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Purification of recombinant green fluorescent protein by three-phase partitioning

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Abstract

The technique of three-phase partitioning (TPP) was used to purify the green fluorescent protein (GFP) in a single step. TPP uses a combination of ammonium sulphate and *tert*-butanol to precipitate proteins from their crude extracts. In the first round of TPP with 20% ammonium sulphate saturation at the ratio of crude to *tert*-butanol 1:1 (v/v), most of the GFP remains in the lower aqueous phase. When subjected to a second round of TPP with 60% ammonium sulphate saturation at the ratio of crude to *tert*-butanol 1:2 (v/v) gives 78% recovery of GFP with a 20-fold purification. The sodium dodecyl sulphate–polyacrylamide gel electrophoretic (SDS–PAGE) analysis of purified preparation shows single band. The fluorescence excitation and emission spectra agreed with values reported in literature.

Keywords: Three-phase partitioning; Green fluorescent protein; Proteins

1. Introduction

The green fluorescent protein (GFP) from the jellyfish *Aequorea* is widely used in cell biology and biotechnology [1,2]. While various suitable bacterial expression systems are available for producing this protein in large amounts, an efficient purification protocol is not available. Yakhnin et al. [3] described a four-step purification procedure with rather poor 36% yield. Narahari et al. [4] used two chromatofocussing steps to obtain a 50-fold increase in purity with 60% recovery. Use of immobilized metal affinity chromatography by Li et al. [5] is perhaps the most convenient procedure. A yield of 86% with a 30-fold purification was reported in this case. All these methods (as well as some earlier attempts) involved column chromatography as at least one of the steps. It is generally agreed that nonchromatographic methods are more easily scalable [6]. In the present work, a one-step nonchromatographic purification procedure for obtaining GFP of high purity with a good recovery is described. The purification utilizes three-phase partitioning (TPP) [7].

Three-phase partitioning has been successfully used in our laboratory for purification of pectinase [8], phospholipase D [9] and protease/amylase inhibitor [10,11], etc. The approach consists of adding ammonium sulphate (at below "salting-out" level for proteins) and *tert*-butanol to the crude extract of a protein. In less than an hour, three phases are formed. The upper *tert*-butanol rich phase generally removes any lipid or hydrophobic material. The interfacial phase is the protein precipitate. The lower aqueous phase contains remaining protein(s) and cell debrii, etc. Varying ammonium sulphate concentration, relative volume of *tert*-butanol and temperature may result in the target protein appearing in the interfacial layer in a quite selective fashion. Sometime (as has been done in the present work), the first TPP removes the contaminant protein(s) and the aqueous phase containing most of the desired protein when subjected to a second round of TPP results in considerable purification of target protein.

2. Materials

Escherichia coli DH5α, plasmid pUC18 and GFP gene were procured from Bangalore Genie (Bangalore, India). All media components were purchased from Hi Media Labs. (Mumbai, India). Ammonium sulphate, *tert*-butanol

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and bovine serum albumin were purchased from E. Merck (Mumbai, India). All buffer salts and Coomassie Brilliant Blue G-250 were obtained from Sisco Research Lab. (Mumbai, India).

3. Methods

3.1. Cloning and culture conditions

GFP gene was cloned in pUC18 plasmid according to the vendor's instructions [12]. Transformation of $CaCl_2$ treated *E. coli* DH5α cells was carried according to Sambrook et al. [13]. Host *E. coli* DH5α cells were grown in Luria broth medium (pH 7.5) containing tryptone (10 g), yeast extract (5 g), sodium chloride (5 g) (LB medium). The strain was maintained on agar slants by periodic transfer. The transformants were grown in LB medium supplemented with ampicillin (100 μg/ml). Inoculum was prepared by inoculating a loopful of transformed *E. coli* cells from plate into 10 ml of LB medium followed by incubation at 37 °C with constant shaking at 120 rpm for 12 h. LB medium (100 ml) was inoculated and incubated at 37 °C, 120 rpm for 16 h before harvesting the cells.

3.2. Preparation of cell extract

Cells were harvested by centrifugation at 8000 × *g* for 10 min at 4 °C. The bacterial cell pellet (0.283 g wet mass) was suspended in 15 ml of 0.02 M Tris–HCl pH 8.0 buffer containing 0.15 M sodium chloride and 0.005 M EDTA. The cells were homogenized by sonication (in an ultrasonic homogenizer Model 150 W from MSE, Sussex, UK) in an ice-water bath for three cycles of 90 s each (15 s pulse with 5 s interval). The homogenate was centrifuged at 8000 × *g* for 10 min at 4 °C. Release of protein was monitored in the supernatant after each cycle. Each cycle consisted of suspension of cell pellet in the buffer, sonication and centrifugation. No fluorescent material was observed in the supernatant after the third cycle.

3.3. 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 and 1 cm path length in arbitrary units [3].

3.4. Estimation of protein

Protein content was estimated by the dye binding method using bovine serum albumin as the standard protein [14].

3.5. Three-phase partitioning of GFP

The crude extract (2 ml containing 226 arbitrary units of GFP) in 50 mM Tris–HCl pH 7.0 was saturated with 20% ammonium sulphate at 25 °C and vortexed gently to dissolve the salt, followed by the addition of *tert*-butanol (2 ml). The mixture was vortexed gently and allowed to stand for 1 h. After this, the mixture was centrifuged (2000 × *g* for 5 min) and the formation of three phases (upper organic phase, middle interfacial precipitate and lower aqueous phase) was observed. The upper *tert*-butanol layer was removed carefully with a Pasteur pipette. After this, the lower aqueous layer was removed by piercing the interfacial precipitate layer using another Pasteur pipette. The interfacial precipitate containing GFP was collected and dissolved in 1 ml of 50 mM Tris–HCl pH 7.0. The dissolved precipitate is dialyzed before protein estimation. In the first round GFP remained in the aqueous phase, so the aqueous layer was subjected to second round of TPP.

3.6. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of the samples using 12% gel was performed according to Laemmli [15] on a Genie gel electrophoresis unit (Bangalore Genie).

4. Results and discussion

Fig. 1 shows the partition of GFP into the interfacial layer and the lower aqueous phase with different percent saturation of ammonium sulphate while using *tert*-butanol equal in volume to the starting crude extract. Fig. 2 gives such data obtained when the ratio of the *tert*-butanol to the crude extract

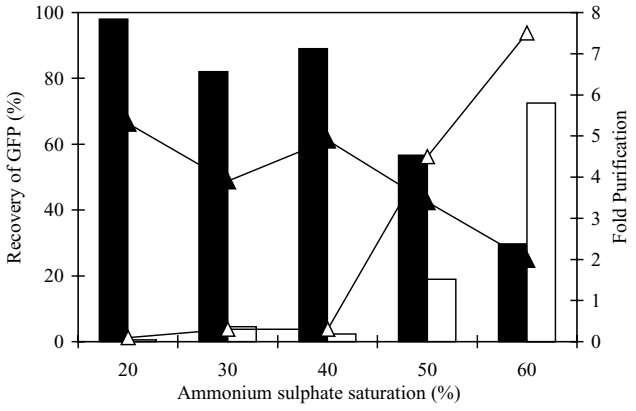

Fig. 1. Three-phase partitioning of GFP at different saturations of ammonium sulphate at 25 °C: 2 ml of crude extract (226 arbitrary units of GFP) in 50 mM Tris–HCl pH 7.0 was saturated with 20% ammonium sulphate, followed by addition of 2 ml *tert*-butanol. The three phases formed were collected separately and analyzed as described in Section 3. Each experiment was carried out six times and the difference in the individual results (in terms of percent yield and specific activities) in each set of corresponding experiments was less than 3%. (■) Recovery of GFP in aqueous phase, (□) recovery of GFP in interfacial precipitate, (▲) fold purification in aqueous phase, (△) fold purification in interfacial precipitate.

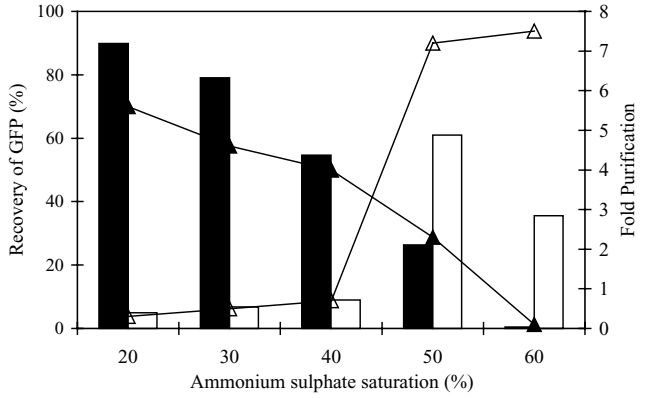

Fig. 2. Three-phase partitioning of GFP at different saturation of ammonium sulphate at 25 °C: 2 ml of crude extract (226 arbitrary units of GFP) in 50 mM Tris–HCl pH 7.0 was saturated with 20% ammonium sulphate, followed by addition of 4 ml *tert*-butanol. The three phases formed were collected separately and analyzed as described in Section 3. Each experiment was carried out six times and the difference in the individual results (in terms of percent yield and specific activities) in each set of corresponding experiments was less than 3%. (■) Recovery of GFP in aqueous phase, (□) recovery of GFP in interfacial precipitate, (▲) fold purification in aqueous phase, (△) fold purification in interfacial precipitate.

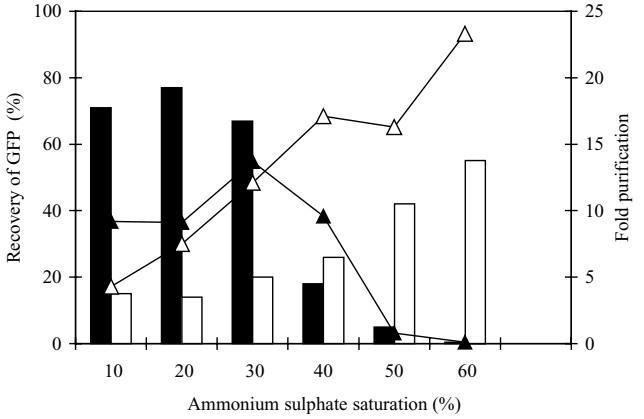

Fig. 3. Second stage TPP of aqueous phase of 20% ammonium sulphate saturation at 25 °C (where crude to *tert*-butanol ratio is 1:1):1.6 ml of aqueous phase (221 arbitrary units of GFP) in 50 mM Tris–HCl pH 7.0 was saturated with 60% ammonium sulphate, followed by addition of 1.6 ml of *tert*-butanol. The three phases formed were collected separately and analyzed as described in Section 3. Each experiment was carried out 4 times and the difference in the individual results (in terms of percent yield and specific activities) in each set of corresponding experiments was less than 3%. (■) Recovery of GFP in aqueous phase, (□) recovery of GFP in interfacial precipitate, (▲) fold purification in aqueous phase, (△) fold purification in interfacial precipitate.

was 2:1 (v/v). Use of 20% ammonium sulphate saturation with 1:1 ratio of crude extract to *tert*-butanol was found to be best conditions and that conditions gave 98% recovery of GFP in aqueous phase (as mentioned in arbitrary units). Similar percent saturation of ammonium sulphate and 1:2 ratio of crude extract to *tert*-butanol, 90% recovery in aqueous phase with 5.6-fold purification was also in the same range. However, as GFP was predominantly recovered in the aqueous phase in the first case, this makes it possible to subject the aqueous phase to a second round of TPP. Fig. 3 shows the results of the second round of TPP with the aqueous phase obtained from the first round of TPP. The maximum fold purification of 23-fold along with 56% recovery of GFP in interfacial phase was obtained with 60% ammonium sulphate saturation. During TPP, the temperature also has been found to play an important role [9]. The above TPPs were done at 25 °C. Table 1 shows the effect of varying temperature. The best results were obtained at 10 °C with 76% recovery in interfacial precipitate and 18-fold purification. However, the temperature at 25 °C and changing the ratio of crude extract to *tert*-butanol to 1:2 (at 25 °C) gave similar results: which were 78% recovery with 20-fold purification (Table 2). SDS–PAGE analysis (Fig. 4) of these two purified preparations obtained under the conditions of crude:*tert*-butanol ratios of 1:1 and 1:2 showed that both preparations are comparable in levels of purity. The estimated molecular mass (SDS–PAGE) was found to be 27 000 Da which agreed well with the reported molecular mass of the protein [4]. Thus any of the two procedures utilizing the two cycles of TPP constitute a fairly simple and efficient purification process for GFP.

Earlier work has shown that proteinase K underwent some structural changes as a result of TPP. Considering the utility of GFP as a 'fluorescent label', it was thought prudent to check whether its fluorescence properties have changed. Fig. 5 shows excitation and emission spectra of purified GFP. It can be seen that the excitation and emission spectra are identical in general specifically with respect to λ_{max} with what is reported in the literature for wild type GFP [1].

Purification costs constitute a fairly large percentage of the overall production costs of proteins/enzymes. TPP is considered a fairly economical process since it does not utilize

Table 1
Effect of temperature on the second round of TPP (60% ammonium sulphate saturation, the ratio of crude to *tert*-butanol 1:1 (v/v)) of lower aqueous phase of first round TPP (20% ammonium sulphate saturation at 25 °C with crude to *tert*-butanol ratio 1:1 (v/v))

Temperature (°C)	GFP in interfacial precipitate (arbitrary units)	Protein (mg) in interfacial precipitate	Recovery of GFP (%)	Fold purification
4	122	0.07	54	17
10	172	0.09	76	18
25	126	0.05	56	23
37	129	0.06	57	21

Recovery of GFP and fold purification is calculated with respect to crude extract.

Table 2
Effect of ratio of crude to *tert*-butanol on the second round of TPP (60% ammonium sulphate saturation, at 25 °C) of lower aqueous phase of first round of TPP (20% ammonium sulphate saturation at 25 °C with crude to *tert*-butanol ratio 1:1 (v/v))

Ratio of crude to *tert*-butanol (v/v)	GFP in interfacial precipitate (arbitrary units)	Protein (mg) in interfacial precipitate	Recovery of GFP (%)	Fold purification
1:1	126	0.05	56	23
1:1.5	74	0.05	33	14
1:2	177	0.09	78	20

Recovery of GFP and fold purification is calculated with respect to crude extract.

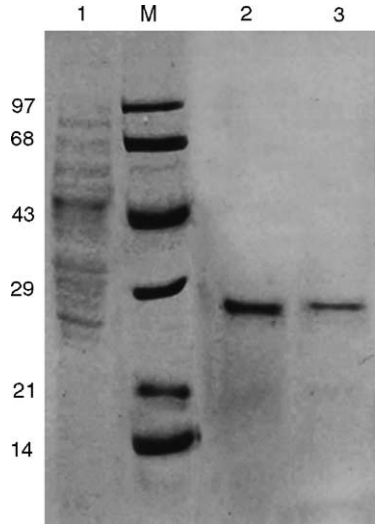

Fig. 4. SDS–PAGE pattern of purified GFP. Lanes: 1, crude extract of GFP (25 μg); 2, 20-fold purified GFP (25 μg); 3, 23-fold purified GFP (15 μg); M, marker proteins (25 μg). Values: $M_r \times 10^{-3}$.

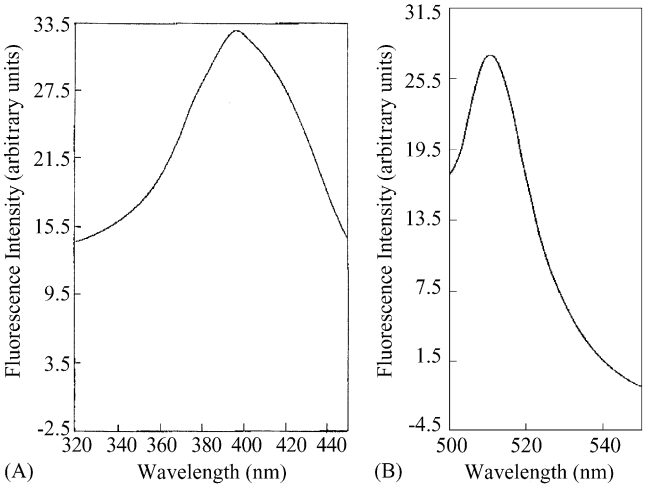

Fig. 5. Fluorescence spectra of GFP. (A) Excitation spectra (at an emission wavelength of 510 nm). (B) Emission spectra (at an excitation wavelength of 395 nm). The spectra were recorded by using 0.014 mg of purified protein in 3 ml of 0.02 M Tris–HCl buffer pH 8.0.

large amounts of salt (always less than used in a 'salting-out' protocol during ammonium sulphate precipitation) [7]. The availability of this simple purification strategy should help in production of GFP in a more efficient and economical way.

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