Partitioning of Phenylalanine Ammonia-lyase from *Rhodotorula glutinis* in Aqueous Two-phase Systems of PEG/salts

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In this work, the partitioning of phenylalanine ammonia-lyase was investigated in different systems of PEG1000, PEG2000, PEG3350, PEG6000 and PEG8000 with $(NH_4)_2SO_4$, Na_2SO_4, Na_2CO_3 and potassium phosphate. The results showed that the partition of phenylalanine ammonia-lyase in PEG/salt aqueous two-phase systems was mainly influenced by hydrophobicity, excluded volume effect and salting-out effect; compared with other PEG/salt systems, PEG1000/Na_2SO_4 was the most effective for phenylalanine ammonia-lyase partitioning. The result further supports that PEG/salt aqueous two-phase system provides a new and applicable route for the purification of phenylalanine ammonia-lyase, as shown in our published paper.[13]

Key words:
Aqueous two-phase system, phenylalanine ammonia-lyase, partitioning, PEG, salt

Introduction

The polymer/polymer or polymer/salt aqueous two-phase systems (ATPS) have been widely used in the separation of enzymes or proteins.[1] Due to the mild environment for enzymes and biologically active proteins formed by ATPS,[2] extraction in these systems has the advantages of high capacity, high activity yields, and it is easy to scale up. More particularly, with low viscosity, low cost, and enhanced enzymes stability,[3] PEG (polyethylene glycol)/salt systems are more popular than other ATPS. However, PEG/salt systems such as PEG/potassium phosphate and PEG/magnesium sulphate are more popular than other systems.[4,5]

As the first enzyme in the phenylpropanoid biosynthetic pathway,[6] phenylalanine ammonia-lyase (PAL, EC4.1.3.5) can catalyse non-oxidative deamination of phenylalanine to cinnamic acid, which leads to the biosynthesis of a large range of phenylpropanoid-derived secondary products in plants.[7] PAL exists in plants,[8] fungi[9] and actinomycetes,[10] but lacks in true bacteria and animal tissues. PAL is one of the few non-hydrolytic enzymes that have commercial applications. It has industrial application in the production of L-phenylalanine (L-Phe) from trans-cinnamic acid (t-Ca), i.e. the reverse of the normal *in vivo* reaction.[11] In addition, PAL is effective in the treatment of certain mouse neoplastic tumors, quantitative analysis of serum L-Phe in patients with monitoring phenylketonuria, and also in the preparation of low Phe diets.[12]

In our published work, the PEG1000/Na_2SO_4 aqueous two-phase system was found to be effective for the purification of phenylalanine ammonia-lyase.[13] In order to study the effects of the influencing factors on the partitioning of phenylalanine ammonia-lyase in the PEG/salt aqueous two-phase system and find an applicable route for PAL purification, different systems of different molecular mass of PEG with several salts were investigated in the present work.

Materials and methods

Chemicals

Polyethylene glycol (PEG) 400, 1000, 2000, 3350, 6000, 8000, $(NH_4)_2SO_4$, Na_2SO_4, Na_2CO_3, K_2HPO_4, KH_2PO_4 and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). L-Phe of biochemical-grade and all other chemicals of analytical-grade were purchased from Beijing Chemical Reagent Company. Yeast extract, beef peptone and glucose were purchased from Beijing Shuangxuan Microbial Production Company (Beijing, China).

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Microorganism

The yeast *Rhodotorula glutinis* AS2.102, obtained from China General Microbiological Culture Collection Center (CGMCC), was used as the microbial source of PAL in this work.

Culture media

Stock medium contained (g L^{-1}), malt extract 10 ºBe, agar 20.

Seed medium contained (g L^{-1}), yeast extract 10, beef peptone 10, glucose 5, NaCl 5, K_2HPO_4 1.

PAL induction medium contained (g L^{-1}), yeast extract 10, beef peptone 10, glucose 5, NaCl 5, K_2HPO_4 1, L-Phe 0.5.

All of the media were sterilized for 20 min at 121 °C.

Growth conditions

The cells on the slant stock medium were cultivated for 24 h at 30 °C, and were inoculated into 50 mL seed medium in a 250 mL Erlenmeyer flask and cultivated on a 150 rpm reciprocal shaker for 24 h at 30 °C. Then, 5 mL seed culture was inoculated into a 250 mL Erlenmeyer flask with 50 mL PAL induction medium and cultivated on a 150 rpm reciprocal shaker for 21 h at 30 °C.

Preparation of the crude extract

The culture medium harvested was centrifuged at 2500 g (Centrifugal TDL-5, obtained from An'ting Scientific Instruments Company, Shanghai, China) for 10 min and the cell suspension was collected. After subsequent washing with 0.9 % sterilized saline and distilled water, the cell suspension (about 20–25 mg cell fresh mass mL^{-1} suspension) was prepared with 25 mmol L^{-1} Tris-HCl buffer (pH 8.8). The cell mixture was sonicated in a Sonicator (JY92-2D, purchased from Ningbo Scientz Biotechnology Co. LTD) for 45 min with ice-cold water. Then the mixture was centrifuged to remove cell debris, and the supernatant obtained (0.013–0.016 U mg^{-1} protein) was referred to as the crude extract.

Assay of PAL activity

The PAL activity unit (U) here is defined as the amount of enzyme required to biotransform 1 μmol L-Phe to t-Ca per minute at 30 °C. PAL specific activity is therefore expressed as the number of U in per milligram of dry cell (U mg^{-1}). The activity is assayed by Hodgins' method.[19] Substrate solution containing 2.5 mL of 50 mmol L^{-1} L-Phe, 0.5 mL of 0.05 % cetylpyridinium chloride and 1.75 mL of 25 mmol L^{-1} Tris-HCl buffer (pH 8.8) was mixed with 0.25 mL of cell suspension at 30 °C. After 10 min, the reaction mixture was centrifuged and the t-Ca concentration in the supernatant was determined at 278 nm with a UNICO (Shanghai, China) UV-2000 spectrophotometer.

Preparation of aqueous two-phase systems

The partitioning of PAL from *Rhodotorula glutinis* was carried out in several ATPS consisting of PEG with different molecular mass and various salts with different concentrations, such as $(NH_4)_2SO_4$, Na_2SO_4, Na_2CO_3 and potassium phosphate. The four salts are the general salts utilized in the ATPS. The research on the phase diagrams of some of these PEG/salt ATPS has been reported by Snyder *et al.*[20] Because PAL is stable at pH 8.0–9.5 and pH 8.8 is generally selected as the appropriate pH for PAL,[11–21] thus all the PAL partitioning was carried out at pH 8.8 in this work and the pH was adjusted by Tris-HCl buffer (pH 8.8). The phosphate stock solution consisted of appropriate amounts of KH_2PO_4 and K_2HPO_4 in order to obtain the desired pH 8.8.

ATPS were prepared in 15 mL centrifuge tubes by adding the appropriate amount of PEG, salts and 2 mL PAL crude extract (25–30 mg protein mL^{-1} crude extract, 0.013–0.016 U mg^{-1} protein). The compositions of the selected phase systems were those at which the volume ratio (Ψ_R) was 1. Ψ_R was defined as the ratio of the volume of top phase to that of the bottom phase. Distilled water and Tris-HCl buffer (pH 8.8) were added to obtain the final mass of 10 g of the system. The compositions were mixed using a Vortex mixer (Vortexgenie2, G-560E, USA) and centrifuged for 5 min at 2000 g. The top phase was carefully separated using a pasteur pipette and the interface layer of each tube was discarded. Volumes of the separated phases were measured. Aliquots from each phase were taken for enzyme assay and protein determination. Partition coefficient (K_E or K_P) was defined as the ratio of enzyme activity or protein concentration in the top phase to that in the bottom phase. Purification factor (P_F) was defined as the ratio of PAL specific activity of each phase to the initial PAL specific activity of crude extract. Activity yield (Y) was defined as the total PAL activity in each phase to the total PAL activity in the crude extract. All experiments were run in triplicate at 30 °C and errors were estimated to be a maximum of ±10 % of the mean value.

Protein determination

The protein concentration was measured by the method of Bradford[22] using bovine serum albumin as a standard.

Results and discussion

PAL partitioning in PEG/$(NH_4)_2SO_4$ ATPS

Table 1 shows the effect of PEG molecular mass and mass fraction on the partitioning of PAL from *Rhodotorula glutinis* in PEG/$(NH_4)_2SO_4$ ATPS at 30 °C and pH 8.8. At PEG molecular mass 1000 and 2000, almost any K_E and K_P in the systems were larger than 1, which indicates that both proteins and PAL in these systems were preferably distributed to the top phase. With the increase of PEG molecular mass, most of the partitioning coefficients reduced to below 1, and PAL shifted to the bottom phase. However, at PEG molecular mass 8000, PAL distributed to the top phase again. That indicates that PEG molecular mass can influence the partition of PAL, but it is not the only reason.

With large subunits, PAL has a fairly hydrophobic character, as revealed by hydrophobic interaction chromatography,[13] which favored PAL partitioning to the top PEG-rich phase. In addition, the negatively charged proteins prefer the top phase in PEG/salt systems.[23] The pI of PAL from *Rhodotorula glutinis* is between 5.1–5.4,[14,15] therefore, PAL exists as an anion at pH 8.8, which resulted in PAL partitioning to the top phase. Due to an excluded volume effect, the partition coefficients generally decreased with increasing PEG molecular mass.[24] This was possibly the reason why PAL partitioning shifted to the bottom phase with the increase of PEG molecular mass. Furthermore, in PEG/salt system, driven by the salting-out forces, most proteins strongly favored the bottom salt-rich phase.[25] However, the salting-out effect was not significant in this salt system, because the PAL did not distribute to the bottom phase with the increase of $(NH_4)_2SO_4$ fraction. On the other hand, the hydrophobic character of PEG increased with its chain length,[26] therefore, the affinity of PAL with high surface hydrophobicity to the top, PEG-rich phase should increase with PEG molecular mass. Probably the effect of hydrophobicity was stronger than that of the excluded volume effect and salting-out effect at PEG8000 on PAL partitioning; the partitioning showed a great increase in the top phase from PEG6000 to PEG8000. Therefore, the partition of PAL at different phase composition and PEG molecular mass was influenced by many factors.

Considering K_E, K_P, P_F and Y, 15.0 % PEG2000/16.0 % $(NH_4)_2SO_4$ was the best system in PEG/$(NH_4)_2SO_4$ ATPS. In this system, $K_E > 1$, whereas $K_P < 1$, which means PAL partitioned in the top phase, while contaminant protein and other undesirable components predominantly distributed to the bottom phase. Therefore, it was a good separation of PAL from other proteins. Y (yield) of 49.6 %

Table 1 – *Effect of PEG molecular mass and mass fraction in PEG/$(NH_4)_2SO_4$ ATPS on partitioning of PAL from Rhodotorula glutinis at 30 °C and pH 8.8*

Phase composition (*w*/%)	K_E	K_P	P_F (top/bottom)	*Y*/% (top/bottom)
18.5%PEG1000–16.0%$(NH_4)_2SO_4$	6.52	1.03	1.94/0.31	31.3/4.8
21.0%PEG1000–20.0%$(NH_4)_2SO_4$	3.91	15.22	0.79/3.06	23.6/6.0
24.0%PEG1000–24.0%$(NH_4)_2SO_4$	3.41	19.76	0.52/2.99	16.0/4.7
14.0%PEG2000–14.0%$(NH_4)_2SO_4$	1.04	0.38	0.89/0.33	27.0/25.9
15.0%PEG2000–16.0%$(NH_4)_2SO_4$	**1.12**	**0.32**	**1.94**/0.56	**49.6**/44.3
18.5%PEG2000–20.0%$(NH_4)_2SO_4$	1.25	1.14	0.97/0.89	48.5/38.8
12.0%PEG3350–14.0%$(NH_4)_2SO_4$	0.25	0.27	0.33/0.36	5.7/23.2
13.0%PEG3350–16.0%$(NH_4)_2SO_4$	0.41	0.22	1.12/0.61	5.9/14.2
15.0%PEG3350–24.0%$(NH_4)_2SO_4$	0.27	0.37	0.54/0.74	6.4/23.8
12.0%PEG6000–14.0%$(NH_4)_2SO_4$	0.16	0.14	0.33/0.29	5.3/32.1
13.0%PEG6000–16.0%$(NH_4)_2SO_4$	0.43	0.11	2.11/0.54	5.4/12.6
15.0%PEG6000–24.0%$(NH_4)_2SO_4$	0.37	0.30	1.11/0.90	5.0/13.6
10.0%PEG8000–14.0%$(NH_4)_2SO_4$	0.75	0.60	1.71/1.36	36.3/48.3
12.0%PEG8000–16.0%$(NH_4)_2SO_4$	1.12	0.68	2.03/1.23	40.8/36.4
16.0%PEG8000–24.0%$(NH_4)_2SO_4$	3.39	12.90	0.60/2.28	50.3/14.8

PAL specific activity of crude extract is $14.58 \cdot 10^{-3}$ U mg^{-1} protein, total protein is 28.26 mg.

and P_F of 1.94-fold were achieved in the top phase. Generally, yield is the most important factor for evaluating an ATPS. Compared with the results reported by Ogata *et al.* (69.9 %),[16] Hodgins (52.8 %)[17] and Godwin *et al*. (80.0 %)[18] obtained in the traditional salting-out step of PAL purification from *Rhodotorula glutinis*, the yield of 49.6 % was rather low. Therefore, 15.0 % PEG2000/16.0 % $(NH_4)_2SO_4$ was not a potential system for partitioning of PAL.

PAL partitioning in PEG/Na_2SO_4 ATPS

The P_F and Y got the highest values, 3.69-fold and 86.7 %, respectively, in the top phase in 11.0 % PEG1000/14.0 % Na_2SO_4 system shown in Table 2. The yield of PAL showed that the partitioning of PAL shifted to the bottom phase at PEG2000, reached the highest at PEG3350, and then decreased with the increase of molecular mass of PEG, but PAL distributed to the bottom phase at PEG8000. It appeared that the larger the molecular mass of PEG, the lower the recovery yield of PAL in the top phase. In this PEG/Na_2SO_4 ATPS, PAL partitioning was influenced by hydrophobicity, excluded volume effect and salting-out effect as in PEG/$(NH_4)_2SO_4$ system. The large submit and pI of PAL and the structure of PEG also influenced the PAL partitioning. Compared with $(NH_4)_2SO_4$ system, the yields of PAL activity in the top phase were fairly high in Na_2SO_4 system at PEG1000 and 2000. The precipitation of PAL at the interface of the phases surely led to the result. Because the concentration of PEG in $(NH_4)_2SO_4$ system was higher than that in the Na_2SO_4 system, but the solubility of PAL was limited in PEG, which resulted in some precipitation of PAL at the interface.

In 7.6 % PEG3350/9.7 % Na_2SO_4 system, the highest yield of 88.6 % was achieved in the bottom phase, higher than the yield of 86.7 % in the top phase of 11.0 % PEG1000/14.0 % Na_2SO_4 system. However, both K_E and K_P were smaller than 1 in this Na_2SO_4 system, which means the PAL and contaminant proteins were predominantly distributed to the bottom phase and could not be selectively partitioned, thus the P_F was smaller than 1. Considering K_E, K_P, P_F and yield, 11.0 % PEG1000/14.0 % Na_2SO_4 system was the best one in PEG/Na_2SO_4 system, with K_E of 12.95, K_P of 0.62, P_F of 3.69-fold and Y of 86.7 %. The K_E and K_P indicated that PAL was significantly separated from other proteins and distributed in the top phase. Activity yield Y = 86.7 % was higher than the reported values.[16–18] P_F of 3.69-fold was similar to the highest one reported by Godwin *et al*. (3.70-fold).[18] Therefore, this system was superior to the reported traditional salting-out step for primary purification of PAL.

The best ATPS in PEG/Na_2SO_4 system was obtained at PEG1000, which maybe suggest that low PEG molecular mass would be more favorable for PAL partitioning. However, the data of

Table 2 – *Effect of PEG molecular mass and mass fraction in PEG/Na_2SO_4 ATPS on partitioning of PAL from Rhodotorula glutinis at 30 °C and pH 8.8*

Phase composition (*w*/%)	K_E	K_P	P_F (top/bottm)	*Y*/% (top/bottom)
11.0%PEG1000–14.0%Na_2SO_4	**12.95**	**0.62**	**3.69/0.18**	**86.7/6.68**
12.5%PEG1000–14.5%Na_2SO_4	10.12	0.73	2.46/0.18	82.4/8.1
14.0%PEG1000–15.0%Na_2SO_4	9.26	1.43	1.36/0.21	65.8/7.1
11.0%PEG2000–14.0%Na_2SO_4	0.88	0.14	2.96/0.47	42.5/48.3
12.5%PEG2000–14.5%Na_2SO_4	0.82	0.21	1.94/0.49	34.6/42.3
14.0%PEG2000–15.0%Na_2SO_4	1.76	0.35	2.42/0.48	50.1/28.5
9.9%PEG3350–10.9%Na_2SO_4	0.09	0.14	0.39/0.61	7.2/80.4
14.0%PEG3350–10.8%Na_2SO_4	0.23	0.18	0.28/0.22	14.3/62.3
17.9%PEG3350–12.9%Na_2SO_4	0.32	0.35	0.29/0.32	16.3/50.8
12.0%PEG6000–9.0%Na_2SO_4	0.09	0.14	0.39/0.60	6.3/70.2
13.0%PEG6000–10.0%Na_2SO_4	0.14	0.18	0.37/0.48	7.7/55.0
15.0%PEG6000–14.0%Na_2SO_4	0.59	0.24	0.56/0.23	20.6/34.9
13.0%PEG8000–8.0%Na_2SO_4	1.29	0.59	2.21/0.98	50.2/38.9
10.0%PEG8000–13.0%Na_2SO_4	0.73	1.03	0.94/1.33	34.2/46.8
14.0%PEG8000–15.0%Na_2SO_4	0.82	0.20	1.88/0.45	32.1/39.2

PAL specific activity of crude extract is $14.81 \cdot 10^{-3}$ U mg^{-1} protein, total protein is 28.88 mg.

PEG400/Na_2SO_4 system did not support this suggestion (The data are not shown in this paper.). PAL and proteins predominantly concentrated on the top phase at all the compositions of these systems, therefore, it was not effective in partitioning of PAL. As Cascone *et al.*[27] reported, the reason might be that the lower PEG molecular mass would virtually attract all proteins to the PEG phase, which would lead to very poor separation and purification from contaminating proteins.

PEG/Na_2SO_4 ATPS was also used to successfully partition recombinant cutinase from *Fusarium solani pisi* and lysozyme from chicken egg with yield of 91 % and 70 %, respectively, reported by Sebastiao *et al*.[28] and Su *et al.*[29] Compared with these two results, 11.0 % PEG1000/14.0 % Na_2SO_4 ATPS with the Y = 86.7 %, proved to be a potential system for further study on PAL purification.

PAL partitioning in PEG/Na_2CO_3 ATPS

PAL partitioning changed with the increase of PEG molecular mass in Na_2CO_3 system. At PEG1000, the fairly high values of K_E and K_P showed that almost all of the PAL and proteins transferred to the top phase, leading to bad separation of PAL from contaminant proteins. Therefore, the PEG1000/Na_2CO_3 systems were not effective for PAL partitioning. At the range of PEG molecular mass 2000–8000, the partition was similar to that in $(NH_4)_2SO_4$ system, i.e; there was a PAL partitioning shift at PEG2000 from top phase to the bottom one, but PAL distributed to the top phase at PEG8000. Similar to $(NH_4)_2SO_4$ system, but unlike Na_2SO_4 system, the best composition appeared at PEG2000, 16.0 % PEG2000/13.0 % Na_2CO_3 with K_E of 0.85, K_P of 4.97, P_F of 4.03-fold and Y = 50.8 %. It suggested that salts had significant effect on PAL partitioning in PEG/salts systems.

The salting-out force arranged as $Na^+ > NH_4^+$ and $PO_4^{3-} > CO_3^{2-} > HPO_4^{2-} > SO_4^{2-} > OH^-$,[30,31] Na_2CO_3 had a much stronger salting-out effect on PAL than $(NH_4)_2SO_4$ and Na_2SO_4, which led to the higher partitioning of PAL in the bottom phase than that in the two systems. In addition, CO_3^{2-} was more favorable for the stability of PAL than SO_4^{2-} and NH_4^+.[21] However, the PAL partition was still influenced by excluded volume effect and hydrophobicity. The excluded volume effect promoted the partitioning to the bottom phase, while the hydrophobicity favored the partitioning to the top phase with increasing PEG molecular mass.[24,26] Because the effects were not in the same direction, a best partitioning was obtained in the bottom phase at PEG2000, not at the smallest or the largest PEG molecular mass.

Table 3 also shows that at the same PEG molecular mass, the yield in the top phase increased

Table 3 – *Effect of PEG molecular mass and mass fraction in PEG/Na_2CO_3 ATPS on partitioning of PAL from Rhodotorula glutinis at 30 °C and pH 8.8*

Phase composition (w/%)	K_E	K_P	P_F (top/bottom)	Y/% (top/bottom)
12.0%PEG1000–11.0%Na_2CO_3	8.62	73.28	0.09/0.72	83.2/9.65
16.0%PEG1000–13.0%Na_2CO_3	6.09	52.65	0.09/0.79	85.6/14.0
18.0%PEG1000–14.0%Na_2CO_3	3.98	27.43	0.10/0.74	75.6/16.9
12.0%PEG2000–11.0%Na_2CO_3	0.57	1.27	0.58/1.31	27.1/47.6
16.0%PEG2000–13.0%Na_2CO_3	**0.85**	**4.97**	0.69/**4.03**	43.0/**50.8**
18.0%PEG2000–14.0%Na_2CO_3	1.51	6.39	0.78/2.50	53.4/35.4
14.0%PEG3350–10.9%Na_2CO_3	0.89	0.83	0.44/0.40	19.1/21.4
16.1%PEG3350–11.9%Na_2CO_3	1.18	1.41	0.39/0.46	19.0/16.1
20.0%PEG3350–14.0%Na_2CO_3	1.13	2.54	0.25/0.56	16.5/14.5
15.0%PEG6000–10.0%Na_2CO_3	0.49	0.52	0.21/0.23	5.2/10.5
17.0%PEG6000–12.0%Na_2CO_3	0.55	1.04	0.17/0.42	6.4/11.7
19.0%PEG6000–14.0%Na_2CO_3	1.50	1.29	0.25/0.21	14.7/9.8
11.7%PEG8000–5.6%Na_2CO_3	1.21	0.40	2.23/0.74	42.8/35.4
19.0%PEG8000–6.0%Na_2CO_3	1.47	1.22	1.10/0.91	51.1/34.7
8.8%PEG8000–12.1%Na_2CO_3	0.57	0.81	0.82/1.17	33.1/57.8

PAL specific activity of crude extract is $13.65 \cdot 10^{-3}$ U mg^{-1} protein, total protein is 28.24 mg.

with the concentration of PEG, owing to the chemical modification by PEG on the stability of PAL. The yield in the bottom phase did not decrease with the concentration of Na_2CO_3, therefore, the salting-out effect was not the predominant force for the partitioning in the bottom phase.

Though P_F of 4.03-fold in the 16.0 % PEG2000/13.0 % Na_2CO_3 system was higher than that in other salt systems, yield of 50.8 % was rather low compared to reported values[16–18] therefore, this selected system was unsuitable for PAL purification.

PAL partitioning in PEG/potassium phosphate ATPS

K_E and K_P at all the compositions of potassium phosphate system shown in Table 4 were fairly higher than 1, which indicated that there was no good separation of PAL from other proteins in the systems. Some higher P_F were obtained in the bottom phase in some systems, but the yields were smaller than that in the top one. However, the effect of the partitioning depended on yield, therefore, there was not an effective one in the potassium phosphate systems for PAL partitioning.

Phosphate anion has low affinity for the PEG-rich phase, which induced a positive electrical potential on this polymer side of the phase to keep interface electroneutral. Since PAL had a negative charge at pH 8.8, it induced its transfer to the top phase.[32] Although the salting-out force of PO_4^{3-} arranges before CO_3^{2-} and SO_4^{2-}, and that of HPO_4^{2-} arranges before SO_4^{2-}, the salting-out effect was not the significant factor to shift the PAL partitioning to the bottom phase. The competition among the forces of interface electrical potential, hydrophobicity, excluded volume effect and salting-out effect in two different directions caused most of PAL to distribute to the top phase. Therefore, the PEG/potassium phosphate system was not effective for PAL partitioning.

However, many enzymes or proteins have been partitioned and purified successfully by using this system. Cisneros *et al.*[33] reported that 81 % of lutein from the green microalga *Chlorella protothecoides* could be recovered by using 22.9 % PEG 8000/10.3 % phosphate system. Chouyyok *et al.*[34] also reported that PEG1000/potassium phosphate system could extract alkaline protease from cell free fermentation broth of *B. subtilis* TISTR 25 with yield of 62.2 % and P_F of 6.1. We have not found an explanation for the difference in the applications of PEG/potassium phosphate ATPS for the purification of these two substances and the PAL of the present study.

Table 4 – *Effect of PEG molecular mass and mass fraction in PEG/potassium phosphate ATPS on partitioning of PAL from Rhodotorula glutinis at 30 °C and pH 8.8*

Phase composition (*w*/%)	K_E	K_P	P_F (top/bottom)	*Y*/% (top/bottom)
17.9%PEG1000–10.5% phosphate	5.98	52.4	0.45/3.94	42.6/7.12
20.0%PEG1000–11.4% phosphate	2.52	39.8	0.31/2.45	30.6/13.08
21.9%PEG1000–12.3% phosphate	1.04	28.00	0.17/4.71	16.7/16.0
17.9%PEG2000–10.8% phosphate	4.70	64.4	0.32/4.40	59.7/12.7
20.0%PEG2000–11.6% phosphate	2.70	33.5	0.35/4.30	52.4/19.6
21.9%PEG2000–12.3% phosphate	3.30	29.0	0.39/3.42	57.6/17.6
17.9%PEG3350–10.8% phosphate	2.20	45.4	0.3/6.18	52.8/24.0
20.0%PEG3350–11.6% phosphate	0.98	29.4	0.3/9.07	34.7/35.6
21.9%PEG3350–12.3% phosphate	2.16	46.6	0.31/6.65	48.6/22.4
17.9%PEG6000–10.8% phosphate	1.56	44.3	0.33/9.29	40.8/26.2
20.0%PEG6000–11.6% phosphate	2.19	36.7	0.44/7.50	49.4/22.6
21.9%PEG6000–12.3% phosphate	2.01	21.3	0.44/4.57	43.7/21.8
17.9%PEG8000–10.8% phosphate	1.54	24.04	0.42/6.36	42.4/27.5
20.0%PEG8000–11.6% phosphate	3.25	28.01	0.46/4.00	73.8/22.7
21.9%PEG8000–12.3% phosphate	1.72	48.10	0.30/8.50	45.5/26.5

PAL specific activity of crude extract is $15.32 \cdot 10^{-3}$ U mg^{-1} protein, total protein is 29.60 mg.

Conclusions

The partitioning of PAL in ATPS is generally influenced by hydrophobicity, excluded volume effect and salting-out effect. A significant difference for PAL partitioning was shown in the PEG/$(NH_4)_2SO_4$, PEG/Na_2SO_4, PEG/Na_2CO_3 and PEG/potassium phosphate systems. Comparing the values of K_E, K_P, P_F and Y in the best system of each PEG/salts ATPS, 15.0 % PEG2000/16.0 % $(NH_4)_2SO_4$, 11.0 % PEG1000/14.0 % Na_2SO_4 and 16.0 % PEG2000/13.0 % Na_2CO_3, respectively, the most effective one was obtained. With P_F of 3.69-fold and Y = 86.7 %, the 11.0 % PEG1000/14.0 % Na_2SO_4 (pH 8.8) system was selected as the most effective one for further study on partitioning of PAL. The Y = 86.7 % is higher than the highest reported value of 80 % in the traditional salting-out step of PAL primary purification from *Rhodotorula glutinis*, obtained by Godwin *et al.*,[18] and a more higher yield has been reported with the addition of salts to the ATPS in our laboratory.[13]

Therefore, the work indicated that the hydrophobicity, excluded volume effect and salting-out effect are the influencing factors for the partition of phenylalanine ammonia-lyase in aqueous two-phase system, and the ATPS provides an applicable route for the purification of PAL and could be an alternative step for the traditional salting-out in the procedures of PAL purification.

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List of Symbols

K_E – partition coefficient of enzyme
K_P – partition coefficient of protein
P_F – purification factor
w – mass fraction, %
Y – activity yield, %
Ψ_R – volume ratio

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