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Green Fluorescent Protein Purification by Organic Extraction[1]

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Green fluorescent protein (GFP) is widely used as an excellent reporter molecule in biochemistry and cell biology. Some biochemical and immunological assays require high-purity GFP. However, the majority of current procedures for GFP purification include multiple time-consuming chromatography steps with a low yield of the desired product or require tag-containing proteins. An alternative method is described for the GFP purification without affinity extensions using organic extraction yielding a highly homogeneous protein indistinguishable in spectroscopic properties from that purified by previous methods. © 1998 Academic Press

Unlike other bioluminescent reporters, green fluorescent protein (GFP)[3] from jellyfish *Aequorea victoria* contains a chromophore intrinsic to its primary structure and does not require any substrates or cofactors for fluorescence. As a reporter protein GFP has been expressed into a variety of cells and organisms. Several improved fast-folding GFP mutants have been described recently for expression in *Escherichia coli* (1–4). These bacterial expression systems are suitable, theoretically, for the preparation of GFP in large quantities. However, we met substantial difficulties in attempts to purify GFP despite the high expression level of this protein in *E. coli*. The main problem was the loss of most of the GFP during multiple chromatography steps. We found an alternative pathway for GFP purification. The two main steps in our procedure are extraction of GFP with ethanol into the organic phase and subsequent protein reextraction into the aqueous phase. After the extraction steps GFP becomes almost homogeneous and one chromatography step is used to purify GFP from nonprotein substances. The tested features of GFP obtained according to our procedure are nearly identical to those described previously for this protein.

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[3] Abbreviations used: GFP, green fluorescent protein; TEA, triethanolamine; CD, circular dichroism.

MATERIALS AND METHODS

Bacterial Strain, Plasmid, and Growth Conditions

The plasmid pBAD-GFP expresses the fast-folding GFP mutant containing a wild-type chromophore under control of the arabinose-induced promoter araBAD (1,5). The JM109 *E. coli* strain was used as a host. The culture was grown in Terrific Broth (Sigma) with 100 mg/liter ampicillin. Two hundred and fifty milliliters of the media was inoculated with 2.5 ml of fresh overnight culture and grown at 37°C to OD_{600} = 0.5 and then induced with arabinose to 0.08%. Growth was continued for 6 h at 25°C after induction. Bright green cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C and stored at −70°C before use.

Isolation of Soluble Protein Fraction

The bacterial pellet was suspended in 60 ml of Buffer A (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA) without any protease inhibitors. The cells were disrupted by sonication in an ice-water bath for 15 min (1-s pulses with 4-s intervals). Insoluble material without green fluorescence was removed by centrifugation at 4°C for 15 min at 16,000 rpm (30,000*g*). Triethanolamine base (TEA) and dry ammonium sulfate were added to the bright green supernatant to final concentrations of 100 mM and 1.6 M, respectively [938 μl of TEA and 14.9 g $(NH_4)_2SO_4$ per 61 ml of the solution]. Such an ammonium sulfate solution was considered as a 40% saturation (saturation is about 4 M). After incubation in ice for 1 h the precipitated proteins were removed by centrifugation for 20 min at 6000 rpm at

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4°C. Dry ammonium sulfate [13.4 g $(NH_4)_2SO_4$ per 67 ml of the solution] was added to the green supernatant to a final concentration of 2.8 M (70% saturation).

Organic Extractions

The entire suspension in 70% ammonium sulfate (with both the supernatant and the precipitate) was twice extracted with a one-fourth and a 1/16th volume of ethanol, respectively, by vigorous shaking for 1 min. The aqueous and ethanol phases were separated by centrifugation for 5 min at 4000 rpm at room temperature. GFP extraction into the organic phase was clearly observed by the bright green fluorescence of the upper phases in daylight or upon UV illumination. Both ethanol phases were collected carefully to avoid disturbance of the interphase.

A one-fourth volume of *n*-butanol was added to the combined ethanol extract. After vigorous shaking for 30 s the formed phases were separated by centrifugation as indicated above. At this step GFP changed almost completely into the lower aqueous phase. The upper organic phase was discarded and an equal volume of chloroform was added to the aqueous phase. After extraction for 30 s the phases were separated as previously performed. The upper aqueous phase containing GFP was collected and the lower organic phase/interphase was extracted with an equal volume of 30% ammonium sulfate saturation in water. Both aqueous phases were combined and considered as the aqueous $(NH_4)_2SO_4$ extract of GFP.

Phenyl-Sepharose Chromatography

The GFP extract from the previous step was loaded directly on 9 ml of phenyl-Sepharose (Pharmacia), equilibrated with a 20% saturation of ammonium sulfate in Buffer B (20 mM Tris–HCl, pH 8.0, 1 mM EDTA). After extensive washing with the starting buffer the GFP was eluted with 3.3 column volumes (30 ml) of a $(NH_4)_2SO_4$ linear gradient from 20 to 0% saturation in Buffer B and then again with Buffer B. Purification was tested by absorbance at 280 nm and visually by moving the green area through the column. A single green peak was eluted at about zero $(NH_4)_2SO_4$.

Spectroscopy

To calculate the recovery of GFP during purification, the active GFP content was measured by fluorescence at 510 nm (with excitation at 395 nm) on the continuous-flow Jasco Intelligent 821-FP spectrofluorometer (Japan Spectroscopic Co.) in arbitrary units. Protein determinations were done by the method of Bradford (15).

Absorbance was measured on a Shimadzu UV-1601 spectrophotometer (Japan). To measure the GFP concentration, the absorption coefficients were determined as 1.0 and 1.1 ml/mg · cm for 280 and 395 nm, respectively. Fluorescence was measured on an Aminco SPF-l000cs spectrofluorometer (USA) at an angle of 90° to the incident light. The GFP concentration was 0.1 mg/ml. Between 0.0001 and 0.1 mg/ml the dependence of the fluorescence on the GFP concentration was linear (data not shown).

The circular dichroism (CD) spectrum was taken on a Jasco-600 spectropolarometer (Japan). The cell pathlength for recording the CD spectrum was 10 mm. The GFP concentration was 0.6 mg/ml. GFP is monomeric at concentrations below 2 mg/ml (9). All spectroscopic measurements were performed at 23°C.

RESULTS AND DISCUSSION

Expression of GFP

We used the fast-folding GFP mutant selected for efficient expression in *E. coli* (1). However, most of the GFP expressed at 37°C in our hands was aggregated into insoluble inclusion bodies with no associated fluorescence. The coexpression of GFP with the *E. coli* chaperones GroEL and GroES did not increase the proportion of soluble GFP (data not shown). In contrast, the drop of the cultivation temperature to 25°C before induction significantly increased the relative amount of soluble and active GFP (2,6). This “room” temperature with the prolonged cultivation was used for all our subsequent GFP cultivations. Expression level was the same in the range of the inductor (L-arabinose) concentrations (between 0.2 and 0.04%) and dropped threefold at 0.01% of arabinose, in good accord with Ref. (5). GFP was extremely resistant to trypsin and thermolysin proteolysis and no protection against proteases was provided at any steps of isolation of this protein.

Extraction of GFP with Ethanol

During an unsuccessful attempt to precipitate GFP from an ammonium sulfate solution with organic solvents, we found that GFP could be almost completely extracted into the ethanol phase. Several extraction conditions were tested. If the ammonium sulfate concentration was below 50% saturation (about 2 M), the ethanol became completely mixed with the salt solution without phase separation. With 50% ammonium sulfate the volume of the upper organic phase exceeded that of the aqueous phase. Ammonium sulfate concentrations of 60% saturation and higher were preferable since the ethanol phase volumes were lower and those of the aqueous phase changed less significantly after several extractions. Ammonium sulfate can be added both as the pH-adjusted saturated solution and in dry form. In the latter case, addition of the water-free triethanolamine base to a 100 mM final concentration was used to compensate for the

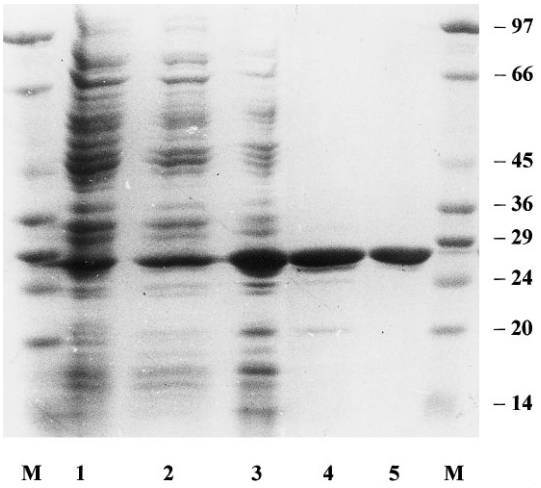

FIG. 1. Analysis of GFP purification by SDS–gradient polyacrylamide (10–20%) gel electrophoresis. The gel was stained by Coomassie blue G250. Lanes: M, molecular mass standards (kDa); 1, 30,000*g* supernatant after sonication; 2, 40% ammonium sulfate supernatant; 3, ethanol extract; 4, aqueous ammonium sulfate extract; and 5, peak after phenyl-Sepharose chromatography.

pH drop. Sometimes, after several rounds of extraction, the ammonium sulfate partly precipitated from the aqueous phase without any effect on the extraction efficiency. GFP partly precipitated at ammonium sulfate concentrations higher than 40% saturation and sometimes gave a bright green interphase during extraction. However, GFP could be easily extracted from the interphase by a little longer extraction (up to 3 min) with a new portion of ethanol. Other conditions had a minimal, if any, effect on extraction. The extraction process was visually tested by fluorescence over the UV transilluminator.

Ethanol was found to be the only tested organic solvent suitable for GFP extraction. GFP could not be extracted with isopropanol or acetonitrile, and methanol did not give a separate phase with ammonium sulfate solutions.

The ethanol extract prepared at room temperature was saturated with water and ammonium sulfate. A substantial part of this salt could be crystallized by extract incubation at 4°C, remaining GFP being completely soluble. However, such desalting interfered with subsequent purification steps. Thus, prolonged time storage and especially cooling of the GFP ethanol extracts should be avoided.

Extraction of GFP Back into Water

Ethanol should be removed from the extract for subsequent work with GFP. For this purpose we used the subsequent extraction of the ethanol phase with a less polar organic solvent. As mentioned above, the ethanol extracts were saturated with ammonium sulfate and water. These components formed a separate phase if more hydrophobic organic solvent was added. Acetonitrile, *n*-butanol, *tert*-butanol, 1-chlorobutane, isoamyl alcohol, chloroform, and dichloroethane were suitable for such an extraction. The aqueous phase volume decreased with the increase of polarity of the second organic solvent. GFP was completely extracted into the salt-containing aqueous phase and this step can be used to concentrate the GFP solution 10-fold. Double extraction with butanol and chloroform described under Materials and Methods used the differences of these solvent densities relative to those of the aqueous salt solutions. Double extraction allowed first to discard the upper ethanol–butanol phase and second to collect the upper aqueous phase with a following washing of the interphase and lower chloroform phase by ammonium sulfate solution in water.

As in the previous step, the GFP extraction back into aqueous phase was visually tested by fluorescence with a UV transilluminator. This step provided the best purification. In the aqueous $(NH_4)_2SO_4$ extract the GFP content was more than 90% of the total proteins according to SDS electrophoresis with a yield of about 60% (Fig. 1 and Table 1).

Purification of GFP by Phenyl-Sepharose Chromatography

After all the extraction steps the GFP preparation contained some nonprotein substances which were not

TABLE 1

Purification of GFP Expressed in *E. coli*

Purification step	Total protein (mg)	GFP fluorescence (arbitrary units)	Fold purification	Yield (%)
30,000*g* supernatant	152	436	1	100
40% $(NH_4)_2SO_4$ supernatant	106	412	1.4	94
Ethanol extract	24	254	3.9	58
Aqueous $(NH_4)_2SO_4$ extract	11	221	10	51
Phenyl-Sepharose peak	6.7	156	14	36

Note. Values present the means of two independent purifications.

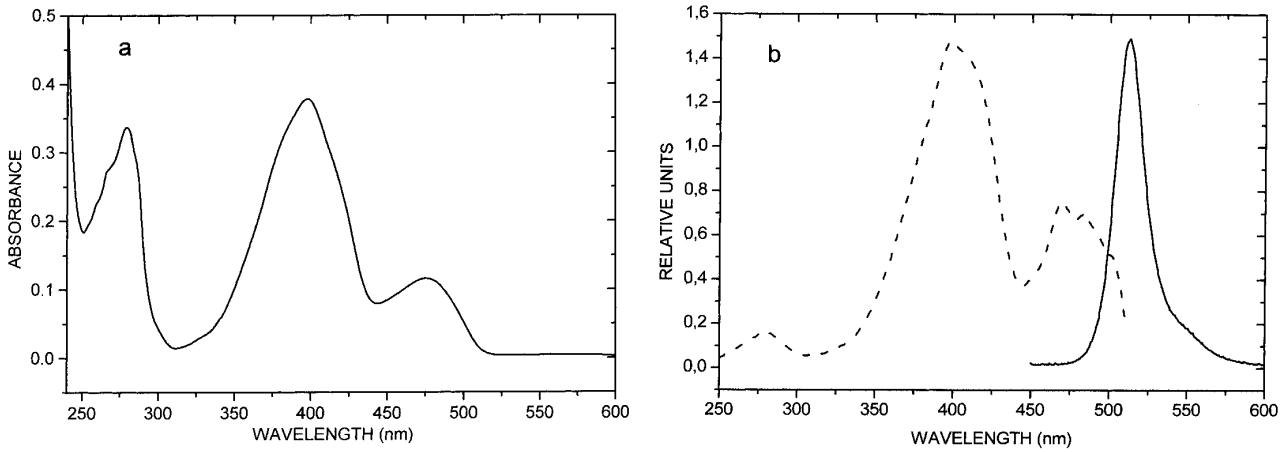

FIG. 2. (a) Absorbance spectrum of GFP. (b) Fluorescence emission spectrum of GFP excited at 395 nm (—); fluorescence excitation spectrum of GFP measured by emission at 510 nm (· · ·).

stained with Coomassie G-250, but had a substantial absorbance at 280 nm (data not shown). Phenyl-Sepharose chromatography was used mainly to remove these impurities. Despite organic solvent contamination and due to the high ammonium sulfate concentration, the GFP extract can be loaded directly on phenyl-Sepharose. Reverse-phase media poorly absorbed the nonprotein contaminations from the GFP extract in contrast to the GFP which was eluted only with the ammonium sulfate-free buffer as one major sharp peak (data not shown). The chromatography step gave some additional purification with an excellent yield (Fig. 1). The overall scheme of GFP purification is shown in Table 1.

Spectroscopic Characterization of GFP

Purified GFP (see above) was tested by spectroscopy (absorbance, fluorescence, and CD). The ratio of the absorbance values at wavelengths of 395 and 280 nm was 1.1 (Fig. 2a). The fluorescence excitation and emission spectra of GFP (Fig. 2b) were the same as that for the wild-type chromophore-containing GFP extracted by traditional methods (1, 2, 7–14). The wavelengths of the excitation and emission maxima were 397 and 512 nm, respectively. The minor excitation peak at 280 nm is presumably due to resonance energy transfer from a single tryptophan residue with an excitation maximum

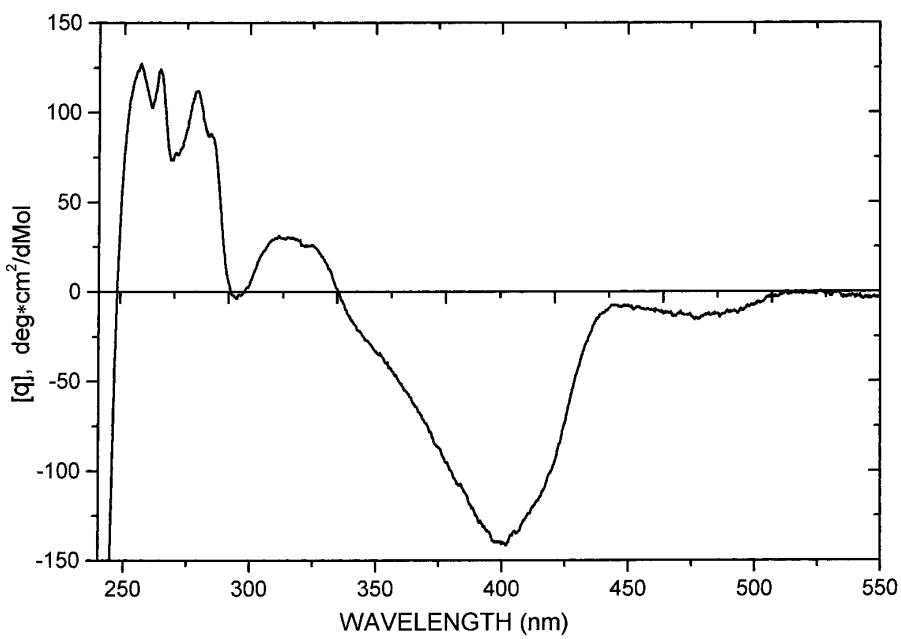

FIG. 3. Near-UV circular dichroism spectrum of GFP.

at 280 nm (see absorbance spectra). The fine structure seen in the excitation spectrum between 450 and 500 nm is probably noise due to a higher resolution of this spectrum recording compared to that of the absorbance. The near-UV CD spectrum of GFP is presented in Fig. 3. It is seen that the GFP CD spectrum conforms to the native spectra (12). Thus, the GFP purified by the above extraction technique has the same spectroscopic (absorbance and fluorescence) and structural (near-UV CD) characteristics as the native GFP.

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