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Application of temperature-induced phase partitioning at ambient temperature for enzyme purification

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ABSTRACT

Aqueous two-phase partition and temperature-induced phase separation using a non-ionic, random copolymer composed of 20% ethylene oxide, 80% propylene oxide (EO_{20} PO_{80}) has been used for purification of glucose-6-phosphate dehydrogenase, hexokinase and 3-phosphoglycerate kinase from bakers' yeast. This $EO_{20}PO_{80}$ copolymer has a cloud point of 18°C, at which temperature it phase separates from water. Enzymes were first partitioned at 4°C in an initial $EO_{20}PO_{80}$–dextran T500 aqueous two-phase system. This system had an upper copolymer-rich phase and a lower dextran-rich phase. After phase separation had occurred the upper $EO_{20}PO_{80}$-rich phase was removed and placed at 24°C. This resulted in formation of a new two-phase system with an upper water phase and a lower phase containing 98% copolymer and 2% water. Enzymes were recovered exclusively in upper water phase leaving a polymer-rich lower phase free of contamination. The phase diagram for the system $EO_{20}PO_{80}$ and dextran T500 at 4°C has been determined.

INTRODUCTION

Aqueous two-phase systems are widely used for separation and purification of biomolecules [1–3]. Use of these systems for purification of biological materials on a large scale has also been described [1,4–7]. In most instances these systems are composed of a poly(ethylene glycol) (PEG)-enriched upper phase and a dextran- or hydroxypropyl starch-enriched lower phase. Both phases contain 80–95% water. Phase separations can also be obtained by using PEG and a high concentration of salt. One problem encountered with these systems is difficulty separating target molecules from polymer solution. In other words, partitioning has the disadvantage that the process begins by adulteration of the target with two new polymers that must also be removed. These systems would also be more cost efficient if the polymer could be readily recycled without costly ultrafiltration or chromatography steps. Temperature-induced phase partitioning avoids these problems.

Many studies have been done on a group of non-ionic, linear polymers composed of poly(ethylene glycol) which are normally soluble in water, but which separate from solution when heated sufficiently [8–11]. The temperature at

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which this phenomenon occurs is known as the cloud point (CP) of the polymer. This phase separation results in a water-rich upper phase and a PEG-rich lower phase. It is possible to vary the temperature at which this separation occurs by changing the molecular mass of the polymer or by addition of various salts to the solution [11,12]. Studies have shown that incorporation of propylene oxide groups, to form a random copolymer of ethylene oxide and propylene oxide, also lowers the cloud point [13]. The degree to which the cloud point is lowered is dependent on the ratio of ethylene oxide to propylene oxide. Non-ionic surfactants containing ethylene oxide groups also exhibit a cloud point in aqueous solution. A surfactant of this type, Triton X-114, has been used for isolation of membrane proteins with temperature-induced phase separation [14].

Temperature-induced phase separation combined with aqueous two-phase partitioning or affinity partitioning offers a viable solution to problems of polymer removal and recycling. Recently, these systems have been introduced for enzyme purification [13,15]. Partition of steroids has also been studied in these systems [16]. Random, non-ionic copolymers of 50% ethylene oxide and 50% propylene oxide (UCON) when mixed with dextran or hydroxypropyl starch form aqueous two-phase systems very similar to a PEG–dextran system which can be used to purify biomolecules [13,15]. Target molecules are first partitioned at room temperature in a system composed of UCON and dextran or hydroxypropyl starch. UCON is enriched in upper phase and dextran or hydroxypropyl starch in lower phase. After separation has occurred, upper UCON-rich phase is removed and its temperature increased above the cloud point of UCON. This results in formation of a new two-phase system with an upper water phase and a lower UCON-rich phase. The cloud point of UCON (50°C) can be lowered to 37°C by addition of salt. In this new two-phase system biomolecules partition exclusively to the upper water phase, allowing the lower UCON-rich phase to be recovered and recycled for a second extraction. A simple purification scheme for biomolecules using temperature-induced phase partition combined with aqueous two-phase partition or affinity partition has been developed [13,15].

This study deals with application of a random, non-ionic copolymer composed of 20% ethylene oxide and 80% propylene oxide ($EO_{20}PO_{80}$) for aqueous two-phase partition and temperature-induced phase separation. Increasing propylene oxide concentration from 50% to 80% reduces the cloud point to 18°C which eliminates the need to add salt and lowers the polymer concentrations required to obtain two-phase separation. When $EO_{20}PO_{80}$ is mixed with dextran in water, it forms an aqueous two-phase system with a dextran-rich lower phase and a copolymer-rich upper phase. Enzyme partition and purification in an $EO_{20}PO_{80}$–dextran system could thus be performed at 4°C. Upper phase can be removed and placed at room temperature, 24°C, which is above the copolymer's cloud point. This results in formation of a new two-phase system with an upper water phase containing the biomolecules, and a lower copolymer-rich phase. We here present results of this study with glucose-6-phosphate dehydrogenase, hexokinase and 3-phosphoglycerate kinase. The phase diagram for the system $EO_{20}PO_{80}$, dextran T500 and water at 4°C has been determined. Partition and purification of glucose-6-phosphate dehydrogenase, hexokinase and 3-phosphoglycerate kinase from bakers' yeast have been performed using a combination of aqueous two-phase partition with $EO_{20}PO_{80}$–dextran T500 and temperature-induced phase separation at 24°C.

MATERIALS AND METHODS

Chemicals

Dextran T500 (M_r 500 000) was obtained from Pharmacia (Uppsala, Sweden) and PEG 4000 (M_r 4000) from Merck (Darmstadt, Germany). UCON 50-HB-5100 was a kind gift from Union Carbide (New York, USA). A random copolymer of 20% ethylene oxide and 80% propylene oxide ($EO_{20}PO_{80}$) was obtained from Berol Nobel (Stenungsund, Sweden). $EO_{20}PO_{80}$ was further purified by dissolving the polymer in water and adding dichloromethane. The polymer was extracted into the dichloromethane phase.

The solution of polymer in dichloromethane was dried with magnesium sulfate. Solvent was evaporated under vacuum at 65°C. After purification, copolymer purity, ratio of ethylene oxide to propylene oxide and molecular weight (M_r 2000) were determined by NMR in [$^2H_6$] dimethylsulphoxide (DMSO) solvent by comparing area of butyl groups (one butyl end group per copolymer molecule) to area of ethylene oxide and propylene oxide groups. All other chemicals were of analytical reagent grade.

Proteins

Glucose-6-phosphate dehydrogenase (G6PDH) from bakers' yeast (EC 1.1.1.49), hexokinase from bakers' yeast (EC 2.7.1.1) and 3-phosphoglycerate kinase (3-PGK) from bakers' yeast (EC 2.7.2.3) were purchased from Sigma (St. Louis, MO, USA).

Yeast extract

Yeast homogenate was prepared from commercial bakers' yeast by sonication after a 1:1 dilution in 50 m*M* triethanolamine–HCl buffer, pH 8.0, containing 2.0 m*M* EDTA. β-Mercaptoethanol was added to the solution to a concentration of $1.23 \cdot 10^{-3}$ *M*. Sonication was for 15 min in a Branson CP-30 sonifier. After testing for enzyme activity and protein concentration, yeast homogenate was centrifuged for 10 min at 12 000 *g* to remove cell debris. The pellet was discarded and supernatant was used for enzyme extraction experiments.

Two-phase systems and temperature-induced phase separation

All polymer concentrations were calculated as % (w/w). Aqueous two-phase systems were prepared from stock solutions of the polymers in water, 20% (w/w) $EO_{20}PO_{80}$, 30% (w/w) UCON, 30% (w/w) PEG 4000 and 20% (w/w) dextran T500. Dextran concentration was determined by polarimetry using an Optical Activity AA-5 automatic polarimeter (Optical Activity, UK) equipped with a sodium lamp set for 589 nm [2].

Polymer solutions were weighed out and mixed with water and buffer. The systems containing $EO_{20}PO_{80}$ were allowed to equilibrate 1 h at 4°C before addition of protein sample. Systems with either PEG or UCON for upper phase polymer were allowed to phase separate at room temperature. Addition of yeast extract was accomplished by adding 2 g extract to a phase system so that the total weight of the system was 10 g including extract. All partition experiments were performed in duplicate, with enzyme assays and protein determination being performed in triplicate. Systems without yeast extract were allowed to sit 30 min at 4°C to achieve phase separation. Systems containing yeast extract were centrifuged 5 min at 125 *g* to accelerate phase separation. The upper $EO_{20}PO_{80}$-rich phase was removed and isolated in a separate container. For systems with $EO_{20}PO_{80}$, separated upper phase was allowed to equilibrate 30 min at 24°C before centrifugation for 10 min at 125 *g*. This resulted in formation of a new two-phase system consisting of an upper water phase and a lower $EO_{20}PO_{80}$-rich phase [13,15,16]. Due to its high viscosity, this lower phase was diluted 5:1 before analysis, while the upper water-rich phase was analyzed without dilution.

Partition of enzymes between the phases was determined by removing appropriate amounts of each phase and assaying for enzyme activity. Partition of protein between the two phases is expressed by the partition coefficient, *K*, defined as

$$K = C_t / C_b$$

where C_t and C_b are concentrations of partitioned substance in moles per liter of top and bottom phases, respectively. For enzymes, *K* is measured as activity in units per ml in the top and bottom phases [2].

In many cases it is also advantageous to calculate the distribution ratio, *G*, defined as

$$G = K \cdot (V_t / V_b)$$

where V_t and V_b are volumes of upper and lower phases respectively. *G* gives the ratio between total amount of protein or enzyme in each phase [2].

Protein and enzyme assays

Protein was determined according to Bradford [17] using Coomassie Brilliant Blue G and mea-

sured at 595 nm with bovine serum albumin as standard.

Enzyme activity was determined photometrically at 340 nm using a Hewlett-Packard 8452A Diode Array Spectrophotometer (Hewlett-Packard, Palo Alto, CA, USA). Descriptions of the assays are found in the following references: glucose-6-phosphate dehydrogenase according to Noltmann *et al.* [18]; hexokinase according to Maitru [19]; 3-phosphoglycerate kinase according to Scopes [20].

Determination of binodal and tie lines

The binodal represents the borderline between one and two phases and the tie line describes composition of the two phases when in equilibrium. The binodal for the system $EO_{20}PO_{80}$ and dextran T500 was determined as described by Albertsson [2] (Fig. 1). Tie lines for this system were determined by removing samples from upper and lower phases after separation was complete and measuring dextran concentration. Since total phase composition was known from weight of polymers added, knowledge of dextran concentration in upper and lower phases allowed determination of tie lines.

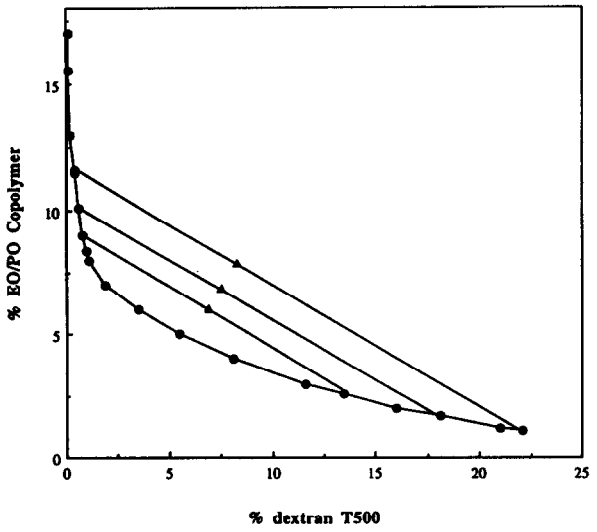

Fig. 1. Phase diagram for $EO_{20}PO_{80}$ (M_r 2000) and dextran T500 (M_r 500 000) and water at 4°C. (●) Points obtained by titration [2] or (▲) analysis of separated phases of the systems.

Cloud point determination and composition of copolymer phase at 24°C

The cloud point (CP) of $EO_{20}PO_{80}$ copolymer was determined visually by placing a 10% solution of polymer in water in a sealed glass tube and placing the tube into an ethanol bath. Temperature was increased in 1°C increments from 4°C, where the solution was clear, to 18°C, where the solution became cloudy.

Composition of lower copolymer phase at 24°C was determined by removing upper water phase and placing lower $EO_{20}PO_{80}$-enriched phase at 80°C under vacuum until a constant weight was reached. To remove any residual water, toluene was added to a concentration of 50% and solution was returned to 80°C and vacuum. After 3 hours constant weight was again obtained. Amount of water contained in this lower phase was calculated by subtracting final weight of sample from starting weight.

RESULTS AND DISCUSSION

Phase diagram

The phase diagram for $EO_{20}PO_{80}$, dextran T500 and water at 4°C is shown in Fig. 1. Ethylene oxide–propylene oxide copolymer was enriched in upper phase while dextran T500 was enriched in lower phase. Comparison of the phase diagram for this system with the phase diagram of PEG 4000–dextran T500 shows that the critical points of these systems are similar, even though the molecular mass of the $EO_{20}PO_{80}$ copolymer (2000) is half that of PEG 4000 [13]. This means that polymer concentrations required to achieve two-phase separations are lower with the $EO_{20}PO_{80}$ copolymer compared with PEG. For a PEG 2000–dextran T500 system the critical point must be higher than for $EO_{20}PO_{80}$–dextran T500, since lowering the polymer molecular mass increases the polymer concentrations required for phase separation [2]. Phase separation at lower polymer concentrations can be explained by increased hydrophobicity of $EO_{20}PO_{80}$ copolymer (20% ethylene oxide and 80% propylene oxide) when compared to PEG (100% ethylene oxide). This increase in hydrophobicity leads to an increase in net repulsive interaction between polymers, and thus to

phase separation at lower polymer concentrations [2,21]. Lower temperatures may result in a slightly lower binodal, but this effect is small [2,22]. Comparison of binodal lines at 8°C, 23°C, 38°C and 50°C for the system PEG 6000/dextran T70 shows that the temperature change has very little effect on the critical point [23].

Cloud point and composition of copolymer phase at 24°C

PEG (100% ethylene oxide) is a linear non-ionic polymer which is water soluble until heated above its cloud point (112°C for PEG M_r 20 000) [8–10]. Decreased solubility at higher temperatures can be explained by conformational changes in the ethylene oxide chain. At lower temperatures polar conformations of the polymer chain are dominating whereas at higher temperatures non-polar conformations are more abundant. The non-polar conformations are less hydrophilic than the polar conformations so that excess of non-polar conformations leads to decreased polymer solubility [11]. The increase in temperature results in polymer separating from water and forming a separate phase. It is possible to manipulate the cloud point of a polymer by addition of salt or by changing polymer molecular mass [11,12]. Changing the ratio of ethylene oxide to propylene oxide will also lower the cloud point [13]. A random, linear non-ionic copolymer (UCON 50-HB-5100, M_r 4000) which is composed of 50% ethylene oxide and 50% propylene oxide has a cloud point of 50°C [13,15,24]. As long as water solubility is retained these copolymers will form aqueous two-phase systems with dextran T500. The polymer used in this study is a linear, non-ionic random copolymer of 20% ethylene oxide and 80% propylene oxide with a relative molecular mass of 2000. The cloud point for a 10% solution of this copolymer was determined to be 18°C, which is below normal room temperature. In order to achieve an aqueous two-phase system with dextran, it was necessary to perform partition experiments below the cloud point of $EO_{20}PO_{80}$. This was accomplished by using a cold room at 4°C for initial partitioning steps. Removal of this initial upper phase from the cold room and placement at room temperature, 24°C, resulted in formation of a new aqueous two-phase system with an upper water-rich phase and a lower copolymer-rich phase. The composition of lower phase formed at 24°C, as determined by vacuum drying, is 98% $EO_{20}PO_{80}$ and 2% water.

Partition of pure glucose-6-phosphate dehydrogenase, hexokinase and 3-phosphoglycerate kinase

Commercially prepared G6PDH, hexokinase and 3-PGK from bakers' yeast were partitioned in two different $EO_{20}PO_{80}$–dextran T500 systems. The first system was 7% copolymer, 4% dextran and 0.01 *M* sodium phosphate buffer (Table I). The *K* values at 4°C for partition between the $EO_{20}PO_{80}$ and dextran phases were 0.75 for G6PDH, 1.01 for hexokinase and 0.71

TABLE I

PARTITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE, HEXOKINASE AND 3-PHOSPHOGLYCERATE KINASE

Primary phase system: 7% $EO_{20}PO_{80}$, 4% dextran T500, 0.01 *M* sodium phosphate buffer, pH 7.0, 20 units glucose-6-phosphate dehydrogenase, 20 units hexokinase and 45 units 3-phosphoglycerate kinase. *K* and *G* values at 24°C are for partition between water and $EO_{20}PO_{80}$ phases formed by the increase in temperature. *Y* = % yield of enzymes recovered in water phase at 24°C.

Enzyme	*K* (4°C)	*G* (4°C)[a]	*K* (24°C)[b]	*G* (24°C)	*Y*
G6PDH	0.75	1.77	40	368	60
Hexokinase	1.01	2.38	141	>1000	61
3-PGK	0.71	1.67	29	272	62

[a] Volume ratio = $(V_t/V_b) = 2.36$.
[b] Volume ratio = $(V_t/V_b) = 9.17$.

for 3-PGK. At 24°C the partition values were much higher for all enzymes. K was 40 for G6PDH, 141 for hexokinase and 29 for 3-PGK. These very high partition values at 24°C reflect the reluctance of biomolecules to go into this hydrophobic phase which contains a high concentration of copolymer [13,15,16]. G values at 24°C were high due to large partition coefficients, small volumes of lower copolymer-rich phase and large volumes of upper water phase in this system. Amount of enzyme recovered in this upper water phase at 24°C was 60% for G6PDH, 61% for hexokinase and 62% for 3-PGK. The percentage of recovered enzyme activity is calculated from the original added enzyme activity at 4°C. No enzyme activity was lost in the initial aqueous two-phase system.

While partition coefficients for the above system were good, to achieve a higher yield it was desirable to find a system with a higher ratio of V_t/V_b at 4°C. For this reason the system 8.5% $EO_{20}PO_{80}$, 2.0% dextran T500 and 0.01 M sodium phosphate buffer was chosen (Table II). This system has a volume ratio of 4.28 for copolymer–dextran phases at 4°C, and 7.69 for water–copolymer phases at 24°C. However, moving further away from the critical point of the phase diagram resulted in lower partition coefficients [2]. At 4°C, K was 0.19 G6PDH, 0.58 for hexokinase and 0.54 for 3-PGK. At 24°C, increased concentration of copolymer served to drive enzymes completely into upper water phase, as no activity was detected in lower $EO_{20}PO_{80}$ phase. Increased G values at 4°C helped to increase enzyme recovery to 73% for G6PDH, 124% for hexokinase and 72% for 3-PGK. Hexokinase recovery was probably over 100% due to inactivation of this enzyme in concentrated, hydrophobic upper phase at 4°C. After partitioning to upper water phase which was virtually free of copolymer at 24°C, enzyme activity increased greatly resulting in a high yield.

Partition of enzymes in three systems with differing hydrophobicities has also been studied (Table III). Systems were chosen with equal tie line lengths so that enzyme partition in the phases could be compared [2]. All systems contained 0.01 M sodium phosphate buffer, pH 7.0. While partition in PEG–dextran and UCON–dextran was performed at 22°C, partition in the $EO_{20}PO_{80}$–dextran system was at 4°C. However, this temperature change should have little effect on enzyme partition [22]. The polymers which formed the upper phase were either PEG 4000 (M_r 4000), UCON 50-HB-5100 (M_r 4000) or $EO_{20}PO_{80}$ (M_r 2000), while bottom-phase polymer was dextran T500 in all systems. Since the amount of ethylene oxide groups was 100, 50 and 20%, respectively, this made it possible to study the effect of both hydrophobicity and molecular mass on enzyme partition. Decreasing the molecular mass of a polymer should increase enzyme partition into

TABLE II

PARTITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE, HEXOKINASE AND 3-PHOSPHOGLYCERATE KINASE

Primary phase system: 8.5% $EO_{20}PO_{80}$, 2% dextran T500, 0.01 M sodium phosphate buffer, pH 7.0, 20 units glucose-6-phosphate dehydrogenase, 20 units hexokinase and 45 units 3-phosphoglycerate kinase. K and G values at 24°C are for partition between water and $EO_{20}PO_{80}$ phases formed by the increase in temperature. Y = % yield of enzymes recovered in water phase at 24°C.

Enzyme	K (4°C)	G (4°C)[a]	K (24°C)[b]	G (24°C)	Y
G6PDH	0.19	1.42	>100	>100	73
Hexokinase	0.58	4.41	>100	>100	124
3-PGK	0.54	4.11	>100	>100	72

[a] Volume ratio = $(V_t/V_b) = 4.28$.
[b] Volume ratio = $(V_t/V_b) = 7.69$.

TABLE III

PARTITION OF G6PDH, HEXOKINASE AND 3-PGK IN THREE-PHASE SYSTEMS WITH DIFFERENT HYDROPHOBICITY IN THE UPPER PHASE IN THE ORDER PEG 4000 < UCON < $EO_{20}PO_{80}$

Systems contained 0.01 *M* sodium phosphate buffer, pH 7.0. For systems with PEG 4000 and UCON *K* values are for partition between upper and lower phase at 22°C. For system with $EO_{20}PO_{80}$ *K* values are for partition between upper and lower phase at 4°C. All systems were chosen so that tie line lengths were equal.

System	*K* G6PDH	*K* hexokinase	*K* 3-PGK
7.0% PEG 4000–7.0% dextran T500	0.21	0.50	0.43
5.1% UCON 50-HB-5100–7.0% dextran T500	0.13	0.50	0.36
7.0% $EO_{20}PO_{80}$–4.0% dextran T500	0.19	0.58	0.54

the phase rich in that polymer [31,32]. Comparison of partition between the PEG 4000 and UCON systems shows that the increase in hydrophobicity lowered the *K* values for G6PDH (0.21 to 0.13) and 3-PGK (0.43 to 0.36), while hexokinase was unaffected (0.50 in both systems). However, in the $EO_{20}PO_{80}$ systems *K* values increased for hexokinase (0.58) and 3-PGK (0.54). *K* for G6PDH (0.19) was higher than in the UCON system, and only slightly lower than in the PEG system. These results are interesting in that they show that decreased molecular mass has more effect on enzyme partition than an increase in hydrophobicity.

Partition and purification of enzymes from yeast homogenate

Partition and purification of G6PDH, hexokinase and 3-PGK from yeast homogenate after centrifugation were performed in a system composed of 8.5% $EO_{20}PO_{80}$, 2% dextran T500, 0.02 *M* sodium phosphate buffer, pH 7.0, and 20% yeast extract (Tables IV and V). Partition was carried out as previously described except that primary phase system at 4°C was centrifuged 5 min at 125 *g* to accelerate phase separation when yeast extract was present. Most protein and cell debris partitioned to lower dextran-rich phase, resulting in increased volume and viscosity for this phase, while upper copolymer-rich phase remained clear. Total protein content and enzyme activity were measured in raw homogenate, homogenate after centrifugation, upper and lower phases at 4°C, and upper and lower phases at 24°C. Specific activity (units per mg protein) was calculated for each enzyme, with specific activity in raw homogenate (uncentrifuged) being set equal to a purification factor of 1. *K* values at 4°C were 0.05 for protein, 0.18 for G6PDH, 0.53 for hexokinase and 0.98 for 3-PGK, with G values of 0.2, 0.75, 2.21 and 4.07, respectively (Table IV).

In previous reports, after removing upper copolymer-rich phase formed during this primary partitioning step, it was necessary to add sodium sulfate to a concentration of 0.2 *M* to lower the cloud point of this phase, since many enzymes are denatured at 50°C [13,15]. However, since the cloud point of $EO_{20}PO_{80}$ is 18°C, no salt addition was necessary and phase separation could be achieved close to room temperature (24°C). The time required for phase separation

TABLE IV

PARTITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE, HEXOKINASE AND 3-PHOSPHOGLYCERATE KINASE FROM YEAST EXTRACT

Primary phase system: 8.5% $EO_{20}PO_{80}$, 2.0% dextran T500, 0.02 *M* sodium phosphate buffer, pH 7.0, and 20% yeast extract. *K* and *G* values at 24°C are not given as there was no detectable enzyme activity or protein in the lower phase at this temperature.

Sample	*K* (4°C)	*G* (4°C)[a]
Protein	0.05	0.20
G6PDH	0.18	0.75
Hexokinase	0.53	2.21
3-PGK	0.98	4.07

[a] Volume ratio = V_t/V_b = 4.0.

TABLE V

PURIFICATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE, HEXOKINASE AND 3-PHOSPHOGLYCERATE KINASE FROM YEAST HOMOGENATE

Primary phase system: 8.5% $EO_{20}PO_{80}$, 2.0% dextran T500, 0.02 *M* sodium phosphate buffer, pH 7.0, and 20% yeast extract. *Y* = % yield of enzymes. *PF* = purification factor at 24°C[a].

Sample	G6PDH		Hexokinase		3-PGK	
	PF	*Y*	*PF*	*Y*	*PF*	*Y*
Raw homogenate	1	–	1	–	1	–
After centrifugation	2.4	71	2.1	64	3.2	96
Upper phase 4°C	4.2	52	13.0	69	15.7	80
Upper phase 24°C	5.3	50	15.3	67	15.4	72

[a] The specific activity of an enzyme (units per mg protein) in the homogenate was equivalent to a purification factor of 1.

was reduced by centrifugation at 125 *g*. At 24°C, no enzyme activity or protein was detected in lower copolymer-rich phase. All of the enzyme activity and protein were contained in the upper water-rich phase free of copolymer. Recovery of enzyme in this upper water phase at 24°C was 50% for G6PDH, 67% for hexokinase and 72% for 3-PGK (Table V). Purification factors in this system were also good, with 5.3 for G6PDH, 15.3 for hexokinase and 15.4 for 3-PGK. These high purification factors are a result of the extremely low protein partition coefficient (0.05) at 4°C, which resulted in most of the protein and cell debris being found in lower dextran-rich phase.

Purification scheme using temperature-induced phase separation

Currently a popular method for large-scale separation of biomolecules is based on a two-phase system composed of PEG and high concentrations (7 to 10%) of a salt such as sodium phosphate or sodium sulfate [1,25–27]. These systems allow recovery and recycling of PEG polymer. Calculations have shown that a purification scheme using aqueous two-phase partitioning is economically competitive with other purification methods [4,5,28–30]. However, the high concentration of salt in PEG-salt systems is a drawback. Disposal of salts presents an ecological problem, and many biomolecules are denatured by these systems. In addition, it is impossible to use an affinity ligand in these systems when binding is based on electrostatic forces.

A scheme for enzyme purification using temperature-induced phase formation has been previously proposed [13,15]. A purification scheme which allows enzyme to be obtained in a water–buffer–salt phase free of cell debris and includes recycling of UCON polymer was presented [13]. After formation of the second phase system at increased temperature, the lower UCON-rich phase was recovered and recycled in a second extraction. In another example an affinity ligand was attached to UCON polymer and used to selectively purify G6PDH from yeast extract, with recovery of G6PDH in a water–buffer–salt solution and recovery of UCON-ligand in final UCON phase [15]. In contrast to these schemes which called for addition of salt to a concentration of 0.2 *M* and a substantial increase in temperature, when using the copolymer $EO_{20}PO_{80}$ formation of a second aqueous two-phase system requires no salt and this system forms readily at room temperature (Fig. 2). The low temperature (24°C) at which temperature-induced phase separation is performed eliminates any heat denaturation of sensitive biomolecules and simplifies use of affinity ligands as no salt is present in the system.

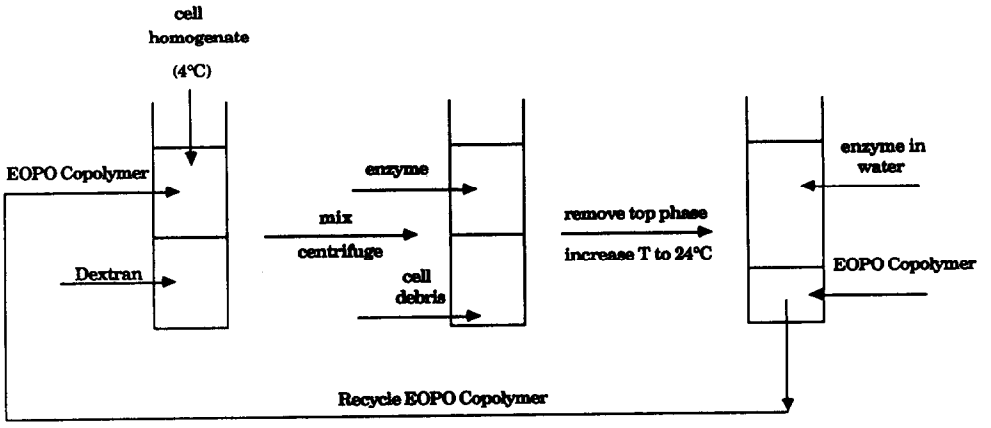

Fig. 2. Enzyme purification scheme using aqueous two-phase partitioning at 4°C and temperature-induced phase separation at 24°C, with recycling of the $EO_{20}PO_{80}$ copolymer.

CONCLUSIONS

A copolymer of 20% ethylene oxide and 80% propylene oxide with a cloud point at 18°C has been used in aqueous two-phase partitioning to purify enzymes from yeast homogenate. This copolymer forms an aqueous two-phase system at 4°C when mixed with dextran T500. The copolymer is enriched in the upper phase and dextran T500 in the lower phase. Proteins partition strongly to the lower dextran phase of this system. After phase separation occurs, the upper copolymer phase containing target enzymes is removed and placed at 24°C, which is above this polymer's cloud point. The temperature increase leads to formation of a new two-phase system composed of a water upper phase and a concentrated copolymer lower phase. Enzymes and proteins partition almost exclusively to the upper water phase of this system. Enzyme yield can be increased by manipulation of polymer concentrations. A simple purification scheme for biomolecules based on this extraction technique is proposed, in which target molecules are recovered in a water–buffer solution and copolymer can be easily recovered and recycled.

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