Recovery of endo-polygalacturonase using polyethylene glycol-salt aqueous two-phase extraction with polymer recycling

You-Ting Wu, Martinha Pereira, Armando Venâncio & José Teixeira*
Centro de Engenharia Biológica-IBQF, Universidade do Minho, Braga 4710, Portugal

Received 15 November 1999; accepted in revised form 6 October 2000

Key words: aqueous two-phase systems, partitioning, extraction, polymer recycling, endo-polygalacturonase, Kluyveromyces marxianus

Abstract

The partitioning behaviour of endo-polygalacturonase (endo-PG) and total protein from a clarified Kluyveromyces marxianus fermentation broth in polyethylene glycol (PEG)-ammonium sulfate and PEG-potassium phosphate (pH=7) aqueous two-phase systems was experimentally investigated. Both the enzyme and total protein partitioned in the bottom phase for these two kinds of systems. The enzyme partitioning coefficient can be lower than 0.01 in PEG8000-(NH₄)₂SO₄ ATPS with a large phase volume ratio and a moderate tie-line length, which implies the possibility of concentration operation using aqueous two phase partitioning. An ion-exchange separation of high purification efficiency was applied to analyze the clarified and dialyzed fermentation broth. A total purification factor of only 2.3 was obtained, which indicated the high enzyme protein content in the total protein of the fermentation broth. Consequently, the main purpose for separating endo-PG is concentration rather than purification. A separation scheme using an aqueous two-phase extraction process with polymer recycling and a dialysis was proposed to recover endo-PG from the fermentation supernatant of K. marxianus for commercial purpose. A high enzyme recovery up to 95% and a concentration factor of 5 to 8 with a purification factor of about 1.25 were obtained using the single aqueous two-phase extraction process. More than 95% polymer recycled will not affect the enzyme recovery and purification factor. Dialysis was used mainly to remove salts in the bottom phase. The dialysis step has no enzyme loss and can further remove small bulk proteins. The total purification factor for the scheme is about 1.7.

Introduction

Pectinases are a group of enzymes that degrade pectin-containing substances and are widely used in the food industry. Recently, pectinase production from Kluyveromyces marxianus fermentation has been extensively studied (Barnby, 1987; Schwan and Rose, 1994; Schwan et al., 1997). Thus, it is of interest to develop new and scalable separation process to recover pectinase enzymes from the fermentation broth of K. marxianus for commercial use.

In literature, there exist some methods for separating pectinase from its fermentation broth. Harsa et al. (1993) had given a general review of them. Most of these separation methods are multi-step (usually involve a number of separation stages such as precipitation, membrane and gel filtration, ion exchange and affinity chromatography, and dialysis) and were developed for characterization purposes rather than commercial implementation. These methods would also be unlikely to meet the requirements for the process, such as easy scale-up, high enzyme recovery and low capital and operating costs. As a result, Harsa et al. (1993) reported their effort in separating pectinase for the commercial purpose only using ion exchange, which should attract due attention for its simple process design and high recovery (up to 93%) of the purified enzyme. However, their method suffered from the disadvantage of long processing time for the large volume of fluid, since the flow in the ion exchange column is limited by the gel.

Aqueous two-phase extraction (ATPE) technique is another useful and scalable separation method for enzymes (Johansson and Tjerneld, 1989; Lin et al., 1996;
Venâncio et al., 1996; Almeida et al., 1998; etc.), cells (Sturesson et al., 1990), nucleic acids (Persson et al., 1999) and other biological substances, and has been widely studied in recent two decades (Albertsson, 1986; Fisher and Sutherland, 1989). However, few attempts of separating the pectinas by using ATPE are found in literature. It is of great interest to develop a separation process based on the ATPE technique.

In this work, the partitioning of endo-polygalacturonase and total protein from a clarified \textit{K. marxi- ans} fermentation broth in polyethylene glycol-salt aqueous two phase systems was investigated, and the influence of polymer molecular weight and concentration, pH and phase ratio on the partitioning coefficient was also examined with the objective of finding a suitable system for separating endo-polygalacturonase from its fermentation supernatant. The ion-exchange separation of high purification efficiency described by Harsa et al. (1993) was also introduced to analyze the fermentation supernatant.

Materials and methods

Materials

Aqueous two-phase systems (ATPS) : Polyethylene glycol with three molecular weights 4000, 8000 and 20000 was obtained from Promega Corporation (Madison, WI, USA). Salts such as (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and potassium phosphate (30.6 g K\textsubscript{2}HPO\textsubscript{4} to 168.6 g KH\textsubscript{2}PO\textsubscript{4}, pH=7) and other chemicals are of analytical reagent grade. The ATPS were prepared from stock solutions: 40\% or 50\% for PEG stocks, and 40\% for salt stocks. All concentrations were given in weight per weight percentage (w/w\%).

Fermentation broth : Fermentation broth was obtained from the shaking incubation of \textit{K. marxi- ans} CCT3172 at 30 °C in the following media \textsubscript{(1]}: 10 g glucose, 3.0 g (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 4.5 g KH\textsubscript{2}PO\textsubscript{4}, 0.25 g MgSO\textsubscript{4}·7H\textsubscript{2}O, 0.25 g CaCl\textsubscript{2} and 1.0 g yeast extract.

Enzyme samples : Two enzyme samples for the partitioning experiments were prepared from the fermentation broth of \textit{K. marxi- ans} after centrifugation: solution A (with an enzyme concentration about 8 times the concentration of the original broth) was prepared by dissolving previously lyophilized centrifuged fermentation broth, and solution B was made from solution A by dialysis using a 14000 MW cut-off membrane. Solution B was only used to assay the effect of dialysis on the separation.

Methods

Enzyme sample (0.2 ml) was added into ATPS to form a 1 g total solution. After strongly shaking for 1 minute, the solution was centrifuged at 3000 rpm for 3 minutes and put in an air bath of 25 °C for over 12 hours. Samples of top and bottom phases were taken with pipettes for the assays of enzyme and total protein in order to determine the partitioning between the phases. The distribution of enzyme and total protein can be described by their respective partitioning coefficients, \( K_e \) and \( K_p \), defined as the ratio of enzyme activity or protein concentration in the top and bottom phases.

The protein concentration was measured with a Coomassie Protein assay reagent (Pierce, Rockford, IL, USA), a method based on the dye-binding technique (Bradford, 1976). High concentrations of PEG, (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and phosphate in the top or bottom phase may interfere with the determination. However, experiments revealed that the determination of total protein was not affected when the sample was diluted to have the concentrations of PEG and salt not more than 2.0 w/w\%. The enzyme activity was determined by measuring the release of reducing groups from polygalacturonic acid using dinitrosalicilic acid reagent (Schwan and Rose, 1994), and the activity unit per volume (U ml\textsuperscript{-1}) was expressed as \( \mu \)mol galacturonic acid equivalents released per minute per ml enzyme solution. Experiments showed that high concentrations of PEG and salt might affect this determination, an appropriate dilution was made: for the PEG rich phase, the sample was diluted to have PEG concentration less than 2.0 w/w\%, while for the salt rich phase the salt concentration below 0.025 M.

The ion-exchange separation was performed on a FPLC apparatus with a 20 cm × 1.6 cm column. The column was packed with 16 ml of cm-Sepharose ion-exchange gel, equilibrated with 0.1 M acetate buffer of pH 4.5 (buffer A) and eluted with a buffer B solution (0.3 M NaCl in 0.1 M acetate buffer, pH 4.5) at a flow rate of 1.0 ml min\textsuperscript{-1}. 
Results and discussion

Partitioning behaviours of endo-polygalacturonase and total protein in PEG-salt ATPS

Two kinds of PEG-salt ATPS: PEG-(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} (PEG4000, 8000 and 20000) and PEG-potassium phosphate (pH=7) (PEG 4000, 8000 and 20000) ATPS, were used. Figure 1 shows the partitioning behaviors of endo-polygalacturonase in PEG-(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} ATPS while Figure 2 gives the results in PEG-phosphate (pH=7) ATPS. It was found in both kinds of ATPS that the enzyme showed a strong tendency to concentrate in the bottom salt-rich phase. It implied that the enzyme was strongly hydrophilic since the hydrophobicity of top PEG-rich phase was larger than that of bottom phase. Meanwhile, increasing the tie-line length (TLL, determined from the phase diagram data (Zaslavsky, 1995)) of the system generally decreased the $K_e$, and since the PEG concentration increased with increasing TLL at the same phase ratio, $K_e$ also decreased with increasing PEG concentration. As for the relation between $K_e$ and PEG molecular weight, $K_e$ decreased when PEG molecular weight changed from 4000 to 8000. However, the partitioning coefficient of enzyme in PEG20000-(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} system became lower than that in PEG8000-(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} system, while most values of $K_e$ in PEG20000-phosphate (pH=7) system were located between those in PEG4000 and PEG8000 systems. The enzyme partition became complex when PEG molecular weight was too large.

The endo-polygalacturonase partitioning under other conditions was also investigated. Figure 3 illustrates the $K_e$ at different pH values in PEG-(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} ATPS, and the $K_e$ with respect to phase volume ratio (R, defined as the volume ratio of top phase to bottom volume) in both PEG-(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and PEG-phosphate systems. From Figure 3, the enzyme was still predominately in the bottom phase and the $K_e$ had a minor change when pH increased from 3 to 7.4. It means that the change in the surface charge of the enzyme (pI ranging from 5.0 to 6.0) was not enough to alter the hydrophilicity of the enzyme. In the case of $K_e$ with respect to phase volume ratio, $K_e$ had a trend to decrease ($R<2$) and then increase ($R=2$) with increasing $R$, and the value was nearly kept constant when $R$ changed from 0.5 to 2 in both PEG-(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and PEG-phosphate ATPS. The phenomena indicates that the concentration process of endo-polygalacturonase with high recovery can be easily done at a higher phase ratio. Figure 4 shows the specific activity and recovery of endo-polygalacturonase in the bottom phase at higher phase volume ratios in a PEG8000-(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} ATPS with a moderate tie-line length. As can be seen, the larger the phase ratio, the higher the specific activity and the lower the recovery. The high enzyme recovery at high phase volume ratio also implies that the enzyme partitioning coefficients became lower than 0.01. Analysis showed that the enzyme free volume might be greatly reduced by the high PEG and salt concentrations (the larger the phase volume ratio, the higher the PEG concentration in the feeding) and the interactions between enzymes became a dominating factor (Abbott et al., 1991).

The partitioning of total protein in the enzyme solution is also of great importance. Figure 5 gives the total protein partitioning coefficient for enzyme solutions $A$ ($K_p$) and $B$ ($K_p^*$) in PEG-(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and PEG-phosphate ATPS. It could be seen for both kinds of ATPS that the total protein in enzyme solution $A$ also mainly partitioned to the bottom phase and was nearly kept unchanged when the TLL of the system increased. As for enzyme solution $B$, since small proteins with molecular weight lower than 14000 Dalton and other small molecules (such as substrates not consumed) were washed out by dialysis, only enzyme and other high molecular weight proteins were left, the total protein became more easily dominant in the bottom phase in PEG-(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} ATPS and the $K_p^*$ ratio.
changed very little in PEG-phosphate ATPS when compared with the case of enzyme solution A.

**Ion-exchange separation**

Since both enzyme and total protein partitioned in the bottom phase, the purification factor (defined as the ratio of enzyme yield to protein yield) was not high enough. It raised difficulties for removing most unwanted proteins from the fermentation broth by aqueous two-phase partitioning. It becomes particular important to know what percentage of the total protein consists of endo-PG.

The ion-exchange of high purification efficiency described by Harsa et al. (1993) were used to characterize the fermentation supernatant. After loading the column with 10 ml dialyzed fermentation supernatant, unbound protein was removed by washing with buffer A solution. No endo-PG activity was found in the spent solution. The bound fraction was eluted with a 50% buffer B and then 100% buffer B. Endo-PG activity was recovered in a single protein peak. The elution liquid was collected and analyzed for activity and protein. Table 1 gives the results and compared with the results of Harsa et al. (1993). Nearly all enzyme activity was recovered in the peak (>95%), and a total purification factor of only 2.3 was obtained, while Harsa et al. reported a purification factor of 327. This had to be attributed to the high enzyme protein in the total protein of the fermentation supernatant. If all the protein recovered in that peak is assumed to be enzyme, endo-PG consists of more than 40% of total protein. Thus, the main purpose for separating endo-PG favors concentration rather than purification, and the aqueous two-phase extraction becomes an interesting method.

---

**Figure 2.** Partitioning of endo-polygalacturonase in PEG-phosphate (pH=7) ATPS at 25 °C. (□) PEG4000; (○) PEG8000; (△) PEG20000. The phase ratio of ATPS is about 1:1.

**Figure 3.** Partitioning of endo-PG at 25 °C. (a) Ke vs. pH in 9.6% PEG8000-9.5% (NH₄)₂SO₄ ATPS with 0.04 M citric acid - Na₂HPO₄ buffer; (b) Ke vs. phase volume ratio in PEG8000-(NH₄)₂SO₄ ATPS with the tie-line length of 15.8 w/w% (□) and in PEG8000-phosphate ATPS with the tie-line length of 17.7 w/w% (○).
Table 1. Endo-PG purification using ion-exchange

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Protein yield (%)</th>
<th>Total Activity (U)</th>
<th>Enzyme Yield (%)</th>
<th>Total purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation broth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>after centrifugation</td>
<td>8.33</td>
<td>0.0758</td>
<td>100</td>
<td>26.49</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Dialysis</td>
<td>10.0</td>
<td>0.0553</td>
<td>73.0</td>
<td>26.40</td>
<td>99.7</td>
<td></td>
</tr>
<tr>
<td>Ion-exchange</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eluted solution</td>
<td>11.0</td>
<td>0.0310</td>
<td>40.9</td>
<td>25.44</td>
<td>96.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Fermentation broth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>after centrifugation*</td>
<td>519</td>
<td>1878.8</td>
<td>100</td>
<td>404.8</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Ion exchange,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eluted solution *</td>
<td>89.5</td>
<td>5.4</td>
<td>0.29</td>
<td>383.7</td>
<td>94.9</td>
<td>327</td>
</tr>
</tbody>
</table>

*The data were cited from Harsa et al. (1993).

Figure 4. Specific activity and recovery of endo-polygalacturonase in bottom phase with respect to phase volume ratio in PEG8000-(NH₄)₂SO₄ ATPS with the tie-line length of 28 w/w%. (○) specific activity; (△) enzyme recovery.

Laboratory-scale recovery of endo-polygalacturonase using PEG-(NH₄)₂SO₄ aqueous two-phase partitioning with polymer recycling

In order to use the aqueous two-phase extraction for enzyme recovery, optimization of aqueous two-phase extraction was studied. From the partition experiments of endo-PG, PEG8000-(NH₄)₂SO₄ ATPS with a moderate tie-line length of 28 w/w% is selected as the optimized medium. Among conditions such as PEG and salt concentrations, pH and phase volume ratio, the most important parameter for extracting endo-PG using ATPE technique is the phase ratio of the ATPS (see Figures 3 and 4), which influence the enzyme recovery as well as the enzyme concentration in the bottom phase. Since a too large phase ratio will also make the ATPE process difficult to operate, a phase ratio of 8 to 11 is appropriate. There is no need to insert a dialysis stage before the ATPE process as judged from data in Figure 5 (since $K_p * < K_p$). It is also not necessary to add buffer into the ATPS (or adjust pH of the system) from the data in Figure 3 and since the

Figure 5. Partitioning of total protein in PEG8000-(NH₄)₂SO₄ for enzyme sample A (○) and B (○) and in PEG8000-phosphate (pH=7) ATPS for enzyme sample A (●) and B (●) at 25 °C. The phase ratio of ATPS is about 1:1.
enzyme is stable in PEG-salt ATPS without buffer for more than one week (data not shown).

Based on the optimized conditions, a simple separation scheme with polymer recycling was proposed, as shown in Figure 6. It consists of an ATPE process, followed by dialysis. The ATPE process is operated between two tie-lines as shown in Figure 7 and contains two steps: the primary step is the concentration of endo-PG in PEG8000-(NH₄)₂SO₄ ATPS with a tie-line length of 28 w/w% and a phase volume ratio of 8 to 11 (point P in Figure 7), and the second step is the concentration of the top PEG rich phase for PEG recycling by adding solid salt and constructing another PEG8000-(NH₄)₂SO₄ ATPS which has a tie-line length of about 58 to 60 w/w% (point Q). Enzyme is recovered in bottom phase of the primary ATPE step (point B). The recovered top phase (point C) in the second ATPE step serves for the polymer recycling for operation point P and bottom phase (point D) for salt crystallization. The second step can not only remove water from the PEG solution, but also can strip off most contaminating substances from the top phase of the first step. The dialysis is mainly used to remove salts in the bottom phase of the first ATPE step.

Several laboratory-scale separation experiments were conducted to estimate the efficiency of the proposed scheme. In the first experiment, after removing the cells from the fermentation broth by centrifugation, 35 ml of the clarified broth was added into a separator and was thoroughly mixed with 51 g of 50% PEG8000 stock and 12.5 g of 40% (NH₄)₂SO₄ stock to form a 100 g total solution, the formed ATPS has a phase ratio of about 11 and the tie-line length of 28 w/w%. After separation, 7.7 ml of bottom phase was recovered and dialyzed using 14000 MW cut-off membrane. In the second experiment, about 69.5 ml clarified broth was mixed with 25.5 g solid PEG and 5.1 g solid salt to form 100 g total system. In the third experiment, the PEG was previously cycled for 3 times in extracting enzyme from its clarified broth, 46.5 g of the recovered PEG solution (PEG concentration is about 52.5%) was mixed with 1.0 g solid PEG, 48.5 ml clarified broth and 4.40 g solid salt. The system still has a phase ratio of 8 to 10 and a tie-line length of about 28 w/w%. The fourth experiment is similar to the third, but using the fourth cycled PEG. The results of these four experiments are summarized in Table 2. As can be seen, in all the experiments, yields of about 78% for total protein and up to 95% for enzyme were obtained after ATPE, and about a five-fold to eight-fold increase in enzyme concentration was realized, based on the original enzyme concentration in the clarified broth. The purification factor for the ATPE process is kept nearly constant at 1.25, no matter whether or not cycled PEG has been used. As it was also confirmed that enzyme activity in the bottom phase is not lost when kept at room temperature for more than one week, it is expected that the coupling of this concentration process with enzyme production using a more efficient strain (meaning fermentation broth with a higher enzyme concentration) will allow to use the bottom phase as a vehicle to supply endo-PG to enzyme users. Furthermore, more than 95% PEG can be recovered for polymer recycling, since the PEG
Table 2. Laboratory-scale endo-PG recovery using PEG8000-(NH₄)₂SO₄ aqueous two-phase extraction

<table>
<thead>
<tr>
<th>Separation step*</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Protein yield (%)</th>
<th>Total Activity (U)</th>
<th>Specific activity (Umg⁻¹)</th>
<th>Enzyme Yield (%)</th>
<th>Accumulative Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarified broth</td>
<td>35</td>
<td>0.406</td>
<td>100</td>
<td>60.9</td>
<td>153.4</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>ATPE using PEG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stock and salt</td>
<td>7.7</td>
<td>0.316</td>
<td>77.8</td>
<td>57.8</td>
<td>182.9</td>
<td>94.9</td>
<td>1.22</td>
</tr>
<tr>
<td>Dialysis</td>
<td>12.9</td>
<td>0.233</td>
<td>57.3</td>
<td>58.2</td>
<td>249.8</td>
<td>95.6</td>
<td>1.67</td>
</tr>
<tr>
<td>Clarified broth</td>
<td>69.5</td>
<td>0.632</td>
<td>100</td>
<td>221.1</td>
<td>349.8</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>ATPE using solid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG and solid salt</td>
<td>7.8</td>
<td>0.486</td>
<td>76.9</td>
<td>209.4</td>
<td>430.9</td>
<td>94.7</td>
<td>1.23</td>
</tr>
<tr>
<td>Clarified broth</td>
<td>48.5</td>
<td>0.441</td>
<td>100</td>
<td>154.2</td>
<td>349.7</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>ATPE using solid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>salt and PEG</td>
<td>8.2</td>
<td>0.345</td>
<td>78.3</td>
<td>150.0</td>
<td>434.8</td>
<td>97.3</td>
<td>1.24</td>
</tr>
<tr>
<td>solution after 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialysis</td>
<td>13.1</td>
<td>0.257</td>
<td>58.2</td>
<td>150.5</td>
<td>585.6</td>
<td>97.6</td>
<td>1.68</td>
</tr>
<tr>
<td>Clarified broth</td>
<td>48.5</td>
<td>0.441</td>
<td>100</td>
<td>154.2</td>
<td>349.7</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>ATPE using solid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>salt and PEG</td>
<td>10.0</td>
<td>0.347</td>
<td>78.7</td>
<td>152.5</td>
<td>438.6</td>
<td>98.9</td>
<td>1.26</td>
</tr>
<tr>
<td>solution after 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The PEG8000-(NH₄)₂SO₄ ATPS used here have a phase ratio of 8:10 and a tie-line length of about 28w/w%.

Figure 7. Schematic view of two working tie lines for the ATPE process operation. P is operation point for the primary ATPE step, and A and B are top and bottom phases; Q, C and D are for the second ATPE step and have the same meanings as the corresponding P, A and B. Enzyme is recovered in B.

concentration in the bottom phase is very low (<1.0 w/w%).

Complementary, the salts of high concentration (about 1 M) and some remaining bulk proteins of low molecular weight (lower than 14000 Dalton) in the bottom enzyme phase were further removed by dialysis. No enzyme loss was found in this step, and an additional purification with a factor of about 1.3 was also obtained. This corresponds to a total purification factor of about 1.7 for the proposed scheme. As to the dilution effect in dialysis step (see Table 2), standard membrane filtration device can be used to avoid it. In addition, the salt recycling is also possible by crystallizing salts from the bottom phase (point D in Figure 7, salt concentration higher than 27 w/w%) of the second ATPE step, as also shown in Figure 6. Even though the salt circulation has not been studied in this paper, it is from theoretical calculation expected that more than 60% of salt can be recovered. All these benefit the lowering of operational costs.

Membrane filtration can also be used to concentrate the supernatant. Harsa (1992) reported the use of an ultra-filtration step with quoted 20000 MW cut-off membrane. However, more than 20% of the enzyme was lost to permeate, presumably due to the less than perfectly sharp cut off. Comparing with membrane filtration, ion-exchange and other multi-step schemes, the extraction scheme proposed here has its own advantages of low capital investment and operating costs, easy scale-up and high enzyme recovery.
Conclusions

The partitioning experiments of endo-polygalacturonase in PEG-(NH₄)₂SO₄ and PEG-potassium phosphate (pH=7) ATPS were extensively investigated, and the influencing factors such as PEG molecular weight and concentration, pH and phase volume ratio of the ATPS were exploited. Both endo-polygalacturonase and total protein in the clarified broth were predominately in the bottom phase of PEG-salt ATPS, and most partitioning coefficient data of enzyme were lower than 0.1. pH shows little influence on the partitioning of endo-polygalacturonase, while PEG molecular weight and concentration and phase volume ratio of the systems become important. The ion-exchange separation of endo-PG with a purification factor of only 2.3 revealed high enzyme protein content in the total protein of the fermentation broth, which favors the concentration using ATPE. In addition to the partitioning experiments, a simple scheme consisting of an ATPE process with polymer recycling and a dialysis was also proposed to recover the endo-polygalacturonase from its clarified broth. An enzyme recovery up to 95% and an about five-fold to eight-fold increase in enzyme concentration with a purification factor of 1.25 were realized using optimized ATPE process. More than 95% PEG was recycled with no reduction in partition factor. The dialysis step is used to remove salts in the bottom enzyme-rich phase and can further remove some small molecular weight bulk protein. This achieves a total purification factor of about 1.7 for the proposed scheme. The scheme proposed here can be scaled up for producing commercial endo-polygalacturonase.

Acknowledgment

You-Ting Wu and Martinha Pereira were financially supported by the Praxis XXI program of FCT (Fundação para a Ciência e Tecnologia) of Portugal. The work was from project CEC (INCO-DC) with the contract number ERB IC18 CT97 0182.


Address for correspondence: Professor José Teixeira, Centro de Engenharia Biológica-IBQF, Universidade do Minho, Braga 4710, Portugal. Tel.: (351) 253-604406; Fax: (351) 253-678986; E-mail: jateixeira@deb.uminho.pt

References