Efficient methods of purification of α-galactosidase from Aspergillus niger:
Aqueous two-phase system versus three-phase partitioning

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Abstract

Aqueous two-phase system (ATPS) and three-phase partitioning (TPP) had been considered as efficient and economical strategies for large-scale purification of the desired protein from a fermentation broth containing a wide variety of biomolecules. The influence of various process parameters on α-galactosidase partitioning in the process of ATPS and TPP were investigated respectively. For ATPS process, highest purification fold (3.25-fold) with 84.41 % activity recovery of α-galactosidase was observed in the system containing 12 % (w/w) PEG 2000 and 11.9 % (w/w) phosphate, at pH5.0 and 25°C. Meanwhile, the optimal TPP system was found at pH5.5 with 60 % (w/v) ammonium sulfate saturation and 1:1.5 crude extract to t-butanol ratio. Consequently, 6.27-fold purification with 97.21 % activity recovery of α-galactosidase was given by the system. There was no change in the optimum pH and temperature observed for partially purified α-galactosidase compared to crude enzyme extract. By comparing two strategies from courses and effects, TPP technique is a more attractive process for the purification of α-galactosidase from Aspergillus niger and it is a good candidate for industrial application.

Keywords: Aqueous two phase system, Three phase partitioning, α-Galactosidase, Purification, Enzyme activity

Abbreviations:
- ATPS — Aqueous two-phase system
- TPP — three-phase partitioning
- pNPG — p-Nitrophenyl-α-D-galactopyranoside
- pNP — p-Nitrophenol
- PEG — Polyethylene glycol

Introduction

In recent years, there has been an increasing interest in α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) due to its application in food and feed industries [1]. It has been discovered and isolated from various plants and microbial sources. Technology of purification
was the key point of the industrial enzyme production. Various traditional methods are used to purify \( \alpha \)-galactosidase, including salting out, ion exchange chromatography, affinity chromatography, membrane filtration, gel permeation. However, most of methods involve multi-step protocol, and they are generally time-consuming, requiring pretreatment and expensive to produce in large-scale.

ATPS separation technique is a simple and efficient procedure to purify proteins and it based on a liquid-liquid phase separation [2]. It is generally believed that effects of hydrophobic, hydrogen bond and ionic interactions are the driving forces of forming aqueous two-phase. Numerous applications of ATPS on proteins (enzymes) separation and purification have been demonstrated in recent years [3,4].

TPP is another quick and efficient approach for the concentration and purification of proteins from complex mixtures, and it has been widely used to purify biomolecules. Three phases are normally formed by adding a salt (generally ammonium) and an organic solvent (generally \( t \)-butanol) to mixtures. Salting out, kosmotropic, isoionic and co-solvent precipitation of proteins are considered to be involved the process of TPP[5,6].

The present work is set out to compare the two approaches of ATPS and TPP on the separation and purification of \( \alpha \)-galactosidase from crude extract of *Aspergillus niger*. The study optimized the influencing parameters of the two methods by evaluating the recovery of enzyme and the degree of purification. Then the purified enzyme was further characterized. To our knowledge, this is the first report on the comparison between two simple and efficient approaches (ATPS & TPP) for purifying \( \alpha \)-galactosidase.

**Materials and methods**

**Microorganism and fungal culture**

Extracellular \( \alpha \)-galactosidase was produced using *Aspergillus niger zju-Y1* [7] grown in defined medium as follows. The fermentation medium (sterilized) consisted of 8.094 g wheat bran; 1.904 g soybean meal; 0.001 g MnSO\(_4\)·H\(_2\)O; 0.001 g CuSO\(_4\)·H\(_2\)O, and well-suited distilled water was adjusted to pH5.5. The culture condition was \( 28 \) \(^\circ\)C for 96 h, 10 % inoculum volume.

**Aqueous two-phase system**

According to the modified version of the method from Naganagouda [8], aqueous two-phase system was carried out following Fig.1. The composition of the ATPS are shown in Table 1, the mixture was then gently shaken for 15 min for thorough mixing and allowed to stand for 30 min at 25 \(^\circ\)C. Then the two phases were separated by centrifugation at 3000\(\times\)g for 30 min at 4 \(^\circ\)C for taking it to reach equilibration. After equilibration, the individual phases were collected and aliquots of the phases were analyzed for enzyme and protein concentration.
Crude extract from Aspergillus niger

**ATPS**
Add Polyethylene glycol (PEG)
VerteX gently to dissolve
Add phosphate
VerteX gently to dissolve & thoroughly mixing
Formation of two phases
Separate upper phase and determine
Bottom phase ↔ Interfacial precipitate

**TPP**
Add ammonium sulphate (30% ~ 70%, w/v)
Adjust pH of system (pH 4.5 ~ pH 6.0)
Add tert-butanol (1:0.5 ~1:2, v/v)
Stand at RT and low speed centrifucation
Formation of three phases
Remove upper organic phase and determine
Bottom aqueous phase

**Analyze α-Galactosidase phase**

Fig. 1 Flow sheet of the steps involved in simultaneous ATPS & TPP of α-galactosidase from *Aspergillus niger*

<table>
<thead>
<tr>
<th>No.</th>
<th>PEG molecular weight</th>
<th>PEG (w/w %)</th>
<th>Phosphate (w/w %)</th>
<th>Crude enzyme extract (w/w %)</th>
<th>(K_{\alpha})-galactosidase</th>
<th>Protein Purification factor (fold)</th>
<th>Activity recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2000</td>
<td>12.0</td>
<td>11.9</td>
<td>76.1</td>
<td>0.17</td>
<td>1.77</td>
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<tr>
<td></td>
<td></td>
<td>8.0</td>
<td>21.4</td>
<td>70.6</td>
<td>0.15</td>
<td>1.81</td>
<td>2.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>28.5</td>
<td>66.5</td>
<td>0.29</td>
<td>1.56</td>
<td>2.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
<td>33.3</td>
<td>63.7</td>
<td>0.51</td>
<td>1.29</td>
<td>1.41</td>
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<tr>
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<td>11.9</td>
<td>76.1</td>
<td>0.23</td>
<td>1.61</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>8.0</td>
<td>21.4</td>
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<td>0.21</td>
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<tr>
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<td></td>
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<td>63.7</td>
<td>0.49</td>
<td>1.37</td>
<td>1.79</td>
</tr>
<tr>
<td>3</td>
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<td>12.0</td>
<td>11.9</td>
<td>76.1</td>
<td>0.19</td>
<td>1.48</td>
<td>2.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.0</td>
<td>21.4</td>
<td>70.6</td>
<td>0.56</td>
<td>1.31</td>
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<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>28.5</td>
<td>66.5</td>
<td>0.71</td>
<td>1.02</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
<td>33.3</td>
<td>63.7</td>
<td>0.76</td>
<td>1.05</td>
<td>0.78</td>
</tr>
</tbody>
</table>

**Table 1.** Composition of PEG–phosphate salt ATPS and effect of PEG molecular weight on partitioning of α-galactosidase and other total proteins

Note: \(K_{\alpha}\)-galactosidase = α-galactosidase activity in top phase/α-galactosidase activity in bottom phase;

\(K_{\text{protein}}\) = concentration of protein in top phase/concentration of protein in bottom phase

**Three-phase partitioning**
As shown in Fig.1 according to method of Dhananjav [9], the crude α-galactosidase extract was saturated with ammonium sulfate (percent saturation at 30, 40, 50, 60 and 70 % w/v) at 25℃. Then solutions were vortexed gently to dissolve the salt. After the pH of system was adjusted to each pH values (4.5, 5.0, 5.5, 6.0) by addition of conc. HCl or NaOH. Next, the
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Crude enzyme extract was added to *t*-butanol with the ratios of 1:0.5, 1:1, 1:1.5 and 1:2, v/v. The mixture was vortexed for 1 min and allowed to stand for 30 min at 25 °C. After centrifuged at 3000 rpm for 10 min and at 4 °C, the three phases (upper *t*-butanol phase, bottom aqueous phase and middle interfacial precipitate phase) could be separated. The lower aqueous layer was collected by piercing and the organic phase was removed carefully with a pasteur pipette. Afterwards the interfacial precipitate was retained and dissolved in 0.5 ml double distilled water. The partitioned enzyme obtained with optimized TPP was used for further characterization studies.

**Partition parameters**

The partition coefficient for α-galactosidase activity in the aqueous two-phase systems (ATPS) was defined as:

\[
K_{\text{α-galactosidase}} = \frac{\text{α-galactosidase activity in top phase}}{\text{α-galactosidase activity in bottom phase}}
\]

(1)

\[
K_{\text{protein}} = \frac{\text{concentration of protein in top phase}}{\text{concentration of protein in bottom phase}}
\]

(2)

\[
\text{Activity Recovery(%)_{ATPS}} = \frac{\text{α-galactosidase in bottom phase}}{\text{total amount of α-galactosidase in crude extract}} \times 100
\]

(3)

\[
\text{Purification Factor}_{\text{ATPS}} = \frac{\text{specific activity in top phase}}{\text{specific activity in crude extract}}
\]

(4)

The partition coefficient for α-galactosidase activity in the three-phase partitioning (TPP) was defined as:

\[
\text{Purification Factor}_{\text{TPP}} = \frac{\text{specific activity in middle phase}}{\text{specific activity in crude extract}}
\]

(5)

\[
\text{Activity Recovery(%)_{TPP}} = \frac{\text{α-galactosidase in middle phase}}{\text{total amount of α-galactosidase in crude extract}} \times 100
\]

(6)

The specific activity represents the ratio of the enzyme activity to the protein concentration in a sample. All results were obtained from three replicate assays.

**Assays of α-galactosidase and Protein estimation**

α-Galactosidase assay was carried out in test tubes by the modified version of the method from Garro [10]. The reaction mixture contains 10 mmol/L pNPG 50 µl, 50 µl 100 mmol/L McIlvaine buffer pH 5.0 and 100 µl cell-free extract, which sum up to a final volume of 200 µl. The mixture was incubated at 50 °C for 10 min, and the reaction was terminated by adding 3 ml of sodium carbonate (0.25 mmol/L). One enzyme unit (U) was defined as the amount of enzyme that releases 1.0 µmol of pNP from its substrate pNPG per min under given assay conditions.

The soluble protein was determined by the Bradford method using bovine serum albumin...
as the standard. The experiment was carried out with the Easy Protein Quantitative Kit (DQ101-01, Trans, CHN) following the manufacturer’s instruction.

**Results and Discussion**

**ATPS of α-galactosidase**
PEG-phosphate system was used in purifying α-galactosidase. The pH of the systems could affect the partition coefficient of enzyme via changing the net charge of α-galactosidase surface. The ATPS system was carried out at pH5.0, which was near to the pI of α-galactosidase derived from *Aspergillus niger*. In addition, an increase in temperature was prone to salting out effect in the bottom phase, which could reduce the solubility of enzyme. Otherwise low temperature might slow down the formation of ATPS. According to Naganagouda [8], room temperature (25) was performed in the ATPS.

According to the Table 1, the values of $K_{α\text{-galactosidase}}$ were less than one in all phase compositions evaluated. It indicated that α-galactosidase partitioned preferentially towards the salt-rich bottom phase. This also demonstrated that α-galactosidase derived from *Aspergillus niger* was a relatively hydrophilic enzyme with hydrophilic amino acid residues on the surface. The highest purification factor and α-galactosidase activity recovery were observed on the phase system with a composition of 12 % (w/w) PEG 2000 and 11.9 % (w/w) phosphate salts. Maximum recovery of 84.41 % with 3.25-fold of purification were obtained after purifying with ATPS. Since α-galactosidases from fungus have high molecular weight (50-130 kDa) [11 12], the partitioning behavior of enzyme were significantly influenced by the molecular weight of PEG according to the report [13]. It is believed that size-exclusion effect was the main cause for changing the partition coefficients. Low $K_{α\text{-galactosidase}}$ is obtained with low concentration of phosphate. It presumed that ion concentration has relationship with surface properties of enzyme. Under the low concentration of phosphate, α-galactosidase had shown more hydrophilic than high concentration. As a result the enzyme tends to partition more in the bottom phase under low concentration of phosphate. To summarize, the major governors of the partitioning of α-galactosidase are PEG molecular weight and PEG and phosphate concentration.

**TPP of α-galactosidase**
The influence of ammonium sulfate saturation was tested by maintaining TPP process at pH5.5 and the ratio of crude extract to t-butanol at 1:1.5. As is seen from Fig. 2A, the maximum purification factor of 6.27-fold along with 97.21% recovery of α-galactosidase activity in interfacial phase was observed on 60% (w/v) ammonium sulfate saturation. Poor recovery of α-galactosidase was observed with the lower salt concentration (< 50% saturation, w/v). Conversely, higher salt saturation (65% w/v) resulted in poorer purification fold due to a reduction in the selectivity of extraction. The ammonium sulfate saturation (50%) was appropriate to precipitate the α-galactosidase from *Aspergillus niger* in the interfacial phase and to remove the other proteins.
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The efficiency of TPP always depends on the $pI$ and molecular mass of the target protein. Therefore different pH levels of the TPP systems could lead to different behaviors exhibited by enzyme. The influence of pH (ranging between 4.0 and 6.0) of the TPP system on the partitioning of $\alpha$-galactosidase was monitored via bioactivity assays (Fig. 2B). The studies were carried out with the condition of salt saturation of 60% (w/v) and the same crude extract to $t$-butanol ratio (1:1.5). It can be seen that the optimal results were obtained at pH5.5. This demonstrates that the TPP systems usually change quickly around the $pI$ of the required enzyme. It is believed that the electrostatic component of the reactions when sulfate anions binding to cationic proteins are involved [14].

The relative amount of $t$-butanol is of critical importance and must be optimized. It is presumed that $t$-butanol was a suitable organic cosolvent for purifying $\alpha$-galactosidase via TPP systems, for the reason that it does not easily permeate inside the desired protein so that less denaturation could occur [15]. The amount of ammonium sulfate and pH in TPP system were fixed to 60% (w/w) and 5.5 respectively. And the ratio of the crude extract to $t$-butanol (v/v) ranges from 1:0.5 to 1:1 (Fig. 2C). The results showed that TPP systems could obtain the best effect (purification factor of 6.27-fold; 97.21% recovery of $\alpha$-galactosidase) with the ratio of crude extract to $t$-butanol (v/v) of 1:1.5. This could be explained that there was not adequate synergy with ammonium sulfate with lower amount of $t$-butanol. Rather, higher concentration of $t$-butanol in TPP system was likely to cause protein denaturation, thus decrease the efficiency of desired enzyme extraction.

**Characterization of $\alpha$-galactosidase**

Several parameters affecting the $\alpha$-galactosidase activity were evaluated in order to characterize the purified enzymes via ATPS or TPP. SDS-PAGE analysis of the partitioned

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**Fig. 2** Influence parameters of TPP on the degree of purification and activity recovery of $\alpha$-galactosidase derived from *Aspergillus niger*. A: Effect of varying saturations of ammonium sulfate, B: Influence of pH, C: Optimization of crude extract to t-butanol ratio
α-galactosidase from ATPS and TPP (Fig. 3) showed that the enzyme was nearly homogenous with the molecular weights corresponding to 58kDa to 108kDa. It is believed that as a cell factory *Aspergillus niger* could secrete more than one α-galactosidase with different molecular weights. According to the reports, the molecular weights of α-galactosidase from fungus are between 50 to 130kDa [11 12]. And the value of the molecular weight here is in the similar range. In addition, the enzyme purification via TPP showed less protein bands than ATPS. Namely, less non-targeted protein was left in the middle phase of TPP than in the bottom phase of ATPS, which was in agreement with the outcome mentioned above.

Fig. 3 10% (v/v) SDS–PAGE analysis of α-galactosidase from *Aspergillus niger*. Lane 1: molecular weight markers (55–170 kDa); Lane 2: crude α-galactosidase extract; Lane 3: TPP purified α-galactosidase (middle phase); Lane 4: ATPS purified α-galactosidase (bottom phase)

To examine the enzyme catalytic activities after purification, the effects of pH and temperature on the enzymatic activity of α-galactosidase were also evaluated before and after the purification with ATPS and TPP. The variation of the relative activity of enzyme at the pH from 3.0 to 7.0 at 37 was shown in Fig. 4A. The optimum of pH was found to be at pH 5 for all samples (crude extract, partially purified enzyme by ATPS or TPP). It also showed that all three samples shared similar pH characteristics. According to Fig. 4B, the three samples also had the same optimum temperature (50) for activity, but the enzyme purified via TPP was slightly more sensitive to high temperature (>60). It is presumed that other proteins in crude extract could enhance the activity of α-galactosidase and have a protective effect for the enzyme. In summary, the results demonstrated that both ATPS and TPP provided the mild aqueous environment for biological molecule separation.

Fig. 4 Effect of pH and temperature on α-galactosidase activity before (Crude extract) and after purification via ATPS and TPP respectively. A: Effect of pH, B: Effect of temperature.
**Comparison between ATPS and TPP**

Compared with conventional purification techniques (take the conventional chromatographic methods as example) (data not shown), both ATPS and TPP based separation strategies had shown significant superiority in α-galactosidase purification. ATPS and TPP strategies always need less operations and chemicals than chromatographic technique, so that they are believed to be efficient and economical approaches [8 9]. In addition, both PEG after ATPS and t-butanol after TPP could be recycled by techniques such as membrane separation or rotary evaporation and so on, which further reduces the cost of purification and it is easy to realize in industrial production.

Furthermore, strategies of ATPS and TPP were compared with various characterizing parameters in the process of purification (Table 2). After ATPS and TPP process, the characteristic (effects of activity on pH and temperature) of α-galactosidases almost remained unchanged. TPP produced a favorable purification increasing effect with the overall α-galactosidase recovery of 97.21% and purification factor at 6.27-fold. Meanwhile it was obtained at 84.41% and 3.25-fold after ATPS process, respectively. With only one step operation, purification factor of α-galactosidase in TPP showed more 92.92% than ATPS.

**Table 2. Difference between TPP and ATPS for purifying α-galactosidase from Aspergillus niger**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ATPS</th>
<th>TPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>No need</td>
<td>No need</td>
</tr>
<tr>
<td>Time required</td>
<td>2h-3h</td>
<td>1h-2h</td>
</tr>
<tr>
<td>Chemicals</td>
<td>PEG; phosphate</td>
<td>Ammonium sulfate; t-butanol</td>
</tr>
<tr>
<td>Enzyme partitioned</td>
<td>Bottom phase</td>
<td>Middle phase</td>
</tr>
<tr>
<td>Purification factor (fold)</td>
<td>3.25</td>
<td>6.27</td>
</tr>
<tr>
<td>Activity recovery (%)</td>
<td>84.41</td>
<td>97.21</td>
</tr>
<tr>
<td>Optimum pH of enzyme</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Optimum temperature of enzyme</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Postprocessing</td>
<td>Need</td>
<td>No need</td>
</tr>
</tbody>
</table>

The chemicals involved ATPS and TPP were totally different. Allowing for two phases formation, PEG and salts with critical concentration were involved in ATPS. Therefore, it required a longer time to dissolve, mix and form two-phases than TPP systems that employed liquid of t-butanol and salt. As is seen from Fig. 5, the enzyme were easier to be obtained from TPP process, due to that the interfacial phases of TPP led to a clearer effect of delamination than ATPS which formed two phases. The TPP process not only purified the protein but also concentrated it into one of the phases. Precipitated α-galactosidase in interfacial phase via TPP is more concentrated than the ATPS purification techniques within a single step. Furthermore, the following purification and concentration processes for ATPS on account of salt rich in the bottom phase must be considered, which needs extra time and cost. TPP strategy for purifying α-galactosidase from Aspergillus niger is more efficient, concentrated and economical than ATPS within single step.
Fig. 5 ATPS and TPP of α-galactosidase from fermented media of Aspergillus niger. ATPS and TPP were carried out as described in the text. (A) ATPS: 1 PEG rich top phase, 2 salt rich bottom phase; (B) TPP: 1 organic top phase, 2 interfacial phase with α-galactosidase precipitate, 3 aqueous phase

Conclusion

According to the results above, both strategies (ATPS and TPP) of purifying α-galactosidase from Aspergillus niger were shown efficient, economical and scalable compare with conventional techniques. Moreover, TPP was considered superior to ATPS for better recovery of α-galactosidase and purification fold, less time consuming, and the absence of extra following purification process. In conclusion, the TPP processes described here have the potential to be carried out as purification protocol for industrial enzyme.

Acknowledgments

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References

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