Purification of green Fluorescent protein overexpressed by a mutant recombinant *Escherichia coli*

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Abstract

Green Fluorescent protein was purified from sonicated recombinant *Escherichia coli* and its mutant obtained after exposure to UV light. The latter overexpresses green Fluorescent protein. The two-step procedure consisted of a two-phase aqueous extraction with PEG/salt and precipitation of the proteins from PEG phase by free Zn$^{2+}$. The recoveries of green Fluorescent protein were 73 and 83% in the cases of recombinant *E. coli* and its mutant, respectively. The corresponding fold purifications were 24 and 9, respectively. In both cases, the purified protein showed a single band on SDS-PAGE corresponding to 28 kDa.

Keywords: Green Fluorescent protein; Protein overexpression; Protein purification; Selective precipitation; Two-phase aqueous extraction

The green Fluorescent protein (GFP) is widely used as a Fluorescent label in biochemistry and biotechnology [1-3]. Given its large number of applications, it is not surprising that large number of efforts have been made for developing suitable bacterial expression systems [4-6] as well as for its purification [7-10]. Recently, we have described a purification procedure for obtaining GFP from a recombinant *Escherichia coli* by three-phase partitioning [11]. Subsequently, it was found that exposure of this recombinant *E. coli* containing GFP plasmid to UV light led to 37-fold higher production of GFP (MS sent for publication). This constitutively overexpressed GFP continued to be soluble and intracellular but was easier to extract inasmuch as it required less amount of sonication during extraction. The present work aims at exploiting this overexpression by developing a purification protocol which utilizes aqueous two-phase extraction followed by precipitation by simple metal ions. Aqueous two-phase extraction is a well-established bioseparation strategy which can be easily scaled up and can directly handle cellular homogenates without preclearing [12,13]. The technique of immobilized metal ion affinity chromatography (IMAC) utilizes the affinity of Cu$^{2+}$, Zn$^{2+}$, and Ni$^{2+}$ to specific residues on the protein surface [14]. The precipitation of protein by free metal ions has also been used [15-18]. Using these non-chromatographic techniques (aqueous two-phase extraction and precipitation with metal ions) in sequence, purification of GFP for both recombinant *E. coli* and mutant recombinant *E. coli* has been possible.

Materials

*Escherichia coli* DH5a, plasmid pUC18, and GFP gene were procured from Bangalore Genie, Bangalore, India. All media components were purchased from Hi Media Laboratories, Mumbai, India. Polyethylene glycol (PEG 6000) was from E. Merck, Mumbai, India. All other chemicals were of analytical grade.

Methods

Cloning and culture conditions

GFP gene was cloned in pUC18 plasmid with lac promoter and transformed in *E. coli* DH5a as described by Jain et al. [11]. The transformants were grown in LB (Luria broth) medium supplemented with ampicillin.
(100ng/ml). Inoculum was prepared by inoculating a loopful of transformed *E. coli* cells from plate into 10 ml LB medium followed by incubation at 37 °C with constant shaking at 120rpm for 12h. LB medium (100 ml) was inoculated and incubated at 37°C, 120rpm for 16h before harvesting the cells. A mutant of the recombinant *E. coli* strain containing GFP plasmid was prepared by UV induced random mutagenesis. This mutant is known to overexpress GFP constitutively (MS sent for publication).

Cells were harvested by centrifugation at 8000g for 10 min at 4 °C (2.03 g wet weight/L). The bacterial cell pellet (0.203 g wet weight) was suspended in 20 ml of 0.02 M Tris-HCl, pH 8.0, buVer (sonication buVer) containing 0.15M sodium chloride and 0.005 M EDTA. The crude extract was prepared by sonication of the cell suspension [11] and used without any centrifugation/preclarification.

**Fluorescence measurements**

The active GFP content was measured by Fluorescence at 510 nm (with excitation at 395 nm) on a Shimadzu RF-5000 spectroFluorometer with a band width of 5 nm and 1 cm path length in arbitrary units (AU) [7]. One AU is defined as the amount of protein producing change of Fluorescence emission intensity of one under above conditions.

**Estimation of protein**

Protein content was estimated by the dye binding method using bovine serum albumin as the standard protein [19].

**Aqueous two-phase partitioning**

The desired phase components were prepared in 10 ml of graduated tubes by adding 2 ml polyethylene glycol solution (22% w/v in 0.02 M Tris-HCl buVer, pH 6.5) and 2 ml of salt solution (10% K$_2$HPO$_4$ w/v and 14% w/v NaCl in 0.02 M Tris-HCl buVer, pH 6.5). After vortexing for a minute, two distinct phases were formed within 5 min at 25 °C. The crude extracts of GFP from recombinant (250 µl containing 70.2 AU) and mutant recombinant *E. coli* (100 µl containing 896 AU) types were added to the systems and vortexed for a minute. After 30 min, the top phase containing this GFP was removed by pipette. The upper PEG phase (2.2 ml for recombinant GFP and 2.1 ml for recombinant mutant GFP) and the lower salt phase (1.9 ml for recombinant GFP and 1.8 ml for recombinant mutant GFP) were analyzed for Fluorescence and protein content.

**Precipitation of GFP with metal ions**

PEG phases containing GFP from recombinant *E. coli* and mutant recombinant *E. coli* types were subjected to metal ion precipitation with 2mM ZnCl$_2$ and vortexed gently. After incubating for 1 h at 25 °C, the solutions were centrifuged (8000g, 10 min) and supernatant was collected. The supernatant was checked for Fluorescence and protein content.

**Polyacrylamide gel electrophoresis**

SDS-PAGE of the samples using 12% gel was performed according to Laemmli [20] on a Genie gel electrophoresis unit (Bangalore Genie, Bangalore, India).

**Results and discussion**

The present work uses a mutant of recombinant *E. coli* as a source of constitutively overexpressed GFP. While the details of the genetic changes which cause this overexpression are being investigated, the organism is a good source for purifying GFP with an improved Fluorescence signal.

Li and Beitle [10] have recently shown that incorporation of salt improves selectivity of some two-phase extraction systems for partitioning of GFP. Fig. 1 shows that 14% (w/v) sodium chloride when incorporated in the system gave improved partitioning of GFP in PEG phase. It is rightly mentioned that presence of salt promotes hydrophobic interactions of proteins with PEG [10]. The fact that this operated favorably and selectively for GFP in their and present case is not surprising if we consider that GFP has been shown to bind to the hydrophobic column of phenyl Sepharose by Yakhnin et al. [7]. It was found that increasing the sodium chloride concentration to 16% did not improve the partitioning and it was not possible to incorporate sodium chloride beyond this concentration. Both Li et al. [9] and Li and
Beitle [10] have shown that GFP could be purified by an immobilized metal affinity chromatography step. Figs. 2-4 show precipitation of GFP by varying concentrations of Cu$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$, respectively. While it was possible to precipitate contaminating proteins in all the three cases to a varying degree, GFP was predominantly found to stay in the supernatant. Significant fold purification was also observed in all the cases but it was better with Zn$^{2+}$ and Ni$^{2+}$. It was decided to work further with Zn$^{2+}$ and Ni$^{2+}$. Fig. 3 shows that use of 5 mM Ni$^{2+}$ gave 100% recovery of GFP and 4.1-fold purification. Fig. 4 shows that use of 5 mM Ni$^{2+}$ gave 100% recovery of GFP and 4.1-fold purification.

Fig. 2. Precipitation of GFP from recombinant E. coli using varying concentrations of Cu$^{2+}$ ions: crude GFP extract (1 ml) in 0.02 M Tris-HCl buffer, pH 7.0, was incubated with various concentrations of CuSO$_4$. After centrifugation, the supernatant and precipitates were checked for fluorescence and protein. The fluorescence of initial crude extract is taken as 100%. Each experiment was carried out in duplicate and the difference in the individual results in each set of corresponding experiments was less than 5%. Bars in the histogram represent the percentage recovery of GFP in supernatant and the continuous curve drawn across the histogram represents the fold purification in the supernatant. Fold purification in this work represents the change in specific activity, relative to specific activity of crude extract.

Fig. 3. Precipitation of GFP from recombinant E. coli using varying concentrations of Ni$^{2+}$ ions: the precipitation was carried out as described in the legend of Fig. 2. The symbols used are the same as in Fig. 2. Fold purification in this work represents the change in specific activity, relative to specific activity of crude extract.

Fig. 4. Precipitation of GFP from recombinant E. coli using varying concentrations Zn$^{2+}$ ions: the precipitation was carried out as described in the legend of Fig. 2. The symbols used are the same as in Fig. 2. Fold purification in this work represents the change in specific activity, relative to specific activity of crude extract.

Fig. 5. Precipitation of GFP from recombinant E. coli using Zn$^{2+}$ with varying pH: 1 ml of crude GFP extract in buffers with different pH (Mes-NaOH buffer for pH 5.0-6.0 and Tris-HCl buffer for pH 6.5-8.0) was incubated with 1 and 2 mM ZnCl$_2$. After centrifugation, the supernatant and precipitates were checked for fluorescence and protein. The symbols used are the same as in Fig. 2. Each experiment was carried out in duplicate and the difference in the individual results in each set of corresponding experiments was less than 5%. Fold purification in this work represents the change in specific activity, relative to specific activity of crude extract.
ing pH during precipitation while using 1 and 2 mM Zn\textsuperscript{2+}. Fig. 6 shows the eVect of varying pH during precipitation with 5 mM of Ni\textsuperscript{2+}. Use of 2 mM Zn\textsuperscript{2+} at pH 6.5 is found to be the best condition resulting into 83\% recovery with 7.6-fold puri\textit{W}cation.

These results showed that each of the two nonchro\-matographic steps (aqueous two-phase extraction and precipitation with metal ions) could lead to partial puri\textit{W}cation of GFP. As two-phase aqueous extraction can be carried out directly without any centrifugation or preclari\textit{W}cation step, it was decided to use this as a Wrst step in exploring the puri\textit{W}cation strategy based upon combination of two-phase extraction and metal precipitation.

Table 1 shows the result of such a strategy with GFP from recombinant \textit{E. coli}. 73\% GFP could be recovered with 24-fold puri\textit{W}cation. The puri\textit{W}ed protein was found to show a single band on SDS-PAGE (Fig. 7A) with a molecular weight of 28kDa which was in agreement with the reported value [8]. Table 2 shows the corre-

Fig. 6. Precipitation of GFP from recombinant \textit{E. coli} using Ni\textsuperscript{2+} with varying pH: 1 ml of crude GFP extract in bu\textit{Ver} with di\textit{Ver}ent pH (Mes-NaOH bu\textit{Ver} for pH 5.0-6.0 and Tris-HCl bu\textit{Ver} for pH 6.5-8.0) was incubated with 5 mM NiSO\textsubscript{4}. After centrifugation, the supernatant and precipitates were checked for Xuorescence and protein. The symbols used are the same as in Fig. 2. Each experiment was carried out in duplicates and the di\textit{Ver}ence in the individual results in each set of corresponding experiments was less than 5\%. Fold puri\textit{W}cation in this work represents the change in speci\textit{W} activity, relative to speci\textit{W} activity of crude extract.

Table 1

<table>
<thead>
<tr>
<th>Puri\textit{W}cation step</th>
<th>GFP Xuorescence (AU)</th>
<th>Protein (ng)</th>
<th>Recovery of GFP (%)</th>
<th>Fold puri\text{W}cation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>70</td>
<td>600</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Aqueous two-phase partitioning (PEG phase)</td>
<td>55</td>
<td>76</td>
<td>79</td>
<td>6</td>
</tr>
<tr>
<td>ZnCl\textsubscript{2} precipitation (using PEG phase)</td>
<td>51</td>
<td>18</td>
<td>73</td>
<td>24</td>
</tr>
</tbody>
</table>

Cell broth (1.67 ml) was centrifuged and bacterial cell pellet (4.72 mg) was suspended in 0.25 ml of sonication bu\textit{Ver}. After sonication, it was introduced to the aqueous two-phase system. The separation of two phases and precipitation was done as described in the Methods section. All experiments were performed in duplicate and the di\textit{Ver}ence in the readings in the duplicates was less than 5\%.
Fig. 7. (A) SDS-PAGE pattern of GFP from recombinant *E. coli*: electrophoresis was carried out using a 12% crosslinked polyacrylamide gel. Lane M, marker proteins; lane 1, crude extract; lane 2, PEG phase; lane 3, salt phase; and lane 4, purified GFP after metal precipitation. (B) SDS-PAGE pattern of GFP from mutant recombinant *E. coli*: lane M, marker proteins; lane 1, crude extract; lane 2, PEG phase; lane 3, salt phase; and lane 4, purified GFP after metal precipitation. Same amount of protein (25 μg) was applied in each lane.

Table 2
Purification of green fluorescent protein (GFP) from mutant of recombinant *E. coli* by aqueous PEG/salt two-phase partitioning followed by precipitation with 2 mM Zn\(^{2+}\) at pH 6.5

<table>
<thead>
<tr>
<th>Purification step</th>
<th>GFP Fluorescence (AU)</th>
<th>Protein (μg)</th>
<th>Recovery of GFP (%)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>896</td>
<td>455</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Aqueous two-phase partitioning (PEG phase)</td>
<td>895</td>
<td>190</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>ZnCl(_2) precipitation (using PEG phase)</td>
<td>745</td>
<td>40</td>
<td>83</td>
<td>9</td>
</tr>
</tbody>
</table>

Cell broth (0.5 ml) was centrifuged and bacterial cell pellet (1.0 mg) was suspended in 0.10 ml sonication buffer. After sonication, it was introduced to the aqueous two-phase system. The separation of two phases and precipitation was done as described in the Methods section. All experiments were performed in duplicate and the difference in the readings in the duplicates was less than 5%.

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References


