was only 20%, although the volume ratio of organic to aqueous phase was ca. 50; (b) some of the salt contained in the aqueous phase is lost; (c) chloroform brings about special demands on the material and safety of the plant; (d) an additional thermal process becomes necessary for the removal of chloroform.

TABLE III

RECYCLING OF THE UPPER PHASE

V_T and V_B are volumes of the upper and lower phase of the affinity extraction, respectively; Ea_{spec} is specific activity.

Number of uses	V_T (ml)	V_B (ml)	Yield (%)		Ea_{spec} (U/mg)
			FDH	PEG-Red	
1	3.70	1.11	75	100	6.04
2	3.74	1.07	80	84	6.05
3	3.47	1.04	78	80	5.81
4	3.74	1.10	88	85	5.88
5	3.61	1.18	67	77	3.54

Therefore, a simple procedure was needed. The protocol adapted is based on the fact that the buffer in the affinity step is potassium phosphate, so the top phase of a PEG—phosphate system could be directly recycled. Investigation showed that the top phase of the two-phase system can be recycled directly, four times, without lowering the quality of the product significantly (Table III).

Scale-up

The above process was carried out in larger scales. The results obtained from these experiments were comparable with those from 5-g systems, including the recycling of the affinity ligand. The top phase of a 5-kg system extracting 1 kg of cell mass at a time was used successfully five times in repetitive experiments (Table IV). By comparing the data given in Table IV, one should note that the systems with 50 and 220 kg were prepared with different batches of *Candida boidinii* cells, grown in slightly different ways, which may account for the differences in specific activity obtained. On investigating the conditions for a scale-up, it was observed that a tiny amount of PEG-Red remained in the lower phase of the PEG—salt system, yielding a lyophilized FDH with a weak red colour. To minimize the amount of PEG-Red in the final product, the bottom phase of the second partition step was re-extracted by adding 1% PEG-10000. In this way, a considerable portion of the ligand was enriched in the upper phase of the resulting one third of the two-phase system. The

TABLE IV

SCALE-UP OF THE AFFINITY EXTRACTION OF FORMATE DEHYDROGENASE

Scale of the phase system (kg)	Yield (%)	Specific activity (U/mg)	Number of experiments	Recycling of top phase
0.005	72 ± 3	5.9 ± 0.06	5	4
5.0	78 ± 2	5.0 ± 0.05	5	4
10.0	70 ± 4	4.7 ± 0.11	2	1
50.0	65	3.7	1	—
220.0	74	3.5	1	—

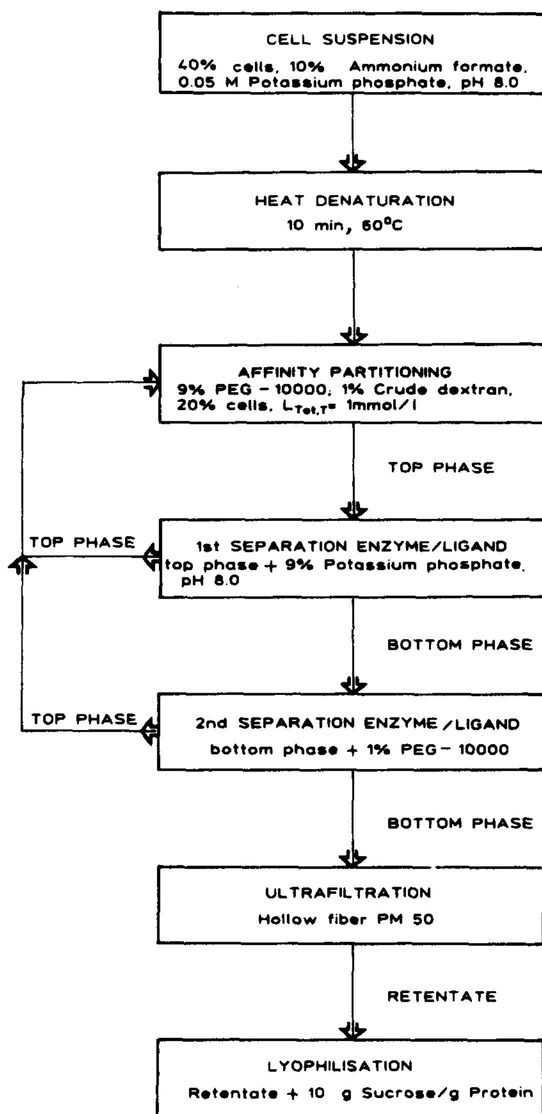


Fig. 4. Process for purification of formate dehydrogenase with affinity extraction as the central step.

top phase of both PEG—salt systems were combined and recycled. Fig. 4 presents a flow sheet of the whole process. In this way, the FDH produced was tested for its application in an enzyme-membrane reactor for continuous NADH regeneration in the production of L-leucine [18]. Fig. 5 shows that the reactor performed without any difficulties over a period of fourteen days, when it was terminated. The small amount of PEG-Red contained in the product showed no significant effect on amino acid synthesis by the coupled-enzyme system.

Economics

To decrease the production costs of FDH still further, the synthesis of

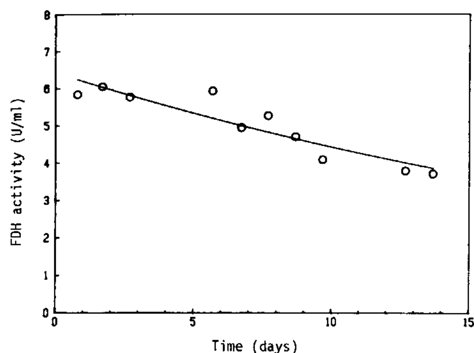


Fig. 5. Enzymic activity of formate dehydrogenase in an enzyme-membrane reactor producing L-leucin continuously.

PEG-Red was simplified and gel filtration was omitted (see Experimental). Although containing a mixture of unbound, mono- and disubstituted PEG, as well as a small amount of free dye, the product was suitable for affinity extraction (Table IV). For the production of 410 g of PEG-Red, a price of US$ 0.17 per g PEG-Red was calculated using eqn. 1 [19]:

$$T = 1.13\,(M + E) + 2.6\,L + I \tag{1}$$

where T = total costs, M = material costs, E = energy costs (US$ 0.05 per kWh), L = labour costs (US$ 12 per h), I = investment costs (13% depreciation per year, operation time 250 days per year).

If affinity partition is used for the purification of enzymes on an industrial scale, large amounts of ligand are necessary. Therefore, a price for PEG-Red was estimated for a scale-up factor of 100, according to eqn. 2 [20]:

$$K_2 = K_1[(m + e)f + lf^b + if^c] \tag{2}$$

where K_1 = total costs on a small scale, K_2 = total costs for a scale-up with a factor f, f = scale-up factor, m, e, l, i = part of the material, energy, labour and investment costs, b, c = exponents characterizing the non-linear increase in costs with the scale-up factor (b = 0.3, c = 0.6).

From these calculations, a price of US$ 2.1 per kg PEG-Red was derived. In Table V, data for a cost calculation are presented according to the process steps. In this case, FDH was purified from 10 kg of cells. Material costs are shown in parentheses if the upper phase is not recycled. It can be seen that, except for the labour costs, the material costs are the highest; this is because they rise linearly with the scale-up factor (see eqn. 2). Therefore, recycling of the top phase is very cost-effective and necessary to meet economic constraints.

Calculating the costs for extraction of 100 kg of cells, a price of US$ 1.3 per 1000 U FDH results, assuming an enzyme yield of 70%. In order to compare the different published processes for the purification of FDH, one has to take into account the purity of the product: the price has to be corrected for the amount of separated proteins, which results in US$ 0.96 per 1000 U FDH and mg removed protein. In Table VI, the economic estimates of three

TABLE V

CALCULATION OF COSTS IN US DOLLARS

Estimated for 10-kg cells of *C. boidinii*. For explanation of symbols see eqn. 1.

Step	*M*	*E*	*L*	*I*	*T*	(%)
Freezing/suspending	0.15	0.70	5.80	0.40	16.40	3
Heat denaturation	15.20	0.30	5.80	0.30	32.80	6
Affinity partition	(19.20)* 10.80	0.40	46.35	3.90	136.00	25
1. Separation of FDH—ligand	4.30	0.45	46.35	3.90	129.80	24
2. Separation	0.50	0.30	22.80	0.00	60.20	11
Ultrafiltration	0.80	0.40	5.80	2.80	19.20	4
Lyophilization	0.40	1.10	5.80	5.80	22.50	4
Cleaning	0.20	—	46.35	—	120.80	23
	(40.60)* 32.30	3.60	185.00	17.20	537.00	

*The values in parentheses are the material costs if the top phase is not recycled.

TABLE VI

COMPARISON OF THE COSTS FROM THREE DIFFERENT PROCESSES FOR THE ISOLATION AND PURIFICATION OF FORMATE DEHYDROGENASE

Process	Total units of purified FDH	Specific activity (U/mg)	K'*	Reference
Affinity partition	1 092 000	4	0.96	—
Extraction	1 391 000	2	1.00	2
Standard	31 000	3	79.00	11

*US$ per 1000 U FDH and mg separated protein.

processes are compiled. The process named "Standard" is based on ion-exchange chromatography [13], whereas "Extraction" works only with aqueous two-phase systems [2]. The calculations are based on data taken from Kroner et al. [2]. Furthermore, it is assumed that all three processes start with a specific activity of 0.6 U/mg in the homogenate. The data show that the costs of affinity partitioning are comparable or even lower than the costs for the known extraction procedure in aqueous two-phase systems without affinity interaction. So, the theory that affinity partitioning a priori would be too expensive for the purification of enzyme on a technical scale can no longer be put forward.

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