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Aqueous two-phase Extraction of Intracellular Lipase with Sn-1,3 Positional Selectivity from *A. niger* GZUF36

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The extraction of intracellular lipase with Sn-1,3 positional selectivity from a new strain of *A. niger* GZUF36 has been exploited by aqueous two-phase system (ATPS). Firstly, crude intracellular lipase solution was prepared by cell disruption. Sonication is the best method for disruption of *A. niger* cell. Then, extraction of the intracellular lipase from the crude enzyme solution was focused on by ATPS. The influence of key parameters such as molecular weight of polyethylene glycol (PEG), PEG 2000 concentration and $(NH_4)_2SO_4$ concentration on the partitioning behavior of lipase was evaluated. The suitable aqueous two-phase system consisted of 20% PEG 2000 and 12% $(NH_4)_2SO_4$ resulted in one-sided partitioning of the intracellular lipase with selectivity of 2.39, recovery of lipase 74.93% and purification factor of 3.42-folds. ATPS extraction may be used as a first step to purify the enzyme from crude enzyme solution.

Key words: Sn-1,3 position selectivity; intracellular lipase; *A. niger* GZUsF36; aqueous two phase system; extraction.

1,3-Diglyceride is a healthy lipid[1, 2]. However its content is low in natural grease. It is necessary to prepare 1,3-DG. So, in previous study, 1,3-DG was synthesized by intracellular lipase with Sn-1,3 regio-selectivity from a new strain of *A.niger* GZUF36 by our team[3]. The lipase is of high Sn-1,3 regio-selectivity and good resistance to organic solvents. This makes it be very attractive for biotransformation. Although the whole-cell lipase from *A.niger* GZUF36 can be used to high selectively synthesize 1,3-DG by glycerolysis[3], It needs to be purified for further learning about its enzymology characterization and broadening the application. However, intracellular lipase needs to be separated from broken cell before purification.

Cell is generally broken by physics protocols, such as high-pressure homogenizer methods[4], sonication[5], grinding[6] and so on, owing to their green and high efficiency.

A separation protocol is crucial to separating a protein. A good separation protocol is generally simple and high efficiency. An aqueous two-phase system (ATPS)[7, 8] is just such protocol. It can be constructed by mixing either two incompatible polymers or a polymer and a salt in water, is proved to be an effective protocol for the separation of lipases.

Conventional protocols[9] for separating proteins are generally adsorbing separation[10], salt

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precipitation and chromatography technology[11, 12]. On one hand, compared with adsorbing separation or chromatography technology, ATPS has the advantages of low cost and saving time. On the other hand, in comparison to the salt precipitation, ATPS has high separation efficiency.

So, in this work, intracellular lipase from *A.niger* GZUF36 was separated by aqueous two-phase extraction with the aim of applying it as a first step to purify the enzyme from broken *A.niger* cell.

MATERIALS AND METHODS

A. niger GZUF36 was screened by our laboratory, collected in China Center for Type Culture Collection. *Á*-NPP was purchased from Alfa Aesar (China) Chemical Co. Ltd. All the chemicals used in this work were of analytical grade and commercially available. Seed culture medium and fermentation medium were referred to our previous report[3].

Cell culture and dry were also referred to our previous report[3].

Preparation of broken cell solution with different methods: (a) Sonication method: Briefly, 1.0 gram freeze-dried *A. niger* cells was suspended in 30 mL of 0.02 M potassium phosphate buffer (pH 7.5), then broken with 20 min pretreatment time, 200 W , 3 s/3 s (work/pause) cycle of ultrasonic pulse in an ice water bath. (b) Grinding in liquid nitrogen: One g freeze-dried cells of *Aspergillus niger* was ground to a fine powder in a clean mortar filled with liquid nitrogen. (c) Grinding in ice bath: One g freeze-dried cells of *A. niger* was continuously ground for 10 min. The broken cell with methods (b) and (c) was suspended in 30 mL of 0.02 M potassium phosphate buffer (pH 7.5), respectively.

Preparation of crude lipase solution: 30 mL broken cell solution above was centrifuged at 10,000 g and 4 °C for 10 min. Then, the supernatant was harvested. The precipitation was resuspended in 0.02 M potassium phosphate buffer at pH 7.5. Next, the resuspension was centrifuged under the same condition and the harvested supernatant was added to the previous one, volume to 50 mL by adding some amount of 0.02 M potassium phosphate buffer, and then stored as crude lipase solution at -20 °C until further use.

ATPS extraction

The phase diagram was plotted at varying PEGs molecular weight and ammonium sulfate concentrations according to the Chien Wei Ooi's report[13]. All ATPS were constructed in graduated tubes. After 3 min of gentle stirring, the systems were centrifuged at 6000 rpm for 5 min. The tubes were equilibrated in a thermostatic bath at 4 æ%C and 1 atm overnight to form clear and transparent two phases. The volumes of the top and bottom phases were measured, after which both phases were put aside for determination of lipase activity and a total protein. To optimize ATPS, effects of key factor including PEGs molecular weight, PEG concentrations and ammonium sulfate concentrations on the extraction of lipase was analyzed while 20 % crude lipase solution remained in ATPS at pH 7.5.

Determination of selectivity, volume ratio of phase, purification factor and recovery of lipase

Selectivity (S) was defined as the ratio of the lipase partition coefficient (K_L) to the protein partition coefficient (K_p):

$$S = \frac{K_L}{K_P} = \frac{A_T}{B_T} \cdot \frac{P_B}{P_T}$$

where K_L and Kp are the ratios of lipase enzyme to protein concentrations found in each phase, respectively. A_T and B_T are the lipase activities (in U/mL) in the top phase and the bottom phases, respectively. P_T and P_B are the total protein concentration (in mg/ml) determined in the top phase and bottom phases, respectively.

V_R is the volume ratio of the top phase to the bottom phase.

The top phase purification factor was defined as the ratio of the specific-lipase activity in the top phase to the initial lipase-specific activity in the crude extract.

The recovery of lipase (Y_L, %) is defined as the ratio of lipase activity in the top phase to the total lipase activity added in the ATPS.

Synthetic activity was measured by *Á*-NPP according to previous reports[14]. Protein concentration was determined with the Bradford method[15]. Bovine serum albumin was used as the standard.

All experiments were performed in triplicates. Data were presented as the mean of

three determinations and their relative standard deviations were all below 5 %.

RESULTS AND DISCUSSIONS

Effects of cell disruption methods on the crude intracelluar lipase activity

A.niger GZUF-36 cell was broken with different methods and prepared crude intracellular lipase solution. As shown in table 1, crude intracellular lipase solution prepared by sonication displayed the highest activity, protein content and specific activity. It suggested that sonication is the best method for disruption of *A.niger* cell due to its uniform and thorough fragmentation. So, the crude enzyme was used for the subsequent experiment of extraction by ATPS.

Table 1. Effects of cell disruption methods on the crude intracelluar lipase activity

Cell disruption method	Lipase activity ($U \cdot g^{-1}$ dry cell)	Protein content ($mg \cdot g^{-1}$ dry cell·)	Lipase specific activity ($U \cdot mg^{-1}$ protein)
Sonication	214.40	32.71	6.55
Grinding in liquid nitrogen	112.12	23.25	4.82
Grinding in ice bath	133.71	22.51	5.94

Phase diagram of polyethylene glycol (PEG)/ ammonium sulfate ATPS

For PEG and ammonium sulfate are used to form ATPS, the phase diagrams of various PEGs with different molecular weight with ammonium sulfate are shown in Figure 1. With an increase in PEG molecular weight, phase separation concentration required by the system decreases. The reason may be that hydrophilicity of PEG lowers with the increase of PEG molecular weight. Therefore, the resistance of separation between polyethylene glycol and ammonium sulfate phases becomes small, and ATPS was easier to form by PEG with larger weight / ammonium sulfate. Then, the effect of only PEG molecular weight with 2000 and 6000 on the ATPS extraction of lipase was studied in the subsequent experiment.

Table 2. Effect of PEG molecular weigh on the distribution of lipase in ATPS

PEG molecular weight	V_R	Selectivity	Y_L (%)
2000	0.99	0.92	47.89
6000	0.85	0.84	18.73

Table 3. Effect of PEG concentration on the distribution of lipase in ATPS

PEG %	V_R	Selectivity	Y_L(%)
8	-	-	-
12	0.84	0.72	36.44
16	1.76	0.90	56.13
20	1.16	1.18	56.74
24	1.69	0.86	48.65

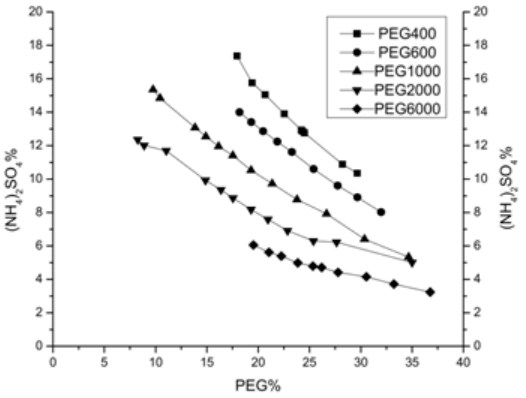

Fig. 1. Phase diagram of PEG/ammonium sulfate

Table 4. Effect of $(NH_4)_2SO_4$ concentration on the distribution of lipase in ATPS

$(NH_4)_2SO_4$ concentration	V_R	Selectivity	Y_L(%)
8	-	-	-
12	1.46	2.39	74.93
16	0.88	1.82	57.53
20	0.63	1.28	39.69
24	0.42	1.00	29.66

Selection of PEG molecular weight for ATPS extraction of lipase from crude enzyme

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 (2000 and 6000) and maintaining the phase compositions (PEG/$(NH4)_2SO_4$) at constant value (16/16, %).

The results are shown in Table 2. It was found that V_R, selectivity and recovery of lipase are all higher in PEG 2000/ $(NH_4)_2SO_4$ system than in PEG 6000/ $(NH_4)_2SO_4$ system, indicating that PEG 2000 was suitable for the extraction of lipase. The reason may be that PEG with high molecular weight has higher molecular particles and greater the smaller surface area than PEG with low molecular weight, resulting in adsorption of hydrophobic groups on lipase weaker. In addition, with the increase in PEG molecular weight, viscosity of ATPS rises, which is un-favour to lipase pass between the two-phase interface[16].

Effect of PEG concentration on the distribution of lipase in ATPS

To study the effect of PEG 2000 concentration (8% to 24%) on the partitioning of lipase, $(NH4)_2SO_4$) was kept at constant value (16 %) in ATPS.

The results are shown in Table 3. When the concentration of PEG 2000 was 10%, ATPS can not be formed. As PEG 2000 concentration increased from 12% to 24%, selectivity increased first and then decreased. when the concentration of PEG 2000 is 20 %, selectivity reached the maximum of 1.18. That may be attributed to the increase in PEG 2000 concentration resulting in the rise of mass transfer resistance of protein, which unfavorable for the extraction of the target protein molecule. V_R and recovery of lipase displayed similar phenomena, and recovery of lipase achieved the maximum of 56.74% at 20% PEG 2000. Therefore, PEG 2000 of 20% was most beneficial to extraction of lipase in ATPS.

Effect of $(NH_4)_2SO_4$ concentration on the distribution of lipase in ATPS

When effect of $(NH_4)_2SO_4$ concentration was studied, PEG 2000 concentration remained 20 % in ATPS.

While the concentration of ammonium sulfate was 8 %, ATPS was not formed. With the increase of ammonium sulfate concentration from 12% to 24%, V_R, selectivity and recovery of lipase in ATPS all showed a decreasing trend (Table 4), all reached the maximum at 20% $(NH_4)_2SO_4$, which was 1.46, 2.39 and 74.93%, respectively. A plausible explanation for the phenomenon is that high concentration of ammonium sulfate would damage the hydrate film on the surface of lipase, resulting in the enzyme salting out. So, 20% $(NH_4)_2SO_4$ was best for the extraction of lipase in ATPS. And then, purification factor of 3.42-folds was calculated in the 20% PEG 2000/12%$(NH_4)_2SO_4$ system.

CONCLUSION

In this study, *A. niger* GZFU36 cell was broken for preparation of crude lipase solution by sonication, grinding in liquid nitrogen and grinding in ice bath, respectively. Sonication was the best method for cell disruption. PEG molecular, PEG concentration and $(NH_4)_2SO4$ concentration have a crucial impact on the extraction of lipase from crude enzyme solution by ATPS. The suitable ATPS extraction was the phase compositions of 20% PEG/ 12% $(NH_4)_2SO4$ with purification factor of 3.42 folds as well as 74.39% recovery of lipase. Aqueous two-phase extraction may be used as a first step to purify the intracellular lipase from crude enzyme solution of broken cell of *A.niger* GZUF36. This study provides the basis for further purification of the enzyme.

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