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Permeation Associated with Three-Phase-Partitioning Method on Release of Green Fluorescent Protein

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Abstract

Transformed cells of *Escherichia coli* expressing recombinant green fluorescent protein (GFPuv) were subjected to two methods of extraction: (1) freezing/thawing/sonication (FTS) cycles prior to the three-phase partitioning (TPP) method, or (2) directly to TPP extraction. The amount of GFPuv released by the FTS plus TPP method varied: 374 μg/mL (first cycle), 93–442 μg/mL (second cycle), 32–359 μg/mL (third cycle), 18–115 μg/mL (fourth cycle). The GFPuv yields by the second method (TPP only) were, 23–54 μg/mL for the first extract and 33-91 μg/mL for the second. The FTS plus TPP method released similar amounts of GFPuv to that extracted by TPP; and provided a better mixture elution through the hydrophobic interaction column: 13–63 μg/mL for FTS plus TPP methods, and 2.5–13 μg/mL for TPP. The results showed that although selective permeation is a more laborious methodology, it was more efficient for obtaining of GFPuv in relation to the direct extraction of the cells for TPP.

Index Entries: Recombinant green fluorescent protein; *Escherichia coli*; protein purification; three-phase-partitioning method.

Introduction

The native form of the green fluorescent protein (GFP), extracted from jellyfish *Aequorea victoria*, emits a brilliant green fluorescent light when exposed to ultraviolet (uv) light (λ-360–400 nm) *(1)*. The recombinant form of the green fluorescent protein, GFPuv, can be successfully expressed in prokaryotic and eukaryotic cells *(2)*.

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The expressed GFPuv in the cytoplasm of *Escherichia coli* strains (DH5-α, JM109, and TB1) can be liberated from the cells by enzymatic digestion (lysozyme) of the cellular wall, by chemical lysis (*t*-butanol, ethanol), or by selective physical permeation through the freezing, thawing, and sonication methods *(1,3–8)*.

Direct extraction from *E. coli* by the three-phase partitioning (TPP) method has been used to extract, concentrate, and purify proteins and enzymes *(5,9–12)*. The TPP method combines several extraction advantages: salting-out, isoeletric precipitation *(13)*, solvent precipitation *(14)*, and osmolytic *(15)* and kosmotropic precipitation of proteins *(16)*.

t-Butanol, which is miscible in water, when added to the concentrated aqueous solution in ammonium sulfate (about 50%) partitions the mixture in two phases: *t*-butanol upper layer and aqueous lower layer. *t*-Butanol associates with the proteins present in the aqueous phase, precipitating them in the interface between the aqueous lower layer and t-butanol upper layer *(9)*. The relationship between *t*-butanol and ammonium sulfate concentration should be appropriate in order to allow synergism between the phases during the precipitation of proteins *(11)*. Yakhnin et al. *(5)* observed that an aqueous solution of 2 *M* ammonium sulfate (approx 50% of saturation) provided a better recovery (90%) of extracted GFPuv in relation to the crude extract, relative to recovery when extracted with ethanol (58%). On the other hand, Sharma and Gupta *(12)* recovered 85% of an amylase inhibitor using 30% saturated ammonium sulfate plus *t*-butanol, representing a purification of 25-fold. When ammonium sulfate is removed, proteins dissolve in the aqueous phase again, maintaining their physical and biologic properties *(15–17)*.

The pH of a solvent determines the charge state of a globular protein. According to Rothstein *(17)*, the charge state of a protein can be manipulated experimentally by varying the solvent properties, especially the pH. Proteins with zero net charge (isoeletric point [p*I*]) show a greater tendency to precipitate, since minimum solubility usually occurs at or near the p*I*. Proteins are soluble in the conditions they evolved in, for *E. coli* cytoplasm, low ionic forces, between 0.1 and 2.0 *M*, and neutral pH value.

The purposes of this work were to compare physical and chemical methods in the isolation, extraction, and purification of GFPuv expressed in the cytoplasm of *E. coli* DH5-α cells, and to determine the stability of GFPuv at different pHs.

Materials and Methods

Transformation

E. coli DH5-α *(3,6)* was transformed with pGFPuv (Clontech), which is a high copy number plasmid *(2)*, by the standard calcium chloride method *(3)*. The transformed cells were stored (at –75ºC) into Luria-Bertani (LB) broth (USB) supplemented with 100 µg/mL of ampicilin (Boehringer Mannheim) and 50% glycerol.

Expression

An overnight culture of *E. coli* in LB broth/ampicillin was transferred to the surface of LB/ampicillin/isopropyl-β-D-thiogalactopyranoside (IPTG) USB) agar and incubated (37°C for 24 h at 100 rpm). Using a hand held uv lamp (395 nm, model UVL-4; UVP), four brilliant fluorescent colonies were selected and transferred, each one to a tube containing LB/ampicillin broth and incubated (37°C for 24 h). The 8 mL suspension of (from four tubes) was mixed and transferred into 200 mL of LB/ampicillin broth. The seeded broth was divided into 50-mL (cultures 1–4) lots and each transferred to a 250-mL Erlenmeyer flask. The flasks were incubated (100 rpm at 37°C for 3 h) to an $OD_{660\,nm}$ of 0.8 (10^8 CFU/mL) when IPTG was added (0.5 m*M* final concentration). After 21 h (100 rpm at 37°C), the GFPuv expressed by induced cells was confirmed under UV light (395 nm). For all cultures, the cellular concentration was obtained by the gravimetric method of the biomass (mg/L) held on the surface of a 0.22-µm membrane (Millipore) and submitted to 105°C for about 24 h, attaining constant weight. The biomass concentration related to isolated GFPuv was expressed by specific productivity (µg of gfp_{uv}/mg of dry cell weight [DCW]).

Extraction of GFPuv by TPP Extraction Method (9)

The cultures were centrifuged (1000*g* for 30 min at 4°C), and the pellets were observed under UV light (395 nm) and resuspended in cold extraction buffer solution (XE: 25 m*M* Tris-HCl, pH 8.0, Trizma® Base [Sigma, St. Louis, MO] 1.0 m*M* β-mercaptoethanol [β-ME] [Pharmacia Biotech, Uppsala, Sweden]; 0.1 m*M* phenylmethylsulfonyl fluoride [PMSF] [USB]. For culture 1, 10 mL of XE was cultured and then divided into three aliquots of 450 µL (1A, 1B, 1C), and the remaining aliquot (8.65 mL) was lyophilized and the pellet resuspended in 1.0 mL of XE (1-D). Cultures 2–4 were resuspended in 1.0 mL of XE.

The samples of culture 1 were subjected to direct extraction by the TPP method. For samples 1A, 1C and 1D, to each 450 µL of cell suspension, 300 µL of 4 *M* $(NH_4)_2SO_4$ (1.6 *M* final concentration) and 750 µL of *t*-butanol (ratio 1:1) were added. For sample 1B, 450 µL of cell suspension was mixed with 250 µL of 4 *M* $(NH_4)_2SO_4$ (1.4 *M* final concentration) and 700 µL of *t*-butanol (ratio 1:1). The mixtures were vortexed for 1.0 min and centrifuged at 6000*g* for 10 min. The three phases formed were collected. After the *t*-butanol upper layer and the white interfacial precipitate were removed, another equal volume of *t*-butanol was mixed with the lower aqueous layer and centrifuged. The upper layer of the system was discarded. The interfacial green layer was collected and dissolved in 450 µL of XE buffer. While the lower layer was fluorescent, it was subjected to repeated TPP. In every TPP extraction, the subsequent white interfaces that showed fluorescence under UV light were dissolved in 450 µL of XE, and 250 µL (sample 1B) or 300 µL (samples 1A, 1C, 1D) of $(NH_4)_2SO_4$ and 700 or 750 µL of *t*-butanol were added, respectively.

Permeation (18)

Selective permeation of the pelleted cultures 2–4 was performed by four cycles of slow freezing (–20°C; 0.83°C/min), followed by thawing (20°C) and sonication (or freezing/thawing/sonication [FTS]) (High Intensity Ultrasonic Processor, Vibram cells, model 100; Sonic & Materials). The freezing/thawing cycles were performed in a freeze-dryer (FTS System™, Secfroid; Lyolab G) chamber (Dura Stop™ MP). With a PT-100 thermocouple inserted into a pellet, the freezing/thawing temperature was registered every minute using the software Lyphoware for Windows. Between the freezing/thawing cycles, the microtube was kept immersed in an ice-salt bath, and a 3.0-mm microtip ultrasonic processor was placed into the sample, which was submitted to threefold pulse sonication at 0°C. Each pulse was at 25 vibration amplitude at alternating cycles of 6.0 s on and 1.0 s off. Thus, the permeate was subjected to TPP extraction.

Partial Purification of GFPuv with Hydrophobic Interaction Column

Extract (250 µL) was mixed with 250 µL of 4 *M* $(NH_4)_2SO_4$ and transferred to the top of a fast-flow, methyl support hydrophobic interaction column (HIC). The column was previously equilibrated with 2 *M* $(NH_4)_2SO_4$. After the column was loaded, GFPuv was retained near the top of the column by affinity binding to the HIC resin. The loaded column was washed first with 250 µL of 1.3 *M* $(NH_4)_2SO_4$ to elute proteins other than GFPuv that bind with low affinity. GFPuv was eluted with 750 µL of buffer solution (10 m*M* Tris-HCl, 10 m*M* EDTA, pH 8.0) and stored at 4°C. The progress of the GFPuv through the column was observed with a UV light, as well as confirmation of the eluted material.

Fluorescence Intensity

The fluorescence intensity of GFPuv was measured in the eluted samples in the spectrofluorophotometer (RF-5301 PC; Shimadzu, Kyoto, Japan), with an excitation filter of 394 nm and an emission filter of 509 nm. Purified recombinant GFPuv purchased from Clontech ("standard GFPuv") was used to generate a standard curve to relate protein concentration to fluorescence intensity. A standard curve was prepared using known amounts (1.52, 2.44, 3.90, 6.25, and 10.00 µg/mL) of standard GFPuv diluted in buffer solution (10 m*M* Tris-HCl, pH 8.0; 1.0 m*M* β-ME; 0.1 m*M* PMSF). The fluorescence intensity of the samples was compared to the standard curve from the standard GFPuv (GFPuv µg/mL = 0.0256 × [fluorescence intensity] + 0.8576; R^2 = 0.99) and was expressed as micrgrams of GFPuv per milliliter.

Total Protein Concentration Released

The total protein concentration released in the eluted samples was compared to purified bovine serum albumin (BSA) in buffer solution (mol wt of 66 kDa; Sigma) at A_{280} in a spectrophotometer and expressed in mil-

ligrams of BSA per milliliter. The total protein concentrations in the buffer solution ranged from 100 to 1000 µg/mL, the maximum A_{280} being 0.615. The relationship between total proteins and BSA was made through the standard curve (total protein µg/mL = 1727.2 × [$OD_{280\,nm}$] - 26.863; $R^2 = 0.99$). The specific GFPuv mass was expressed as micrograms of GFPuv BSA.

Electrophoresis

Samples of the extracted GFPuv were run on a 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The first 30 min was performed at 50 V (70 mA/100 W). The voltage was increased to 200 V for the last 40 min. The protein bands were visualized with Coomassie Brilliant Blue gel staining.

Excitation and Emission Spectra

The spectral ranges of the excitation and emission for the extracted and purified GFPuv were compared to standard GFPuv: 500-µL aliquots in buffer (10 m*M* Tris-HCl, 10 m*M* EDTA, pH 8.0) containing (1) standard GFPuv (2.52 µg/mL), (2) extracted GFPuv (8.68 µg/mL), and (3) extracted/purified GFPuv (8.84 µg/mL) each transferred to a quartz cuvet (3 × 10 mm light path length × 45 mm height). Using a spectrofluorometer, samples were first excited from 300–450 nm, and emission intensity was recorded at 509 nm at every 0.2-nm excitation wavelength and then excited at 394 nm and the emission scanned from 450 to 550 nm.

Stability of GFPuv at Different pH Values

Samples of the standard GFPuv and extracted/purified GFPuv (approx 5.0 µg of gfp_{uv}/mL) were exposed (24 h at 4°C) to different pH values (4.0 ± 0.2 to 8.0 ± 0.2) in buffer solutions: (1) 10 m*M* acetate (pH 4.0 and 5.0), (2) 10 m*M* phosphate (pH 5.5, 6.0, and 7.0), and (3) 10 m*M* Tris (pH 8.0). To each 1175 µL of buffer solution, a 25 µL sample was mixed. The mixture (1200 µL) was stored at 4°C overnight, and centrifuged (6000*g* for 30 min). Precipitation was not observed. The pH values were measured and correlated with the corresponding GFPuv concentration by the standard calibration curve (Fig. 3). The pHmeter AR-20 (Fisher) was previously adjusted with standard buffers (Synth) of known pH values: 4.0, 7.0, and 9.0.

Expression of the Results

The GFPuv contents were expressed as micrograms of GFPuv per milliliter of sample. The amount of total proteins was expressed as milligrams of BSA per milliliter of sample. The specific mass of GFPuv as related to the total protein concentration (BSA) was expressed as milligrams of GFPuv per milligram of BSA. The specific productivity was obtained from the relationship between the GFPuv concentration and dried cell weight (micrograms of GFPuv per milligram of DCW).

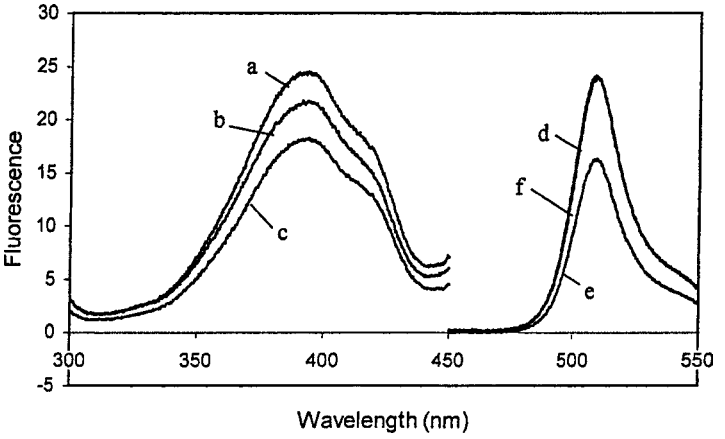

Fig. 1. The spectrum of excitation (maximum peak: 394 nm) for (a) standard GFPuv, (b) extracted GFPuv, and (c) extracted/purified GFPuv is shown. Also shown is the spectrum of emission (maximum peak; 509 nm) of (d) standard GFPuv, (e) extracted GFPuv, and (f) partially purified.

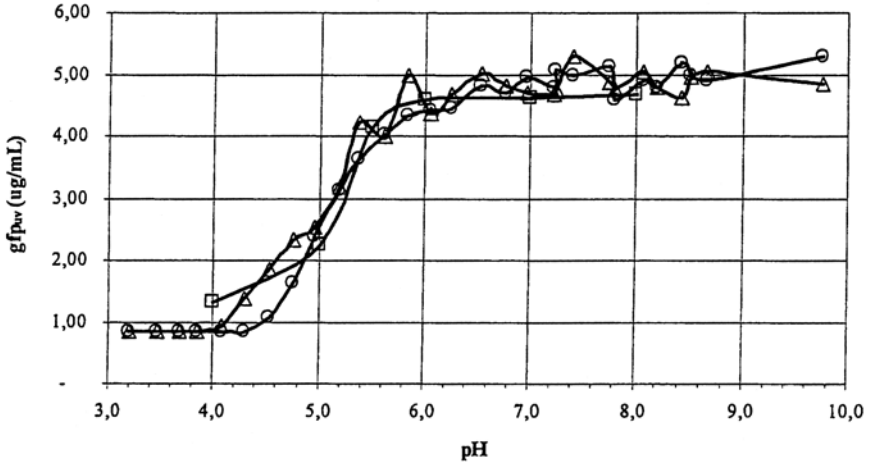

Fig. 2. GFPuv extracted from *E. coli* at different pH values: (—Δ—) extracted GFPuv; (—○—) extracted/purified with HIC; (—□—)standard GFPuv.

Results and Discussion

The characteristic spectra of excitation and emission for the standard GFPuv, and extracted and purified (with HIC), are shown in Fig. 1. Optimal excitation was at 394 nm and emission at 508.8 nm, for the samples tested, similar to standard GFPuv.

The effect of pH on the samples (standard and extracted/purified GFPuv) is shown in Fig. 2. A quite good stability of the molecule was

observed at pH of about 5.0–5.2, the inflection region of the curves, related to GFPuv concentration vs pH. According to Rothstein *(17)*, the solubility of a protein in aqueous solution is minimum at the pH at which it is isoeletric, the p*I* of the protein. For this range of pH value, where p*I* is located, the protein has a zero net charge *(1,2)*. This is observed by an abrupt decrease in the fluorescence intensity and solubility. Ward *(1)* verified that the p*I* for the native GFP was between pH 4.7 and 5.1, corresponding with our finding on GFPuv extraction efficiency. According to Scopes *(16)*, the p*I* for most plants and bacteria proteins is characterized between pH 4.5 and 5.0, while the p*I* of most animal proteins is between 5.5 and 6.0.

The maximum and stable fluorescence intensity of GFPuv was shown in the pH range of 6.0–9.8, declining abruptly between 5.5 and 4.5. For pH values <4.5, the fluorescence intensity was minimum (Fig. 2). Bokman and Ward *(19)* verified that native GFPuv maintains stable fluorescence in the pH range of 5.5–12.0; however, fluorescence intensity decreases between pH 5.5 and 4.4, and drops sharply above 12.0. The red-shifted green fluorescent variants (EGFP and GFP-S65T) exhibit a narrow range of pH stability between 7.0 and 11.5, drops sharply above 11.5, and decreases between 7.0 and 4.5, thus retaining about 50% of fluorescence at pH 6.0 (W. W. Ward, personal communication, 2000).

The GFPuv yields obtained by TPP extraction at different concentrations of $(NH_4)_2SO_4$, are shown in Table 1. Samples 1A, 1C, and 1D were extracted with 1.6 *M* $(NH_4)_2SO_4$. Sample 1B was extracted with 1.4 *M* $(NH_4)_2SO_4$.

The pool concentration of 98 μg of GFPuv/mL in sample 1B was about 1.2, 4.0, and 2.0 times greater, respectively, than obtained for samples 1A (85 μg of GFPuv/mL), 1C (25 μg of GFPuv/mL), and 1D (47 μg of GFPuv/mL). However, the specific mass for 1B (7.4 μg of GFPuv/mg) was, respectively, 2.0, 5.0, and 2.0 times lower than attained for 1A (15 μg of GFPuv/mg), 1C (40 μg of GFPuv/mg), and 1D (13 μg of GFPuv/mg), because the concentrations of impurities extracted with GFPuv were 3-20 times larger. Therefore, the final concentration of 1.6 *M* $(NH_4)_2SO_4$ in the aqueous phase favored the GFPuv extraction yield, in accordance with Dennison and Lovrient's *(9)* observations.

Even though sample 1D was 20 times more concentrated (lyophilized and rehydrated) than 1A, 1B, and 1C, the thickest white interface (from the first TPP step) improved the specific mass in the pool (13 μg of GFPuv/mg of BSA), which was close to that of sample 1A (15 μg of GFPuv/mg) and three times lower than that of sample 1C (40 μg of GFPuv/mg). This yielded the lowest total protein concentration (0.63 mg of BSA/mL).

The GFPuv contents obtained from FTS permeation cycles applied to the pelleted culture followed by TPP extraction are given in Table 2. For every permeation and extraction step applied to the sample 2, GFPuv concentrations were larger than those obtained for samples 3 and 4, although the GFPuv contents in the pool of FTS cycles were close for all samples. However, the specific masses obtained from sample 3 by TPP extractions

Table 1
GFPuv Contents Obtained from Direct Application of TPP Extractions to the Pelleted Culture 1[a]

Sample	Extraction[c]	$(NH_4)_2SO_4$ (*M*)	GFPuv (µg/mL)	Extraction (%)	Total protein BSA (mg/mL)	Specific mass (µg GFPuv/mg BSA)	Specific productivity (µg GFPuv/mg DCW)
1A	First	1.6	33	15	8.3	4.0	23
	Second	1.6	91	42	7.1	13	62
	Third	1.6	92	42	5.2	18	63
	Pool	—	85	—	5.5	15	58
	HIC	—	2.5	—	1.5	1.6	1.7
1B	First	1.4	44	16	—	—	—
	Second	1.4	83	30	10	8.3	57
	Third	1.4	54	20	16	3.4	37
	Fourth	1.4	93	34	5.1	18	63
	Pool	—	98	—	13	7.4	67
	HIC	—	3.7	—	1.2	3.1	2.5
1C	First	1.6	23	36	0.65	35	15
	Second	1.6	33	53	0.85	39	23
	Third	1.6	7.2	11	0.25	28	4.9
	Pool	—	25	—	0.63	40	17
	HIC	—	5.2	—	0.33	16	3.6
1D	First[b]	1.6	54	36	6.0	9.1	37
	Second[b]	1.6	77	52	2.6	30	53
	Third[b]	1.6	17	12	2.0	8.8	12
	Pool	—	47	—	3.6	13	32
	HIC	—	13	—	2.7	4.8	8.8

[a]Samples 1A, 1C, and 1D, with 1.6 *M* $(NH_4)_2SO_4$ in aqueous phase, sample 1B with 1.4 *M* $(NH_4)_2SO_4$ in aqueous phase and sample, 1D lyophilized and rehyrated.

[b]Yield (%) recuperation of GFPuv in crude extract (pool) after eluted through HIC column = (HIC/Pool) × 100.

[c]Pool = mixture of equal volumes of permeates or extracts; HIC = hydrophobic interaction column (µg GFPuv/mL of eluted material).

[d]Yield = HIC/Pool

Table 2
GFPuv Contents Obtained from Association of Physical Permeation by FTS Cycles Applied to Pelleted Cultures Followed by TPP Extractions

Treatment[a]	GFPuv (μg/mL)	Yield (%)	Total protein BSA (mg/mL)	Specific mass (μg GFPuv/mg BSA)	Specific productivity (μg GFPuv/mg DCW)
Sample 2 (500 μL of 10 m*M* Tris-HCl pH 8.0)—dry mass of 1.40 mg/mL					
Permeation					
First FTS	833	42	95	8.8	595
Second FTS	504	25	77	6.6	360
Third FTS	351	18	40	8.7	251
Fourth FTS	175	8.8	25	7.0	125
Pool	198	—	—	—	—
Extraction					
First FTS	374	29	35	11	267
Second FTS	442	34	24	18	316
Third FTS	359	28	8.4	43	257
Fourth FTS	115	8.9	3.6	32	82
Pool	218	—	23	9.4	156
HIC	63	—	3.5	18	45
Sample 3 (1000 μL of 10 m*M* Tris-HCl pH 8.0)—dry mass of 0.92 mg/mL					
Permeation					
First FTS	315	33	87	3.6	342
Second FTS	411	43	136	3.0	446
Third FTS	137	14	31	4.3	149
Fourth FTS	82	8.7	12	6.5	89
Pool	946	—	94	10	1028
Extraction					
First FTS	255	41	1.7	150	277
Second FTS	257	41	5.6	45	279
Third FTS	96	15	4.6	21	104
Fourth FTS	18	2.8	1.0	17	19
Pool	196	—	2.4	83	213
HIC	52	—	32	1.6	56
Sample 4 (1000 μL of 10 m*M* Tris-HCl pH 8.0)—dry mass of 1.44 mg/mL					
Permeation					
First FTS	429	66	62	6.9	294
Second FTS	174	27	28	6.1	119
Third FTS	39	6.0	8.6	4.5	27
Fourth FTS	7.8	1.2	4.6	1.7	5.3
Pool	233	—	24	9.6	160
Permeated mixture					
First extraction	20	14	2.2	9.2	14
Second extraction	93	64	1.0	90	64
Third extraction	32	22	1.6	21	22
Pool	43	—	2.4	18	29
HIC	13	—	3.4	3.7	8.7

[a]Pool = mixture of equal volumes of permeates or extracts; HIC = hydrophobic interaction column (μg of GFPuv/mL of eluted material).

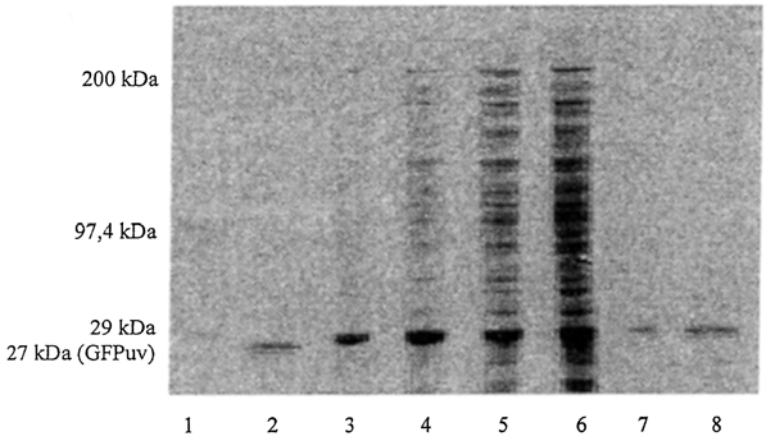

Fig. 3. SDS-PAGE of sample 3. Lane 1, molecular mass weighed 18–200 kDa (Gibco); lane 2, standard GFPuv (20 μg/mL); lane 3, permeate of First-cycle FTS (32 μg/mL); lane 4, permeate of second-cycle FTS (60 μg/mL); lane 5, permeate of third-cycle FTS (39 μg/mL); lane 6, permeate of fourth-cycle FTS (46 μg/mL); lane 7, mixture of permeate after TPP extraction (13 μg/mL); and lane 8, mixture of eluted extracts from column HIC (60 μg/mL).

increased up to 42% (150 μg of GFPuv/mg for first FTS/TPP) resulting in the largest sample concentration in the pool (83 μg of GFPuv/mg).

The combination of first and second FTS cycles followed by TPP extractions resulted in yields>50% GFPuv, respectively: 67% for sample 2, 77% for sample 3, and 93% for sample 4, indicating that the third and fourth FTS plus TPP steps can be eliminated.

In sample 4, the pool obtained from the first through fourth FTS cycles (equal volumes) and subjected to the first through third TPP extractions showed 10 times less total proteins (2.4 mg of BSA/mL) and specific mass two times larger (18 μg of GFPuv/mg) than for sample 2 (9.4 μg of GFPuv/mg), owing to the effectiveness of TPP extractions.

The samples run on gel electrophoresis showed a progressive increase in impurity from the first up to the fourth FTS permeation (Fig. 3, lanes 4–6), owing to a simultaneous increase in the intracellular release of molecules other than GFPuv by successive FTS cycles (Fig. 1). A significant removal of molecules other than GFPuv, by TPP extraction of permeated sample 3, was confirmed in lanes 7 (before HIC) and 8 (after HIC) (*see* Fig. 3). A single band between 27 (standard GFPuv) and 29 kDa (standard mol wt) can be visualized. Therefore, the HIC procedure did not improve TPP effectiveness on the GFPuv purification, confirming Sharma and Gupta's *(11,12)* and Yakhnin's *(5)* observations.

Conclusion

The recombinant GFPuv, which was expressed by transformed cells of *E. coli* DH5-α, showed similarities in fluorescence, stability, and solubility

to standard GFPuv. The slow FTS permeation followed by TPP extraction provided better GFPuv yields compared with those obtained by directly applied TPP extraction.

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