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Selective Permeation and Organic Extraction of Recombinant Green Fluorescent Protein (gfpuv) from Escherichia coli

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Abstract

Background: Transformed cells of Escherichia coli DH5-α with pGFPuv, induced by IPTG (isopropyl-β-d-thiogalactopyranoside), express the green fluorescent protein (gfpuv) during growth phases. E. coli subjected to the combination of selective permeation by freezing/thawing/sonication cycles followed by the three-phase partitioning extraction (TPP) method were compared to the direct application of TPP to the same culture of E. coli on releasing gfpuv from the over-expressing cells.

Material and Methods: Cultures (37°C/100 rpm/ 24 h; μ = 0.99 h⁻¹ - 1.10 h⁻¹) of transformed (pGFP) Escherichia coli DH5-α, expressing the green fluorescent protein (gfpuv, absorbance at 394 nm and emission at 509 nm) were sonicated in successive intervals of sonication (25 vibrations/pulse) to determine the maximum amount of gfpuv released from the cells. For selective permeation, the transformed previously frozen (-75°C) cells were subjected to three freeze/thaw (−20°C/ 0.83°C/min) cycles interlaid by sonication (3 pulses/6 seconds/25 vibrations). The intracellular permeate with gfpuv in extraction buffer (TE) solution (25 mM Tris-HCl, pH 8.0, 1 mM β-mercaptoethanol β-ME, 0.1 mM PMSF) was subjected to the three-phase partitioning (TPP) method with t-butanol and 1.6 M ammonium sulfate. Sonication efficiency was verified on the application to the cells previously treated by the TPP method. The intra-cell releases were mixed and eluted through methyl HIC column with a buffer solution (10 mM Tris-HCl, 10 mM EDTA, pH 8.0).

Results: The sonication maximum released amount obtained from the cells was 327.67 µg gfpuv/mL (20.73 µg gfpuv/mg total proteins – BSA), after 9 min of treatment. Through the selective permeation by three repeated freezing/thawing/sonication cycles applied to the cells, a close content of 241.19 µg gfpuv/mL (29.74 µg gfpuv/mg BSA) was obtained. The specific mass range of gfpuv released from the same cultures, by the three-phase partitioning (TPP) method, in relation to total proteins, was higher, between 107.28 µg/mg and 135.10 µg/mg.

Conclusions: The selective permeation of gfpuv by freezing/thawing/sonication followed by TPP separation method was equivalent to the amount of gfpuv extracted from the cells directly by TPP; although selective permeation extracts showed better elution through the HIC column.
Background

The fluorescent green protein, gfpuv extracted from Aequorea victoria shows a maximum excitation under ultraviolet light (UV) in the interval of $\lambda = 395–498$ nm and emission in the interval of $\lambda = 490–520$ nm, with the maximum peak at $\lambda = 508–509$ nm. The recombinant fluorescent green protein, gfpuv, expressed by transformed (pGFP, Clontech) cells of Escherichia coli, was developed by introducing point mutations in an in vitro wild gfp DNA, replacing three amino acids (Phe$^{99}$ for Ser; Met$^{153}$ for Thr; and Val$^{163}$ for Ala, based on the amino acid numbering of wild gfp). The resulting gfpuv is expressed two to three times faster in E. coli strains and shows 18 times brighter fluorescence than the native gfp: the maximum peaks for fluorophore excitation being at 395 nm and centered at 508–509 nm for emission [1].

The recombinant gfpuv is a globular protein, compact and acidic, made up of 27 kDa monomers (with 238 amino acids), having a propensity to dimerize. The protein fluorescence is stable between pH 5.5 and 11.5, with the maximum at pH 8.0.

The first step for the gfpuv purification process was the intracellular release and isolation of the protein into an aqueous extraction buffer – TE (25 mM Tris-HCl, pH 8.0, 1 mM β-mercaptoethanol, “β-ME”, 0.1 mM phenylmethysulfonylfluoride, “PMSF”). During extraction procedures, the proteolysis of the C-terminal extremity of gfpuv was inhibited by working at cool temperatures ($\leq 4^\circ$C), and in the presence of PMSF. The addition of β-ME and the maintenance of pH at 8.0 enhances protein stability [1].

Several methods are used currently for cellular partial (permeation) or total cellular disruption. For sake of convenience, they may be classified as (i) physical mechanical (ultra sonication, bead mill, blade and high-pressure homogenizers) and non-mechanical treatments (thermal shock, freeze and thaw); and a combination (freezing/thawing cycles interlaid with sonication); (ii) chemical methods for selective protein fractionation by the use of (a) organic solvent (alcohol, toluene, acetone); (b) inorganic acids and bases (pH variation) and salts (salting in and salting out phenomena); (c) combination of salting out (ammonium sulfate and cosolvent (ethanol, t-butanol)); (d) organic water soluble polymers (polyethylene glycol "PEG" molecular weight 4,000 or greater); (iii) affinity methods (chromatography-HIC: hydrophobic interaction chromatography; electrophoresis; affinity phase partitioning); (iv) biological methods with enzyme digestion of microbial membranes (lysozyme); (v) combination of those techniques (physical permeation plus chemical cellular disruption followed by affinity method).

During the isolation procedures from the bacterial cells, the stability of the required protein structure is of fundamental importance. Through the fractionation technique employed as the first step, the maintenance of the protein structure and activity is more important than the purity attained. A protocol selected to release the proteins from the intracellular compartments must provide the best recovery and purity, avoid proteolysis by the addition of protease inhibitors, and minimum binding to either particulates, high molecular weight proteins or non-protein substances that may prevent the recovery of the protein.

For the purpose of gfpuv purification, Yakhnin et al [2] proposed a two-step procedure for the extraction of gfpuv with ethanol into the organic phase and subsequent protein re-extraction into the aqueous phase, to reduce the loss of most of the gfpuv during multiple chromatography steps. The ethanol extractions were made from the entire suspension in 70% ammonium sulfate ((NH$_4$)$_2$SO$_4$). The re-extraction in the aqueous (NH$_4$)$_2$SO$_4$ was performed with n-butanol added to the ethanol extract. The authors observed that one chromatography final step was enough to purify gfpuv from nonprotein substances.

The addition of a polar miscible solvent, such as ethanol or acetone, to an aqueous extract containing proteins has a variety of effects that combined, lead to protein precipitation. The principal effect is the reduction in water activity. The solvating power of water for a charged, hydrophobic protein molecule is decreased as the concentration of organic solvent increases, which causes a bulk displacement of water plus the partial immobilization of water molecules through hydration of the organic solvent molecules. The most hydrophobic molecules or proteins may be soluble in 100% organic solvent [3].

The most advantageous salt for protein isolation and further purification, through fractionation methods, is ammonium sulfate, (NH$_4$)$_2$SO$_4$, which forms saline bridges with polar regions, stabilizing the molecule [2–8]. The main advantages of ammonium sulfate fractionation method, through salting out at high salt concentration (2–3 M ammonium sulfate), are (a) the stabilization of protein precipitate over long-term storage, (an easy way of packaging and commercialization); (b) the prevention of proteolysis and bacterial action and (c) the conservation of the protein precipitates, either before centrifuging or as a pellet, a good form in which a sample may be kept for further purification steps [3].

The addition of t-butanol to the ammonium sulfate solution of proteins consolidates the technique known as three-phase-partitioning (TPP), which is characterized by three distinct phases: an upper t-butanol, a lower aqueous
phase containing (NH₄)₂SO₄ at high concentration, and a middle layer [4–7]. The mixing of the upper phase with lower phase creates a third intermediate phase of precipitate material. The concentration of the required protein in the third phase depends on the (NH₄)₂SO₄ concentration. t-butanol stabilizes protein structure, inhibits proteases and protein/protein interactions [4,5]. These are similar qualities shown by ammonium sulfate during the salting out phenomenon.

(NH₄)₂SO₄ at high concentration promotes hydration of polar regions and dehydration of the hydrophobic regions on the protein surface, without direct interaction to molecular structure. Salting out depends on hydrophobic regions on the surface of proteins, where solubility is strongly reduced. At high salt concentration, the salt ions become solvated easier than polar regions on the protein surface. Furthermore, the freely available water molecules become scarce and the bare polar regions that have their water solubility reduced tend to aggregate and form clusters of different sizes [2,3,5].

In contrast, proteins and particulate material with a majority of non-polar surface residues may remain in solution even at the higher salt concentration. With the TPP method, those residues in contact with t-butanol are precipitated and form the first fraction of a floating intermediate phase, characterized by white cluster that is difficult to re-dissolve even in extraction buffer (TE). For the TPP method, the first white middle layer is a collection of high molecular weight contaminants and debris, precipitating at up to 25% ammonium sulfate saturation with conventional salting out fractionation method.

The binding of t-butanol to the hydrophobic regions of the protein molecules in the homogenate depends on the both the concentrations of the (NH₄)₂SO₄ and the required protein, which floats into the third phase at the TPP technique. At that time, when t-butanol is added a second time to the homogenate in the presence of a high concentration of ammonium sulfate, a protein is precipitated as an interface between the organic and aqueous phases. Therefore, the t-butanol stabilizes protein aggregates in the middle layer at the second TPP, preventing protein/protein interaction [5]. The thin middle layer is easily dissolved in the extraction buffer (TE, with added EDTA) and may be stored (4°C) for latter purification steps or subjected to further fractionation method by hydrophobic interaction chromatography (HIC – affinity chromatography).

TPP that is an emerging bioseparation technique presents the advantages of conventional salting out, isoionic precipitation, co-solvent precipitation and osmolytic and kosmotropic precipitation of proteins. It is easily scalable and can be used directly with crude suspensions [6,7].

Consequently, TPP was the technique chosen for direct fractionation of gfpuv.

In this study, the bacteria cellular release was blended in 450 µl of TE (25 mM Tris-HCl, β-ME, PMSF). The homogenate was mixed with 300 µl of 4 M ammonium sulfate and 750 µl of t-butanol (ratio 1:1) was added (Ward personal communication) [8]. TPP was performed at room temperature. The sample was vigorously stirred, centrifuged, and the three phases formed were collected separately. The large amounts of gfpuv present in the lower aqueous layer (containing (NH₄)₂SO₄), was subjected to TPP several times. The intermediate middle layer containing gfpuv was precipitated. The precipitated gfpuv was collected and re-suspended in extraction buffer (TE, with added EDTA) and then fractionated on to the HIC column.

Sharma & Gupta [6] employed the TPP method for direct one step purification of protein inhibitor from the crude wheat germ extract. The authors observed best results (25-fold purification with an activity recovery of 85%) with a 1:1 ratio of t-butanol to crude extract and 30% ammonium sulfate (w/v) at 20°C. Sharma & Gupta [7] purified phospholipase from Daucus carota by a single step of the TPP procedure and obtained 13-fold purification with an activity of 72%.

The purpose of the research was to compare the association of selective permeation by freezing/thawing/sonication (FTS) cycles applied to the transformed cells of E. coli followed by the three-phase-partitioning extraction (TPP) method and the direct application of TPP to the same culture of E. coli on releasing gfpuv from the over-expressing cells.

Material and methods

Transformation

The Escherichia coli DH5-α [2] were transformed with pGFPuv (Clontech, CA, USA) [9], by the standard calcium chloride method [5]. The transformed cells of Escherichia coli DH5-α with pGFPuv (Clontech, CA, USA) [2,5] were stored at (-75°C) into LB/amp broth with glycerol added in the proportion of 1:1.

Expression

A 24 h (37°C/100 rpm) culture of E. coli was developed in 25 mL Luria Bertani (LB; Difco, USB/Mexico, OH, USA) medium, supplemented with ampicillin (100 µg/mL Boehringer, Mannheim, Germany). When the broth cultures attained a OD₆₆₀nm = 0.7–0.8 (10⁸ CFU/mL isopropyl-β-D-thiogalactopyranoside (IPTG; dioxane free, USD/Italy,
OH, USA) was added to a final concentration of 0.5 mM [10], as inductor of gfpuv expression by the mutant gfpuv, which is under tight control of the lacZ protein β-galactosidase promoter/repressor.

**Growth conditions**

The growth culture was centrifuged (1000 × g/30 min/4°C), the supernatant was decanted, the pelleted cells were observed under UV light (λ = 395 nm) and resuspended in 1.0 mL cold extraction buffer solution (TE: 25 mM Tris-HCl, pH 8.0 – Trizma® Base, Sigma, MO, USA; 1.0 mM β-mercaptoethanol “β-ME”, Pharmacia Biotech, Sweden; 0.1 mM phenylmethylsulphonylfluoride “PMSF”, USB, Switzerland), dispersed and frozen at -75°C. The length of time the pelleted cells were held frozen was not monitored. (Ultra Freezer-Kelvinator Series 100 – Manitowoc, WI, USA). (Figure 1. The pelleted E. coli cells) The uniform dispersed suspension was thawed (4°C), subjected to blending and pelleted again by centrifugation.

The supernatant was read for total protein (OD280nm) and isolated gfpuv (absorbency = 395 nm; emission = 509 nm) released. The fluorescent pellet was resuspended and dispersed into 1.0 mL TE. Both, supernatant and pelleted cells, were stored at 4°C and processed further:

(i) Sonication to the total permeation of gfpuv: A pelleted frozen (-75°C) 24 h culture of E. coli was dispersed into 1.0 mL TE and sonicated (High Intensity Ultrasonic Processor, Vibram cells, model VC 100, Sonic & Materials, CT, USA). A 3 mm microtip ultrasonic processor was inserted in the tube of dispersed suspension (kept immersed in an ice-salt bath) and sonicated with 3, 6, 12, 24, 48, 96, 192, 384-fold pulses, over total intervals of 18s, 54s, 126s, 270s, 558s, 1134s, 1728s, 2880s. Each pulse was at 25 vibration amplitude at alternating cycles of 6 seconds on and 1.0 second off. Between sonication performed intervals, the suspension was centrifuged, a 1.0 mL aliquot of the supernatant was read for total protein and released gfpuv (see Table 1).

(ii) Three-Phase-Partitioning (TPP) extraction method[4–8]. A pelleted frozen (-75°C) 24 h culture of E. coli was dispersed into 1.0 mL TE (4°C) and subjected to direct isolation by TPP. To each aliquot of 450 µL, 300 µL of 4 M (NH₄)₂SO₄ and 750 µL of t-butanol were added [6–8]. The mixture was vortexed for 1.0 min, allowed to settle for complete phase separation and centrifuged (6,000 × g/3 min). The t-butanol upper layer and the white interfacial precipitate were removed and discarded. 750 µL of t-butanol was mixed into the lower aqueous layer. The mixture was allowed to settle and centrifuged. The upper layer was discarded. The interfacial green layer was collected and dissolved in 450 µL TE buffer solution and kept at 4°C for spectrophotometer and spectrofluorometer readings and partial purification through hydrophobic interaction chromatography (HIC). While the lower layer was still fluorescent, it was subjected to repeated TPP by mixing in 750 µL t-butanol, centrifugation and intermediate phase separation. (Figure 2)

(iii) Freezing/Thawing/Sonication (FTS) selective permeation of gfpuv[10]: A pelleted frozen (-75°C) 24 h culture of E. coli was dispersed into 1.0 mL TE. The re-suspended pellets were subjected to three repeated cycles of slow freezing (0.83°C/min until samples reached -20°C) and thawing (0.83°C/min, at room temperature), in a freezer-dryer (FTS System™, Secfroid, Lyolab G, NY, USA) chamber (Dura Stop™ MP). With PT-100 probes, inserted into the pelleted suspension, the freezing/thawing tempera-

### Table 1: Sonication of over expressing cells of E. coli.

| Time (seconds) | Total proteins (BSA) (mg/mL) | gfpuv (µg/mL) | gfpuv/BSA (µg/mg) |
|----------------|-----------------------------|--------------|-------------------|
| 0              | 0.87                        | 25.79        | 29.53             |
| 18             | 1.62                        | 39.30        | 24.30             |
| 54             | 3.04                        | 65.89        | 21.68             |
| 126            | 6.80                        | 167.56       | 24.63             |
| 270            | 6.19                        | 184.48       | 29.82             |
| 558            | 15.81                       | 327.67       | 20.73             |
| 1134           | 9.83                        | 222.68       | 22.64             |
| 1728           | 20.29                       | 395.14       | 19.47             |
| 2880           | 14.05                       | 268.28       | 19.10             |

**Figure 1**

Pelleted cells of E. coli, expressing green fluorescent protein (gfpuv).
tures were registered every minute through a software “lyphoware” for Windows (Figure 3). The phenomenon of superfusion on the freezing of the samples was observed to happen between (-11°C) and (-14°C), after which the samples reached (-20°C) at 0.83°C/min [10].

Between the freezing/thawing cycles, the sample was subjected to three-fold pulse sonication in an ice-water bath over a total interval of 23s. Between the freezing/thawing/sonication cycle, cells were pelleted by centrifugation, and the supernatant was read for total protein and released gfp (see Table 03).

(iv) Three-Phase-Partitioning (TPP) extraction method associated to the FTS procedure. The pellets from the 3rd FTS cycle were re-suspended into 450 µL of TE and subjected to the TPP extraction method.

(v) Direct extraction by the TPP method. Each pelleted frozen (-75°C) 24 h culture (I & J) of E. coli was dispersed into 1.0 mL of TE and vortexed for total uniform dispersion. The suspension was divided into two groups, as follows:

(a) A 450 µL sample of each culture was subjected to: (i) direct 2-step extraction by the TPP method; (ii) followed by sonication procedure (3-fold pulse/ 25 vibration amplitude/ alternating cycles of 6 seconds on and 1.0 second off) in an ice-water bath for 23s and (iii) a final 1-step TPP extraction.

(b) The other 450 µL sample of each culture was previously subjected to: (i) the sonication procedure (three-fold 6s pulse at 25 vibration amplitude) in an ice-water bath for 23s; (ii) followed by one-step TPP extraction method.

(vi) Fast Flow methyl hydrophobic interaction chromatography (HIC) for ion exchange separation and concentration of gfp from the extraction mixtures. Equal volumes (1:1) of the TE containing gfp and 4 M (NH₄)₂SO₄ were mixed, blended and centrifuged. At room temperature, 1.0 mL of the clarified supernatant was loaded onto a methyl HIC column fast flow, pre-equilibrated with 2 M (NH₄)₂SO₄. gfp was adsorbed at the top of the methyl hydrophobic matrix. The column was loaded with 250 µL of 1.3 M (NH₄)₂SO₄ which carried the gfp throughout the column bed. The protein was eluted with 1.0 mL buffer solution (10 mM Tris-HCl, 10 mM EDTA, pH 8.0).

Concentrations
The supernatant was read for total protein (based on BSA) and isolated gfp released. The fluorescent bright green intact cells with easily defined outlines were observed under an Olympus (BX-60-IV FLA System Attachment, NY, USA) microscope, through filters for the detection of fluorescent cells [U-MWG (510–550 nm); U-MWU (330–385 nm); U-MNV (400–410 nm)]. Recombinant green fluorescent protein (gfp) absorbs at UV (330–385 nm) and blue (400–410 nm) emission, with a maximum absorbance peak at 395 nm and a minor at 470 nm, emitting maximum green light at 509 nm. The spectrum was confirmed through Fluorescence Spectrophotometer F-2000 – Hitachi (OH, USA) spectra.

Standard Curves
The fluorescence intensity of gfp detected in a cleared bacterial cell lysate was measured using the Fluorescence Spectrophotometer, with an excitation filter of 394 nm and an emission filter of 505 nm. The fluorescence intensity of the experimental samples was compared to the standard curve (gfp µg/mL = 0.001* (fluorescence intensity) - 0.1133; R² = 0.995) to determine the amount of gfp released from the cells. The standard curve was prepared using known amounts (between 6.66 µg/mL and 0.59 µg/mL) of purified recombinant gfp (Clontech) diluted in the same buffer solution (10 mM Tris-HCl, pH 8.0, 1.0 mM β ME, 0.1 mM PMSF).

The total protein released in the medium from E. coli cells was expressed in mg/mL and compared with total protein concentrations expressed as purified bovine serum albumin (BSA, mol wt. 66 kDa, Sigma, MO, USA) at λ = 280 nm in a spectrophotometer. The total protein concentrations in the buffer solution ranged from 100 to 1000 µg/mL, the maximum OD₃₁₄ nm being 0.615; and the comparative relationship between total proteins and BSA was made through the standard curve (total protein µg/mL = 1727.2*(OD₃₁₄) - 26.863; R² = 0.9943).
Results

In Tables 1, 2 and 3, the results were expressed by total protein (BSA, mg/mL), released gfpuv (µg/mL) content, gfpuv yield (% µg/µg) in relation to the total released gfpuv content obtained; the specific gfpuv mass being expressed by µg of gfpuv in relation to mg of total protein (µg gfpuv/mg BSA).

(i) Sonication of the cells to complete permeation and isolation of gfpuv contents (Table 1)

Sonication was performed on cells of E. coli DH5-α expressing a high concentration of gfpuv to verify the maximum protein content that can be released from the cells. After 558s sonication interval treatment, a maximum concentration of 327.67 µg gfpuv/mL was obtained, equivalent to the specific mass of 20.73 µg gfpuv/mg BSA.

It was interesting to observe that the values of specific mass had varied from 21.68 µg/mg to 29.82 µg/mg, indicating that during the sonication procedure the amount of released gfpuv was proportional to the other proteins and molecules extracted from the sonicated cells.

(ii) Isolation by three-phase partitioning (TPP) method, using ammonium sulfate (final concentration 1.6 M) and t-butanol (Table 2)

In this work, the pelleted sample was diluted in 450 µl of extraction buffer (TE: 25 mM Tris-HCl, pH 8.0, β-ME, PMSF). It was followed by addition of 300 µl of 4 M (NH₄)₂SO₄ and, after that, 750 µl t-butanol (ratio 1:1). The sample was stirred for 1.0 min, centrifuged at 10,000 x g / 3 min and the three phases formed were collected separately. The first white middle layer (an white disc in-
Table 2: Three-phase-partitioning (TPP) method applied directly to cells of E. coli with specific growth rate constant (µ, h⁻¹) of 0.99 h⁻¹ for culture A, 1.05 h⁻¹ for cultures B, C, D, E and of 1.10 h⁻¹ for cultures F, G, H.

| Sample | gfpuv (µg/mL) | BSA (mg/mL) | gfpuv/BSA (µg/mg) |
|--------|---------------|--------------|-------------------|
| A1     | 354.08        | 3.30         | 107.28            |
| A2     | 444.05        | 3.29         | 135.10            |
| A (Pool)| 378.53        | 3.98         | 95.11             |
| B1     | 46.68         | 2.60         | 18.05             |
| B2     | 38.59         | 2.24         | 17.26             |
| B (Pool)| 45.66         | 2.42         | 18.87             |
| C      | 39.60         | 1.11         | 35.65             |
| D      | 15.92         | 0.41         | 38.86             |
| E      | 28.12         | 0.73         | 38.70             |
| F1     | 15.78         | 0.52         | 30.42             |
| F2     | 11.56         | 0.22         | 52.69             |
| F (Pool)| 5.47          | 0.67         | 8.12              |
| G      | 19.42         | 0.79         | 24.53             |
| H1     | 16.69         | 0.82         | 20.44             |
| H2     | 9.16          | 0.19         | 47.67             |
| H      | 4.78          | 0.67         | 7.09              |

Cultures B, C, D and E showed (Table 2) expressions in µg gfpuv/mL and SM (µg gfpuv/mg BSA), respectively, 45.66 µg/mL and 18.87 µg/mg for culture B; 39.60 µg/mL and 35.65 µg/mg for culture C; 15.92 µg/mL and 38.86 µg/mg for culture D; 28.12 µg/mL and 38.70 µg/mg for culture E. The TPP method extracted lower amounts of contaminants for concentrations lower than 30 µg gfpuv/mL, when the specific mass gradually increased, as shown in cultures F, G and H. The TPP method not only extracted gfpuv but also carried out a selective isolation of the protein.

For higher fluorescence (> 30 µg gfpuv/mL), the formed intermediate white ring proceeding from the contaminant precipitation was observed to entrap low amount of gfpuv which was lost. On the other hand, the TPP method cleaned the isolated gfpuv solution, making subsequent elution through the HIC easier.

Cultures F, G and H showed (Table 2) half the expression of gfpuv as follows: 15.78 (F), 19.42 (G) and 16.69 (H) µg gfpuv/mL, versus 46.68 (B1), 38.59 (B2), 39.60 (C), 15.92 (D) and 28.12 (E) µg gfpuv/mL. However, the specific masses of these cultures B, C, D and E, F, G, H were equivalent, showing that lower concentrations of gfpuv improved their isolation from the cells and contaminants.

Sharma [6] used the three-phase-partitioning (TPP) method to purify crude extract of protease/amylase inhibitors at 20°C; t-butanol was employed at a ratio of 1:1, and (NH₄)₂SO₄ at 30%; and the recovery was about 85% and 25-fold purification.

(iii) Permeation by physical procedures (freezing/thawing/sonication procedures) associated to the TPP method (Table 3)

The freezing (-75°C) permeated 69.85 µg gfpuv/mL (A1), five times less than the TPP method (354.08 µg gfpuv/mL, sample A1, Table 2); however, the specific contents were similar, respectively 86.25 and 107.28 µg gfpuv/mg BSA. Therefore, slow freezing allowed a cleaner permeate than the extraction by TPP, which released four times more contaminants (3.30 mg BSA/mL) than permeation did (0.81 mg BSA/mL).

It was observed that, for culture A1 (Table 2), with a high concentration of gfpuv expressed, the TPP method extracted between 354.08 and 444.05 µg gfpuv/mL from the cells, 70% more than the slow freezing (-75°C). This unit operation was followed by three repeated cycles of freezing/thawing/sonication (FTS), which caused a total permeation of 241.19 µg gfpuv/mL, distributed as follows (Table 3): 29% (69.85 µg/mL) by freezing (-75°C), 24% (57.83 µg/mL), 38% (91.78 µg/mL), and 9% (21.73 µg/mL) after the 1st, 2nd and 3rd FTS cycles, respectively. Penna et al. [10] verified that for increasing amounts of over-expressed...
Table 3: Permeation of E. coli cells through independent repeated cycles (1°, 2°, 3°) of slow freezing/ thawing/ sonication (FTS), followed by two three-phase partitioning (TPP) extractions, and elution throughout hydrophobic HIC chromatography column. The specific growth rate constant ($\mu$) of 0.99 h⁻¹ for culture A, 1.05 h⁻¹ for cultures B, C, D, E and 1.10 h⁻¹ for cultures F, G, H.

| Sample | (-75°C) | 1° FTS | 2° FTS | 3° FTS | Pool | TPP |
|--------|---------|--------|--------|--------|------|-----|
|        | gfp<sub>uv</sub> µg/mL | BSA mg/mL | gfp<sub>uv</sub>/BSA µg/mg | gfp<sub>uv</sub> µg/mL | BSA mg/mL | gfp<sub>uv</sub>/BSA µg/mg | gfp<sub>uv</sub> µg/mL | BSA mg/mL | gfp<sub>uv</sub>/BSA µg/mg | gfp<sub>uv</sub> µg/mL | BSA mg/mL | gfp<sub>uv</sub>/BSA µg/mg | gfp<sub>uv</sub> µg/mL | BSA mg/mL | gfp<sub>uv</sub>/BSA µg/mg | gfp<sub>uv</sub> µg/mL | BSA mg/mL | gfp<sub>uv</sub>/BSA µg/mg |
| A1     | 69.85   | 0.81   | 86.25  | 57.83  | 2.80  | 20.62 | 91.78  | 3.89  | 23.61 | 21.73  | 0.61  | 35.87  | 44.49  | 1.10  | 40.32  |
| A2     | 15.23   | 1.95   | 9.24   | 57.83  | 2.80  | 20.62 | 91.78  | 3.89  | 23.61 | 21.73  | 0.61  | 35.87  | 44.49  | 1.10  | 40.32  |
| A3     | 2.95    | 0.20   | 14.81  | 57.83  | 2.80  | 20.62 | 91.78  | 3.89  | 23.61 | 21.73  | 0.61  | 35.87  | 44.49  | 1.10  | 40.32  |
| Pool   | 44.79   | 1.75   | 25.57  | 14.79  | 0.20  | 24.81 | 44.59  | 1.15  | 23.97 | 21.58  | 1.66  | 35.87  | 44.49  | 1.10  | 40.32  |
| B      | 6.81    | 0.35   | 19.72  | 7.75   | 1.77  | 4.36  | 4.69   | 0.79  | 6.27  | 3.48   | 0.48  | 6.84   | 13.77  | 1.54  | 8.93   |
| C      | 5.39    | 0.59   | 9.09   | 13.73  | 2.00  | 6.86  | 2.77   | 0.57  | 4.88  | 3.37   | 0.59  | 5.75   | 13.46  | 1.33  | 10.13  |
| D      | 5.39    | 0.47   | 11.37  | 7.02   | 0.92  | 7.65  | 2.82   | 0.59  | 4.76  | 1.38   | 0.20  | 6.93   | 12.44  | 0.62  | 20.10  |
| E      | 7.00    | 0.38   | 18.70  | 16.54  | 1.75  | 9.43  | 3.71   | 0.57  | 6.54  | 1.26   | 0.14  | 9.13   | 13.40  | 0.96  | 13.97  |
| F      | 2.52    | 0.21   | 11.93  | 17.62  | 2.24  | 7.87  | 4.50   | 1.18  | 3.82  | 2.57   | 0.82  | 3.12   | 5.35   | 1.19  | 4.50   |
| G      | 1.54    | 0.47   | 3.28   | 20.39  | 1.45  | 14.02 | 4.47   | 0.74  | 6.04  | 3.11   | 0.51  | 6.14   | 5.96   | 0.91  | 6.52   |
| H      | 4.06    | 0.23   | 17.43  | 23.95  | 4.02  | 5.96  | 4.70   | 1.04  | 4.50  | 4.27   | 0.45  | 9.56   | 7.03   | 0.57  | 12.26  |

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gfp\textsubscript{uw} in cells, selective permeation by FTS was proportion-
al to the release of 10–40% gfp\textsubscript{uw}.

The 1\textsuperscript{st} and 2\textsuperscript{nd} cycles removed about four times as much BSA in relation to the freezing at -75\degree C, decreasing fourfold the specific contents (Table 3). The concentration of 44.49 \mu g/gfp\textsubscript{uw}/mL extracted by TPP from the cells, after the FTS cycles, represented 15.6\% of the total gfp\textsubscript{uw} (241.19 + 44.49 = 285.68 \mu g/mL) released by the association of FTS followed by TPP, for culture A, resulting in a ratio of about 1 (mixture) of equal parts of the successive FTS samples re-

step) equivalent concentrations for the same cultures (B: 7.35\%; E: 4.10\%; F: 8.64\%; G: 6.24\%; H: 8.00\%;)

(ii) For amounts lower than 20 \mu g/gfp\textsubscript{uw}/mL, the TPP method was applied directly to the intact cells (Table 1), resulting in concentrations of gfp\textsubscript{uw}/mL and specific mass, for the cultures, respectively of (B, pool) 45.66 \mu g/gfp\textsubscript{uw}/mL and 18.87 \mu g/mg; (C) 39.60 \mu g/gfp\textsubscript{uw}/mL and 35.65 \mu g/mg; (D) 15.92 \mu g/gfp\textsubscript{uw}/mL and 38.86 \mu g/mg; (E) 28.12 \mu g/gfp\textsubscript{uw}/mL and 38.70 \mu g/mg. Comparatively, the application of the TPP method to concentrations previously permea-
ted by FTS cycles (Table 2) resulted in total (sum of every step) equivalent concentrations for the same cultures (B: 3: 18.77; E: 30.75; F: 29.74; G: 49.83; H: 53.51) through combined FTS with TPP procedures was as follows:

(i) freezing (-75\degree C): A: 22\%; B: 16.54\%; C: 18.25\%; D: 28.72\%; E: 23.09\%; F: 8.47\%; G: 3.10\%; H: 7.60\%;

(ii) 1\textsuperscript{st} FTS cycle: A: 28.37\%; B: 18.82\%; C: 46.49\%; D: 37.40\%; E: 53.79\%; F: 59.25\%; G: 40.92\%; H: 44.76\%;

(iii) 2\textsuperscript{nd} FTS cycle: 27.07\%; B: 11.97\%; C: 9.38\%; D: 15.02\%; E: 12.07\%; F: 15.13\%; G: 8.97\%; H: 8.78\%;

(iv) 3\textsuperscript{rd} FTS cycle: A: 10.59\%; B: 8.00\%; C: 11.41\%; D: 7.35\%; E: 10.40\%; F: 6.4; G: 6.24\%; H: 8.00\%;

(v) TPP extraction: A: 11.76\%; B: 44.69\%; C: 14.49\%; D: 11.51\%; E: 7.00\%; F: 8.51\%; G: 40.78\%; H: 30.89\%.

For cultures F, G and H, preliminary freezing at -75\degree C permeated the lowest amounts, about 3–8\% gfp\textsubscript{uw} (Table 3); however the 1\textsuperscript{st} FTS cycle caused greatest removal – about 40\% of the protein, promoting the most efficient permea-
tion. Final TPP extraction applied to the cultures was important in the residual removal of the required protein up to 40\% of total gfp\textsubscript{uw} obtained for cultures B, G, H.

(iv) Three-partitioning phase (TPP) technique combined with sonication (Table 4)

To cultures over-expressing high concentrations of gfp\textsubscript{uw}, the TPP method was applied 2–3 times directly to the pel-

ets, while the white middle layer and the lower aqueous phase still containing gfp\textsubscript{uw} were visually detected by flu-

orescence with a UV lamp (\lambda = 395 nm).

Sonication of the pelleted cultures before the TPP application was employed to increase the release of gfp\textsubscript{uw} from the cells. Otherwise, sonication turned the homogenate un-
clear and hindered the TPP efficacy on fractioning pro-
teins. The interaction of high weight proteins entrapped gfp\textsubscript{uw} to the white intermediate phase, which was the layer

not soluble into extraction buffer, led to the loss of the protein. Therefore, the content of BSA was increased and
carried with the gfpuv extraction. For culture I, sonication plus TPP combined procedures extracted similar amounts of gfpuv other than TPP directly applied to the cells. However, specific contents from TPP extracts (45–48 µg gfpuv/mg) were at least twice higher than those obtained from sonication, in addition to TPP (25–28 µg gfpuv/mg). Sonication, applied to the cells after previous TPP treatment, caused an increase of extracted gfpuv content but affected the specific contents 3–6 fold. For samples 3 and 4 (culture I), BSA at 10 to 15% was present in nearly 50% of all gfpuv extracted by the TPP method directly applied to the pellets, and the specific mass was the highest range obtained from 45–48 µg gfpuv/mg. After sonication of those pellets, the specific content decreased between 4–8 µg gfpuv/mg, since up to 60% of BSA was shifted with gfpuv.

For culture J, the application of sonication previously to the TPP method did not interfere with the amount of the gfpuv obtained. Through sample 8, it was observed that the 3rd application of the TPP method directly to the pellets did not carry out the gfpuv fractionation. After the sonication of the pellets, the extraction of 12.50% of the protein through the TPP technique was followed by a reduction of the specific content to 35.46 µg gfpuv/mg.

### Table 4: Permeation of the pellets by freezing at -75°C. Three Phase Partitioning (TPP) method applied to the pelleted culture "I" (samples 1, 2, 3, 4), culture "J" (samples 5, 6, 7, 8, 9, 10), directly (TPP) or following sonication of the pellets (S+TPP).

| Pellet | Cycle Final | BSA (mg/mL) | yield (%) | gfpuv (µg/mL) | yield (%) | gfpuv (µg/mL) | Total | Specific mass |
|--------|-------------|-------------|-----------|---------------|-----------|---------------|-------|--------------|
| Sample |             |             |           |               |           |               |       |              |
| 1      | (-75°C)     | 31.49       | 62.05     | 337.91        | 57.50     | 10.73         |
|        | 1st S+TPP   | 5.33        | 10.53     | 137.52        | 23.40     | 25.74         |
|        | 2nd S+TPP   | 13.92       | 27.42     | 112.28        | 19.11     | 587.71        | 8.07  |
| 2      | (-75°C)     | 45.35       | 68.04     | 358.35        | 58.12     | 7.90          |
|        | 1st S+TPP   | 5.82        | 87.30     | 165.33        | 26.82     | 28.39         |
|        | 2nd S+TPP   | 15.47       | 23.21     | 92.854        | 15.06     | 616.53        | 6.00  |
| 3      | (-75°C)     | 10.87       | 28.78     | 91.75         | 23.41     | 8.42          |
|        | 1st TPP     | 4.01        | 10.65     | 194.30        | 49.67     | 48.41         |
|        | 2nd S+TPP   | 22.89       | 60.59     | 105.34        | 26.93     | 391.20        | 4.60  |
| 4      | (-75°C)     | 8.71        | 46.03     | 73.207        | 26.85     | 8.40          |
|        | 1st TPP     | 2.96        | 15.65     | 134.53        | 49.34     | 45.39         |
|        | 2nd S+TPP   | 7.25        | 38.30     | 64.94         | 23.82     | 272.68        | 8.96  |
| 5      | (-75°C)     | 75.14       | 66.45     | 1633.78       | 46.89     | 21.74         |
|        | 1st S+TPP   | 12.12       | 10.72     | 645.28        | 18.52     | 53.24         |
|        | 2nd S+TPP   | 14.82       | 13.10     | 929.12        | 26.67     | 62.69         |
|        | 3rd S+TPP   | 10.99       | 9.72      | 275.78        | 7.92      | 3483.96       | 25.10 |
| 6      | (-75°C)     | 35.54       | 49.78     | 1262.9        | 37.08     | 35.53         |
|        | 1st S+TPP   | 11.74       | 16.44     | 998.66        | 29.32     | 85.07         |
|        | 2nd S+TPP   | 13.99       | 19.56     | 873.14        | 25.64     | 62.41         |
|        | 3rd S+TPP   | 10.11       | 14.16     | 271.34        | 7.97      | 3406.04       | 26.84 |
| 7      | (-75°C)     | 52.66       | 60.55     | 1606.06       | 37.88     | 30.50         |
|        | 1st S+TPP   | 16.45       | 18.91     | 1528.08       | 36.04     | 92.90         |
|        | 2nd S+TPP   | 13.25       | 15.23     | 875.68        | 20.65     | 66.09         |
|        | 3rd S+TPP   | 4.60        | 5.29      | 230.03        | 5.43      | 4239.85       | 49.97 |
| 8      | (-75°C)     | 14.77       | 32.50     | 584.69        | 25.87     | 39.60         |
|        | 1st TPP     | 7.76        | 17.08     | 581.31        | 25.72     | 74.90         |
|        | 2nd TPP     | 14.94       | 32.88     | 811.9         | 35.92     | 54.35         |
|        | 3rd S+TPP   | 7.96        | 17.53     | 282.44        | 12.50     | 1977.90       | 35.46 |
| 9      | (-75°C)     | 18.41       | 34.54     | 721.81        | 21.39     | 39.21         |
|        | 1st TPP     | 12.62       | 23.68     | 1199.2        | 35.54     | 95.03         |
|        | 2nd TPP     | 9.22        | 17.29     | 921.12        | 27.30     | 99.95         |
|        | 3rd S+TPP   | 13.04       | 24.47     | 531.49        | 15.75     | 2842.13       | 40.75 |
| 10     | (-75°C)     | 15.20       | 27.80     | 567.94        | 17.88     | 37.36         |
|        | 1st TPP     | 13.23       | 24.18     | 1461.90       | 46.03     | 110.47        |
|        | 2nd TPP     | 9.14        | 16.70     | 696.6         | 22.02     | 76.53         |
|        | 3rd S+TPP   | 17.13       | 31.31     | 446.35        | 14.02     | 2729.42       | 26.05 |
Slow freezing cycle at -75°C was responsible for the gfpuv permeation up to 58% for samples 1 and 2 (culture I), and the average of 37–47% for samples 5, 6 and 7 (culture J). However, simultaneously, near 62–68% of all proteins (BSA) were carried and mixed to the required protein. These samples corresponded to high concentrations of gfpuv between 338 μg gfpuv/mL (sample 1) and 1634 μg gfpuv/mL (sample 5). For culture I, independently of the concentrations removed of gfpuv and BSA, the specific mass was equivalent to 8–10 μg gfpuv/mg, also for samples 3 and 4, when the freezing removal of gfpuv varied from 23–26%.

For culture J, even though the concentration of gfpuv ranged from 568 μg/mL (sample 10) to 1634 μg/mL (sample 5), 39% (samples 8 and 9), corresponded to half gfpuv extracted – respectively, 584.69 μg/mL and 721.81 μg/mL.

The uncontrolled slow freezing (-75°C) speed of the pellets caused variations in the permeation of the pelleted cells.

(v) Three-partitioning phase (TPP) technique directly applied (Table 4)

For samples 3 and 4 (culture I), TPP directly applied to previously frozen cells, extracted two times more gfpuv (49.34–49.67%) than prior freezing permeation (23.41–26.85%), corresponding to a five-fold increase of the specific contents (from 8.4 μg/mg to 45.39–48.41 μg/mg), which showed simultaneous ability by the TPP method of extraction, purity and concentration of gfpuv in the aqueous phase. The sonication following procedure displaced 6 times more BSA mixed to gfpuv reducing up to 10 times the specific contents (sample 3), affecting the purity of the homogenate.

The TPP method applied directly to samples 8, 9, and 10 (culture J) attained the best specific content ranging from 74.90 μg/mg to 110.47 μg/mg for the first application and from 54.35 μg/mg to 76.53 μg/mg for the second turn. Sonication following TPP application to the extracted pellets caused a three-fold decrease of specific contents (26.05 μg/mg, sample 10), confirming that sonication had a negative effect on the isolation and purification procedures of gfpuv.

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