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Short communication

Purification and in situ immobilization of lipase from of a mutant of *Trichosporon laibacchii* using aqueous two-phase systems

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ABSTRACT

The crude intracellular lipase (cell homogenate) from *Trichosporon laibacchii* was subjected to partial purification by aqueous two-phase system (ATPS) and then in situ immobilization by directly adding diatomites as carrier to the top PEG-rich phase of ATPS. A partition study of lipase in the ATPS formed by polyethylene glycol–potassium phosphate has been performed. The influence of system parameters such as molecular weight of PEG, system phase composition and system pH on the partitioning behaviour of lipase was evaluated. The ATPS consisting of PEG 4000 (12%) and potassium phosphate (K_2HPO_4, 13%) resulted in partition of lipase to the PEG-rich phase with partition coefficient 7.61, activity recovery 80.4%, and purification factor of 5.84 at pH of 7.0 and 2.0% NaCl. Moreover, the in situ immobilization of lipase in PEG phase resulted in a highest immobilized lipase activity of 1114.6 U g^{-1}. The above results show that this novel lipase immobilization procedure which couples ATPS extract and enzyme immobilization is cost-effective as well as time-saving. It could be potentially useful technique for the purification and immobilization of lipase.

1. Introduction

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are the most important and extremely versatile group of enzymes widely used for biotechnological and industrial applications as additives in food, fine chemicals, detergents, waste water treatment, cosmetics, paper and pulp, pharmaceuticals and leather industry [1,2]. The lipases from *Trichosporon laibacchii* are intracellular lipolytic enzyme that showed (S)-preference [3] in the resolution of 2-arylpropionic acids such as ketoprofen.

It is important to develop industries desire procedures which are less time consuming and give high enzyme yields with considerable purity. Partitioning in aqueous two-phase systems (ATPSs) is a powerful method which has been widely used for separation and purification of proteins in downstream processing due to the smaller time involved and the higher yields and purification levels achieved [4–6]. ATPSs are formed by mixing two flexible chain polymers in water or one polymer and a salt (phosphate, citrate, etc.). The ATPSs that are currently in use are PEG/salt/water or PEG/dextran/water systems [7].

To break the major bottleneck in ATPSs commercialisation, it needs to develop processes with complete recirculation of phase-forming materials to reduce material cost [8]. Guan et al. [9] have successfully immobilized penicillin acylase (PA) directly from the top PEG phase of an ATPS thus resulting in the recycling of the top PEG phase. They used a PEG 2000/phosphate system to achieve an in situ immobilization of PA with 87% recovery. Bradoo also [10] used similar method to recover enzymes from the phosphate phase by immobilization directly on accurel thus resulting in the recycling of the bottom salt phase.

In this work, the objective was to investigate a novel approach for the separation and purification of intracellular lipase, which couples aqueous two-phase partitioning and enzyme immobilization. Therefore, the effect of process parameters, such as polyethylene glycol (PEG) molecular weight, pH and concentration of phase-forming materials on partitioning of lipase, was studied in PEG/phosphate ATPS. Moreover, we also investigated the effect of top PEG phase on the immobilization of lipase.

2. Materials and methods

2.1. Production of lipase

Intracellular lipase was produced from a mutant of *T. laibacchii* CBS5791 by fermentation at 23 °C, pH 6.5, with an agitation of 200 rpm, aeration of 0.5 vvm for 13 h in a 15 l fermentor (DS-Y, Zhenjiang Dasen Fermentation Instruments Co. Ltd., China). The mutant strain was obtained by mutagenesis with two kinds of mutagens, including $C_4H_{10}O_4S$ and ultraviolet. The compositions of seed medium and fermentation medium were the same as that descried in our previous work [11].

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2.2. Preparation of the crude lipase

After the fermentation period, the cells were harvested by centrifuging at 5000 rpm for 10 min and then resuspended in phosphate buffer (pH 6.5) to a final concentration of 150 g l^{-1}. Cell suspensions were disintegrated by high pressure homogenizer (JG-IA type, Scientz Co., Ltd., China) at 70 MPa for 3 cycles at 4 °C. The cell homogenate obtained was collected and used directly for ATPS studies.

2.3. Chemicals

PEG, with respective average molecular weights of 1000, 2000, 4000, 6000 and 20,000 (PEG 1000, PEG 2000, PEG 4000, PEG 6000 and PEG 20,000), were purchased from Shanghai Chemical Reagent Co. Ltd. of China. All chemicals were of analytical grade.

2.4. Partial purification of intracellular lipase by aqueous two-phase systems

PEG/potassium phosphate systems were made up by weighing appropriate amounts of 50% stock solutions of PEG dissolved in deionised water and 40% stock solution of potassium phosphate dissolved in buffer of appropriate pH in 20-ml graduated test tubes. All the concentrations of the phase forming components in this paper are given as percent weight/weight (w/w). Crude lipase (cell homogenate) was added to corresponding system at a constant weight of 1.8 g. Finally deionised water was added to give a total weight of 10.0 g. The systems were mixed for a few seconds on a vortex mixer (866 Type, Beijing Zhong Xi Yuan Da Co. Ltd., China), then centrifuged at room temperature for 5 min at 3000 rpm to expedite the phase separation. The top phase was carefully pipetted out and weighed. Samples removed carefully from the top and bottom phases, were diluted appropriately, and then analyzed for protein estimation and lipase activity. The partition of lipase was described by the partition coefficient (K), the recovery yield of lipase in top phase (Y), volume ratio (R), and purification fold (PF), respectively. The following equations were used for evaluation:

$$R = V_T/V_B; \quad K = U_T/U_B; \quad Y = RK/(1+RK); \quad \mathrm{PF} = (U_T/T_{pro})/U_I$$

where U_T and U_B are the lipase activity (U ml^{-1}) in the top and bottom phases, respectively; V_T and V_B are the volumes of the top and bottom phases, respectively; T_{pro} expresses the total protein mass (mg) in the top phase, U_I (U mg^{-1} pr) is the specific activity of lipase in crude lipase (cell homogenate).

2.5. In situ immobilization of lipase from PEG-rich phase using adsorption-crosslinking method

Diatomites were used as carrier for the lipase immobilization. Appropriate amounts of the carrier (0.8 mg carrier per lipase activity unit) were pretreated with ethanol and distilled water and then were incubated with the phosphate buffer (pH 7.8) for 2 h. To this, 20 ml of top phase containing lipase was added. This new mixture was incubated in a shaker (Beijing Zhong Xi Yuan Da Co. Ltd., China) at 30 °C and 150 rpm for 2 h. After that, glutaraldehyde (GA) was added to the above mixture to give a final concentration 0.2% (v/v), and then the resulted mixture was stirred for 2 h at 25 °C. Finally, the immobilized lipase was obtained after filtration at vacuum, washing with phosphate buffer and drying by vacuum.

2.6. Lipase activity assay and protein estimation

Lipase activity was assayed by using "olive emulsification method" [12]. One activity unit (U) of lipase was defined as the amount of enzyme required to catalyze substrate olive oil to hydrolyze to produce 1 μmol of free fatty acid per min at 40 °C and pH 8.0.

Protein estimation was carried out following a known method [13] using bovine serum albumin as standard protein. For protein estimation in samples containing PEG, blank was prepared using PEG and the absorbance was subsequently nullified.

3. Results and discussion

3.1. Effect of PEG molecular weight on the lipase partition

In order to study the effect of PEG molecular weight on the partitioning of lipase, partition experiments were performed in different ATPSs by varying the molecular weight of PEG (1000, 2000, 4000, 6000 and 20,000) and maintaining the phase compositions (PEG/phosphate) at constant value (15/15, %) and pH at 7.0. The partition coefficients, the activity recovery, and the purification fold are shown as the functions of PEG molecular weight in Fig. 1. Fig. 1 shows that the partitioning of lipase in PEG/phosphate system was strongly dependent on the molecular weight of the PEG. It was found that K decreased with an increase in PEG molecular weight from 4000 to 20,000. The increase of K value was observed for PEG 1000, 2000 and 4000, and the maximal K value was obtained at PEG 4000. A similar phenomenon was also observed by Bassani et al. [14]. This observation may be explained using PEG–protein interaction and PEG exclude volume effect [15]. For systems with PEG 1000–6000, the lipase partition mainly depend on PEG-enzyme interaction, while in PEG 20,000 system, PEG excluded volume effect dominate the partitioning of lipase rather than PEG–protein interaction due to the high molecular weight of this PEG. The excluded volume theory indicates that an increase in PEG molecular weight induces a decrease of the protein solubility in the phase where the protein is situated.

Patil and Raghavarao [16] also reported that an increase in PEG molecular weight results in an increase in the polymer chain length, which led to the decrease in the free volume. As a result, the biomolecules will selectively partition to the bottom phase. Possibly, in PEG of high molecular weight the steric hindrance is major decreasing the partition coefficient. Fernandez Lahore et al. [17] have also reported that the use of PEG 20,000 is unsuitable for purification purpose. For the activity recovery and purification fold, the similar trend was also observed from Fig. 1. Y and PF were found to increase with an increase in molecular weight of PEG from 1000 to 4000. However, beyond PEG molecular weight 4000, there was a decrease in these parameters. Hence, PEG 4000 system was thus selected for further studies. From Fig. 1, it may be noted that for PEG 4000, K, Y and PF were 4.7, 80.5% and 2.45, respectively.

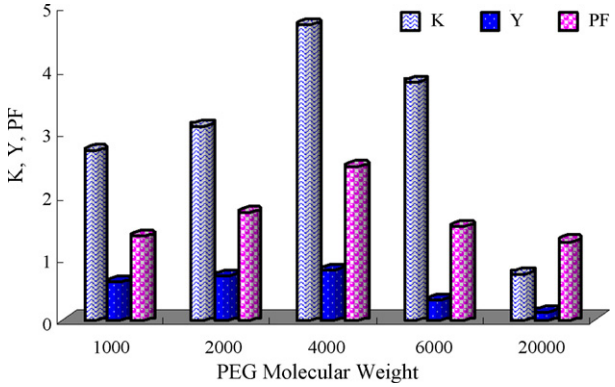

Fig. 1. Partition behaviour of lipase in PEG/phosphate system as a function of PEG molecular weight.

Table 1
Effect of concentration of phase-forming materials on lipase partitioning.

ATPS (%, w/w)		*R*	*K*	*Y* (%)	PF
PEG 4000	KH_2PO_4				
8	11	0.46	2.93	57.4	1.47
10	11	0.49	6.58	76.3	2.39
12	11	0.57	7.97	82.0	3.08
14	11	0.63	6.92	81.3	3.35
16	11	0.67	5.84	79.6	2.94
18	11	0.71	2.52	64.1	1.55
12	9	0.64	2.95	65.4	1.89
12	11	0.58	7.70	81.7	3.14
12	13	0.54	8.51	82.1	3.75
12	15	0.48	7.31	77.8	3.11
12	17	0.41	5.83	70.5	2.65
12	19	0.34	3.71	55.8	1.24

3.2. Effect of different combinations of PEG 4000/phosphate on partitioning of lipase

Lipase partitioning at varying PEG concentration (8–18%) at a constant phosphate concentration (11%) and its partitioning at varying phosphate concentration (9–19%) at a constant PEG concentration (12%) were both studied (Table 1). The results of Table 1 show that both the concentrations of PEG and phosphate had significant influence on the partitioning of lipase in ATPS. Partition coefficient and purification fold increased with an increase of PEG concentration varied from 8 to 14% by maintaining phosphate concentration (11%). At PEG 4000 concentration of 12%, the partition coefficient reached its highest values of 7.97 with purification fold of 3.08 and activity recovery of 82.0%. While for above 12% of PEG concentration, the partition coefficient decreased instead. Based on the partitioning theory, the increase of PEG concentration will result in the increase of viscosity and the interfacial tension between the two phases of the ATPS, and hence the resistance increased for lipase molecules to transfer into the top PEG phase. However, the volume of top phase will increase (*R* value increased) with an increase of PEG concentration, so, the activity recovery changed mildly.

Data in Table 1 also show that for different phosphate concentration (9–19%) systems with the same PEG concentration (12%), the ratio of phase volume (*R*) decreased with an increase of phosphate concentration. It was obvious that the reduction of *R* value will prompt the enrichment of lipase in the top phase. As a result, the partition coefficient reached its maximal value of 8.51 at 13% of phosphate concentration with the highest activity recovery of 82.1%. The PF increased with an increase of phosphate concentration from 9 to 13%, and decreased when phosphate concentration was in the range of 15–19%, the highest PF was obtained in 13% of the salt concentration. This may be attributed to the static actions between inorganic salt and different proteins molecules, and these static actions were different due to the difference in surface hydrophobic character of different proteins. Hence, the target protein molecules are easy to separate from other proteins in ATPS. Thus, the optimal phosphate concentration of 13% was selected for the further study based on the *K* and PF.

It can be observed from Table 1 that among various phase compositions of PEG and phosphate, PEG 4000/K_2HPO_4 system of 12/13 (%) indicated highest partition coefficient (8.51), activity recovery (82.1%) and purification fold (3.75). Hence, this system composition was selected for the further study.

3.3. Effect of pH on partitioning of lipase

In order to investigate the influence of pH on lipase partition in the selected phase system (PEG 4000/K_2HPO_4, 12/13 (%)), experiments were performed in the pH range of 3.0–9.0, and the results are presented in Fig. 2. From Fig. 2, it was found that the increase in pH from 7.0 to 9.0 reduced the partition coefficient from 7.94 to 4.45 and activity recovery from 81.1 to 70.6%, and the purification fold also decreased in this pH range. The reduction in these results may be explained by Albersson equation. According to the Albersson equation [18]:

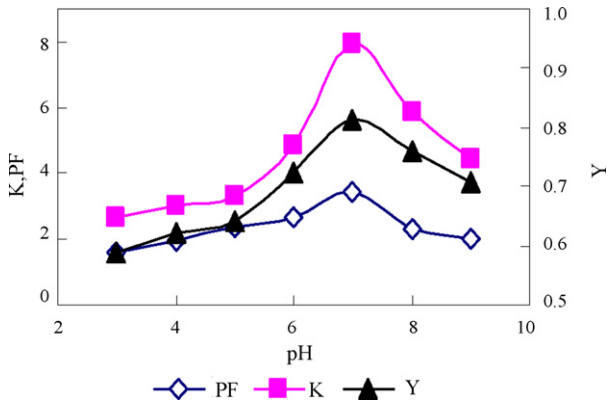

Fig. 2. Partition behaviour of lipase in PEG/phosphate system as a function of pH.

$$\ln K = \ln K_0 + F\Delta\Psi Zp/RT \quad (1)$$

protein partition is driven by two effects: the electrostatic component ($F\Delta\Psi Zp/RT$) determined by the electrical protein charge, and a hydrophobic component (K_0) which has a maximal effect when the pH medium is near the isoelectric pH. When pH increased from 3.0 to7.0, the hydrophobic component is greater than the electrostatic forces, therefore the PEG has tends to interacts with the protein which results in the increase of the partition coefficient and purification fold.

For the activity recovery, it can be seen form Fig. 2 that high pH (7.0–9.0) is more suitable. The activity recovery is above 70% in the pH range of 7.0–9.0. This may be due to the most favorable environment at this pH range where lipase is most stable. Sharma et al. [19] have reported that some of the lipases were found to be stable at higher pH range. In above pH range, the partition coefficient of lipase was found to decrease with an increase in pH, which indicated that the increase in pH level resulted in a decrease of lipase partitioning to the top phase, thereby resulting in lower activity recovery of lipase in top phase. Thus from the above studies, pH 7.0 was selected for the PEG 4000/phosphate system. After 2.0% NaCl was added to the system, a satisfactory result was obtained with partition coefficient 7.61, activity recovery 80.7%, and purification factor of 5.84, where purification fold was increased significantly.

3.4. Effect of the composition of top PEG phase of ATPS on the immobilization

In order to investigate the effect of the composition of top PEG phase of ATPS on lipase immobilization, a series of PEG/phosphate aqueous two-phase systems with different top phase composition were prepared to extract the lipase from cell homogenate and then immobilize the lipase directly from the top phase. The results are presented in Table 2. From Table 2, it was found that the phosphate concentration had an obvious influence on lipase immobilization, which resulted in the immobilized lipase activity ranging from 1114.6 to 809.9 U g^{-1} when phosphate concentration in the top phase ranged from 4.38 to 7.57%. This is because that the presence of phosphate at low concentration might well favor the enzyme immobilization reaction [20]. However, PEG concentration in the top phase had only a small effect on the lipase immobilization, which lead to the immobilized lipase activity varied from 809.9

Table 2
Effect of top PEG phase of ATPS on the lipase immobilization.

ATPS composition		Top phase composition (w/w, %)		Lipase activity of top phase (w/w, %)		Immobilized lipase
PEG	Phosphate	PEG	Phosphate	Total lipase (U)	Specific activity (U mg^{-1} pr)	Activity (U g^{-1})
12	11	22.5	5.23	156.7	45.39	1096.8
12	10	19.4	6.21	104.35	39.73	885.9
12	9	16.1	7.57	93.1	34.51	809.9
14	11	27.4	4.38	163.68	48.58	1114.6
13	11	25.2	4.87	149.29	46.69	1033.1
11	11	23.3	5.49	142.9	38.28	1023.1
10	11	18.6	6.42	112.24	34.66	811.49
Crude lipase				116	14.5	

to 1114.6 U g^{-1} when PEG concentration of the top phase ranged from 16.1 to 27.4%.

4. Conclusion

It can be concluded that the new process which combined aqueous two-phase partitioning with enzyme immobilization could be potentially useful technique for the purification and immobilization of lipase. The satisfactory process conditions were found using PEG 4000/potassium phosphate (12/13, %) at pH of 7.0 and 2.0% NaCl, which ensured partition coefficient 7.61, activity recovery 80.4%, and purification factor of 5.84. Moreover, the in situ immobilization of lipase in PEG phase resulted in a highest immobilized lipase activity of 1114.6 U g^{-1}. This process is cost-effective as well as time-saving because it is fast and simple and involves a smaller number of steps in which immobilization step is directly in the top PEG enriched phase to achieve an in situ immobilization. Further it is relatively straightforward to scale-up and allows for recycling of the phase components thus making the whole process more cost-effective. The approach developed here for lipase from *T. laibacchii*, may be applicable for other lipases such as *Candida* sp. lipases, *Pseudomonas* sp. lipases, and *Porcine pancreas* lipases.

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