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Separation of Biopolymers by Partition in Aqueous Two-Phase Systems

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Separation of biopolymers by partition in aqueous two-phase systems

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ABSTRACT

Aqueous two-phase systems are useful tools for fractionation and studies of cell components including proteins and nucleic acids. The systems consist of two non-miscible liquid layers which both have high content of water. The purification achieved by partition of biosubstances between the two liquid phases depends on the possibility to steer the partition in a selective way. The partition can be affected by hydrophobic and affinity ligands, as well as charged groups, bound to soluble polymers.

I. INTRODUCTION

Aqueous two-phase systems have been used for more than thirty years as a tool in biochemistry and biology.[1-3] The unique property of this liquid-liquid two-

phase systems is the high water content (80-99 % w/w) in both phases. Thus, they are excellent environments for constituents of the biological cell, e.g. organelles, membranes, nucleic acids and proteins. Differences in the partition of the biomaterials between the phases can be utilized for separation purposes. Aqueous two-phase systems have a great potential for biotechnical purification because of the ease of scaling up liquid-liquid extraction processes. Furthermore, the systems have found use in studies of interaction between cell constituents, e.g. between proteins.

II. SYSTEM COMPOSITION

Two kinds of aqueous systems are frequently used. They are either based on two polymers or on one polymer and a salt. This presentation will only deal with the former type. The mutual exclusions of two polymers (in a solvent), resulting in two liquid phases, is a general phenomenon. A large number of polymer pairs, yielding two phases in water, have been described[1] including dextran-poly(ethylene glycol), dextran-Ficoll, dextran-hydroxypropyldextran, Ficoll-poly(ethylene glycol) and polyvinylalcohol-poly(ethylene glycol). The concentrations of polymers necessary to obtain the two phases are shown, in Figure 1, for the pair dextran and poly-(ethylene glycol). The phase diagram also shows the composition of the phases and how the volume ratio can be adjusted. By increasing the temperature the border-

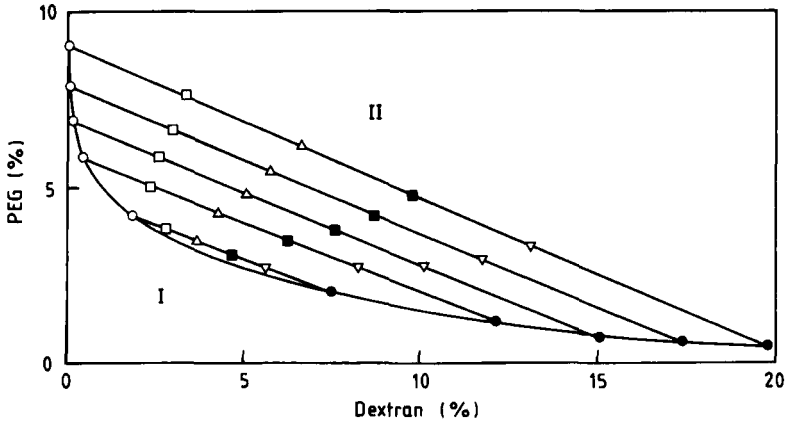

FIGURE 1
Phase diagram for water - dextran (M_w=500,000) - PEG (M_r=8000) at 0 °C. I, one-phase region; II, two-phase region. The compositions of the top phases (○) and the bottom phases (●), belonging to the same two-phase system, are connected with a straight line (tie-line). The points on the tie-lines represent total composition of two-phase systems having the volume ratios (top/bottom) 5 (□), 2 (△), 1 (■), and 0.5 (▽). The diagram is based on data from reference (1).

line between the one- and two-phase regions (the binodial curve) moves towards higher polymer concentrations.

III. ELECTROSTATIC EFFECTS

The partition of biopolymers, as well as cell particles, within an aqueous two-phase system, can be influenced by the presence of salts. It has been studied especially in systems based on dextran and poly(ethylene glycol), PEG. In such systems PEG is found mainly in the lighter (upper) phase while dextran is concentrated to

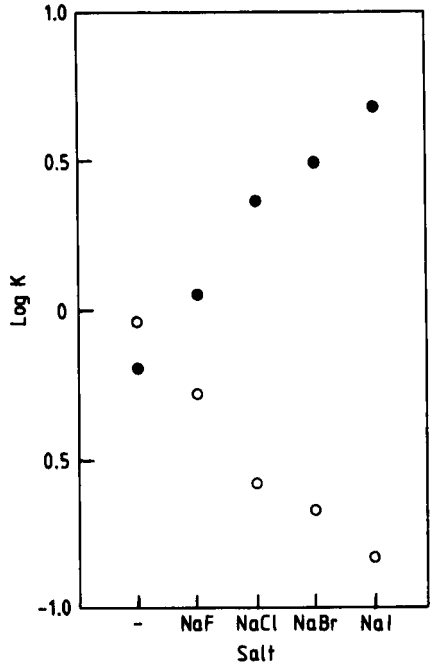

FIGURE 2
Partition of (positively charged) lysozyme (●) and (negatively charged) ovalbumin (○) in two-phase systems containing different sodium halides. System composition: 8 % dextran (M_w=500,000), 8 % PEG (M_r=3400), 0.5 mM sodium phosphate buffer, and 25 mM salt. pH = 6.9 and temperature 23 °C.

the lower phase. The partition of a biopolymer, e.g. a protein, can be described by a partition coefficient, K, defined as the ratio between the protein concentration in upper and lower phase. The effect of a number of salts on the partition of two proteins are shown in Figure 2. Proteins and pH value have been chosen in such way that one protein (ovalbumin) is negatively charged while the other one (lysozyme) has a positive net

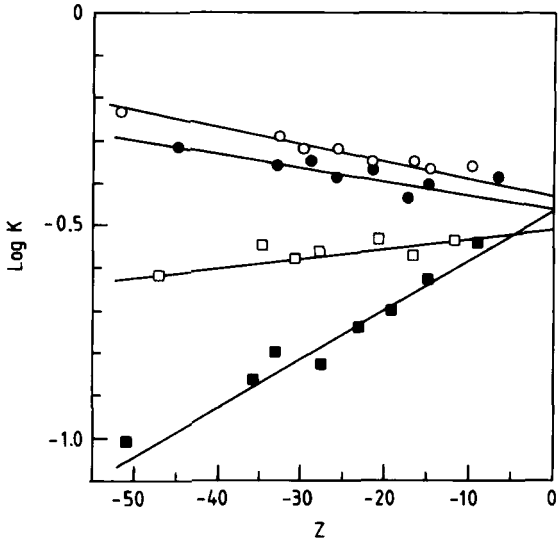

FIGURE 3
Partition of bovine serum albumin as function of its net charge, Z. System composition: 5 % dextran (M_w=500,000), 4 % PEG (M_r=8000), 2 g albumin per liter and salt: 50 mM K_2SO_4 (○), LiCl (●), potassium acetate (□), or KCl (■). The pH values were adjusted by addition of acid or base and Z was obtained from the titration curve of the protein. Temperature 2 °C.

charge. The presence of different salts affects the partition of the two proteins in opposite directions (towards lower or upper phase) depending on the sign of net charge, Z, of the protein. In several cases a linear dependence between logarithmic partition coefficient, log K, and net charge, Z, of proteins has been observed,[4,5] Figure 3. This is expressed in Eq. (1).

$$\log K = \log K_0 + \gamma Z \qquad (1)$$

The constant K_0 is the partition coefficient of the protein at the isoelectric point, while γ is a factor which depends on the salt used (in excess). From theoretical considerations[1], this behaviour can be deduced from the assumption of difference in the affinity of the ions of the salt for the two phases. Hypothetical partition coefficients, K_+ and K_-, can be postulated for the cation and anion, respectively. They describe the partition the ions should have if they could partition independently of each other. Because of the claim of electroneutrality, cations and anions must have the same partition coefficient, K_{salt}, which is the weighed geometrical mean of the K_+ and K_- values, Eq. (2):

$$K_{salt} = (K_+^m \cdot K_-^n)^{1/(m+n)} \qquad (2)$$

when the salt consists of the ions A^{+m} and B^{-n}. The difference in affinity for the phases also causes the ions, close to the interface (between the phases), to orient and form an electrical double layer (Stern layer). This gives rise to a potential difference, the so called interfacial potential, $\Delta\Psi$, which is related to hypothetical ionic partition coefficients via Eq. (3):

$$\Delta\Psi = \frac{R\ T}{(m+n)\ F} \ln \frac{K_-}{K_+} \qquad (3)$$

where R is the gas constant, T is the absolute temperature and F is the Faraday constant. The effect of the partition of a protein, with the net charge Z, is given by Eq. (4):

$$\log K = \log K_0 + \frac{Z}{m+n} \log \frac{K_-}{K_+} \quad (4)$$

or

$$K = K_0 \left[\frac{K_-}{K_+} \right]^{Z/(m+n)} \quad (5)$$

The partition coefficient can therefore be resolved into two factors: one, K_0, which describes the general solvation of the protein molecule in the two phases, and another, K_Z, which depends on the net charge of the partitioned biopolymer and the electrolyte condition of the system, Eq. (6):

$$K = K_0 \cdot K_Z \quad (6)$$

The separatory capacity of a two-phase system for two biopolymers, 1 and 2, can be measured as the difference in their log K values, e.g. the logarithmic separation factor, log β, defined by Eq. (7):

$$\log \beta = \log K_1 - \log K_2 \quad (7)$$

The log β can be expressed[6] in the two K_0 values ($K_{0,1}$ and $K_{0,2}$) and the net charges (Z_1 and Z_2) of the two biopolymers, via Eq. (1), yielding Eq. (8):

$$\log \beta = \log K_{0,1} - \log K_{0,2} + \gamma (Z_1 - Z_2) \quad (8)$$

This relation shows that the separation due to a difference in the log K_0 values can work additively or

subtractively with the charge difference term depending on the sign of the γ factor. The most favourable separation conditions occur then when the charge difference and the γ value are as large as possible.

Especially strong effects on the partition of proteins and nucleic acids have been obtained by binding (covalently) ionic groups to (a fraction of) one of the phase-forming polymers.[7,8] If positively charged groups are bound to PEG a high K_+ value can be foreseen. The effect of positive and negative PEG-bound groups on the partition of two enzyme activities present in yeast extract is shown in Figure 4. The partition of proteins is, in this kind of systems, strongly depending on pH. Because of the relatively low concentration of polymer-bound charged groups, the concentration of other electrolytes, like salts and buffer, must be kept very low. However, a certain amount of salt is normally necessary to include in order to avoid repulsion between the charged PEG molecules which otherwise would counteract phase separation.

IV. HYDROPHOBIC EFFECTS

Hydrophobic groups, e.g. higher fatty acids, can be constrained mainly to one of the phases by being attached to the corresponding polymer.[9,10] By using increasing concentrations of the polymer-bound hydrophobic groups, proteins containing hydrophobic pockets can be increasingly extracted into the actual phase. The parti-

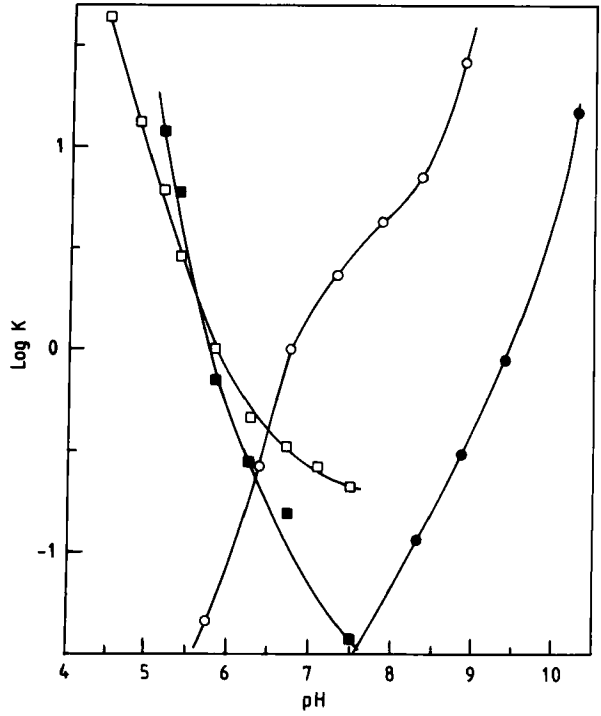

FIGURE 4
Partition of the enzymes fumarase (open symbols) and enolase (filled symbols), present in an extract of pig heart, in systems containing either the positively charged trimethylamino-PEG (○, ●) or the negatively charged carboxymethyl-PEG (□, ■). System composition: 6.4 % dextran (M_w=500,000), 6.6 % derivatized PEG (M_r=8000) and soluble extract from pig heart (60 g tissue used per liter system). Titration with acid or base was used to adjust the pH values. Temperature 2 °C.

tion coefficient of the protein approaches a saturation value. This K value depends on the number of binding sites and the binding strength but it is, partly, also due to ligand-ligand interaction which gives rise to micelle formation. Good correlations with other methods

have been found when hydrophobicity of proteins is measured as the maximal increase in log K value caused by the ligand.[11]

V. USE OF AFFINITY LIGANDS

In the same way as for hydrophobic partition more specific ligands can be attached to one of the phase-forming polymers. This way of selectively influencing the partition of certain macromolecules or particles has been called affinity partition.[12,13] Because of the absence of a matrix, problems occuring in affinity chromatography (e.g. multiple attachment) can be avoided. A number of applications of affinity partition have been collected in Table I.

The effectivity of the affinity extraction depends on the partition of the (polymer-bound) ligand, the strengths of the ligand-protein binding in the two phases and the average number of ligand-polymer molecules bound per protein molecule. A theory for affinity partition was presented by Flanagan and Barondes.[12] They used a thermodynamic approach which is closely related to using a set of equilibrium conditions and mass balance equations, eq. (9)-(14). If the upper phase is indexed '1' and the lower phase is indexed '2' then the ratio between the concentrations of free ligand-polymer, L, in the phases is given by the partition coefficient K_L, Eq. (9). In the same way, the partition of free pro-

TABLE I

Collected works on affinity partition of proteins and nucleic acids.

Extracted biopolymer	Ligand	Reference
Albumin, serum	fatty acids, dyes	14-16
Alcohol dehydrogenase	triazine dyes	17
Colipase	lecithin	18
α-Fetoprotein	triazine dyes	19-21
Formaldehyde dehydrogenase	NADH, dyes	228
Formate dehydrogenase	NADH, dyes	22,23
Glucose 6-phosphate dehydrogenase	triazine dyes	17, 24-27
Glutamate dehydrogenase	triazine dyes	28
Glutamate oxaloacetate dehydrogenase	triazine dyes	28
Glutamate pyruvate transaminase	triazine dyes	28
Glyceraldehyde phosphate dehydrogenase	triazine dyes	29
Glycerol kinase	triazine dyes	28
Lactate dehydrogenase	triazine dyes	30-32
Malate dehydrogenase	triazine dyes	28,31
S-23 Myeloma protein	dinitrophenol	12
Nitrate reductase	triazine dyes	33
$\Delta_{5\to6}$ 3-Oxosteroid isomerase	estradiol	34
Prealbumin	Remazol yellow GGL	20,21, 35
Phosphofructokinase	Cibacron blue F3G-A	36,37
3-Phosphoglycerate kinase	triazine dyes	17,29
Phosphoglycerate mutase	triazine dyes	17,29
Pyruvate kinase	triazine dyes	28,31
Trypsin	p-aminobenzamidine	38
DNA (rich in GC base pairs)	a phenazinium dye	39,40
DNA (rich in AT base pairs)	malachite green	41

tein, P, is described by a partition coefficient K_P, Eq. (10).

$$[L]_1 / [L]_2 = K_L \quad (9)$$

$$[P]_1 / [P]_2 = K_P \quad (10)$$

If a one-one complex exists between the protein and the

ligand-polymer the dissociation constants D_1 and D_2 in the two phases are defined by Eq. (11) and (12).

$$[P]_1 \cdot [L]_1 / [PL]_1 = D_1 \quad (11)$$

$$[PI_2 \cdot [L]_2 / [PL]_2 = D_2 \quad (12)$$

The mass balances for ligand-polymer and protein, respectively, in a system with the volumes V_1 and V_2, are given by Eq. (13) and (14). C_L and C_P are the total (overall) concentrations of ligand-polymer and protein, respectively, added to the system.

$$V_1[L]_1 + V_2[L]_2 + V_1[PL]_1 + V_2[PL]_2 = (V_1+V_2)C_L \quad (13)$$

$$V_1[P]_1 + V_2[P]_2 + V_1[PL]_1 + V_2[PL]_2 = (V_1+V_2)C_P \quad (14)$$

From the above equations it is possible to express the experimentally observed (overall) partition coefficient, K_{obs}, for the protein, Eq. (15), as a function of total concentration of ligand-polymer, C_L.

$$([P]_1 + [PL]_1) / ([P]_2 + [PL]_2) = K_{obs} \quad (15)$$

A few extractions curves calculated according to the model are shown in Fig. 5. The limiting K_{obs} value (in presence of excess of ligand-polymer) is given by Eq. (16).

$$(K_{obs})_{max} = K_P \cdot K_L \cdot D_2/D_1 \quad (16)$$

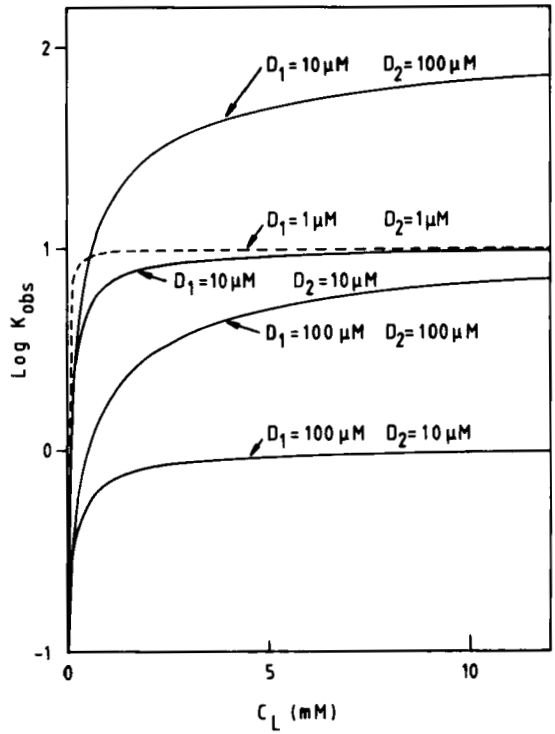

FIGURE 5
Calculated log K_{obs} values of a protein (0.1 mM) as function of total concentration of polymer-bound ligand, C_L in a two-phase system with equal volumes of the phases. K_L=100, K_P=0.1 and the dissociation constants for the complex in upper phase, D_1, and in lower phase, D_2, have been given various values.

When the protein molecule form complexes containing more than one ligand-polymer the maximal partition coefficient reaches more extreme values. With n ligand-PEG molecules in the complex the maximum partition coefficient is given by Eq. (17).

$$(K_{obs})_{max} = K_P \cdot (K_L \, D_2/D_1)^n \qquad (17)$$

The experimentally observed values of the increase in partition coefficients, $(K_{obs})_{max}/K_p$, of enzymes, caused by introducing PEG-bound reactive dyes as affinity ligands, have in some cases (e.g. for glucose-6-phosphate dehydrogenase[24]) been 10^4. The measured K_L was close to 10^2 which should indicate n = 2 if the dissociation constants D_1 and D_2 are assumed to be of equal magnitude.

For the practical extraction the most important factors are the degree of purification, the capacity of the system and the recovery. The percentual recovery, R_1, of target protein in the upper phase is related to the K_{obs} value via Eq. (18),

$$R_1 = K_{obs}\, V_1/(V_2 + K_{obs} \cdot V_1) \qquad (18)$$

The physical capacity of the aqueous two-phase systems for proteins is high. Up to 150 g of bulk protein have been included without any noticable negative effects on the affinity extraction of enzymes. The affinity extraction capacity is limited by the concentration of polymer-bound ligand. When PEG (with M_r = 8000) is used as monosubstituted ligand carrier, the value of C_L can be as high as 12 mM when $V_1=V_2$. With PEG, M_r = 3400, C_L can reach 25 mM under the same conditions. The calculated extraction curves, Figure 6, show that C_p of the target protein can be at least 12 g/l (if its molecular weight is 120,000) with a recovery of more than 90 per cent if only the dissociation constants are in the medium "affinity" region 10^{-4} - 10^{-6} M and the K_L is

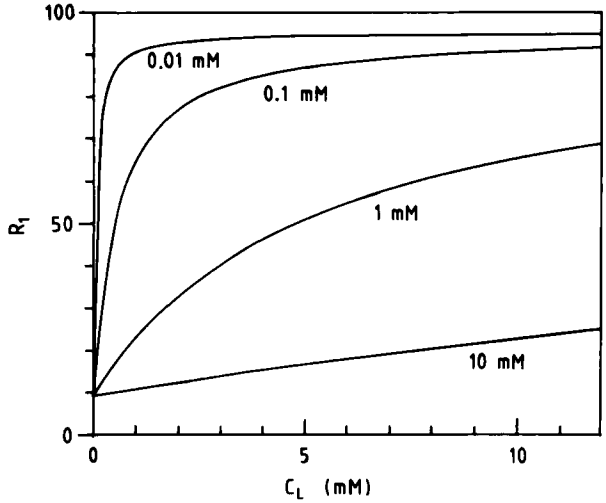

FIGURE 6
Calculated recovery, R_1 of a protein in the top phase as function of the total concentration of ligand-PEG, C_L. The concentration of protein is 0.1 mM and its partition coefficient K_P = 0.1. Ligand-PEG with K_L = 200 forms a 1-1 complex with the protein. The dissociation constant of the complex is 0.01, 0.1, 1, or 10 mM. The volumes of top and bottom phase are equal.

extreme enough (K_L = 200). This satisfactory extraction can be achieved despite the value of n has been chosen to be only one in the calculations.

The calculated yields and purities of an enzyme (with K_P=0.01) extracted from a protein mixture (K = 0.01 for all the bulk proteins), of which the target enzyme constitutes 0.1 per cent, are shown in Table II. The ligand-PEG has been assumed to have K_L = 100 and excess of ligand is used. Either one or two mole ligand-PEG can bind per mole enzyme. The ligand is further

TABLE II

Extraction of an enzyme into the upper phase using saturating amount of ligand-PEG. K = 0.01 for bulkprotein and K_{obs}=1 (if n=1) or 100 (if n=2) for the target enzyme which constitutes 0.1 % of the original protein mixture (F=0.1). V is volume ratio between upper and lower phase. R_1 is the total percentual recovery of enzyme in the upper phase and F is the purity of target enzyme in this phase (in per cent of extracted protein).

n	Number of washing steps with lower phase of equal volume	V = 1		V = 2		V = 5	
		R_1	F	R_1	F	R_1	F
1	-	50.0	4.8	66.7	8.3	83.3	1.7
	1	25.0	99.2	44.4	97.5	69.4	84.2
	2	12.5	100.0	29.6	100.0	57.9	100.0
2	-	99.0	9.1	99.5	4.8	99.8	2.1
	1	98.0	99.9	99.0	99.2	99.6	90.2
	2	97.1	100.0	98.5	100.0	99.4	100.0

assumed to have unique specificity for the enzyme. The effectivity of the extraction can be modulated by adjusting the relative volumes of upper and lower phase and by using repeated "washings" with pure lower phases.

VI. MULTISTAGE PROCEDURES

Extractions in several steps may also include change of ligand-polymer in the extracting phase in order to recover several enzymes in separate phases,[31] Table III. Two ligands, in opposite phases, can also be used to improve the selectivity[24], Table IV. So far, not much work has been done with highly specific affinity ligands. These, however, should both give very good yield and product of high purity as shown above.

TABLE III

Sequential extraction of enzymes from muscle proteins by using a system containing 6 % (w/w) dextran 500, 7.1 % (w/w) PEG 8000, and 47 mM sodium phosphate buffer, pH 7.9. Temperature 0 °C. Clearified extract from swine muscle was included in the first system. Dye-ligand-PEG was used in the upper phases (0.2, 0.3 and 1 % of total PEG for Procion blue, yellow and navy, respectively). MK = myokinase, LDH = lactate dehydrogenase, MDH = malate dehydrogenase, PK = pyruvate kinase.

No of extractions	Procion dye ligand	Enzyme recovery in upper phases (in % of total anmount)			
		MK	LDH	MDH	PK
2	Blue MX-2G	69	6	15	20
2	Yellow HE-3G	28	72	76	4
1	Navy H-4R	4	6	7	69

TABLE IV

Extraction of glucose-6-phosphate dehydrogenase from yeast proteins by affinity partition in five steps. System composition: 9 % (w/w) dextran 70, 5.5 % PEG 40,000 and 45 mM sodium phosphate buffer, pH 7.0. Temperature, 0 °C. Procion Olive MX-3G PEG and Procion Yellow HE-3G dextran were used as ligand-polymers. Adapted from reference 24.

Extraction Nr	Procion dye ligand		Target enzyme		Phase for recovery
	Top	Bottom	Recovery (%)	Purification factor	
1	Olive	-	92	4.4	Top
2	Olive	-	79	6.6	Top
3	Olive	-	74	7.5	Top
4	Olive	Yellow	60	20	Bottom
5	Olive	Yellow	56	32	Bottom

A popular way to perform multistep extraction is counter-current distribution according to Craig.[42] Also with this technique the use of two ligands in opposite phases can strongly improve the fractionation. One or both of the ligands can be used as concentration gradients. Such gradients have been used to obtain zone sharpening.[43] The aqueous two-phase system can also be used for column chromatography by adsorbing the dextran phase to especially prepared chromatographic beads.[44]

VII. TECHNICAL USE

Partition within aqueous two-phase system is a method well suited for technical use. Large scale extraction of enzymes from cell homogenate using PEG-salt systems is well established[45-47]. Also a few examples of affinity extraction in large scale have published.[23,30] The extraction process can be used with good economy since the ligand-PEG can be recovered in good yield (96 %).[30]

VIII. CONCLUSIONS

The aqueous two-phase systems offer a simple and effective way for purification of proteins and other cell components. By creating selective partition systems, e.g. by using affinity ligands, effective and rapid extractions can be carried out with high yield and purity of the target material. The simplicity of using the system makes this purification method well suitable for preparations in large scale.

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