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Influence of Lactic Acid on the Formation of Aqueous Two-Phase Systems Containing Poly(ethylene glycol) and Phosphates

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In this paper, which forms part of a study on aqueous two-phase systems (ATPS) for separation of low-molar-mass organic acids in the biotechnology industry, we present experimental results on the phase equilibria of ATPS containing poly(ethylene glycol) (PEG) and either K_2HPO_4 or KH_2PO_4 or a fixed mixture of the two phosphates. The measurements were made at a temperature of 298.15 K and a pressure of 1 bar. The PEG had a mass-average molar mass of either 10 000 $g \cdot mol^{-1}$ or 20 000 $g \cdot mol^{-1}$ and a polydispersity index of 1.05. The influence of 2-hydroxy-propanoic acid (lactic acid) on the formation of the ATPS was studied, and the partition coefficient for this compound between the coexisting phases was found to be close to unity.

Introduction

Aqueous two-phase systems (ATPS), containing either two structurally different polymers or a polymer and an inorganic salt, have interesting applications in the field of biotechnology for the separation and purification of valuable biomolecules. To develop and optimize such processes, it is essential to have a thorough and systematic understanding of the liquid−liquid equilibria in ATPS media and of the effects of key factors such as temperature, pH, and the concentrations of nutrients, salts, and fermentation products. Several reviews have been published[1–3] of the liquid−liquid equilibria (LLE) in ATPS, and we have published a study of the LLE of the ATPS containing poly(ethylene glycol) (PEG) and poly(ethyleneimine) (PEI) and have considered in particular the effect of pH.[4]

The objective of the present study was 2-fold: first, to examine the formation of ATPS containing PEG and either K_2HPO_4 or KH_2PO_4 or a mixture of the two salts in a prescribed ratio; and second, to study the influence of 2-hydroxy-propanoic acid (lactic acid) on the phase behavior of this ATPS. Lactic acid, because of its hydrophilic nature, might modify the hydration of the nonionic PEG (which has a tendency to interact with water through hydrogen bonding), and thus it may be expected to have a significant influence on the phase behavior of these ATPS. Though there are data published in the literature on the influence of lactic acid on the phase formation of ATPSs comprising two polymers (e.g., PEG + dextran,[5] dextran + ethylene oxide propylene oxide (EOPO) random copolymers,[6] PEG + polyethylenimine (PEI) conjugates, and EOPO + PEI[7]), to the best of our knowledge, a study of the role of lactic acid on the phase formation of the PEG + phosphate ATPSs has not been reported in the past.

High molar mass PEGs of (10 000 and 20 000) $g \cdot mol^{-1}$ were chosen because they form ATPS at lower concentrations and hence of lower viscosities, thereby favoring fermentation processes in such media. Furthermore, as demonstrated by Li et al.,[8] cells have higher specific growth rates in ATPS formed from higher molar mass PEGs.

ATPS composed of PEG and phosphate salts are among the most exploited for the bioseparation of valuable substances. Recently, such systems have been used for purification of potato polyphenol oxidase[9] and xylose reductase,[10] partitioning of amino acids,[11] β-glucosidase[12] and pectinolytic enzymes,[13] extraction and purification of *C*-phycocyanin,[14] polycetides,[15] bioaffinity extraction of glucoamylase,[16] and extraction and determination of morphine.[17] In general, the systems studied comprise PEG with a molar mass up to 6000 $g \cdot mol^{-1}$ and mixtures of monobasic and dibasic phosphates.[18–24] There are, however, few papers describing the phase behavior of ATPS formed from higher molar mass PEGs and phosphate salts.[25]

Material and Methods

Materials. PEG 10 000 and PEG 20 000 were obtained from Fluka. They were narrowly distributed technical products, with a polydispersity index of about 1.05 as determined in the present study by gel permeation chromatography. The phosphates, anhydrous K_2HPO_4 and KH_2PO_4, were obtained from Fluka, with a minimum purity of 99 %. Concentrated lactic acid solution (mass fraction 90 % in water) was obtained from Acros Organics. The polymers and the salts were used without further purification. Double-distilled water was used for the preparation of the stock solutions.

Stock solutions of the two polymers were prepared with mass fractions of about 0.5. For the K_2HPO_4, the mass fraction of the stock solution was about 0.3, while for KH_2PO_4, a mass fraction of about 0.2 was chosen. A stock solution of the two phosphates in a 1:1 ratio by mass was also prepared with a total mass fraction of 0.3.

Since concentrated lactic acid solution contains high levels of dimers, it was first diluted 10-fold and then boiled for (8 to 10) h under reflux to hydrolyze the dimers. The resulting solution, containing about 130 $g \cdot L^{-1}$ of lactic acid, was further diluted to obtain a stock solution of known concentration for use in the preparation of the quaternary ATPS. Pure, crystalline L-(+)-lactic acid (Sigma-Aldrich, 99.5 %) was used to prepare

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standard solutions for the HPLC analyses of lactic acid in the equilibrium phases of the quaternary ATPS.

All stock solutions were prepared gravimetrically on an analytical balance with 0.0001 g accuracy. The expanded uncertainty of all gravimetrical measurements was ± $5.3 \cdot 10^{-4}$ g with coverage factor 2.

Methods. ATPS Preparation. All ATPS were prepared gravimetrically by mixing appropriate quantities of the necessary stock solutions with distilled water in 50 mL beakers. For the determination of the binodal curves, a glass vessel with a volume of 125 mL was used. It was provided with an external jacket through which water at a constant temperature of (298.15 ± 0.1) K was circulated.

For the phase volume measurements, a 30 g sample, with a predetermined overall composition, was stirred vigorously for 15 min and then transferred into a graduated (0.1 mL) test tube fitted with a stopper, with a total volume of 30 mL. The tubes were placed in a water bath thermostatted at (298.15 ± 0.1) K, and the system was left to settle for 15 h, during which time it separated into two clear and transparent liquid phases separated by a well-defined interface.

For the preparation of the quaternary ATPS, stock solutions for PEG 10 000, PEG 20 000, and the K_2HPO_4 were made as above but with the addition of 53.0 $g \cdot L^{-1}$ of lactic acid. This was chosen as exemplifying the maximum lactic acid concentration (about 50 $g \cdot L^{-1}$) that can be achieved in a biotechnological process, e.g., during carbohydrate (mainly lactose) fermentation of cheese whey. Some measurements were also made on systems containing smaller concentrations of lactic acid.

Determination of the Binodal Curves. Formation of the ATPS was observed visually by the appearance of turbidity in the agitated mixture. If there was no turbidity, then either PEG or/and salt of known amounts were added until the ATPS was formed.

The binodal curve was determined by dropwise addition of water to the well-stirred ATPS prepared as described above. Water was added until turbidity disappeared and the solution became transparent, which indicated the transition from two-phase to one-phase behavior. All solutions were maintained at (298.15 ± 0.1) K in a temperature-controlled bath. The quantity of water added was determined gravimetrically and used to recalculate the compositions of the last point in the two-phase region and the first point in the homogeneous one-phase region. The mean value of these two was taken as a point on the binodal. This typically introduced an ambiguity not worse than ± $0.005 \cdot w_i$ in the binodal composition.

The binodal curves of the quaternary ATPS were determined in the same way. However, lactic acid stock solution was used throughout in place of pure water to maintain a constant lactic acid concentration $c_{LA} = 53.0\ g \cdot L^{-1}$.

Determination of the Equilibrium Compositions (Tie-Lines), pH, and Lactic Acid Partition Coefficient. For the determination of tie-lines, pH, and lactic acid partition coefficient, ATPS of known overall composition were prepared as described above. The volumes V_{top} and V_{bottom} of the top and bottom phases of all the ATPS studied were estimated from the graduation of the test tubes with uncertainties of ± 0.1 mL, and volume ratios were calculated. The ATPS were then transferred into separating funnels and thermostatted for an additional (6 to 8) h to ensure equilibrium. After that period, the top and bottom phases were carefully separated, and the pH of the equilibrium phases was measured (pH meter OP-265/1, Radelkis, Hungary) with an uncertainty of ± 0.05 at a coverage factor of 2.

Table 1. Binodal Curves for PEG 10 000 (1) + Phosphate (2) + Water (3) at T = 298.15 K

K_2HPO_4		KH_2PO_4		$0.5K_2HPO_4 + 0.5KH_2PO_4$	
$10^2 \cdot w_1$	$10^2 \cdot w_2$	$10^2 \cdot w_1$	$10^2 \cdot w_2$	$10^2 \cdot w_1$	$10^2 \cdot w_2$
38.48	1.827	37.97	3.835	38.38	2.273
34.43	2.355	35.79	4.282	36.08	2.505
32.71	2.607	34.65	4.401	34.10	2.789
29.94	2.812	32.83	4.901	32.23	3.049
28.65	2.958	29.53	5.649	30.30	3.321
26.53	3.279	31.76	5.044	28.70	3.696
24.81	3.628	28.01	6.100	27.24	4.016
23.48	3.858	26.22	6.451	25.84	4.180
21.77	4.116	24.36	6.975	24.09	4.462
19.67	4.567	23.11	7.340	22.76	4.759
17.30	4.982	22.04	7.704	20.20	5.281
16.31	5.277	20.77	8.097	19.01	5.641
15.13	5.543	19.66	8.332	17.67	6.180
14.08	5.847	18.57	8.654	15.76	6.682
12.27	6.292	17.63	8.938	12.85	7.519
10.58	6.740	16.40	9.416	10.72	8.186
8.919	7.240	14.92	9.883	9.572	8.627
6.859	7.853	13.34	10.44	8.449	9.081
5.615	8.384	12.43	10.77	7.115	9.782
3.959	8.858	11.61	11.08	5.524	10.36
2.075	9.559	10.07	11.80	4.036	11.07
1.293	10.11	8.789	12.29	3.469	11.45
0.769	10.41	7.342	12.94	2.117	12.17
0.711	10.51	6.173	13.47	1.564	12.38
0.148	11.26	4.527	14.32	0.807	12.85
0.095	14.75	3.035	15.40	0.228	14.30
0.069	18.60	2.222	16.06	0.096	15.94
0.051	21.92	1.383	16.69	0.060	22.81
0.039	24.31	0.686	17.32	0.046	25.28
0.025	28.11	0.174	18.11	0.027	26.84

Equilibrium phase compositions (tie-lines) for four of the (PEG + phosphate + water) ternary systems were obtained from the binodal curves, together with the measured mass ratios of coexisting phases of known overall composition. The calculation procedure, essentially as described previously by Mechuk et al.,[26] is detailed further below.

For the quaternary ATPS, the partitioning of the lactic acid between coexisting phases was determined by HPLC analysis. For this purpose, the equilibrium phases were first diluted with known amounts of distilled water and then injected onto the HPLC column. The HPLC system (Perkin-Elmer series 10) was equipped with a refractive-index detector. The column (Aminex HPX-87H, Bio-Rad, Richmond, CA, USA) was maintained at the ambient temperature of (296 ± 2) K. The mobile phase was 5 mM sulfuric acid at a flow rate of 0.6 $mL \cdot min^{-1}$. All analyses were based on three replicate chromatographic measurements made on each sample, and the results were reproduced within ± $0.01 \cdot c_{LA}$, while the corresponding uncertainty was ± $0.015 \cdot c_{LA}$.

Results and Discussion

The influence of the molar mass of the polymer, and the concentrations of salt and lactic acid, on the LLE of the ATPS was studied as discussed below.

Influence of the Salts. The measured binodal curves of the (PEG + phosphate + water) ternary systems are given in Tables 1 and 2 for PEG 10 000 and PEG 20 000, respectively. The results are shown graphically in Figures 1 and 2, and we see that, for both polymers, the largest two-phase regions are formed with K_2HPO_4. As expected, the two-phase region of the ATPS with the 1:1 by mass ratio of the two phosphates is smaller than that formed with K_2HPO_4 and larger than that with KH_2PO_4. Nevertheless, the binodal curve of the ATPS containing the mixed phosphates is much closer to that with K_2HPO_4,

Table 2. Binodal Curves for PEG 20 000 (1) + Phosphate (2) + Water (3) at $T = 298.15$ K

K_2HPO_4		KH_2PO_4		$0.5K_2HPO_4 + 0.5KH_2PO_4$	
$10^2 \cdot w_1$	$10^2 \cdot w_2$	$10^2 \cdot w_1$	$10^2 \cdot w_2$	$10^2 \cdot w_1$	$10^2 \cdot w_2$
37.69	1.940	38.60	3.344	35.46	2.763
35.18	2.139	36.27	3.719	33.62	2.950
32.96	2.442	35.38	3.899	29.98	3.493
30.66	2.726	34.07	4.168	27.99	3.760
27.96	3.195	31.79	4.708	24.92	4.365
25.20	3.560	29.96	5.156	23.38	4.594
24.59	3.598	27.69	5.717	21.45	5.032
23.45	3.822	26.22	6.116	20.64	5.183
21.64	4.133	24.08	6.724	19.24	5.458
19.42	4.406	22.71	7.174	17.84	5.802
17.76	4.724	20.55	7.652	16.01	6.190
15.88	5.087	19.09	8.139	14.78	6.514
14.79	5.280	18.13	8.288	13.65	6.788
12.84	5.771	16.66	8.909	12.69	7.089
11.35	6.105	15.19	9.361	11.19	7.524
9.690	6.527	13.71	9.804	10.10	7.878
7.898	7.025	12.50	10.24	9.246	8.256
6.491	7.438	11.01	10.75	8.261	8.607
5.070	7.810	9.594	11.27	7.327	8.962
4.327	8.081	8.570	11.74	6.421	9.269
3.513	8.282	7.173	12.17	4.853	9.755
2.887	8.561	6.457	12.61	3.314	10.35
0.834	9.440	5.139	13.02	2.182	10.81
0.392	9.915	4.062	13.60	0.965	11.69
0.112	10.664	2.803	14.20	0.495	12.25
0.087	11.356	1.940	14.87	0.143	13.07
0.069	15.634	0.983	15.65	0.098	15.68
0.060	18.798	0.349	16.47	0.088	17.20
0.054	20.402	0.037	17.64	0.065	21.08
0.039	25.021	0.021	19.10	0.049	23.23

and the two appear to be converging at a PEG concentration somewhat above the greatest value studied here. At this stage, we are not able to offer an explanation of this observation. The hypothesis that differences in the pH are responsible was not substantiated by our experimental measurements or by theoretical calculations of the pHs taking into consideration the dissociation constants of the two salts and their overall concentrations.

Further measurements illuminating the influence of the ratio of the two phosphates on the formation of the ATPS are reported in Table 3. The results given here pertain to an ATPS containing an approximate mass fraction of 14 % PEG 20 000 and 9 % total phosphates in different ratios. There is a slight difference in the pH of the equilibrium phases which declines with decreasing proportion of K_2HPO_4 at constant total phosphate concentration. A possible explanation of this can be found in the fact that the two phosphates distribute unevenly between the phases. Table 3 also gives the measured volume ratios in these experiments.

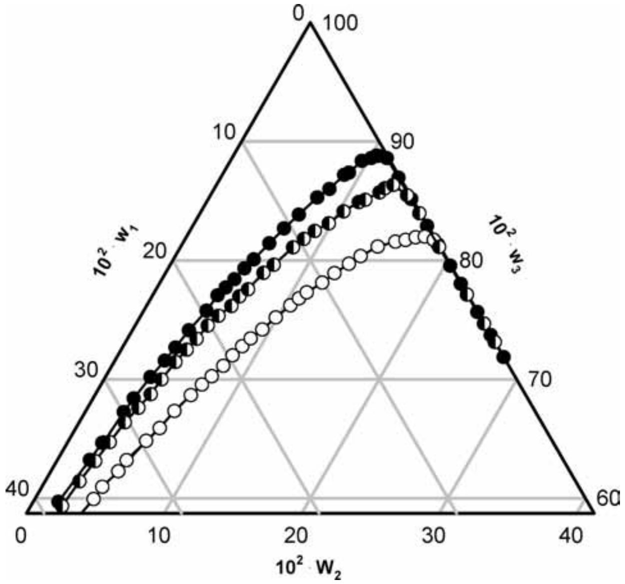

Figure 1. Binodal curves for PEG 10 000 (1) + phosphate (2) + water (3) showing the influence of the phosphate. ●, K_2HPO_4; ○, KH_2PO_4; ◐, K_2HPO_4:KH_2PO_4 = 1:1; and - - -, calculated from eq 6.

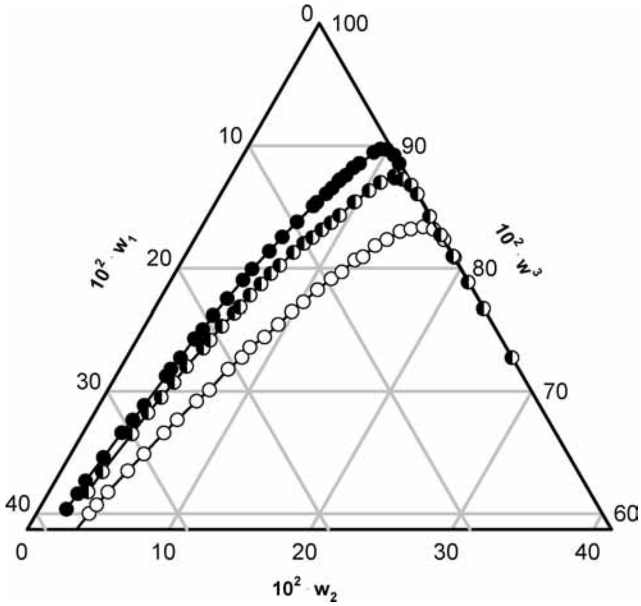

Figure 2. Binodal curves for PEG 20 000 (1) + phosphate (2) + water (3) showing the influence of the phosphate. ●, K_2HPO_4; ○, KH_2PO_4; ◐, K_2HPO_4:KH_2PO_4 = 1:1; and - - -, calculated from eq 6.

Influence of the PEG Molar Mass. The influence of the molar mass of the PEG on the formation of the ATPS with K_2HPO_4 is shown in Figure 3. For PEG 10 000 and PEG 20 000 the two-phase boundaries are rather close and actually converge at higher polymer concentration. Figure 3 also shows the experimental data of Mishima et al.[20] for PEG 20 000, which are in an excellent agreement with the present results, and also for PEG 4000. These data show that the two-phase region expands with increase of the molar mass of the PEG. The influence of the polymer molar mass is clearly less pronounced above 10 000 $g \cdot mol^{-1}$ and a reasonable inference is that there may be a value above which the molar mass no longer plays a significant role. Similar behavior was found for the ATPS containing KH_2PO_4.

Equilibrium Phase Compositions (Tie-Lines) for the Ternary ATPS. Following Merchuk et al.,[26] tie-lines were constructed connecting each feed state with the equilibrium top and bottom phases into which it splits by consideration of both the measured binodal curve and a mass balance criterion. We denote the mass fraction of PEG in the feed, top phase, and bottom phase by $w_{1,F}$, $w_{1,T}$, and $w_{1,B}$, respectively, and the corresponding mass fractions of the phosphate by $w_{2,F}$, $w_{2,T}$, and $w_{2,B}$. The following mass balance equations may then be written

$$w_{1,F} = \alpha \cdot w_{1,T} + (1 - \alpha) \cdot w_{1,B} \tag{1}$$

$$w_{2,F} = \alpha \cdot w_{2,T} + (1 - \alpha) \cdot w_{2,B} \tag{2}$$

where α is the fraction of the total mass in the top phase. In addition, we have the equation of the binodal curve $w_2 =$

Table 3. Influences of Phosphate Ratio and Lactic Acid Concentration for ATPS Formed from a Feed Containing 14.08 Mass % PEG 20 000 + 9.39 Mass % Total Phosphates

mass fraction ratio	$c_{LA} = 0$			$w_{LA}/g \cdot L^{-1} = 5.3$			$w_{LA}/g \cdot L^{-1} = 10.6$		
KH_2PO_4:K_2HPO_4	pH top phase	pH bottom phase	volume ratio V_{top}/V_{bottom}	pH top phase	pH bottom phase	volume ratio V_{top}/V_{bottom}	pH top phase	pH bottom phase	volume ratio V_{top}/V_{bottom}
0:10	9.03	8.85	1.08	8.33	8.04	1.00	7.78	7.60	1.00
2:8	7.57	7.4	1.22	7.31	7.15	1.13	7.06	6.88	1.17
4:6	6.93	6.79	1.63	6.73	6.57	1.38	6.46	6.33	1.56
5:5	6.65	6.52	1.50	6.44	6.33	1.56	6.14	6.05	1.86
6:4	6.32	6.26	2.33	6.15	6.05	2.12	5.84	5.73	2.45
8:2	5.74	5.70	1.63	5.44	5.32	15.67		homogeneous	
10:0		homogeneous			homogeneous			homogeneous	

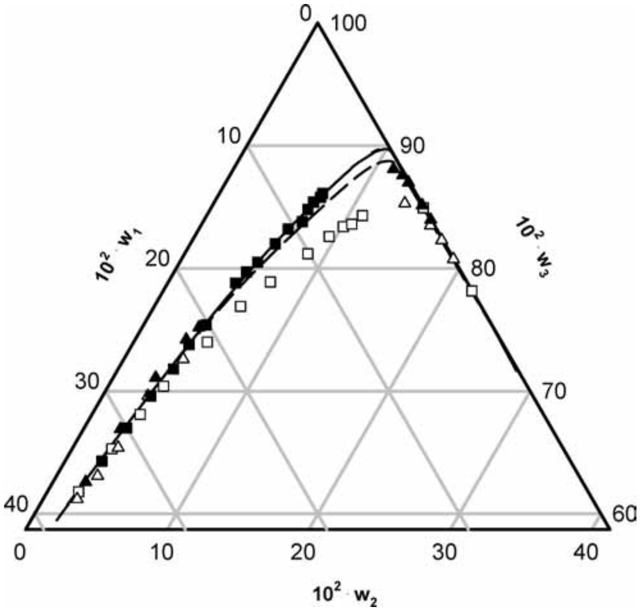

Figure 3. Binodal curves for PEG (1) + K_2HPO_4 (2) + water (3) showing the influence of the molar mass of the polymer. This work: - - - -, PEG 10 000; −, PEG 20 000. Ref 25: □, △, PEG 4000; ■, ▲, PEG 20 000.

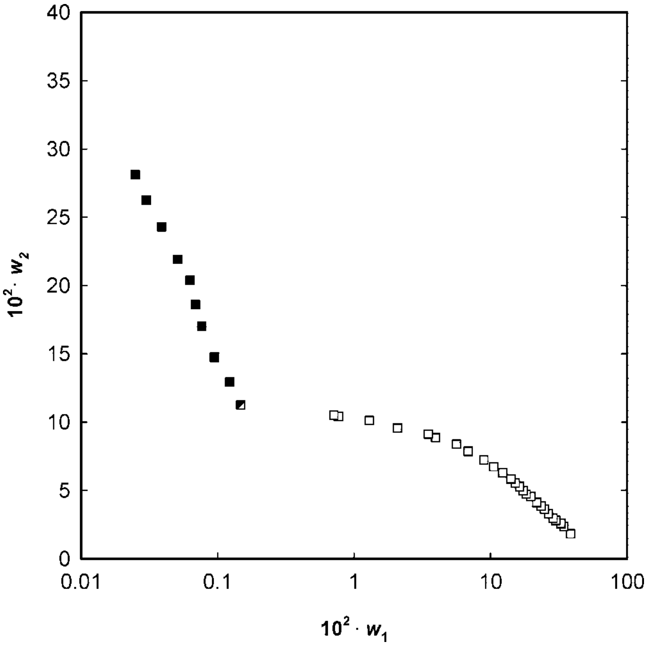

Figure 4. Binodal curve of PEG 10 000 (1) + K_2HPO_4 (2) + water (3) showing the change in slope at low PEG concentration. □, PEG-rich branch; and ■, PEG-poor branch.

Table 4. Intersection Points on the Binodal Curves of PEG + Phosphates + Water

PEG	Phosphate	$10^2 \cdot w_1$	$10^2 \cdot w_2$
10 000	K_2HPO_4	0.153	10.7
10 000	0.5 K_2HPO_4 + 0.5 KH_2PO_4	0.225	13.3
20 000	K_2HPO_4	0.096	9.89
20 000	0.5 K_2HPO_4 + 0.5 KH_2PO_4	0.152	12.0

$f(w_1)$ which may be written for the top and bottom phases as follows

$$w_{2,T} = f(w_{1,T}) \quad (3)$$

$$w_{2,B} = f(w_{1,B}) \quad (4)$$

Given a correlation $w_2 = f(w_1)$ of the experimental binodal curve and mass ratios α for various feed compositions, eqs 1 to 4 may be solved for the coexisting phase compositions. It should be pointed out, however, that the determination of the equilibrium compositions (tie-lines) following the above simple gravimeric method[26] is dependent on the correlation obtained for the binodal curve.

For the PEG + phosphate ATPS examined in the present study, it was not possible to correlate the entire binodal curve by any single functional relationship with a reasonable number of parameters. This difficulty arises from the remarkable and rapid change in the slope and curvature of the binodal curves found near the consulate point. This behavior can be seen in Figures 1 to 3 for small values of w_1 and more clearly on the semilog scale used in Figure 4. In view of this behavior, a piecewise correlation of each binodal was adopted in which

$$w_2 = a + b\ln(w_1) \quad (5)$$

represented the bottom phase (PEG-poor) branch and

$$w_2 = \sum_{i=0}^{3} c_i\{\ln(w_1)\}^i \quad (6)$$

represented the top phase (PEG-rich) branch, and a, b, and c_i (i = 0, 3) are a set of coefficients. The intersection points between the separate branches were determined from the semilogarithmic plots and are given in Table 4. However, in the case of the systems containing KH_2PO_4, the change of slope was not well defined and the entire binodal curve could be adequately represented by eq 6. It is important to note that the only role of these correlations in the calculation of the tie-lines is to permit a smooth interpolation of the experimental points.

Tie-line data for four systems are given in Table 5, where we report both the experimentally determined mass ratios α for various feed compositions and the corresponding calculated equilibrium phase compositions. The results for the PEG 20 000 ATPS with K_2HPO_4 and KH_2PO_4 and with the 1:1 ratio of the two salts are shown in Figure 5a to c. Analogous and very similar results for the ATPS containing PEG 10 000 with K_2HPO_4 were obtained. Mass ratios were not measured for the other two systems containing PEG 10 000.

Influence of the Lactic Acid. Binodal curves for the quaternary ATPS (PEG + K_2HPO_4, + lactic acid + water), with a fixed overall concentration of lactic acid c_{LA} = 53.0

Table 5. Tie-Lines for PEG + Phosphates + Water at $T = 298.15$ K

PEG 20 000 (1) + K_2HPO_4 (2) + Water (3)

feed and mass ratio α (experimental)			top phase (calculated)		bottom phase (calculated)	
$10^2 \cdot w_1$	$10^2 \cdot w_2$	α	$10^2 \cdot w_1$	$10^2 \cdot w_2$	$10^2 \cdot w_1$	$10^2 \cdot w_2$
5.01	8.31	0.2837	16.57	4.99	0.43	9.63
6.02	9.02	0.2930	20.23	4.32	0.13	10.96
6.99	10.07	0.2997	23.84	3.73	$2.03 \cdot 10^{-3}$	12.78
8.16	11.23	0.2476	34.32	2.31	$1.05 \cdot 10^{-3}$	14.16
9.07	12.02	0.2398	39.81	1.69	$8.62 \cdot 10^{-5}$	15.28

PEG 20 000 (1) + KH_2PO_4 (2) + Water (3)

feed and mass ratio α (experimental)			top phase (calculated)		bottom phase (calculated)	
$10^2 \cdot w_1$	$10^2 \cdot w_2$	α	$10^2 \cdot w_1$	$10^2 \cdot w_2$	$10^2 \cdot w_1$	$10^2 \cdot w_2$
4.01	13.95	0.1615	13.73	9.81	2.13	14.75
4.50	14.42	0.2172	18.67	8.22	0.57	16.14
5.03	14.98	0.2246	22.05	7.23	0.1	17.23
5.48	15.74	0.2006	27.36	5.82	$4.12 \cdot 10^{-3}$	18.23
5.99	16.01	0.1616	37.13	3.56	$3.19 \cdot 10^{-3}$	18.41

PEG 20 000 (1) + ($0.5K_2HPO_4 + 0.5KH_2PO_4$) (2) + Water (3)

feed and mass ratio α (experimental)			top phase (calculated)		bottom phase (calculated)	
$10^2 \cdot w_1$	$10^2 \cdot w_2$	α	$10^2 \cdot w_1$	$10^2 \cdot w_2$	$10^2 \cdot w_1$	$10^2 \cdot w_2$
5.08	10.22	0.3684	14.04	6.72	0.13	12.15
5.08	11.38	0.2615	19.11	5.51	0.12	13.47
7.05	12.05	0.2938	23.76	4.57	0.10	15.16
8.49	13.51	0.2773	30.40	3.44	0.09	17.37
10.02	15.05	0.2876	35.78	2.65	0.07	19.85

PEG 10 000 (1) + K_2HPO_4 (2) + Water (3)

feed and mass ratio α (experimental)			top phase (calculated)		bottom phase (calculated)	
$10^2 \cdot w_1$	$10^2 \cdot w_2$	α	$10^2 \cdot w_1$	$10^2 \cdot w_2$	$10^2 \cdot w_1$	$10^2 \cdot w_2$
11.23	7.66	0.6053	18.47	4.79	0.13	12.06
14.94	11.00	0.4054	36.61	2.02	0.08	17.13
15.04	9.59	0.4865	30.83	2.76	0.09	16.05
15.21	8.44	0.5526	27.44	3.25	0.10	14.86
15.30	7.56	0.6053	25.21	3.59	0.11	13.66

$g \cdot L^{-1}$, are reported in Table 6. The results (projected onto the PEG−phosphate−water triangular diagram) are compared with the corresponding acid-free mixtures in Figures 6 and 7 for PEG 10 000 and PEG 20 000, respectively. Evidently, the two-phase region of the quaternary ATPS is smaller than that of the ATPS without lactic acid.

In further test, a number of mixture pairs were prepared. Each pair had identical concentrations of PEG and K_2HPO_4, but one of the pairs contained lactic acid with $c_{LA} = 53.0\ g \cdot L^{-1}$ while the other was acid-free. Two such pairs were considered PEG 10 000 and a further two pairs for PEG 20 000, and the results are plotted in Figures 6 and 7, respectively. In the absence of lactic acid, all these points lie within the two-phase region. However, in the case of PEG 10 000, we see that the addition of lactic acid to a concentration of $53.0\ g \cdot L^{-1}$ was sufficient for the point more rich in water to fall within the homogeneous region. In the case of PEG 20 000, both points remained within the two-phase region, but the point more rich in water was very close to the binodal when lactic acid was present.

Clearly, lactic acid shrinks the two-phase region in these systems. Hence, with increasing c_{LA}, greater concentrations of PEG and/or phosphate will be required to form an ATPS, and the effect is more pronounced in the case of PEG 10 000. These factors would need to be taken into account in the design of a fermentation process for the production of lactic acid in these kinds of ATPS media.

The influence of lactic acid as a one-phase favoring agent was also demonstrated in two additional sets of experiments carried out for ATPS containing an approximate mass fraction of 14 % PEG 20 000 and 9 % phosphates.

In the first series, reported in Table 3, the ATPS studied had different salt ratios and lactic acid concentrations. All ATPSs with $c_{LA} = 5.3\ g \cdot L^{-1}$ exhibited the same qualitative phase behavior as those without lactic acid, but the volume ratio changed dramatically as the salt ratio approached the limit of the two-phase region for the given overall concentrations. For the ATPS with $w_{LA} = 10.6\ g \cdot L^{-1}$, the system with a 8:2 salt ratio was in the homogeneous region. A further increase of the lactic acid concentration to $53\ g \cdot L^{-1}$ resulted in all but one of the systems (salts ratio 0:10) being homogeneous.

In the second series, the ATPS with the given concentrations of PEG 20 000 and phosphate (now K_2HPO_4 only) were studied at different overall lactic acid concentrations, and measurements were made of pH, partition coefficient, and volume ratio. The results are given in Table 7. Here a 10-fold increase in lactic acid concentration led to a marked and commensurate reduction in the pH and a factor of 3 increase in the volume ratio. The latter is not surprising because, as seen in Figure 2, K_2HPO_4, in comparison with KH_2PO_4, extends the two-phase region. The lactic acid partition coefficient, K, defined as the ratio of the lactic acid concentration in the top phase to that in the bottom phase, was close to unity until about a mass fraction of 4 % lactic acid beyond which it decreased slightly.

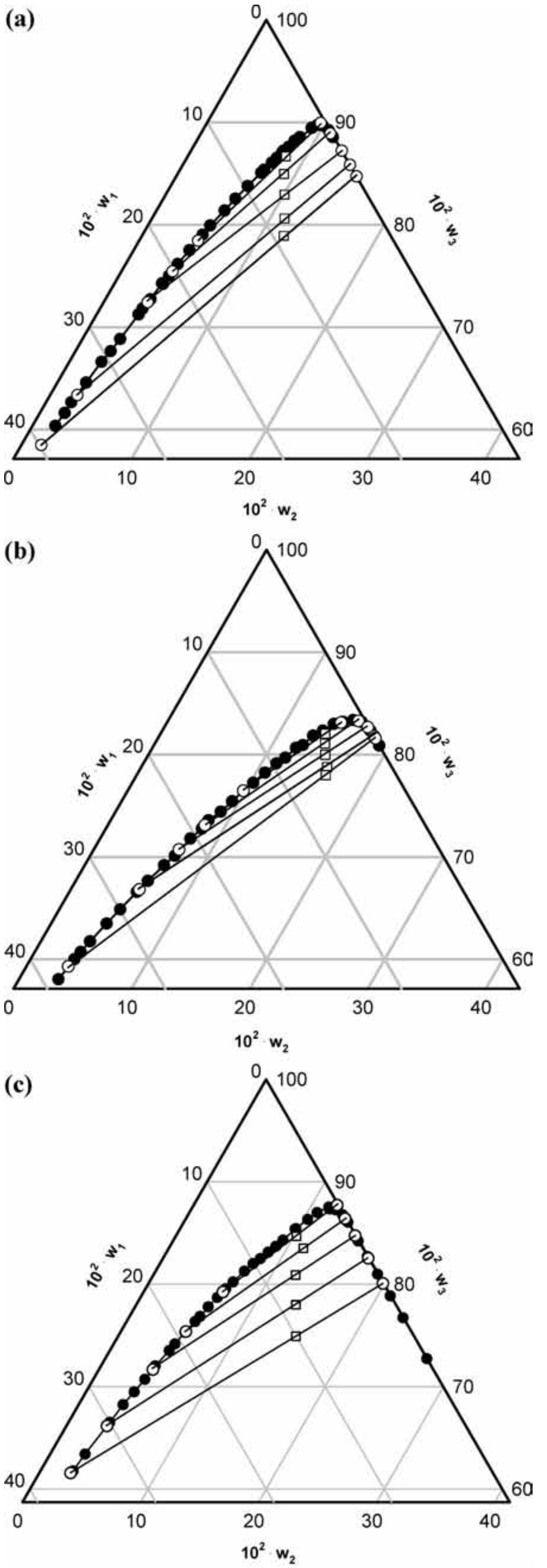

Figure 5. (a) Tie-lines for PEG 20 000 (1) + K_2HPO_4 (2) + water (3). ●, Experimental binodal; —, calculated binodal and tie-lines; □, feed; and ○, calculated equilibrium points. (b) Tie-lines for PEG 20 000 (1) + KH_2PO_4 (2) + water (3). ●, Experimental binodal; —, calculated binodal and tie-lines; □, feed; and ○, calculated equilibrium points. (c) Tie-lines for PEG 20 000 (1) + (K_2HPO_4 + KH_2PO_4) (2) + water (3). ●, Experimental binodal; —, calculated binodal and tie-lines; □, feed; and ○, calculated equilibrium points.

Table 6. Binodal Curves for the Quaternary ATPS PEG (1) + K_2HPO_4 (2) + Water (3) + Lactic Acid (4) with c_{LA}= 53.0 g·L^{-1} at T = 298.15 K

PEG 10 000		PEG 20 000	
$10^2 \cdot w_1$	$10^2 \cdot w_2$	$10^2 \cdot w_1$	$10^2 \cdot w_2$
39.34	4.142	37.87	3.907
36.59	4.589	36.75	4.128
32.36	5.141	35.62	4.350
31.51	5.276	34.48	4.572
29.53	5.602	33.37	4.793
27.09	6.073	32.27	5.015
25.71	6.220	31.19	5.236
23.39	6.573	30.14	5.458
22.66	6.748	29.10	5.680
21.55	7.094	28.07	5.901
20.20	7.417	27.05	6.123
19.01	7.649	26.04	6.344
17.74	7.954	25.03	6.566
16.09	8.305	24.02	6.788
15.20	8.584	23.02	7.009
13.74	8.859	20.03	7.674
12.17	9.336	19.05	7.896
10.87	9.718	18.07	8.117
9.676	9.985	15.17	8.782
8.106	10.34	14.22	9.004
6.858	10.74	12.35	9.447
5.955	11.09	10.52	9.890
4.887	11.47	9.625	10.11
3.522	11.94	6.267	11.00
2.646	12.33	4.779	11.44
1.672	12.59	3.469	11.88
0.862	13.08	2.366	12.33
0.439	13.58	1.481	12.77
0.244	14.66	0.804	13.21
0.183	15.96	0.129	13.88

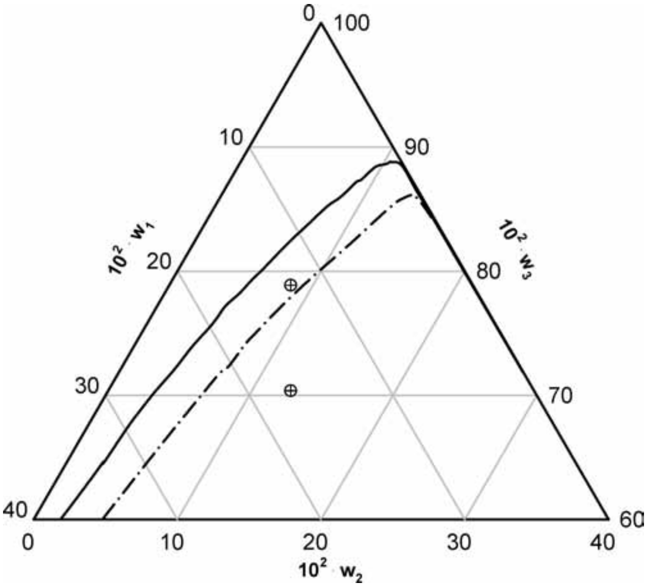

Figure 6. Binodal curve of PEG 10 000 (1) + K_2HPO_4 (2) + water (3) + lactic acid (4) showing the influence of lactic acid. —, c_{LA} = 0; - • - • -, c_{LA} = 53.0 g·L^{-1}; +, test systems with c_{LA} = 0; and ○, test systems with c_{LA} = 53.0 g·L^{-1}.

Conclusions

The present study illuminates the influence of lactic acid on the phase formation of the (PEG + phosphates) ATPS. It is established that the acid acts as a pronounced one-phase favoring agent so that, with increasing lactic acid concentration, the two-phase region of the ATPS shrinks and, for fixed PEG and phosphate concentrations, the phase volume ratio changes. We also find that the lactic acid partition coefficient is close to unity. These factors will be important for the design of fermentation

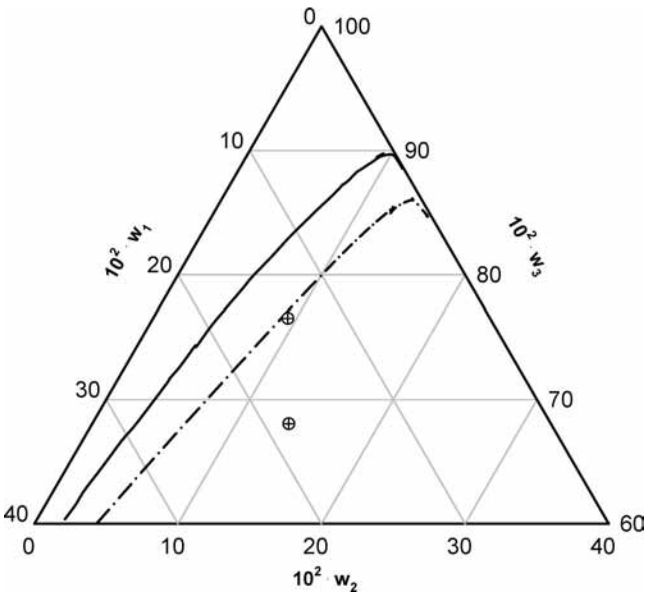

Figure 7. Binodal curve of PEG 20 000 (1) + K_2HPO_4 (2) + water (3) + lactic acid (4) showing the influence of lactic acid. —, $c_{LA} = 0$; - • - • -, $c_{LA} = 53.0\ g\cdot L^{-1}$; +, test systems with $c_{LA} = 0$; ○, test systems with $c_{LA} = 53.0\ g\cdot L^{-1}$.

Table 7. Influence of Lactic Acid Concentration: pH, Partition Coefficient (*K*), and Volume Ratio for ATPS Formed from a Feed Containing 14.08 Mass % PEG 20 000 + 9.39 Mass % K_2HPO_4

$c_{LA}/(g\cdot L^{-1})$	$c_{LA}/(g\cdot L^{-1})$	$c_{LA}/(g\cdot L^{-1})$	pH	pH		volume ratio
feed	top phase	bottom phase	top phase	bottom phase	*K*	(V_{top}/V_{bottom})
5.30	5.28	5.31	8.33	8.04	0.99	1.00
10.6	11.69	10.59	7.84	7.66	1.1	1.00
21.2	24.42	24.88	7.18	7.07	0.98	1.08
31.8	31.38	34.72	6.68	6.56	0.9	1.27
42.4	38.78	51.83	6.1	6.01	0.75	1.63
53.0	51.28	67.49	5.25	5.23	0.76	3.00

processes. Further important questions relating to cell growth and product inhibition can be answered only after carrying out fermentation studies. Thus, although the lactic acid partition coefficient is close to unity, the real fermentation process will establish with certainty whether or not the PEG + phosphate ATPS can be used as a favorable media for lactic acid biotransformation.

Literature Cited

(1) Albertsson, P. A. *Partitioning of Cell Particles and Macromolecules*, 3rd ed.; Wiley-Interscience: New York, 1986.

(2) Walter, H.; Brooks, D. E.; Fisher, D. *Partitioning in Aqueous Two-phase Systems;* Academic Press: New York, 1985.

(3) Zaslavsky, B. Y. Aqueous Two-phase Partitioning. *Physical Chemistry and Bioanalytical Applications*; Marcel Dekker: New York, 1995.

(4) Yankov, D. S.; Stateva, R. P.; Trusler, J. P. M.; Cholakov, G. St. Liquid−Liquid Equilibria in Aqueous Two-Phase Systems of Poly-(ethylene glycol) and Poly(ethyleneimine): Experimental Measurements and Correlation. *J. Chem. Eng. Data* **2006**, *51*, 1056–1061.

(5) Katzbauer, B.; Cesi, V.; Narodoslawsky, M.; Moser, A. Extractive Lactic Acid Fermentation Using Aqueous Two-Phase Systems. *Chem. Biochem. Eng. Q* **1995**, *9*, 79–87.

(6) Planas, J.; Varelas, V.; Tjerneld, F.; Hahn-Hagerdal, B. Amine-Based Aqueous Polymers for the Simultaneous Titration and Extraction of Lactic Acid in Aqueous Two-Phase Systems. *J. Chromatogr. B* **1998**, *711*, 265–275.

(7) Planas, J.; Kozlowski, A.; Harris, J. M.; Tjerneld, F.; Hahn-Hagerdal, B. Novel Polymer-Polymer Conjugates for Recovery of Lactic Acid by Aqueous Two-Phase Extraction. *Biotechnol. Bioeng.* **1999**, *66*, 211–218.

(8) Li, C.; Ouyang, F.; Bai, J. Extractive Cultivation of *Lactococcus lactis* Using a Polyethylene Glycol/MgSO4.7H2O Aqueous Two-Phase System to Produse Nisin. *Biotechnol. Lett.* **2000**, *22*, 843–847.

(9) Vaidya, B. K.; Suthar, H. K.; Kasture, S.; Nene, S. Purification of Potato Polyphenol Oxidase (PPO) by Partitioning in Aqueous Two-Phase System. *Biochem. Eng. J.* **2006**, *28*, 161–166.

(10) Mayerhoff, Z. D. V. L.; Roberto, I. C.; Franco, T. T. Purification of Xylose Reductase from Candida mogii in Aqueous Two-Phase Systems. *Biochem. Eng. J.* **2004**, *18*, 217–223.

(11) Shang, Q. K.; Lia, W.; Jia, Q.; Li, D. Q. Partitioning Behavior of Amino Acids in Aqueous Two-Phase Systems Containing Polyethylene Glycol and Phosphate Buffer. *Fluid Phase Equilib.* **2004**, *219*, 195–203.

(12) Gautam, Sh.; Simon, L. Partitioning of ss-glucosidase from Trichoderma reesei in Poly(ethylene glycol) and Potassium Phosphate Aqueous Two-Phase Systems: Influence of pH and Temperature. *Biochem. Eng. J.* **2006**, *30*, 104–108.

(13) Lima, A. S.; Alegre, R. M.; Meirelles, A. J. A. Partitioning of Pectinolytic Enzymes in Polyethylene Glycol/Potassium Phosphate Aqueous Two-Phase Systems. *Carbohydr. Polym.* **2002**, *50*, 63–68.

(14) Patil, G.; Raghavarao, K. S. M. S. Aqueous Two-phase Extraction for Purification of C-Phycocyanin. *Biochem. Eng. J.* **2007**, *34*, 156–164.

(15) Esmanhoto, E.; Kilikian, B. V. ATPS Applied to Extraction of Small Molecules-Polycetides-and Simultaneous Clarification of Culture Media with Filamentous Microorganisms. *J. Chromatogr. B* **2004**, *807*, 139–144.

(16) de Gouveia, T.; Kilikian, B. V. Bioaffinity Extraction of Glucoamylase in Aqueous Two-Phase Systems Using Starch as Free Bioligand. *J. Chromatogr. B* **2000**, *743*, 241–246.

(17) Li, Sh.; He, Ch.; Gao, F.; Li, D.; Chen, Zh.; Liu, H.; Li, K.; Liu, F. Extraction and Determination of Morphine in Compound Liquorice Using an Aqueous Two-Phase System of Poly(ethylene glycol)/K_2HPO_4 Coupled with HPLC. *Talanta* **2007**, *71*, 784–789.

(18) Zafarani-Moattar, M. T.; Gasemi, J. Liquid-liquid Equilibria of Aqueous Two-Phase Systems Containing Polyethylene Glycol and Ammonium Dihydrogen Phosphate or Diammonium Hydrogen Phosphate. Experiment and Correlation. *Fluid Phase Equilib.* **2002**, *198*, 281–291.

(19) Zafarani-Moattar, M. T.; Sadeghi, R. Liquid-liquid Equilibria of Aqueous Two-Phase Systems Containing Polyethylene Glycol and Sodium Dihydrogen Phosphate or Disodium Hydrogen Phosphate. Experiment and Correlation. *Fluid Phase Equilib.* **2001**, *181*, 95–112.

(20) Mishima, K.; Nakatani, K.; Nomiyama, T.; Matsuyama, K.; Nagatani, M.; Nishikawa, H. Liquid-Liquid Equilibria of Aqueous Two-Phase Systems Containing Polyethylene Glycol and Dipotassium Hydrogenphosphate. *Fluid Phase Equilib.* **1995**, *107*, 269–276.

(21) Haghtalab, A.; Mokhtarani, B. The New Experimental Data and a New Thermodynamic Model Based on Group Contribution for Correlation Liquid-Liquid Equilibria in Aqueous Two-Phase Systems of PEG and (K2HPO4 or Na2SO4). *Fluid Phase Equilib.* **2004**, *215*, 151–161.

(22) Haghtalab, A.; Mokhtarani, B. On Extension of UNIQUAC-NRF Model to Study the Phase Behavior of Aqueous Two-Phase Polymer-Salt Systems. *Fluid Phase Equilib.* **2001**, *180*, 139–149.

(23) Shang, Q. K.; Li, W.; Jia, Q.; Li, D. Q. Partitioning Behavior of Amino Acids in Aqueous Two-Phase Systems Containing Polyethylene Glycol and Phosphate Buffer. *Fluid Phase Equilib.* **2004**, *219*, 195–203.

(24) Se, R. A. G.; Aznar, M. Liquid-Liquid Equilibrium of the Aqueous Two-Phase System Water + PEG 4000 + Potassium Phosphate at Four Temperatures: Experimental Determination and Thermodynamic Modelling. *J. Chem. Eng. Data* **2002**, *47*, 1401–1405.

(25) Mishima, K.; Matsuyama, K.; Ezawa, M.; Taruta, Y.; Takarabe, S.; Nagatani, M. Interfacial Tension of Aqueous Two-Phase Systems Containing Poly(ethylene glycol) and Dipotassium Hydrogenphosphate. *J. Chromatogr. B* **1998**, *711*, 313–318.

(26) Merchuk, J. C.; Andrews, B. A.; Asenjo, J. A. Aqueous Two-Phase Systems for Protein Separation Studies on Phase Inversion. *J. Chromatogr. B* **1998**, *711*, 285–293.

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