Recovery of human interferon alpha-2b from recombinant Escherichia coli using alcohol salt based aqueous two-phase system

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A B S T R A C T

The purification of intracellular human recombinant interferon-alpha2b (IFN-α2b) from *Escherichia coli* (E. coli) was studied using alcohol/salt aqueous two-phase system (ATPS). The influences of nine biphasic systems comprising alcohol-based top phase (ethanol, 1-propanol and 2-propanol) and salt-based bottom phase (ammonium sulfate, di-potassium hydrogen phosphate and monosodium citrate) on IFN-α2b purification were studied. The results showed that the optimum condition for purification of IFN-α2b was achieved in ATPS composed of 18% (w/w) 2-propanol with 22% (w/w) ammonium sulfate in the presence of 1% (w/w) sodium chloride (NaCl). The purified IFN-α2b recorded a purification factor (P) of 16.24 with the yield of 74.64%.

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1. Introduction

The interferon (IFN) is glycoproteins which produced by cell’s immune system in order to eliminate the existence of pathogens. It was first identified in 1957 by Isaacs and Lindenmann [1]. When the viruses enter or invade an individual cell body, the infected cell will respond by producing IFN molecules and releasing it to the neighboring cells. Then, IFNs will react with specific receptors of the neighboring cells to activate the cytoplasmic signals that initiate cellular defensive actions [2,3]. The human IFN can be divided into three major types: alpha, beta and gamma. The alpha type IFN, IFN-α2b was studied in this paper. It was found that IFN-α2b can be effective in treating many diseases, e.g., Hairy cell leukemia, chronic myelogenous leukemia, Condylar acuminat and Chronic hepatitis B [4,5]. As the market of IFN-α2b will continue to grow at a fast pace, the development of an efficient and a cost-effective recovery process is a crucial strategy in IFN production as it will help to reduce the cost of production significantly [6].

The alcohol/salt aqueous two-phase system (ATPS) was first studied by Greve and Kula in 1991 [7]. The alcohol/salt ATPS is extracted via alcohol/salt ATPS can be easily recovered by removal of alcohol using evaporation method [8,9]. Besides, alcohol/salt ATPS offers other advantages like low toxicity to environment and inexpensive cost [10] compared to conventional ATPS, which is used of unrecovered polymer, for example, poly-ethylene-glycol (PEG) for the purification and recovery of biomolecules. A lot of purification studies based on alcohol/salt ATPS are available nowadays, for example, the glycyrrhinin extraction from *Glycyrrhiza uralensis Fisch* using alcohol/salt ATPS achieved a 92% yield of glycyrrhinin with purification factor (P) of 2.6 [9]. In addition, it has also been applied for the extraction of other biological products such as nucleic acids [12], salvianolic acid B [10], betalains [13] and geniposide [14]. The partition of lipase from *Burkholderia pseudomallei* in alcohol/salt ATPS has been reported recently [11]. In the study, the purified lipase from *B. pseudomallei* achieved P of 13.5 folds. However, up-to-date no study has been attempted for recovery of IFN-α2b using alcohol/salt ATPS from *E. coli*. ATPS operated challenged with *E. coli* feedstock would be expected to differ from *B. pseudomallei* feedstock [11]. In this study, the recovery of IFN-α2b using alcohol/salt ATPS from *E. coli* was performed to validate the feasibility and applicability of the system in purification of IFN-α2b. It was found that the yield value (74.64%) of IFN-α2b obtained in this alcohol/salt-based ATPS study was higher than that achieved by previous IFN-α2b purification literature reported using PEG/salt ATPS (40.70%) [15]. Partitioning of target protein into one specific phase can be achieved by manipulating the ATPS properties, the ATPS variables such as system consist of...
different alcohols and salts, different concentration of alcohol/salt ATPS, and addition of sodium chloride (NaCl) were investigated in this study. The purpose of this study is to present a simple and effective method of purifying IFN-α2b via alcohol/salt ATPS from E. coli crude extract.

2. Materials and methods

2.1. Materials

Ammonium sulfate and monosodium citrate were sourced from Sigma Aldrich (St. Louis, USA). Ethanol, 1-propanol, 2-propanol and di-potassium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). Protein assay kit was obtained from Bio-Rad (California, USA). All chemicals used in this paper are analytical grade.

2.2. Bacterial strains and plasmid

Recombinant E. coli K12 culture, Rosetta-gami2 (DE3) strain which was chosen for IFN-α2b producing host cell as it combines the advantageous features of Origami and Rosetta, which will enhance the disulfide bond formation and expression of eukaryotic proteins. IFN-α2b gene was inserted into pET26b plasmid which consists the resistance gene in opposition to kanamycin, this recombinant plasmid was then inserted into E. coli Rosetta-gami2 (DE3) strain with chloramphenicol resistance gene.

2.3. Fermentation of recombinant E. coli

Inoculum was prepared by incubating the recombinant E. coli in 250 mL baffled shake flasks containing 50 mL Terrific Broth (TB) with 34 mg/L chloramphenicol and 30 mg/L kanamycin. The inoculum was incubated at 37 °C and agitated at 250 rpm for 16 h in an incubator shaker (Certomat® BS-1 B. Braun, Germany). After the recombinant E. coli culture inoculated with 8% (v/v) inoculum, the culture was then incubated at 37 °C, with agitation speed at 250 rpm for 4 h. The IFN-α2b production was then induced by adding 1 mM isopropyl-β-D-thio-galactoside (IPTG, Cat. No. 420322, Calbiochem). Once the IPTG added, the temperature was changed to 30 °C with agitation speed at 250 rpm, the cells were then harvested after 8 h and stored at −20 °C until further processing.

2.4. Cell disruption

Osmotic shock was performed to rupture the recombinant E. coli culture cell body, therefore releasing the periplasmic IFN-α2b contained within. The cell pellets were suspended in a buffer solution comprising 20% (w/v) sucrose, 0.03 M Tris–HCl, 5 mM sodium EDTA (pH 8.0). The mixture was rigorously shaken for 5 min to enhance osmotic shock process. Subsequently, the mixture was centrifuged for 10 min at 4000 rpm and 4 °C. Next, the cell pellets were re-suspended and shaken rapidly in cold ultra-pure for 10 min. The mixture was centrifuged again for 10 min at 4000 rpm and 4 °C. The supernatant from centrifuged mixture was collected.

2.5. ATPS

The predetermined quantities of alcohol and dissolved salts were prepared in a 15 mL centrifugal tube. The 1 g of crude extract was then added into the mixture to make the ATPS to reach a final total weight of 10 g. The phase system was mixed thoroughly by gentle agitation and then subjected to centrifugation at 4000 rpm for 10 min to speed up the phase separation. After the phase separation, the top and bottom phases were collected, and then the concentrations of total protein and IFN-α2b within the system were analyzed. It was always not easy to evaluate the degree of purification by monitoring partition coefficient of IFN-α2b only. Therefore, the Pt (top phase) of the ATPS was evaluated as well (Fig. 1). All the experiments were carried out for three times (triplicates) at room temperature. The phase diagrams associated with alcohol/salt systems were referred from previous literature reported by Ooi et al. [11].

2.6. Assay of IFN-α2b

IFN-α2b was quantified by employing the biochip and Fully Automated Surface Plasma Resonance Detection System (BIAcore 3000, GE Healthcare, Sweden) as described in detailed by Ramanan et al. [16]. CM-5 Biosensor Chip (GE Healthcare, Sweden) was bound with anti-IFN mouse monoclonal antibody (MMHA-2, catalog No. 407290, Merck, USA). Samples were mixed with HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20) in 1:1 ratio and run through the biochip at 5 μl/min flow rate, using response unit (RU) as an output measurement. The IFN-α2b standards (Cat. No. ORP-16029, Affinity Bioreagents, USA) was calibrated using ELISA Kit (Cat. No. RPN2759, GE Healthcare, UK).

2.7. Protein analysis

The total protein concentration was analyzed by following the user manual of Bio-Rad protein assay kit. In this study, bovine serum albumin (BSA) was employed as standard protein.

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples from the crude extract and the top phase in ATPS were analyzed by using SDS–PAGE method. The crude extract sample was separated in an acrylamide gel consisting of 12% resolving gel and 4.5% stacking gel. Top phase and bottom phase sample were performed in acrylamide gel consisting of a 20% resolving gel and 4.5% stacking gel. Electrophoresis was run at 110 V and 36 mA for approximately 75 min. After the electrophoresis, the gel was stained and de-stained as described in a previous publication [17].

2.9. Calculation

The volume ratio (Vt) was defined as the ratio of volume in the top phase (Vt) to the volume of the bottom phase (Vb):

\[ V_t = V_t / V_b \]

Fig. 1. Purification of IFN-α2b in alcohol/salt ATPS. Schematic representation of the purification of IFN-α2b from fermentation broth. IFN-α2b is indicated as (●), impurities are indicated as (○) while cell is indicated as (∙).
The top-phase purification factor ($P_T$) is as the ratio of specific IFN-α2b concentration in the top phase of ATPS to the specific IFN-α2b concentration prior to the purification process. The $P_T$ was calculated according to the following equation:

$$P_T = \frac{(\text{IFN concentration/protein concentration})_{\text{in top phase}}}{(\text{IFN concentration/protein concentration})_{\text{in crude stock}}}$$

(2)

The partition coefficient ($K$) of the IFN was calculated as the ratio of the IFN concentration in two phases:

$$K = \frac{C_T}{C_B}$$

(3)

where $C_T$ and $C_B$ are the IFN concentration (mg/mL) in the top phase and bottom phase of ATPS, respectively.

Yield of IFN in top phase was determined using the following equation:

$$Y_T(\%) = \frac{100}{1 + [1/(V_R + K)]}$$

(4)

where $K$ is the partition coefficient and $V_R$ is the volume ratio.

3. Results and discussion

3.1. Selection of alcohol/salt ATPS

The results of $P_T$ and yield of IFN-α2b in different alcohol/salt ATPS were shown in Table 1. Phase diagrams of alcohols against different salts were referred to Ooi et al. [11]. These systems were chosen based on the respective phase diagrams which form different salts were referred to Ooi et al. [11]. These systems were chosen based on the phase diagrams of alcohol/salt systems. The results were expressed as the means of triplicate readings with an estimated error of ±5%.

A higher yield (%) was achieved in alcohol/K$_2$HPO$_4$ system with the tested alcohols but the respective $P_T$ of IFN-α2b were relatively lower. The $P_T$ results may have influenced by the varying level of pH, as the ammonium sulfate, monosodium citrate and dipotassium hydrogen phosphate pH systems were in the different range of 6.0–6.5, 8.0–8.3 and 9.0–9.5, respectively. Partitioning behavior of IFN-α2b is expected to be sensitive to pH level in ATPS since the IFN is charged molecules [18]. As the pH increase, the IFN yield was observed to be higher but $P_T$ become lower. This shows that contaminant proteins and IFN protein were partitioned toward top phase when pH increased; the charge of the proteins and ammonium sulfate systematically. As shown in Table 2, the highest $P_T$ of IFN-α2b was observed in 18% (w/w) 2-propanol and 22% (w/w) ammonium sulfate system, with $P_T$ of 15.45 and yield of 64.03%. In general, the $P_T$ of IFN-α2b generally decreased when the concentration of 2-propanol in the system increased. The increasing concentration of alcohol in the top phase had caused gradual dehydration of top phase and favoring the partition of IFN-α2b to the bottom phase [11]. Therefore, the 18% (w/w) 2-propanol with 22% (w/w) ammonium sulfate system was selected for next study.

3.2. Optimization of 2-propanol/ammonium sulfate ATPS

The $P_T$ of IFN-α2b in 2-propanol/ammonium sulfate ATPS was investigated by varying difference in concentrations of 2-propanol and ammonium sulfate systematically. As shown in Table 2, the highest $P_T$ of IFN-α2b was observed in 18% (w/w) 2-propanol and 22% (w/w) ammonium sulfate system, with $P_T$ of 15.45 and yield of 64.03%. In general, the $P_T$ of IFN-α2b generally decreased when the concentration of 2-propanol in the system increased. The increasing concentration of alcohol in the top phase had caused gradual dehydration of top phase and favoring the partition of IFN-α2b to the bottom phase [11]. Therefore, the 18% (w/w) 2-propanol with 22% (w/w) ammonium sulfate system was selected for next study.

<table>
<thead>
<tr>
<th>2-Propanol (% w/w)</th>
<th>Ammonium sulfate (% w/w)</th>
<th>$P_T$</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>20</td>
<td>6.29</td>
<td>87.58</td>
</tr>
<tr>
<td>16</td>
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<td>7.06</td>
<td>87.15</td>
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<td>24</td>
<td>8.51</td>
<td>86.70</td>
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<td>18</td>
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<td>7.87</td>
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<td>61.83</td>
</tr>
<tr>
<td>22</td>
<td>24</td>
<td>2.14</td>
<td>60.36</td>
</tr>
</tbody>
</table>

The optimization of 2-propanol/ammonium sulfate system was investigated by 15 different combination systems. The IFN-α2b was measured in terms of $P_T$ and yield. The results were expressed as the means of triplicate readings with an estimated error of ±5%.

<table>
<thead>
<tr>
<th>Concentration of NaCl % (w/w)</th>
<th>$P_T$</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.16</td>
<td>61.83</td>
</tr>
<tr>
<td>1</td>
<td>16.24</td>
<td>74.64</td>
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<tr>
<td>2</td>
<td>15.22</td>
<td>70.17</td>
</tr>
<tr>
<td>3</td>
<td>13.39</td>
<td>72.18</td>
</tr>
<tr>
<td>4</td>
<td>13.73</td>
<td>74.47</td>
</tr>
</tbody>
</table>

The 18% (w/w) 2-propanol with 22% (w/w) ammonium sulfate system was used to study the effect of NaCl addition; range of 0% (w/w) to 4% (w/w) NaCl concentration was assayed. The IFN-α2b was measured in terms of $P_T$ and yield. The results were expressed as the means of triplicate readings with an estimated error of ±5%.
3.3. The effect of NaCl on PF of IFN-α2b

The 18% (w/w) 2-propanol with 22% (w/w) ammonium sulfate system was chosen to study the effect of addition NaCl on PF, at the range of 0% (w/w) to 4% (w/w). According to Zalavsky, addition of neutral salt into ATPS will cause electrical potential difference between two phases and affect the partitioning of biomolecules, the electrostatic potential may driven negatively charged IFN-α2b partition to the top phase [20]. On top of that, the addition of NaCl increase the hydrophobic difference between both phases by decreasing the amount of bound water, this strengthen the interaction between the IFN-α2b’s hydrophobic surface area and the propanol’s hydrophobic chain thus leading IFN-α2b protein partition to the top phase which is alcohol-rich [15]. Based on the result, maximum PF (16.24) was achieved at 1% (w/w) NaCl (Table 3).

3.4. SDS–PAGE analysis of IFN-α2b

The purity of the highest recovered IFN-α2b was assessed with a SDS–PAGE (Fig. 2). The results demonstrated that the feedstock (lane 5) exhibited dense intensity of various bands, indicating a lot of proteins content. Meanwhile, the product recovered from the top phase of optimized alcohol/salt-based ATPS (lane 3) exhibited a major band fell at molecular weight of approximately 19 kDa, indicating the IFN-α2b was successfully partitioned to top phase. In general, IFN-α2b has molecular weight of about 19 kDa [21]. Several bands between 34 and 43 kDa were present in both top (lane 3) and bottom phases (lane 2). This explained by the fact that the salt-rich bottom phase was unable to attract all of the unwanted proteins into the bottom phase.

4. Conclusion

Recombinant E. coli IFN-α2b was successfully purified by 2-propanol/ammonium sulfate-based ATPS. The optimum condition was achieved in 2-propanol 18% (w/w) with ammonium sulfate 22% (w/w), and 1% (w/w) concentration of NaCl ATPS system. High PF of 16.24 with yield of 74.64% were achieved in single step downstream process. The comparison between different purification studies and the yield of IFN-α2b extraction achieved was shown in Table 4. With convincing results of high PF and yield, alcohol/salt ATPS could potentially be an alternative to the conventional ATPS for the purification of IFN-α2b from microbial sources.

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References