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A LOW-COST AQUEOUS TWO PHASE SYSTEM FOR ENZYME EXTRACTION

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Summary

Several low-cost maltodextrins form two liquid phases with polyethylene glycol (PEG) in aqueous solution. We have measured physical properties for three PEG-maltodextrin two-phase systems and demonstrated their bioseparation capabilities by extracting alcohol dehydrogenase from yeast extracts with a PEG bound textile dye.

Introduction

Aqueous two-phase extraction has been used to separate and purify a wide variety of proteins and biological materials on both the laboratory and pilot plant scale (Walter et al. 1985). The technique has several advantages: direct scale-up, rapid mass transfer, compatibility with affinity techniques and its ability to handle crude cell lysates.

Two-phase aqueous systems are produced by combining two incompatible water soluble polymers or a polymer and a salt in water, above a critical concentration. Albertsson (1986) describes a wide variety of these aqueous two-phase systems. PEG-salt systems, although low in cost, are of limited utility due to the detrimental effects high salt concentrations (generally > 12 mass%) can have on sensitive biological structures and the inability to apply affinity techniques. Two-polymer systems, on the other hand, are gentle and compatible with affinity techniques permitting highly specific separations (Johansson 1984). The limited commercial use of aqueous two-phase systems in protein purification has been in part due to the high cost of fractionated dextran. Kroner et al. (1982) have tried to circumvent this economic problem by using a moderately priced crude dextran. Unfortunately, the viscosity of the lower, crude dextran-rich, phases were very high. Tjerneld et al. (1986) have used hydroxypropyl starch, which is similar in cost to crude dextran, and PEG to form two-phase systems with reduced lower phase viscosities. PEG-maltodextrin systems, initially used by Mattiasson (1986) for the separation of particles, have not been studied in any detail despite their significant cost advantage.

Materials and Methods

Polymer Solutions. Poly(ethyleneglycol), number average molecular weight 8000 purchased from Sigma Chemical Company (St. Louis, MO)[1], was used as the top phase-forming polymer in all cases. Low molecular weight

[1]The mention of any trade name is not an endorsement by the National Bureau of Standards.

maltodextrins (MD), derived from corn starch with reported average molecular weights of 1200 (Maltrin M150), 1800 (M100), and 3600 (M040) (estimated by HPLC analysis)[2] were used as the lower phase polymer and were obtained from Grain Processing Corporation (Muscatine, IA). Fractionated dextran, average molecular weight 480,000, was purchased from Sigma Chemical Company and was used as a bottom phase forming polymer for comparison. The phase systems were prepared from aqueous stock solutions of 33-40 mass% polymer in all cases.

A solution capable of buffering the pH over the range 4 to 8 was formulated from acetic acid, MOPS, and MES[3] and used in each partitioning experiment at a final concentration of 10 mM for each species (30 mM total). The pH was adjusted to final values with 1 M NaOH. PEG-Cibacron blue FGF (PEG-blue) was prepared as described by Johansson (1984) with the omission of the DEAE adsorption. Five to 10 additional chloroform/water extractions were made, which removed nearly all of the unreacted dye but retained the unmodified PEG. All other chemicals used in these experiments were reagent grade.

Yeast Extract. Yeast enzyme concentrate (YEC) was purchased from Sigma Chemical Company (St. Louis, MO). Stock solutions were made at 10 mg lyophilized powder per ml. The specific activity of ADH in this mixture was found to be 17 U/mg protein using the assay of Vallee (1955).

Yeast Homogenate. Yeast homogenate (YH) was prepared by combining 5 g low temperature dried yeast (*S. cerevisiae*, Sigma Chemical Co.) with 25 g dry ice and grinding for 5 minutes with a mortar and pestle. The resulting yeast paste was taken up in 50 ml of water and kept on ice for immediate use or frozen at -20°C for future use. The specific activity of ADH in YH varied between 5 and 10 U/mg protein.

Physical Properties. All measurements were performed at 25° C. Phase diagrams for PEG 8000-M150, M100, M040 and dextran 500 were determined from 4 or 5, 10 g total weight, two-phase systems for each PEG-maltodextrin or dextran polymer type. Systems were mixed, equilibrated and completely separated by centrifugation at 4000 x g for 15 minutes. The PEG and maltodextrin concentrations in each phase were determined by a combination of refractometry and polarimetry. The specific rotations of the M040, M100, and M150 polymers were found to be 191, 157, and 163,(deg. g^{-1} dm^{-1}), respectively. Densities of the samples were measured using a frequency-type densimeter, and viscosities were determined using a cone and plate viscometer.

Extraction of Alcohol Dehydrogenase. The polymer stock solutions, buffer, water and yeast enzyme concentrate or homogenate were weighed, combined, and mixed thoroughly by gently vortexing for 10 seconds. The ADH activity and total protein in the entire system was then determined. The emulsion which formed was then centrifuged as described above and both the ADH activity and total protein content were determined in each phase. The partition coefficient K, is defined as the ratio of enzyme activity or concentration in the top and bottom phases. For systems

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[2]Grain Processing Corporation, Bulletin 11005, Muscatine, IA.

[3]Abbreviations Used: ADH, yeast alcohol dehydrogenase; MD, maltodextrins; MES,(2-[N-Morpholino]ethanesulfonic acid); MOPS,(3-[N-Morpholino]propanesulfonic acid); PEG, polyethylene glycol; YEC, yeast enzyme concentrate; YH, homogenized yeast cells.

containing particulates, a partition fraction, K_f, is defined as the ratio of activity or concentration in the top phase to that in the entire phase system.

Protein Assay. Protein was determined by the Coomasie Blue binding assay (Bradford 1976) with alcohol dehydrogenase as the standard. For systems containing PEG-blue, identical phases containing no protein were used as control.

Results and Discussion

The phase diagrams for three types of PEG-maltodextrin systems and the PEG-dextran 500 system are shown in Figure 1. The concentration of maltodextrin required to form two phases with PEG is greater than that of dextran and increases as the molecular weight of the maltodextrin decreases. The phase diagrams are therefore shifted toward the right as

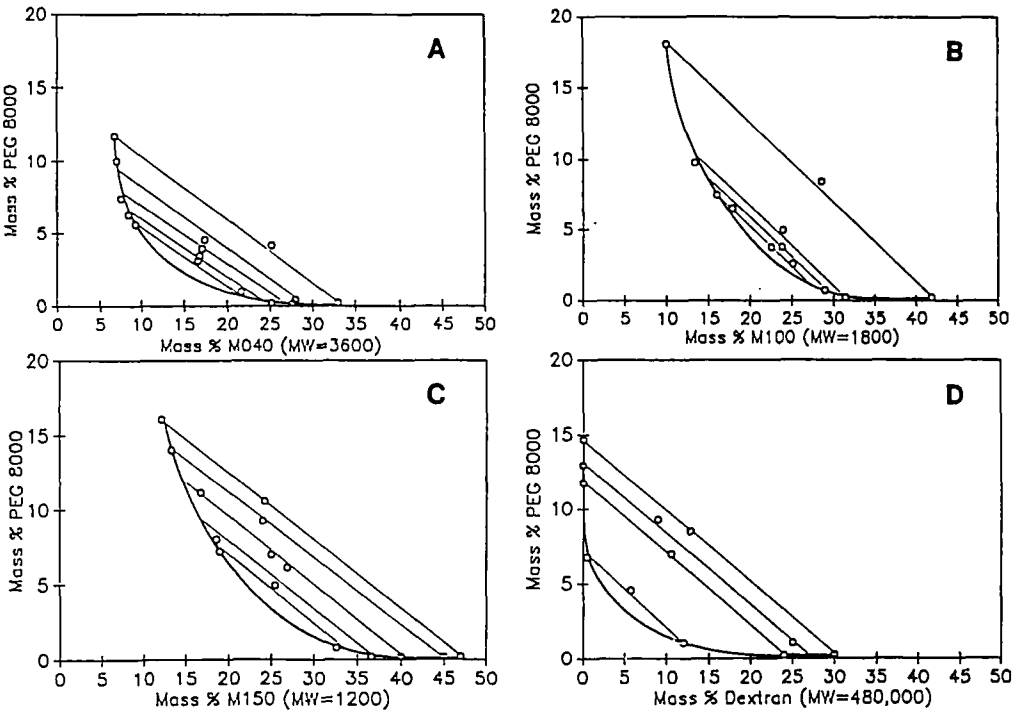

Figure 1. Binodial curves determined at 25° C for 4 different lower-phase polymers. (A)PEG-M040; (B)PEG-M100; (C)PEG-M150; (D)PEG-dextran.

System	Peg 8000 (Mass %)	Malto-dextrin 1800 (Mass %)	Viscosity (cps)	Density (g/cm³)
1 Total	3.75	22.46	-----	-----
1 Top	6.5	17.9	8.06	1.0829
1 Bottom	2.9	25	15.9	1.1146
2 Total	4.93	23.95	-----	-----
2 Top	9.7	19.4	8.83	1.7051
2 Bottom	~0	31	28.5	1.1411
3 Total	3.75	23.79	-----	-----
3 Top	7.5	16.0	7.66	1.0783
3 Bottom	~0	29	17.3	1.1208
4 Total	8.40	28.57	-----	-----
4 Top	18.1	10.0	20.60	1.0757
4 Bottom	~0	42	66.0	1.1747

System	PEG 8000 (Mass %)	Dextran 480,000 (Mass %)	Viscosity (cps)	Density (g/cm³)
1 Total	6.99	10.57	-----	-----
1 Top	11.8	0.2	5.98	1.0171
1 Bottom	0.2	24	295.0	1.0930
2 Total	8.50	12.78	-----	-----
2 Top	14.7	0.3	8.79	1.0226
2 Bottom	0.3	30	565	1.1140
3 Total	4.57	5.77	-----	-----
3 Top	12.8	0.5	3.21	1.0101
3 Bottom	1.1	12	51.5	1.0526
4 Total	9.31	8.89	-----	-----
4 Top	13.0	0.04	7.27	1.0198
4 Bottom	1.1	25	487	1.1033

Table 1. Comparison of physical properties of 4 PEG-M100 phase systems (A) and PEG-dextran systems (B)

the maltodextrin molecular weight decreases. PEG is almost totally excluded from the bottom phase of a PEG-maltodextrin system, while the concentration of maltodextrin in the top phase is relatively high when compared to systems formed with dextran.

The lower phase of the PEG 8000-M040 system formed a gel at higher polymer concentrations at room temperature. This severely limits the usefulness of the M040 as a phase forming polymer. The PEG-M100 phase system formed stable solutions at room temperature and remained clear for several days but became cloudy after prolonged standing at 4° C at the higher polymer concentrations. The PEG 8000-M150 systems remained clear over a period of two days at 4° C. Concentrated stock solutions of M100 (30-35 mass%), remained clear for two days at 4° C and then turbid. Solutions could be cleared by gentle heating and stirring.

The physical properties of 4 PEG-M100 phase systems are compared with those of 4 PEG-dextran systems in Table 1. The bottom phase viscosities of the maltodextrin systems were less than half those formed with dextran, and comparable to PEG 8000-hydroxypropyl starch systems (Tjerneld et al. 1986). The upper phase viscosities of the maltodextrin systems are slightly greater by 2 to 5 cps as compared to similar dextran or hydroxypropyl starch systems, but phase separation and mixing were not hindered. The partition coefficient of the dye ligand PEG-blue in the system 22.5% w/w M100, 4.0% PEG 8000, and 30 mM buffer (pH 7) is 9.1 ± 1.5 is very similar to that in a system containing 6.6% dextran 500, 4.0% PEG 8000 and 30 mM buffer, 9.4 ± 0.2. This value was independent of pH over the range 4 to 8.

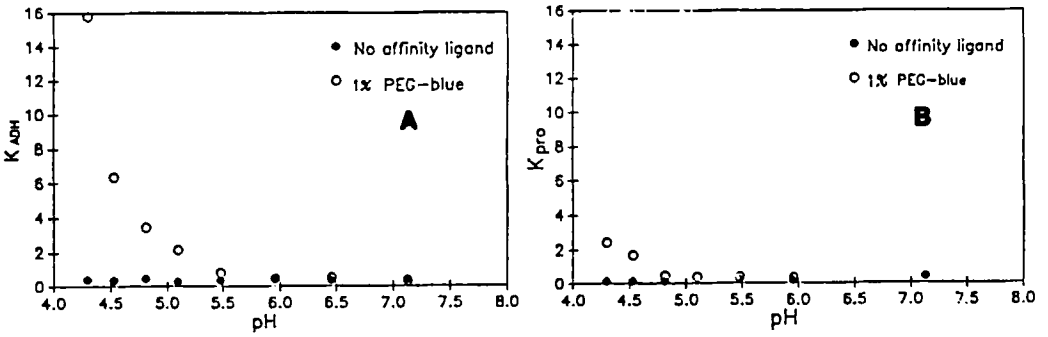

Figure 2. Extraction of ADH (A) and total protein (B) from YEC with PEG-blue (1%) and without at 4° C.

PEG-M100 System

	K_f	K_{ADH}	K_{PRO}	Phase Vol. ratio	Purification Factor
YEC	0.92	11.4	1.2	2.5	2.9
YH	0.47	0.38	0.03	2.5	8.3
PEG-dextran System					
YEC	0.92	12.7	2.5	2.6	1.9
YH	0.45	0.36	0.08	2.5	4.2

Table 2. Extraction of ADH from yeast enzyme concentrate (YEC) and yeast homogenate (YH) with 2% PEG-blue, pH 5.1, 20% phase system volume replaced with YH or 1mg/ml YEC at 4° C.

Phase systems formed with maltodextrin and containing PEG-blue were tested for their ability to purify ADH and to determine the pH optimum for this extraction of ADH from YEC or YH preparations. ADH partitioned predominantly into the bottom phase in the absence of an affinity ligand (Figure 2A). When the affinity ligand PEG-blue was incorporated into the phase system at a concentration of approximately 1% of the total PEG concentration, the partition coefficient of ADH became strongly dependent on the system pH (Figure 2A) and ligand concentration. The partition coefficient of total protein in the system also increased with decreasing pH when the dye ligand was present, but not as dramatically as the ADH (Figure 2B). The optimum pH for the purification of ADH was found to be near 5.0. At lower pH the partition coefficient increased but recovery decreased due to lability of the enzyme and dye-protein precipitation at the interface. Above pH 5, the partition coefficient decreased rapidly, resulting in low recoveries in the top phase unless a very large volume ratio was used.

Direct comparisons between PEG-M100 and PEG-dextran systems for enzyme purification were made by extracting ADH from both YEC and a slurry of disrupted yeast cells (YH). In all cases 2% of the PEG systems was replaced by PEG-blue. These data are shown in Table 1. The K_f and K_{ADH} were virtually identical for either PEG-dextran or PEG-maltodextrin and YEC. The partition coefficient for total protein, K_{PRO}, in the PEG-dextran system was 2-3 times higher, while the purification factors were 50% greater in the PEG-maltodextrin system. With the crude yeast cell homogenate as the starting material, the partition coefficients for both total protein and ADH drop dramatically, while the purification factors in both cases are increased.

Protein partitioning for both two-phase systems with PEG-blue was influenced by ligand concentration, system pH and the presence of cell debris. In the absence of an affinity ligand, the partition coefficient for ADH was slightly greater in the system containing dextran as than in the system containing maltodextrin. This is consistent with the observation that greater molecular masses of bottom phase forming polymer increase the partition coefficient. If the affinity ligand PEG-blue is incorporated into each system, the partition behavior of the ADH and the total protein is very similar in both systems. At a pH of 5, the partition coefficient of ADH was increased sufficiently so that high yields in the top phase were possible without resorting to large phase volume ratios.

The purification factor for ADH from the crude yeast cell homogenate, 8.8 times, is over 3 times as great as the published purification with a PEG-salt system. The amount of activity recovered in the top phase varied between 45 and 55 percent and was dependent on the manner in which the cells were disrupted. We are currently investigating procedures for recovering the purified enzyme from the PEG phase. Preliminary results show that is possible to partition ADH ($K > 0.03$) into the ammonium sulphate bottom phase of a second aqueous two-phase system and then to remove the salt by ultrafiltration or dialysis.

The main drawback to the widespread use of polymer-polymer two-phase aqueous extraction has been the high cost of fractionated dextran and high bottom phase viscosities. Tjerneld (1986) has shown that

chemically modified starches i.e. hydroxypropyl starch, can be used successfully in two phase aqueous systems and that the cost of $3-3.5/ L of phase system is commercially viable. The maltodextrins are more than an order of magnitude less expensive at $0.60/kg than hydroxypropyl starch ($21/kg) and more than two orders of magnitude less expensive than fractionated dextran ($500/kg). Some of the lower cost is offset by the fact that slightly higher concentrations of maltodextrin are required to form two-phase systems. We have estimated the cost for one kilogram of each of the following phase systems which have similar physical properties: 4% PEG 8000 - 7% Dextran 500, 4% PEG 8000 - 22% M100, and 4% PEG 8000 - 14% hydroxypropyl starch. The respective costs are: $7 / kg, $0.2 / kg, and $3 / kg. It is quite obvious that the maltodextrin systems offer a significant cost advantage. Furthermore, maltodextrins are available in bulk quantity as spray dried white powders and are generally recognized as being safe for human consumption.

Conclusion

The PEG-maltodextrin aqueous two-phase system offers the combined advantages of low-cost, reduced lower phase viscosities, high phase density differences and lack of toxicity. When coupled with low-cost affinity ligands i.e. triazine dyes, PEG-maltodextrin aqueous two-phase systems offer an efficient large scale procedure for the recovery of industrially important enzymes.

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