

Evaluation of Recombinant Green Fluorescent Protein, Under Various Culture Conditions and Purification with HiTrap Hydrophobic Interaction Chromatography Resins

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Abstract

To determine the influence of various culture conditions, transformed cells of *Escherichia coli* expressing recombinant green fluorescent protein (GFPuv) were grown in nine cultures with four variable conditions (storage of inoculated broth at 4°C prior to incubation, agitation speed, isopropyl- β -D-thiogalactopyranoside [IPTG] concentration, and induction time). The pelleted cells were resuspended in extraction buffer and subjected to the three-phase partitioning (TPP) extraction method. To determine the most appropriate purification resin, protein extracts were eluted through one of four types of HiTrap hydrophobic interaction chromatography (HIC) columns prepacked with methyl, butyl, octyl, or phenyl resins and analyzed further on a 12% sodium dodecylsulfate polyacrylamide gel. With Coomassie staining, a single band between 27 (standard GFPuv) and 29 kDa (molecular weight standard) was visualized for every HIC column sample. TPP extraction with HIC elution provided about 90% of the GFPuv recovered and eight-fold GFPuv enrichment related to the specific mass. Rotary speed and IPTG concentration showed, respectively, greater negative and positive influences on GFPuv expression at the beginning of the logarithmic phase for the set culture conditions (37°C, 24-h incubation).

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Index Entries: Recombinant green fluorescent protein; GFPuv; hydrophobic interaction chromatography; sodium dodecylsulfate polyacrylamide gel electrophoresis; three-phase partitioning extraction.

Introduction

The recombinant green fluorescent protein, GFPuv, expressed by *Escherichia coli* DH5- α , exhibits fluorescence intensity 18 times greater than the wild-type GFP protein of the jellyfish *Aequorea victoria*. GFPuv is a compact, globular, acidic protein monomer (pI 4.6–5.4) consisting of 238 amino acids, with a mol wt between 27 and 29 kDa, that has a propensity to dimerize (1). The fluorescence of GFPuv requires no cofactor, and GFPuv is a widely utilized genetic marker because it can be easily monitored in a variety of applications.

Because GFPuv exhibits stability to extreme conditions such as exposure to heat and chemical denaturants (disinfectants) in a wide pH range, its expression by prokaryotes, followed by extraction and purification, should be studied for its potential utility as a marker in validation procedures. In addition, the protein extracted from *E. coli* and further purified by hydrophobic interaction chromatography (HIC) resins should be analyzed qualitatively (2) by sodium dodecylsulfate polyacrylamide gel (SDS-PAGE) to define the best purification method. SDS-PAGE with Coomassie or silver staining provides a sensitive method to determine the most appropriate HIC support for the purification of GFPuv.

The aims of the present work were (1) to determine what culture conditions influence the expression of GFPuv by *E. coli*, and (2) to select the most appropriate HIC medium for the separation of three-phase partitioning (TPP)-extracted GFPuv from total cell extract by analyzing the purity of GFPuv. The HIC column was selected by using comparative qualitative analysis with SDS-PAGE and postelectrophoresis staining with either Coomassie or silver stain.

Material and Methods

Transformation of E. coli

Transformation of *Escherichia coli* DH5- α with a high copy plasmid, *pGFPuv* (Clontech Laboratories, Palo Alto, CA), was performed using the standard calcium chloride method (3,4).

Experimental Design

To evaluate the effectiveness of different parameters to improve the expression of GFPuv by *E. coli*, nine culture conditions were set up using a fractional factorial (2^{4-1}) design at two levels. The variables were as follows:

1. Effect of storing the starting culture in Luria Bertani (LB) broth (USB, Cleveland, OH) supplemented with 100 $\mu\text{g}/\text{mL}$ of ampicillin (amp) (Boehringer, Mannheim, Germany) at 4°C for 24, 36, and 48 h ("storage") prior to incubation at 37°C.

Table 1
Expression of GFPuv by *E. coli*
from Nine Cultures (Groups)
Set Up Using a Fractional Factorial (2^{4-1})
Design at Two Levels^a

Culture Group	x_1	x_2	x_3	x_4
1	–	–	–	–
2	+	–	–	+
3	–	+	–	+
4	+	+	–	+
5	–	–	+	+
6	+	–	+	–
7	–	+	+	–
8	+	+	+	+
9	0	0	0	0

Independent Variables	(–)	0	(+)
x_1 Storage 4°C (h)	24	36	48
x_2 Agitation speed (rpm)	100	150	200
x_3 OD ₆₆₀ nm ^b	0.01	0.40	0.80
x_4 IPTG (mM) ^c	0.050	0.275	0.500

^aThe cultures were incubated at 37°C on a rotary shaker for 8 ($x_5 = -1$) and 24 h ($x_5 = +1$).

^bThe addition of IPTG at different cell densities.

^cThe final concentration of IPTG added.

- Effect of rotary speed (100, 150, and 200 rpm).
- Effect of the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) (USB) at set cell densities (OD₆₆₀ between 0.01 and 0.8) corresponding to cultures at 10^3 – 10^7 CFU/mL.
- Concentration of IPTG with final concentrations of 0.05, 0.275, and 0.5 mM.
- Incubation of the culture at 37°C for 8 and 24 h (Table 1).

Inoculum

A 24-h culture of transformed *E. coli* (LB/amp broth; 37°C, 100 rpm) was transferred onto the surface of LB/amp/IPTG agar and incubated at 37°C for 24 h. Isolated green fluorescent colonies (illuminated with a handheld long UV lamp, 360–395 nm; Model UVL 4; UVP, Upland, CA) were picked and transferred to 25 mL of LB/amp broth in 250-mL Erlenmeyer flasks (starter cultures). The starter cultures were incubated at 37°C and 100 rpm until a cell density of 0.0054–0.026 absorbance units (10^4 – 10^5 CFU/mL) was obtained as measured by OD₆₆₀ with a spectrophotometer (Beckman DU-600; Beckman Coulter, Fullerton, CA). An inoculum of

1.0 mL was transferred to each of 32 Erlenmeyer flasks (250 mL) containing 25 mL of LB/amp broth. The flasks of inoculated broth (expression cultures) were stored at 4°C prior to being incubated on a rotary shaker at 37°C (Tecnal model TE 240; SP, Brazil).

Induction by IPTG

The expression cultures were incubated at 37°C until the broth cultures attained a set OD₆₆₀ for the addition of IPTG (Table 1). Expression cultures from corresponding flasks (two flasks/h) were assayed every hour for cell density: (1) OD₆₆₀ with LB/amp broth in the reference cell; (2) dried biomass related to GFPuv expression (µg of GFPuv/mg of dry cell weight [DCW]); from cells retained on the surface of a 0.22-µm membrane (Millipore, SP, Brazil) and dried at 105°C for approx 24 h to attain steady weight.

Extraction of GFPuv by TPP Method (5–7)

The TPP method was applied to induced *E. coli* cultures overexpressing GFPuv. Through the TPP extraction, proteins (other than GFPuv) and other molecules were precipitated and separated from GFPuv. At the end of 24 h of incubation, the cultures were centrifuged at 1000g for 30 min at 4°C and the pellets resuspended in 4 mL of cold extraction buffer (XE: 25 mM Tris-HCl, pH 8.0, Trizma® Base [Sigma, St. Louis, MO] 1.0 mM β-mercaptoethanol [β-ME] [Amersham Biosciences, Uppsala, Sweden]; 0.1 mM phenylmethylsulfonyl fluoride [PMSF] [USB]). From every suspension in XE, each of eight 450-µL aliquots was subjected to one-step extraction by the TPP method. To each 450 µL of cell suspension, 300 µL of 4 M (NH₄)₂SO₄ (1.6 M final concentration) and 750 µL of *t*-butanol (ratio 1:1) were added. The mixtures were vortexed for 1.0 min and centrifuged at 6,000g 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 was discarded. The interfacial green layer was collected and dissolved in 1.0 mL of XE buffer. Every eight TPP-extracted aliquots from the same pellet cell culture were pooled. The final concentration of GFPuv into TPP extraction aliquots varied up to 42.60 µg of GFPuv/mL (Table 2), for a mean specific productivity of 27.39 µg of GFPuv/mg of DCW.

Purification of GFPuv Through HIC Columns

Protein extracts were eluted through HiTrap 1-mL columns, pre-packed with one of four HIC resins: methyl support (Macro-Prep HIC supports; Bio-Rad, Hercules, CA); HiTrap HIC fast flow (FF) columns (Amersham Biosciences, Piscataway, NJ) butyl 4 FF; octyl 4 FF; phenyl 6 FF (low sub) sepharose. From every pelleted cell culture, 250-µL aliquots TPP extract were mixed with 250 µL of 4 M (NH₄)₂SO₄ and transferred to the top of each of the four FF support columns. The columns were previously

Table 2
Enrichment and Recovery of TPP-Extracted GFPuv Aliquots in Eluted Samples from Methyl HIC Columns^a

Eluted samples from methyl HIC columns													
TPP-extracted GFPuv aliquot ^b				First group				Second group					
GFPuv (µg/mL)	BSA (mg/mL)	SM (µg/mg)		GFPuv (µg/mL)	BSA (mg/mL)	SM (µg/mg)	GFPuv (times)	GFPuv (%)	GFPuv (µg/mL)	BSA (mg/mL)	SM (µg/mg)	GFPuv (times)	GFPuv (%)
1.15	4.79	0.24		1.10	0.29	3.84	16.02	95.52	1.19	0.20	5.91	24.67	103.40
1.21	0.89	1.37		1.13	0.15	7.57	5.54	92.88	1.21	0.48	2.51	1.84	99.70
2.03	1.74	1.16		1.47	0.41	3.60	3.09	72.72	1.62	0.53	3.04	2.61	80.05
3.65	3.72	0.98		3.29	0.71	4.65	4.75	90.27	3.67	0.54	6.83	6.98	100.57
5.33	0.81	6.56		4.51	0.34	13.46	2.05	84.63	5.03	0.65	7.70	1.17	94.38
6.86	0.77	8.89		6.68	0.20	33.67	3.79	97.50	6.82	0.36	18.90	2.12	99.43
8.18	1.89	4.33		8.28	0.24	35.12	8.11	101.22	8.71	0.43	20.39	4.71	106.50
10.17	3.40	2.99		9.82	0.53	18.51	6.20	96.60	10.27	0.45	22.89	7.66	100.97
11.30	2.91	3.89		10.99	0.40	27.75	7.14	97.29	10.94	0.33	33.40	8.59	96.88
12.56	0.90	13.97		14.33	0.36	40.32	2.89	108.36	12.82	0.51	24.91	1.78	102.05
15.50	5.56	2.79		11.43	0.24	46.72	16.77	73.73	11.53	0.29	39.81	14.29	74.40
17.35	1.79	9.72		18.59	0.31	59.22	6.10	107.13	11.93	0.49	24.28	2.50	68.77
19.79	4.70	4.21		15.98	0.29	55.91	13.28	72.67	15.08	0.60	25.21	5.99	76.20
20.09	3.49	5.75		20.41	0.62	32.82	5.71	101.61	16.96	0.36	47.55	8.27	84.41
20.80	4.83	4.30		16.48	0.31	52.54	12.21	79.26	15.69	0.45	34.55	8.03	75.46
21.31	2.22	9.58		22.33	0.58	38.27	3.99	99.56	17.60	0.50	35.22	3.67	82.60
22.13	7.92	2.79		17.27	0.70	24.70	8.84	74.16	17.66	0.47	37.36	13.38	75.79

(Continued on next page)

Table 2 (Continued)
Enrichment and Recovery of TPP-Extracted GFPuv Aliquots in Eluted Samples from Methyl HIC Columns^a

Eluted samples from methyl HIC columns													
TPP-extracted GFPuv aliquot ^b				First group				Second group					
GFPuv (µg/mL)	BSA (mg/mL)	SM (µg/mg)		GFPuv (µg/mL)	BSA (mg/mL)	SM (µg/mg)	GFPuv (times)	GFPuv (%)	GFPuv (µg/mL)	BSA (mg/mL)	SM (µg/mg)	GFPuv (times)	GFPuv (%)
22.14	9.53	2.32		17.86	0.27	65.88	28.36	80.67	16.26	0.53	30.40	13.09	73.45
23.76	2.49	9.55		18.83	0.36	53.00	5.55	79.25	19.58	0.32	61.89	6.48	82.43
26.66	4.43	6.02		21.36	0.49	43.64	7.25	80.13	21.18	0.56	37.77	6.28	79.43
27.07	5.70	4.75		27.06	0.28	95.82	20.17	89.96	38.83	0.45	85.45	17.99	136.25
27.83	4.32	6.44		29.52	0.35	84.87	13.18	100.78	25.67	0.24	105.86	16.43	92.24
28.51	2.07	13.80		26.35	0.48	54.35	3.94	92.43	22.72	0.42	54.58	3.96	79.69
28.82	1.99	14.47		25.53	0.55	46.12	3.19	79.74	24.68	0.67	36.96	2.55	85.67
29.26	1.34	21.86		27.30	0.37	74.74	3.42	93.31	27.15	0.44	61.75	2.82	92.81
29.81	2.41	12.35		27.01	0.32	84.48	6.84	90.64	24.01	0.39	62.10	5.03	80.56
31.49	6.16	5.11		31.17	0.38	81.39	15.91	99.00	27.95	0.36	78.66	15.38	88.75
31.90	8.23	3.88		28.90	0.44	65.78	16.97	90.57	27.59	0.47	58.76	15.16	86.49
32.85	1.62	20.25		30.31	0.37	82.80	4.09	92.27	28.01	0.35	79.81	3.94	85.27
34.13	4.28	7.97		31.66	0.55	57.33	7.19	88.12	29.14	0.60	48.66	6.10	85.36
38.34	2.85	13.47		39.46	0.74	53.33	3.96	102.92	37.28	0.39	95.39	7.08	97.23
42.60	1.56	27.39		43.97	0.37	119.72	4.37	103.23	38.83	0.54	71.95	2.63	91.16

^a SM, Specific mass (µg of GFPuv/mg of BSA); GFPuv (%) = Recovery (%), (eluted GFPuv sample/TPP-extracted GFPuv aliquot) × 100; GFPuv (times) = Enrichment (E), (specific mass of eluted GFPuv sample/specific mass of TPP-extracted GFPuv aliquot).

^b TPP-extracted GFPuv aliquot before HIC fast flow.

equilibrated with 2 M $(\text{NH}_4)_2\text{SO}_4$. After the columns were loaded, GFPuv was retained near the top of the columns by affinity binding to the HIC resin. The loaded columns were washed first with 250 μL of 1.3 M $(\text{NH}_4)_2\text{SO}_4$ to elute proteins other than GFPuv that bind with low affinity. GFPuv was eluted with 750 μL of buffer solution (10 mM Tris-HCl; 10 mM EDTA, pH 8.0) and stored at 4°C. The progress of GFPuv through the column was observed with a handheld UV light, as well by the analysis of the eluted material. The fluorescence intensity (excitation/emission maxima at 394/509 nm) of eluted samples was related to μg of GFPuv/mL by the standard curve (Eq. 1).

$$\mu\text{g of GFPuv/mL} = 0.0256 \times (\text{fluorescence intensity}) + 0.8576; R^2 = 0.99 \quad (1)$$

Electrophoresis (7,8)

The eluted GFPuv samples were run on a 12% SDS-PAGE gel along with samples of TPP-extracted GFPuv. The first 30 min was performed at a fixed voltage of 50 V, which was increased to 200 V for the last 40 min. The protein bands were visualized with Coomassie Brilliant Blue. The same samples were run on another 12% SDS polyacrylamide gel at a fixed voltage of 350 V (approx 1 h) and stained with silver nitrate solution, using the PhastSystem (Amersham Biosciences).

Measurement of Fluorescence Intensity

The fluorescence intensity of GFPuv was measured in the eluted samples in a spectrofluorophotometer (RF-5301 PC; Shimadzu, Kyoto, Japan), with an excitation filter of 394 nm and an emission filter of 509 nm. Known amounts of purified recombinant GFPuv (standard GFPuv; Clontech) diluted in buffer (10 mM Tris-HCl, pH 8.0; 1.0 mM βME ; 0.1 mM PMSF) were used to generate a standard curve (Eq. 1) in order to relate the protein concentration with fluorescence intensity of the TPP-extracted/HIC-purified aliquots.

Total Protein Concentration

The total protein concentration released (4,7) in the eluted samples was compared relative to purified bovine serum albumin (BSA) (mol wt 66 kDa; Sigma) in buffer solution at A_{280} in a spectrophotometer and was expressed in mg of BSA/mL. The total protein concentrations in the buffer solution ranged from 100 to 1000 $\mu\text{g/mL}$ with the maximum $A_{280} = 0.615$. The relationship between total proteins and BSA was made through the standard curve ($\mu\text{g of BSA/mL} = 1727.2 \times [A_{280}] - 26.86$; $R^2 = 0.99$). The specific GFPuv mass was expressed as $\mu\text{g of GFPuv/mg of BSA}$.

Statistical Growth Variables

The effects of the culturing variables, interaction coefficients (95%), correlation matrix for estimated parameters, respective confidence inter-

Table 3
Extracted Concentrations of GFPuv and Effects of Expression Derived from *E. coli* Cells
Cultivated at Set Conditions for Standard Period of 8 h ($x_5 = -1$) of Incubation at 37°C

Assay ^a	Group ^b	Storage x_1	RPM x_2	IPTG (mM) x_3	OD ₆₆₀ IPTG x_4	Incubation (h) x_5	DCW (mg/mL)	Concentration (μ g GFPuv/mL) (mean $\pm$ CI) ^c	Specific productivity (μ g GFPuv/mg DCW) (mean $\pm$ CI) ^c
1	1	-1	-1	-1	-0.99	-1	0.24	1.16 $\pm$ -0.11	4.85 $\pm$ 0.47
2	2	1	-1	1	-0.98	-1	0.43	1.42 $\pm$ 0.19	3.18 $\pm$ 0.51
3	2	1	-1	1	-0.98	-1	0.43	1.68 $\pm$ 0.22	3.91 $\pm$ 0.52
4	2	1	-1	1	-0.98	-1	0.47	1.16 $\pm$ 0.07	2.44 $\pm$ 0.16
5	3	1	1	-1	-0.98	-1	1.76	15.24 $\pm$ 3.28	9.20 $\pm$ 1.54
6	7	-1	1	-1	0.99	-1	1.68	3.98 $\pm$ 0.57	2.36 $\pm$ 0.34
7	7	-1	1	-1	0.99	-1	1.68	3.98 $\pm$ 0.57	2.36 $\pm$ 0.34

^a The order in which the experiments were carried out.

^b The set of conditions for every experiment carried out.

^c $p < 0.05$; $n = 10$ observations.

vals (CIs), significance levels ($p < 0.05$) and regression variance analysis (analysis of variance [ANOVA]) (8) were calculated using the SGWIN program (Statgraphics Plus for Windows version 3; Statistical Graphics, Rockville, MD). The four variables (x) considered in regression analysis were taken as dimensionless values over the same (–1) to (+1) range. The maximum (+1), intermediate (0), and minimum (–1) codified ranges for each independent variable, shown in Table 1, were as follows: x_1 = storage at 4°C (24, 36, 48 h); x_2 = rotary speed (100, 150, and 200 rpm); x_3 = effect of IPTG added at set OD₆₆₀ (0.01, 0.40, and 0.80); x_4 = final concentrations of IPTG (0.05, 0.275, and 0.5 mM); x_5 = incubation time at 37°C for 8 h (x_5 = –1) and 24 h (x_5 = +1). The intermediate levels in code units were given by the following equation: codified variable (x_n) = $\{[(\text{effect studied}) - (\text{maximum level} + \text{minimum level})/2]/[(\text{maximum level} - \text{minimum level})/2]\}$.

Results

Optimal Conditions for Expression of GFPuv During Growth of E. coli

The expression of GFPuv, which is under tight control of the *lacZ* protein β -galactosidase promoter/repressor (1), was continuously induced with a concentration varying from 0.05 to 0.5 mM (w/v) IPTG added to the transformed *E. coli* culture at different growth phases, when no lag phase was exhibited. Independent of the growth phase for the IPTG addition, the remaining stationary phase (for 24 h of incubation) was long enough for the overexpression of GFPuv by *E. coli*. On the other hand, for 8 h of incubation, the expression of GFPuv by *E. coli* was dependent on the timing of the addition of IPTG at the beginning of the log phase (Tables 3 and 4).

The influence of the set culture conditions (variables) on the expression of GFPuv (Tables 3 and 4) was analyzed by applying a multiple linear regression to the data related to the concentrations of expressed, extracted, and purified GFPuv obtained from the induced *E. coli* cultures. ANOVA analysis outlines the results by fitting a multiple linear regression model to describe the relationship between μg of GFPuv/mL and the independent variables. Since $p < 0.01$ in the ANOVA, there is a statistically significant relationship between the variables at the 99% confidence levels. The attained quadratic polynomial model is Eq. 2:

$$\begin{aligned} \mu\text{g of GFPuv/mL} = & 10.28 - 1.13x_1 - 5.65x_3 + 8.28x_5 - 1.76x_1x_5 \\ & - 5.38x_2x_5 + 1.21x_4x_5 \end{aligned} \quad (2)$$

for the independent variables x_1 = period (h) of storage at 4°C, x_2 = rotary speed (rpm), x_3 = cell densities (OD₆₆₀) on the addition of IPTG, x_4 = concentration of IPTG (mM) added, and x_5 = culture incubation at 37°C set at 8 h and 24 h.

With 8 h of incubation at 37°C (x_5 = –1), the expressed GFPuv is proportional to the positive coefficient related to rotary speed (x_2 = +1) and to the negative coefficient of the culture cell density (OD₆₆₀, x_3 = –1) at which IPTG is added to the cultures of *E. coli*. This observation can be confirmed

Table 4
Extracted Concentrations of GFPuv and Effects of Expression Derived from *E. coli* Cells
Cultivated at Set Conditions for Standard Period of 24 h ($x_5 = +1$) of Incubation at 37°C

Assay ^a	Group ^b	Storage x_1	RPM x_2	IPTG (mM) x_3	OD ₆₆₀ IPTG x_4	Incubation (h) x_5	DCW (mg/mL)	Concentration (μ g GFPuv/mL) (mean $\pm$ CI) ^c	Specific productivity (μ g GFPuv/mg DCW) (mean $\pm$ CI) ^c
1	1	-1	-1	-1	-0.98	1	1.98	33.54 $\pm$ 1.07	16.97 $\pm$ 0.54
2	1	-1	-1	-1	-0.99	1	2.03	30.92 $\pm$ 1.57	15.24 $\pm$ 0.77
3	2	1	-1	1	-1.00	1	2.14	25.64 $\pm$ 1.84	12.00 $\pm$ 0.86
4	2	1	-1	1	-0.99	1	2.19	29.12 $\pm$ 1.80	13.30 $\pm$ 0.82
5	3	1	1	-1	-0.99	1	2.06	14.54 $\pm$ 0.82	7.05 $\pm$ 0.40
6	3	1	1	-1	-0.99	1	2.12	16.32 $\pm$ 1.11	7.68 $\pm$ 0.52
7	4	-1	1	1	-0.99	1	2.16	22.73 $\pm$ 1.29	10.52 $\pm$ 0.60
8	4	-1	1	1	-0.98	1	1.95	23.46 $\pm$ 1.30	10.86 $\pm$ 0.56
9	5	1	-1	-1	0.12	1	2.05	19.24 $\pm$ 2.20	9.38 $\pm$ 1.08
10	5	1	-1	-1	0.31	1	2.09	21.76 $\pm$ 0.58	10.43 $\pm$ 0.28
11	5	1	-1	-1	-0.35	1	2.14	22.20 $\pm$ 1.38	10.28 $\pm$ 0.65
12	5	1	-1	-1	-0.096	1	2.16	17.32 $\pm$ 0.44	8.03 $\pm$ 0.20
13	6	-1	-1	1	-0.56	1	1.98	31.98 $\pm$ 2.02	16.19 $\pm$ 1.02
14	6	-1	-1	1	-0.53	1	2.02	30.64 $\pm$ 1.46	15.17 $\pm$ 0.73
15	6	-1	-1	1	0.37	1	2.07	24.78 $\pm$ 1.88	11.97 $\pm$ 0.91
16	7	-1	1	-1	1.00	1	2.15	11.47 $\pm$ 0.82	5.33 $\pm$ 0.38
17	7	-1	1	-1	0.69	1	2.09	9.84 $\pm$ 2.74	4.71 $\pm$ 1.31
18	7	-1	1	-1	0.73	1	2.15	7.26 $\pm$ 0.47	3.38 $\pm$ 0.22
19	8	1	1	1	0.75	1	2.22	9.57 $\pm$ 0.51	4.30 $\pm$ 0.23
20	8	1	1	1	0.026	1	2.10	9.67 $\pm$ 0.33	4.61 $\pm$ 0.23
21	9	0	0	0	0.49	1	2.41	12.76 $\pm$ 0.91	5.29 $\pm$ 0.38
22	9	0	0	0	0.13	1	2.19	20.16 $\pm$ 1.45	9.19 $\pm$ 0.66
23	9	0	0	0	0.013	1	2.31	18.71 $\pm$ 1.00	8.11 $\pm$ 0.43

^aThe order in which the experiments were carried out.

^bThe set of conditions for every experiment carried out.

^c $p < 0.05$; $n = 10$ observations.

when storage is kept at 4°C for a maximum of 48 h ($x_1 = +1$), the final IPTG concentration added at a minimum concentration of 0.05 mM ($x_4 = -1$), and the GFPuv concentration is calculated through the fitted Eq. 3:

$$\mu\text{g of GFPuv/mL} = 3.84 + 5.39x_2 - 5.65x_3 \quad (3)$$

By analyses of the extreme conditions, the highest concentration of GFPuv extracted (15.24 μg of GFPuv /mL, Table 3) related to maximum 200-rpm rotary speed ($x_2 = +1$), minimum cell densities ($\text{OD}_{660} = 0.01$, $x_3 = -1$) on the addition of IPTG; the worst condition that provided no expression of GFPuv was evident with the minimum 100-rpm rotary speed ($x_2 = -1$) with the addition of IPTG at the highest OD_{660} of 0.8 ($x_3 = +1$); and the equivalent effect on the expression of ~ 4 μg of GFPuv /mL was verified for both x_2 and x_3 at minimum levels (100 rpm and OD_{660} of 0.01) or at maximum levels (200 rpm and OD_{660} of 0.8).

For 24 h incubation at 37°C ($x_5 = +1$), setting the storage at 4°C to a minimum of 24 h ($x_1 = -1$) and the maximum IPTG concentration of 0.5 mM ($x_4 = +1$), we observe through the fitted Eq. 4:

$$\mu\text{g GFPuv/mL} = 22.65 - 5.38x_2 - 5.65x_3 \quad (4)$$

that the influence of both negative coefficients for rotary speed ($x_2 = -1$) and cell OD_{660} density ($x_3 = -1$) on GFPuv expression resulted in the highest concentration of 33.54 μg of GFPuv /mL (Table 4) when rotary speed (100 rpm, $x_2 = -1$) and OD_{660} cell density (0.01 OD_{660} , $x_3 = -1$) were kept at minimum levels. Otherwise, at maximum levels for both variables, 200-rpm rotary speed ($x_2 = +1$) and 0.8 OD_{660} ($x_3 = +1$) cell densities for the addition of IPTG to the culture, the expression of GFPuv by *E. coli* decreased three times between 9.57 and 9.67 μg of GFPuv /mL (Table 4).

Comparing the highest expression of GFPuv by *E. coli* after incubation at 8 h ($x_5 = -1$) and 24 h ($x_5 = +1$) at 37°C, we observed that although GFPuv expression began following the addition of IPTG (maximum and minimum concentrations), the rotary speed was shown to exhibit a remarkable influence on the increase in cell density at the minimum level of 0.01 OD_{660} at the beginning of the logarithmic phase, when the newly divided cells are induced by IPTG to express GFPuv. Maximum rotary speed of 200 rpm ($x_2 = +1$) for a shorter incubation, 8 h ($x_5 = -1$), almost compensated, without success, the minimum speed at 100 rpm ($x_2 = -1$) for a longer incubation, 24 h, at 37°C ($x_5 = +1$), unless the 8 h ($x_5 = -1$) incubation provided half the concentration of GFPuv obtained for 24 h of incubation.

Another remarkable positive effect on GFPuv expression was the storage of the starter culture at 4°C for 24 h ($x_1 = -1$) and 48 h ($x_1 = +1$). Expression of the highest concentrations of GFPuv was shown to be independent of the time in storage.

Separation Efficiency of HIC on GFPuv Purification

The selected HIC supports present similar hydrophobic characteristics, appropriate for purification of intermediate to weakly hydrophobic

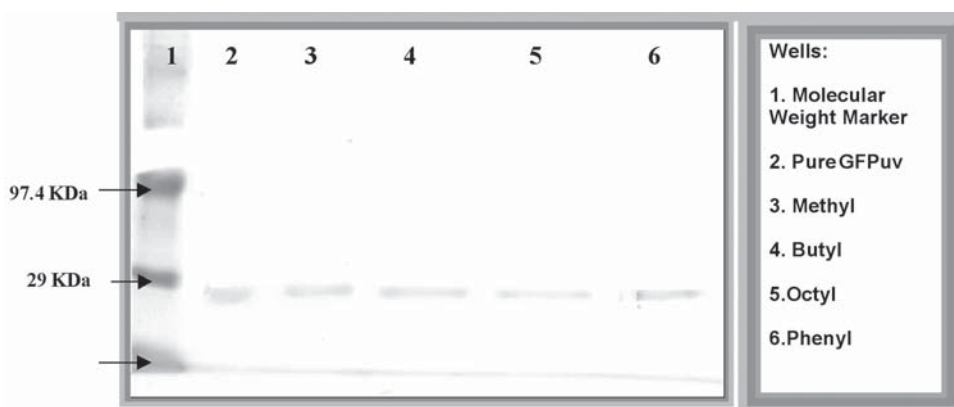


Fig. 1. Polyacrylamide gel run by SDS-PAGE. Protein was stained with Coomassie Brilliant Blue. The eluted samples from HIC columns applied to the wells were as follows: lane 1, mass molecular weight markers 18–200 kDa (Gibco); lane 2, standard GFPuv (20 $\mu\text{g}/\text{mL}$); lane 3, sample eluted from methyl column (0.32 μg); lane 4, sample eluted from butyl column (0.30 μg); lane 5, sample eluted from octyl column (0.29 μg); lane 6, sample eluted from phenyl column (0.26 μg).

proteins (9). The selection of these various resins allowed screening of the most appropriate HIC support for the purification of the TPP-extracted GFPuv from a complex mixture of proteins typically found in cell extracts. HIC is ideally suitable after salt precipitation of the TPP-extracted GFPuv from the pelleted cells, since HIC uses a decreasing salt gradient as the mobile phase, preserving the conformation of GFPuv and, hence, its fluorescence (2,6,9).

TPP-extracted GFPuv from pelleted cells of *E. coli* was eluted through four HiTrap FF HIC columns: methyl, butyl, octyl, phenyl Sepharose. The fluorescence intensity (excitation/emission maxima at 394/509 nm) of eluted samples was related to μg of GFPuv /mL, and then the same samples were run on a 12% SDS-PAGE gel (Figs. 1 and 2).

A single band between 27 (standard GFPuv) and 29 kDa (standard molecular weight) for every HIC column sample (Fig. 1) was visualized by Coomassie staining. As seen in every lane in Fig. 1, the gels proved the effectiveness of TPP extraction for GFPuv, and the bands observed were similar for every sample eluted through a column. With SDS-PAGE, it was not possible to distinguish which HIC column improved the purity of the extracted GFPuv. However, using the more sensitive silver staining, the butyl HIC eluted samples showed some bands >29 kDa (Fig. 2).

Table 5 shows that the amount of BSA eluted with GFPuv from the methyl and butyl HIC columns was half that recovered in the samples eluted from the octyl and phenyl columns, for a range of respective mean concentrations of GFPuv from 27.92 (methyl column) to 28.66 $\mu\text{g}/\text{mL}$ (phenyl support), corresponding to a total average of $28.25 \pm 0.36 \mu\text{g}$ of GFPuv /mL.

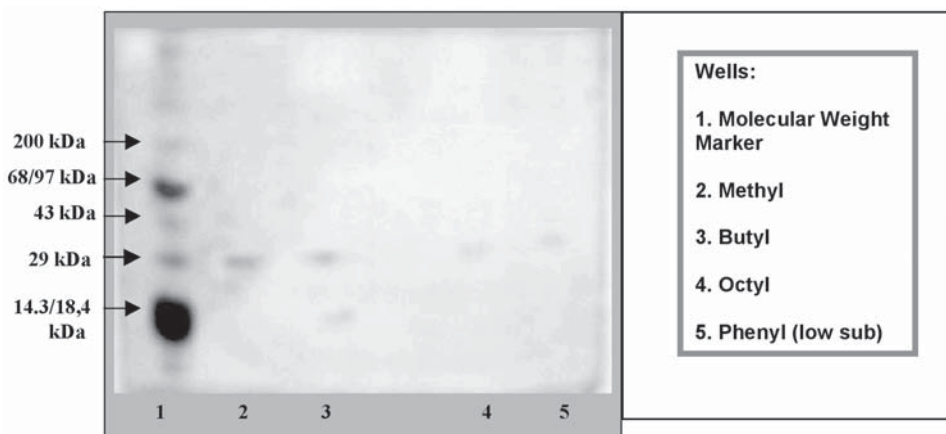


Fig. 2. Polyacrylamide gel run by SDS-PAGE (PhastSystem). Protein was stained with silver nitrate. Gel visualization was altered by nitrate oxidation. The eluted samples from HIC columns applied to the wells were as follows: lane 1, mass molecular weight markers, 18–200 kDa (Gibco); lane 2, sample eluted from methyl column (0.085 μg); lane 3, sample eluted from butyl column (0.080 μg); lane 4, sample eluted from octyl column (0.077 μg); lane 5, sample eluted from phenyl column (0.069 μg).

Table 5
Protein Concentration in Eluted Samples
from HIC Columns Expressed in Terms of GFPuv (μg GFPuv/mL),
BSA (mg BSA/mL), and Specific Mass (μg GFPuv/mg BSA)^a

		Eluted Samples				
HIC Column		1	2	3	4	5
Methyl	GFPuv (μg/mL)	28.33	27.13	26.58	28.27	29.31
	BSA (mg/mL)	1.65	0.45	0.49	0.20	0.37
	SM (μg/mg)	17.12	59.98	54.60	138.35	78.95
Butyl	GFPuv (μg/mL)	27.72	23.78	29.89	29.06	31.85
	BSA (mg/mL)	0.87	0.25	0.33	0.26	1.09
	SM (μg/mg)	31.85	96.00	90.30	110.69	29.12
Octyl	GFPuv (μg/mL)	27.56	25.37	29.05	29.62	28.27
	BSA (mg/mL)	2.63	0.27	1.01	0.60	0.43
	SM (μg/mg)	10.50	95.52	28.78	49.00	65.34
Phenyl	GFPuv (μg/mL)	22.92	25.93	30.50	31.07	32.88
	BSA (mg/mL)	3.18	0.39	0.76	1.05	0.44
	SM (μg/mg)	7.22	65.87	40.05	29.46	74.01

^aThe range of GFPuv concentration analyzed was about 20–30 $\mu\text{g}/\text{mL}$. SM, specific mass.

The same HIC column was used five times with five different samples from the same TPP-extracted 4-mL aliquot. The GFPuv concentration, eluted from the four columns and from five runs through each of the columns, was shown to be equivalent for all four HIC supports. In relation to the removal of BSA from the eluted sample, for the first, second and third runs, the butyl column provided the higher specific masses; for the fourth and fifth runs the methyl support increased the specific mass up to 138.35–78.95 µg of GFPuv / mg of BSA. The use of the same HIC columns after five elutions of the TPP-extracted aliquot did not interfere with the evaluation of their performance.

For further work, the methyl HIC column was chosen for the purification of the TPP-extracted GFPuv samples. GFPuv recovery and enrichment from the eluted samples were also determined (Table 2).

Enrichment and Recovery of GFPuv in Eluted Samples from Methyl Columns

The number of hydrophobic groups covering the surface of the methyl matrix is higher than the other matrices, resulting in a higher capacity for protein retention. Although the protein may contain a large percentage of hydrophobic residues throughout its structure, only residues at the surface contribute to the hydrophobic character of the native protein.

The purification efficiency of GFPuv eluted from the methyl HIC columns was evaluated through (1) the enrichment (E, times) and (2) recovery (%) indices (7,9):

Enrichment (E):

$$\text{Enrichment} = \frac{\text{Specific mass of eluted purified GFPuv sample}}{\text{Specific mass of TPP-extracted GFPuv aliquot}} \quad (5)$$

The enrichment index measures the level of purity of the GFPuv samples by the increase in specific mass (µg of GFPuv / mg of BSA) owing to the removal of proteins (quantified as BSA) other than GFPuv (5,8). Enrichment is expressed in terms of number of times (E) the specific mass of the eluted purified GFPuv sample (µg of GFPuv / mg of BSA) is greater than the corresponding specific mass of the TPP-extracted GFPuv aliquot (µg of GFPuv / mg of BSA) before HIC, by the reduction of BSA concentration and maintaining the initial concentration of GFPuv.

Recovery (%):

$$\text{Recovery} = \frac{\text{Eluted GFPuv sample (µg of GFPuv/mL)}}{{}^1\text{TPP-extracted GFPuv aliquot}} \quad (6)$$

in which, ¹ is a 1-mL aliquot of TPP-extracted GFPuv loaded onto the HIC column. The recovery index measures the percentage (%) of the amount of TPP-extracted GFPuv that was present in the purified GFPuv sample after passage through the HIC columns.

Each TPP-extracted aliquot was divided into two samples that were independently loaded onto the methyl support (Table 2), providing a total

of 64 eluted samples in duplication: the first group of 32 samples plus the second group of 32 samples.

The recovery index varied from 72.67 to 107.13%, and the methyl support showed a very high efficiency in the recovery of GFPuv. From 64 assays, seven samples of the first group and seven samples of the second group presented recovery of GFPuv between 70 and 80%. The majority of the samples (about 78%) showed a recovery index >80% of GFPuv in the eluted samples. Therefore, the range for GFPuv recovery index from the eluted samples was from $90.88 \pm 3.62\%$ for the first group to $89.32 \pm 4.60\%$ for the second group. Even for samples with concentrations lower than 10 μg of GFPuv/mL (from 1.15 to 8.18 μg of GFPuv/mL, in seven TPP-extracted aliquots), the recovery was confirmed to be higher than 80%.

The enrichment index varied from (1) 1.17 – 2.05 times for a 5.33 μg of GFPuv/mL aliquot corresponding to (2) 24.67 – 28.36 times for 1.15 and 22.14 μg of GFPuv/mL TPP-extracted aliquots, respectively. The specific mass of each 25 eluted samples of the first plus the second groups was enriched up to 9.5-fold. Otherwise, a total of 12 eluted samples belonging to the first group (6 samples) and the second group (6 samples) provided GFPuv enrichment between 10- and 20-fold, and one sample of each group showed 28- and 24-fold enrichment, respectively. Therefore, the average index of enrichment provided by the methyl HIC support over TPP-extracted aliquots ranged from an 8.46 ± 2.13 increase (first group) to a 7.60 ± 2.00 increase (second group). Taking into consideration the total proteins in the samples, expressed in mg of BSA/mL, three and four eluted samples of the first and second groups, respectively, contained concentrations >0.5 mg of BSA/mL, and three samples of the first group had up to 0.7 mg of BSA/mL, independent of the initial specific mass of the TPP aliquot eluted through the methyl HIC medium.

Discussion

The present work verified that the influence of the range of IPTG, from 0.05 to 0.5 mM, added to the cultures was independent of cell density (OD_{660} between 0.01 and 0.8); however, the induction timing and the addition of IPTG at the beginning of exponential phase of growth favored the expression of GFPuv by *E. coli*. Shi and Su (10) examined the highest IPTG concentration and optimal induction time on the expression of GFP on the extracellular surface of *E. coli* JM109 (pUMC101) cells. They verified that the timing of induction showed a significant effect on cell growth when the culture was induced at a density lower than $\text{OD}_{660} < 1.0$ for concentrations >0.05 mM IPTG.

The effectiveness of the TPP extraction of GFPuv from *E. coli* was analyzed by electrophoresis and confirmed to be the same in every lane for samples eluted from all HIC columns. The performance of the HIC columns was not affected after five elutions per column.

The enrichment in GFPuv in the eluted samples was dependent on the amount of total proteins (expressed in mg of BSA/mL). After elution

through the methyl HIC columns, the recovery and enrichment of GFPuv in the eluted samples exhibited very high indices, about $90.10 \pm 4.11\%$ of GFPuv recovery and 8.03 ± 2.07 -fold enrichment of GFPuv related to the specific mass. Therefore, the HIC procedure did improve the effectiveness of TPP extraction on GFPuv purification, confirming Sharma and Gupta's (6) and Yakhnin et al.'s (11) observations.

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