A NOVEL METHOD FOR EXTRACTION OF α-AMYLASE FROM AQUEOUS TWO-PHASE SYSTEM OF POLYMER/SALT BY NANOPARTICLES

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Abstract - The purpose of this study is to analyze the α-amylase enzyme partitioning in polymer/salt aqueous two-phase system (ATPS) in the absence and presence of silica nanoparticles (NPs). The binodal curve of magnesium sulfate ($MgSO_4$) salt with polyethylene glycol 4000 (PEG 4000) at 23 °C has been plotted for studying the enzyme partitioning behavior. The partition coefficient of α-amylase and yield recovery has been measured and compared to the case of NPs addition. A dramatic increase in both partition coefficient and yield recovery of top phase is observed by NPs addition confirming their effective role in enzyme partitioning in polymer/salt ATPS.

Keywords - Atps, Partitioning, Silica Nps, α-Amylase Enzyme

I. INTRODUCTION

The organic-aqueous systems formed by an organic solvent and an aqueous solution are not suitable for the separation of biological molecules because of the low solubility of the proteins in these systems[1]. ATPS, as a novel replacement for conventional liquid-liquid extraction methods, is more appropriate for continuous operations and have been used in developing biological processes for recovery and purification of many biological products such as proteins, genetic materials, NPs, cells, and organelles[2].

Four types of ATPS including (polymer + polymer)[3], (polymer + salt)[4], surfactant-based[5], and ionic liquid-based [6]have been addressed in literature among which (polymer + polymer) and (polymer + salt) are the most common ones[7]. In the separation process of these systems, ATPS uses the incompatibility between aqueous solvents of two polymers or a polymer and a high-ionic strength salt. By mixing the two polymers, a large mass is formed resulting in their separation in two different phases[8].

The most common polymers used in these processes are PEG and dextran (DEX). The two-phase separation phenomena could also be observed between a polymer and a high-ionic strength salt (such as PEG and phosphate, sulfate, citrate) [9, 10]. The two-phase separation can be used in proteins detachment from cellular debris or proteins purification from other ones.

In most cases, solvents and suspended particles are transported to the bottom phase with higher polar property, while the protein is transported to the top phase (usually containing PEG), which is less polar and more hydrophobic[5]. The proteins separation is possible by manipulating the partition coefficient, which is obtained by changing the average molecular weight of polymers, the type of ion used in the system, and the ionic strength of the salt phase[11, 12].

Alternatively, a polymer can be modified with hydrophobic groups to alter the protein partitioning which is a soft method of protein purification in which denaturation (loss of biological activity) is usually not observed[13]. This phenomenon results in the protein protection because of the high amount of water and the low value of surface tension. Besides, the polymers themselves may also have stabilizing effects[14].Some of the effective factors influencing the protein partitioning in ATPS are molecular weight, size, and concentration of polymer, ionic strength of salt and pH of the solution[15].

In cases with a higher molecular weight of polymers, a lower concentration is required for the two-phase formation and a greater difference between the molecular weight of the polymers leads to the more asymmetric phase diagram curves [3].

In this paper, the phase diagram of PEG4000/$MgSO_4$ATPS was drawn based on the experimental data. Then, the partition behavior of α-amylase enzyme with the two-phase aqueous solution was analyzed. Finally, the influence silica NPs addition was investigated on the partition coefficient and yield recovery.

II. MATERIAL AND METHODS

A. Material

The properties of the materials used in this study are summarized in Table I. All of the materials were used without further purification (99 %).Deionized water (DIW) was used in the preparation of all solutions.

Material	Chemical Formula	Molecular Weight (g/mol)	Company
PEG 4000	$HO(C_2H_4O)_nH$	1800	Merck
Silicon Dioxide	SiO_2	60.08	synthesized
Magnesium Sulfate	$MgSO_4.7H_2O$	246.48	Merck
α-amylase Enzyme	-	51-54 kDa	Sigma-Aldrich

Table I. Material properties

B. Methods

Phase equilibrium diagram

A phase diagram can be plotted for any polymer or polymer/salt system and can be used in predicting the volume and composition of the two phases. The phase diagram in each system determines the operational ranges of it and is unique at a specified temperature and pressure. A binodal curve in ATPS represents the boundary between the single-phase region (bottom of the curve) and the two-phase region (top of the curve), and the final concentration of the components of top and bottom phases could be determined by the interconnection between the tie lines of two nodes. By moving over the tie lines, the systems with different overall compositions and volume ratio will be specified. In an equivalent condition, the systems on a same tie line have the same final concentration at the top and bottom phases. At the critical point, the composition and volume of the top and bottom phases are the same.A cloud point method at 23 ° C was used to draw the binodal curve.

First, a solution with a specified salt concentration (between 40 and 50 depending on the salt solubility) was prepared and poured into a burette as an additive. Then an aqueous polymer solution with a certain concentration (between 40 and 50% of the polymer) was poured into a basher. At first, both solutions were transparent. The salt solution was then added to the polymer solution in the basher and mixed by a magnetic stirrer. A single-phase solution is transparent and the light is passed through it, while is not passed through a two-phase solution. Therefore, the salt addition was stopped at the moment the solution began to opacity and the volume of the poured salt solution was recorded. This point is considered as the first point of the binodal curve.To determine the next point of the graph, DIW was added droply to the opaque solution until a transparent single-phase solution was obtained. Then the salt solution was added again to the obtained solution until reaching an opaque solution. This process was continued to obtain other points of the graph and the binodal curve versus weight percent of the component could be drawn from the recorded volumes and solution densities. A reverse method could also be used for verification of the first method in a way that the polymer solution was added droply to the salt solution in the basher until reaching an opaque solution [5]. After drawing the experimental data, the binodal curve is usually fitted to some correlations. One of these correlations introduced by Merchuk is given in equation (1)[16]:

$$Y = a\exp(bX^{0.5} - cX^3) \quad (1)$$

Where Y and X are the weight percent of polymer and salt for the binodal experimental data, respectively, and a, b and c are the fitting parameters. To evaluate the fitting of results experimental data with the proposed model, the determination coefficient (R^2) and the root mean square deviation (RMSD) given in equation (2) and (4), respectively, were used.

$$R^2 = 1 - \frac{\sum_1^n (Y_{exp} - Y_{mod})^2}{\sum_1^n (Y_{exp} - \bar{Y})^2} \quad (2)$$

Where

$$\bar{Y} = \frac{\sum_1^n Y_{exp}}{n} \quad (3)$$

$$RMSD = \sqrt{\frac{\sum_1^n (Y_{exp} - Y_{mod})^2}{n}} \quad (4)$$

Where Y_{exp}, Y_{mod}, and n are experimental data, model-matched data, and number of experimental data, respectively.

Several samples with different concentrations of components (selected polymer and salt) were made in the two-phase region. It was tried to prepare samples being able to separately investigate the effect of weight percent of salt and polymer on enzyme partitioning. For example, following steps were passed for preparing a 40 g solution in the two-phase region at 23 ^oC with a weight percent of 6, 24, and 70 for $MgSO_4$, polymer, and DIW, respectively. 2.4 g of salt was poured in 28 g of DIW and mixed with a magnetic stirrer for about 10 minutes. Then 9.6 g polymer was added to the solution and stirred for about 20 minutes to obtain a two-phase opaque mixture. 15 g of the obtained mixture (as a control sample) was poured into a decantor for phase separation. A low amount of α-amylase enzyme (0.01 g) was added to the rest of the obtained mixture (25 g) and stirred for another 20 minutes. Then it was poured in another decantor. Both original and control samples were kept for 24 hours at 23 ^oC to complete the phase separation process and obtain two transparent phases.It should be noted that both samples are located on a same tie line and the mass fraction of the components in the top and bottom phases are equal at the equivalent condition. After dividing the mixture into two separate phases, the two phases were easily separated and the weight of the top and bottom phases were recorded.Theaqueous two-phase system of PEG 4000/$MgSO_4$is shown in Fig. 1. In the case of analyzing the effect of the presence of NPs on partitioning, a small amount of NPs (between 0.05 to 0.1 g) was added to the initial two-phase mixture and 20 minutes was required for

NPs dispersion. Then the control sample was put aside and other steps were performed similarly.



Fig. 1.Aqueous two-phase system of PEG 4000/$MgSO_4$

The partition coefficient(K) can be calculated from the equation (5) where C_T and C_B are the equilibrium concentration of enzyme in thetop and bottom phases, respectively.

$$k = \frac{C_T}{C_B} \qquad (5)$$

Regarding the definition of partition coefficient, the yield recovery or extraction efficiency for the top and bottom phases are obtained from equation (6) and (7), respectively.

$$Y_T(\%) = \frac{100}{1+V_B/(V_T K)} \quad (6)$$

$$Y_B(\%) = \frac{100}{1+(V_T K)/V_B} \quad (7)$$

Since the concentration of polymer, salt and enzyme are unknown in each phase after the phase separation, the Merchuk weight method was used to obtain the polymer and salt concentration. Given that the composition of the top and bottom phase on the binodal diagram, Merchuk proposed by simultaneously solving equations (8) to (11), the unknown parameters could be calculated.The points M, T, and B represent the mixture, top phase, and bottom phase, respectively, and X and y represent the weight fraction of salt ($MgSO_4$) and polymer (PEG), respectively.

$$Y_T = \left(\frac{Y_M}{\alpha}\right) - \left(\frac{1-\alpha}{\alpha}\right) Y_B \qquad (8)$$

$$X_T = \left(\frac{X_M}{\alpha}\right) - \left(\frac{1-\alpha}{\alpha}\right) X_B \qquad (9)$$

$$Y_T = f(X_T) \qquad (10)$$

$$Y_B = f(X_B) \quad (11)$$

The tie line length (TLL) is obtained from equation (12):

$$TLL = \sqrt{(Y_T - Y_B)^2 + (X_T - X_B)^2} \quad (12)$$

The spectrophotometer (UV-2100, UNICO) was used to measure the enzyme concentration in the top and bottom phases. For this purpose, the maximum absorption wavelength of α-amylase was determined by scanning the diluted alpha-amylase solution using a spectrophotometer (M501, CamSpec), and the wavelength of 280 nm was specified as the wavelength at which the maximum absorption occurred.

α-amylase enzyme partitioning in PEG4000/$MgSO_4$ ATPS

The α-amylase enzyme partitioning in PEG 4000/$MgSO_4$ ATPS was analyzed by preparing 3 samples with the compositions given in Table II. w_1 and w_2 represents the $MgSO_4$ and PEG 4000 concentrations, respectively. The component concentration in top and bottom phases are calculated from the Merchuk model (equation (1)) and the pH of each sample were measured by pH meter (Metroh 50B). Finally, the effect of NPs addition on partition coefficientwas investigated.

Sample num	Feed		Top phase		Bottom phase		α	TLL
	$w_{1\%}$	$w_2\%$	$w_1\%$	$w_2\%$	$w_1\%$	$w_2\%$		
1	12.1	37.4	0.5	51.2	43.4	0.0	0.7	66.8
2	20	25	0.6	50.0	39.4	0.0	0.5	63.3
3	12.1	25	1.9	38.3	31.1	0.3	0.7	47.9

Table II. Data obtained from Merchuk model for PEG 4000/$MgSO_4$ATPS

III. RESULTS AND DISCUSSION

Phase equilibrium diagram

The experimental data of the phase equilibrium diagram (the boundary between single-phase and two-phase regions) obtained by the cloud point method at 23 °C is shown in Table III.

The data given in Table III were fitted to the three-parameter correlation of Merchuk's model (equation (1)) to determine the binodal curve for the ATPS of polymer/salt. The binodal curve is shown in Fig. 2.

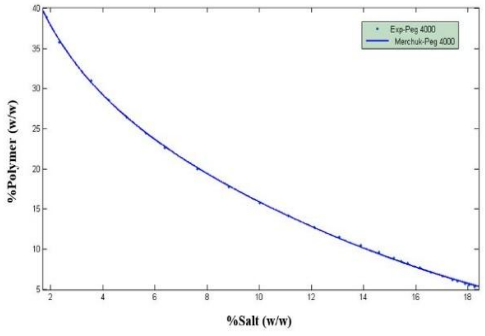


Fig. 2. The Binodal diagram with experimental data for the ATPS of PEG 4000/$MgSO_4$

Salt weight percent (w_1%)	Polymer weight percent (w_2%)
5.30	18.24
5.52	18.03
5.69	17.89
6.01	17.58
6.22	17.41
6.66	17.02
7.19	16.56
7.68	16.15
8.24	15.68
8.50	15.44
8.88	15.16
9.60	14.59
10.48	13.88
11.50	13.07
12.74	12.10
14.14	11.11
15.80	10.02
17.74	8.83
20.00	7.62
22.65	6.37
24.42	5.66
26.41	4.92
28.58	4.21
30.96	3.55
35.78	2.32
38.89	1.85

Table III. Experimental data of phase equilibrium at 23 °C, $MgSO_4.7H_2O$ and PEG 4000

The fitting results to the Meruchuk correlation are given in Table IV.The high values of R^2 along with the relatively low value of RMSD show the good fitting of experimental to Merchuk correlation, confirming the effectiveness of this correlation inPEG4000/$MgSO_4$ ATPS.

ATPS	a	B	C	R^2	RMSD
PEG 4000/$MgSO_4$	69.68	-0.4314	1.14E-04	0.9998	0.1454

Table IV. Fitting parameters, R^2values, and RMSD values

α-amylase partitioning in the absence of silica NPs

The α-amylase enzyme partitioningin the given samples in Table II (in the absence of silica NPs) has been examined and the physical characteristics of the phases and the yield values of top phase are given in Table V. As it is obvious, the greater value of partition coefficient than unit (an average value of 1.4) indicates the more tendency of enzyme partitioning in the top phase leading to the higher yield of top phase (an average value of 73%). Besides, increasing the polymer/salt concentration at a constant value of salt/polymer results an increment in the partition coefficient.

Sample Num.	Phase Density		pH		K	Y_T(%)
	Top	Bottom	Top	Bottom		
1	1.06	1.18	5.1	5.0	1.5	83.3
2	1.04	1.20	5.0	5.0	1.4	64.1
3	1.05	1.27	5.4	5.2	1.3	71.9

Table V. Physical characteristics and partitioning results of α-amylase in PEG 4000/$MgSO_4$ ATPS at 23 ° C in the absence of silica NPs

α-amylase partitioning in the presence of silica NPs

The effect of silica NPs addition on enzyme partitioning inthe samples provided in Table II are given in Table VI.Addition of silica NPs leads to a significant augmentation in the partition coefficient (average value of 3.1) and the phase extraction efficiency (average value of 86%), which is a very desirable phenomenon. This is due to the role of NPs as a linkage between PEG 4000 and α-amylase enzyme leading to the formation of spherical clusters including NPs, polymer, and enzyme. Because of the presence of polymers in the clusters, they move up toward the top phase and consequently, most of the enzyme will be partitioned in the top phase[17].

Sample Num.	Phase Density		pH		K	Y_T(%)
	Top	Bottom	Top	Bottom		
1	1.08	1.28	5.8	6.2	2.8	91.7
2	1.05	1.27	6.8	6.2	3.1	80.5
3	1.05	1.20	6.4	6.5	3.3	87.8

Table VI. Physical characteristics and partitioning results of α-amylase in PEG 4000/$MgSO_4$ ATPS at 23 ° C in the presence of silica NPs

IV. CONCLUSION

In this paper, the partitioning behavior and yield recovery of α-amylase enzyme in PEG4000/$MgSO_4$ ATPS were examined in the presence and absence of silica NPs at 23 °C. The average value of partition coefficient was 1.4 and 3.1 in the absence and presence of silica NPs, respectively.

The greater than unit value indicates the more tendency of enzyme to partition in the top phase and the significant difference between two average coefficients indicates the effective role of silica NPs for enzyme partitioning in polymer/salt ATPS. Besides, a significant increase (about 15%) was also observed in yield recovery by NPs addition. Hence, silica NPs are a good candidate in polymer/salt ATPS.

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