0273-2289/02/98-100/0791/$13.00

Intracellular Release of Recombinant Green Fluorescent Protein (gfp_{uv}) from *Escherichia coli*

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Abstract

The recombinant green fluorescent protein (gfp_{uv}) was expressed by *Escherichia coli* DH5-α cells transformed with the plasmid pGFPuv. The gfp_{uv} was selectively permeabilized from the cells in buffer solution (25 m*M* Tris-HCl, pH 8.0), after freezing (–70°C for 15 h), by four freeze (–20°C)/thaw cycles interlaid by sonication. The average content of released gfp_{uv} (experiment 2) was 7.76, 34.58, 39.38, 12.90, and 5.38%, for the initial freezing (–70°C) and the first, second, third and fourth freeze/thaw cycles, respectively. Superfusion on freezing was observed between –11°C and –14°C, after which it reached –20°C at 0.83°C/min.

Index Entries: gfp_{uv}; *Escherichia coli*; physical permeabilization; superfusion phenomenon; freeze/thaw/sonication procedures.

Introduction

The purified and partially characterized wild green fluorescent protein *gfp* extracted from *Aequorea victoria* yields a bright green fluorescence when light stimulated *(1)*. The recombinant form of gfp_{uv} *(2)*, which has been successfully expressed in either eukaryotic or prokaryotic cells, can be vizualized by standard ultraviolet (UV) light (360–400 nm). The gfp_{uv}, which is an independent species, can be successfully inserted in sporulated bacteria and expressed on outgrowing of germinated cells and is a tool for

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rapid biologic indicator of survivors in sterilization processes. Because of its properties and marked intrinsic fluorescence, the gfp_{uv} has been considered a novel genetic reporting system *(2)*.

The recombinant gfp_{uv} 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*) *(3)*. The resulting gfp_{uv} is expressed two to three times faster in *Escherichia coli* strains and has 18 times brighter fluorescence than the native *gfp*; the maximum peaks for chromophore excitation are at 395 nm and centered at 508–509 nm for emission *(1–4)*.

The *gfp* molecule is a globular protein, compact and acidic, made up of monomers with a molecular weight of 27 kDa, the recombinant form having a greater propensity to dimerize than the native *gfp (3)*. The polymer has in its primary structure 238 amino acids with C-terminal sequence made up of His-Gly-Met-Asp-Glu-Tyr-Lys, susceptible to attack by carboxypeptidases and nonspecific proteases. During selective permeabilization, the proteolyses of the C-terminal extremity of gfp_{uv} can be inhibited working at cool temperatures (O to 4°C), and in the presence of phenylmethylsulfonyl fluoride (PMSF) *(1,4)*.

The important advantage of gfp_{uv} is its high stability and resistance to heat (≤70°C) and chemical denaturation, provided by its very regular tridimensional β-barrel with 11 strands on the outside of the cylinder. The gfp_{uv} chromophore group, in the geometric center of the cylinder, sets an irregular α-helical segment, formed by a sequence of the three most important amino acids (Ser^{65}, Tyr^{66}, Gly^{67}) *(4)*. The gfp_{uv} has an isoelectric point between 4.6 and 5.4 and maintains its fluorescence at pH 5.5–11.5, the optimum pH being 8.0. The addition of β-mercaptoethanol *(5)* and the maintenance of pH at 8.0 enhance protein stability *(1–3)*.

The gfp_{uv} expressed in the cytoplasm of *E. coli* strains (DH5-α, JM109, and TB1) can be released from the cells by enzymatic (i.e., lysozyme) digestion, chemical lysis, or physical permeabilization (freeze/thaw/sonication methods) *(5,6)*. Bacterial cells can be nonmechanically permeabilized by slow freezing and thawing rates in cycles, damaging the cellular membrane by crystallized water, and provoking leakage of intracellular proteins. By selective permeabilization, small proteins can be released from a complex mixture of nucleic acids and cell fragments, facilitating posterior recovery and purification of the protein *(7)*. In large-scale operations, freezing and thawing usually precede other disruption steps, simplifying the downstream operation for the purification of the protein of interest, and reducing the total recovery cost *(8)*. Although the freeze/thaw operations are slower than others and cause permeation of soluble proteins with variable specific gfp_{uv} contents, they prevent total cell disruption and extraction of viscous endogenous cytoplasmic material *(6–9)*.

The aim of the present study was the expression of the recombinant gfp_{uv} in the cytoplasm of *E. coli* DH5-α cells, to apply freeze/thaw cycles interlaid by sonication to the cell pellets for the purpose of permeating the

cell wall and liberating selective soluble protein high in specific gfp_{uv} contents, and to monitor freezing/thawing rates and temperature variations to verify the superfusion phenomenon and to certify the –20°C attainment in the cell pellets.

Material and Methods

Transformation

The cells of *E. coli* DH5-α cells *(5,9)* were transformed with $pGFP_{uv}$ (Clontech), which is a high-copy-number plasmid *(2)*, by the standard calcium chloride method *(5)*. The transformed cells were stored at –70°C in Luria Bertani (LB)/amp broth with glycerol added in the proportion of 1:1.

The gfp_{uv} gene is fused to the *lacZ* initiation codon from pUC 19, which adds an additional 24 amino acids to the N-terminus of gfp_{uv} and permits high expression from the *lac* promoter. The mutant gfp_{uv} is under tight control of the *lacZ* protein β-galactosidase promoter/repressor and can be induced continuously with a final concentration of 0.5 m*M* (w/v) isopropyl-1-thio-β-D-galactoside (IPTG) (dioxane free; USD/Italy) in LB (Difco) medium supplemented with ampicillin (100 µg/mL) (Boehringer, Mannheim, Germany). The pGFPuv vector also contains the *bla* gene for ampicillin (amp) selection *(2,6,7)*.

Expression

A 24-h (at 37°C) culture (LB/amp/broth) of *E. coli* was transferred onto the surface of LB/amp/IPTG agar and incubated at 37°C for 24 h. Using an UV lamp (λ = 395 nm) (model UVL 4; UVP), each single, isolated, brightly fluorescent colony from a plate was selected, picked up, and transferred to a culture tube by dispersal into 2.0 mL of LB/amp broth, for a total of 20 tubes, which were incubated (37°C for 24 h). The 40-mL suspension (from the 20 culture tubes) was homogenized, its optical density (OD) (λ = 660 nm) between 0.5 and 0.8 was taken with a spectrophotometer (Beckman DU-600), and it was incorporated into 400 mL of LB/amp broth. This homogenized suspension was distributed into 50-mL lots in a 250-mL flask. The flasks were shaken (100 rpm at 37°C for 3 h) until the broth cultures attained an OD_{660nm} of 0.7–0.8 (10^8 CFU/mL), when IPTG was added to a final concentration of 0.5 m*M*. After 21 h (100 rpm at 37°C), the gfp_{uv} expressed by induced cells was confirmed at λ = 395 nm.

Concentration

Growth culture was harvested by centrifugation (1000*g* for 30 min at 4°C). The supernatant was poured out, and the pelleted cells were observed under UV light, resuspended in 10 mL of cold buffer solution (10 m*M* Tris-HCl, pH 8.0; Trizma® Base) (Sigma, St. Louis, MO), dispersed, homogenized, and centrifuged. The clear nonfluorescent supernatant was discarded and the cells, which showed intense fluorescence, were submit-

ted to freezing at –70°C (Ultra Freezer, Kelvinator Model 100; Manitowoc, WI) until the extraction was carried out. Under an Olympus (BX-60-IV FLA System Attachment) microscope, through filters for the detection of fluorescent cells (U-MWG [510–550 nm], U-MWU [330–385 nm], U-MNV [400–410 nm],) the bright green intact cells with easily defined outlines were observed (Fig. 1). Green fluorescent protein (*gfp*) absorbs UV (330–385 nm) and blue (400–410 nm) lights, with a maximum peak of absorbance at 395 nm and a minor at 470 nm, and emits maximum green light at 509 nm, with a shoulder at 540 nm *(10)*.

Selective Permeabilization

The thawed pelleted cells were transferred to a 2.0-mL microtube with 1.0 mL of cold extraction buffer (25 m*M* Tris-HCl, pH 8.0, plus 1.0 m*M* β-mercaptoethanol [Pharmacia Biotech, Sweden] and 0.1 m*M* PMSF [USB, Switzerland]), homogenized, and pelleted again by centrifugation. Five hundred microliters of the poured-out supernatant was reserved from every step for further reading with a spectrofluorometer and spectrophotometer to compare the isolated gfp_{uv} yield with the pure gfp_{uv} (Clontech) and total protein released (based on bovine serum albumin [BSA]). The fluorescent pellet was resuspended with the addition of 500 µL of the extraction buffer (about 20X pellet volume).

The resuspended pellets were submitted to at least four 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) chamber (Dura Stop™ MP). With PT-100 probes inserted into the pelleted suspension, the freezing/thawing temperatures were registered every minute through a software "lyphoware" for Windows. Between the freeze/thaw cycles, the microtube was kept immersed in an ice-salt bath, and a 3-mm microtip ultrasonic processor was placed in the sample, which was submitted to three-fold pulse sonication (High Intensity Ultrasonic Liquid Processor, Vibram Cell, VC100, Sonic and Materials, Newton, CT) at 0°C over a total interval of 23 s. Each pulse was at 25 vibration amplitude at alternating cycles of 6 s on and 1.0 s off. Between the freeze/thaw/sonication cycle, cells were pelleted by centrifugation, and the supernatant was reserved for spectrofluorometer and spectrophotometer readings. For the first group of assays, at the end of four repeated freeze/thaw cycles, the samples were submitted to four sequential sonication procedures. The release of the gfp_{uv} into the medium was followed by the measurement of fluorescent intensity of samples for every experimental step, after a standard curve established by known amounts of the pure recombinant gfp_{uv}.

Standard Curves

The fluorescence intensity of gfp_{uv} detected in a cleared bacterial cell lysate was measured using the Fluorescence Spectrophotometer F-2000 (Hitachi), with an excitation filter of 394 nm and an emission filter of 505 nm.

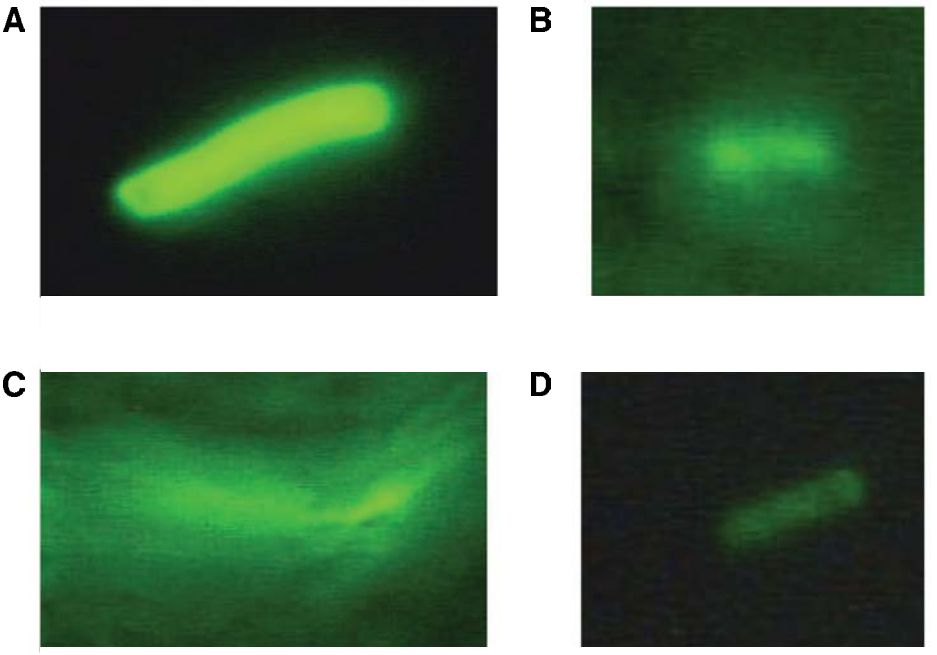

Fig. 1. *E. coli* DH5-α cells expressing gfp_{uv} (**A**), permeating gfp_{uv} (**B**) diffused in the medium (**C**), and fading fluorescent permeable cells no longer releasing gfp_{uv} (**D**).

The fluorescence intensity of the experimental samples was compared to the standard curve (gfp_{uv} µg/mL = 0.001 × [fluorescence intensity] – 0.1133; R^2 = 0.995) to determine the amount of gfp_{uv} released from the cells. The standard curve was prepared using known amounts (between 6.66 and 0.59 µg/mL) of purified recombinant gfp_{uv} (Clontech) diluted in the same extraction buffer solution (25 m*M* Tris-HCl , pH 8.0, plus 1.0 m*M* β-mercaptoethanol and 0.1 m*M* PMSF).

The total protein released in the medium from *E. coli* cells was expressed in milligrams per milliliter and was compared with total protein concentrations expressed as purified BSA (mol wt 66 – kDa; Sigma) at λ = 280 nm in a spectrophotometer. The total protein concentrations in the buffer solution ranged from 100 to 1000 µg/mL, the maximum OD_{280nm} being 0.615, and the comparative relationship between total proteins and BSA was made through the standard curve (total protein µg/mL = 1727.2 × [OD_{280nm}] – 26.863; R^2 = 0.9943).

Statistical Analyses

The results are expressed in Tables 1 and 2 by total protein (mg/mL), released gfp_{uv} (µg/mL) content, and gfp_{uv} yield (%, µg/µg) in relation to the total released gfp_{uv} content obtained; the specific gfp_{uv} is expressed as micrograms of gfp_{uv} in relation to milligrams of total protein. In Table 1, experi-

Table 1

Influence of Freeze/Thaw Cycles and Sonication Treatments on gfp_{uv} Selective Permeabilization in Relation to Total Protein, Perceptual Yield of gfp_{uv}, and Specific Contents of gfp_{uv} Released

Experiment 1					
Cycle		Total protein (mg/mL)	gfp_{uv} (μg/mL)	Specific gfp_{uv} (μg/mg)	gfp_{uv} Yield[c] (% [w/w])
Freeze	(–70°C)	3.95	13.29	3.36	6.98
Freeze/thaw[a]	First	7.62	11.78	1.55	6.19
	Second	14.67	10.01	0.68	5.26
	Third	5.20	10.99	2.11	5.77
	Fourth	3.81	8.56	2.25	4.50
Sonication[b]		6.39	135.70	21.22	71.30
Total					100.00

[a]Freeze (–20°C)/Thaw (0.83°C/min).

[b]Sonication (four cycles of 3 pulses and 23 s each).

[c]gfp_{uv} yield (%[μg/μg]) in relation to total gfp_{uv} released.

ment 1 corresponds to the application of four repeated freeze/thaw cycles followed by four sonication cycles of the expressing cell pellets obtained from the same growth culture. Each experiment in Table 2 corresponds to a new cellular growth, with an increase in aeration from experiment 2 to experiment 4; the cells of experiments 1 and 2 were obtained from the same growth conditions. The specific growth rates ranged from 0.99 (experiments 1 and 2) to 1.10 h^{-1} (experiments 3 and 4). The results in Table 2 were attained from associated application to the cell pellets of a maximum of six repeated freeze/thaw interlaid by sonication cycles.

Results and Discussion

Bacterially expressed gfp_{uv} was characterized in clarified intracellular permeates without further purification.

An Olympus BX60 microscope was used to observe the expression of gfp_{uv} by *E. coli* cells, to follow the morphologic alterations of permeated cells and consequent leakage of the gfp_{uv} to the medium. Figure 1 shows intact cells expressing gfp_{uv} (Fig. 1A), cells permeating the protein (Fig. 1B), gfp_{uv} diffusing from permeable cells to the medium (Fig. 1C), and smaller permeable cells, which retained a residue of gfp_{uv} but no longer released the protein (Fig. 1D). Following microscopic observations and fluorescence intensity levels, it was observed that a substantial fraction of the overexpressed protein was released from the cells that were not totally broken down. The yields of permeate amounts of gfp_{uv} in the buffer-clarified solutions are shown in Tables 1 and 2.

Table 2
Total Protein, gfp_{uv} Concentration, Yield Percentage of gfp_{uv} and Specific gfp_{uv} Obtained After Different Cell Permeabilization Treatment

Experiment 2

Cycle		Total protein (mg/mL)	gfp_{uv} (μg/mL)	Specific gfp_{uv} (μg/mg)[a]	Yield (% [w/w])[b]
Freeze	(–70°C)	3.58	32.95	9.21	7.76
Freeze/	First	0.98	146.77	150.01	34.58
thaw/	Second	1.45	167.07	114.82	39.38
sonication[c]	Third	5.51	54.77	9.939	12.90
	Fourth	2.42	22.83	9.438	5.38
	Fifth	—	—	—	—
	Sixth	—	—	—	—
Total					100.00

Experiment 3

Cycle		Total protein (mg/mL)	gfp_{uv} (μg/mL)	Specific gfp_{uv} (μg/mg)[a]	Yield (% [w/w])[b]
Freeze	(–70°C)	—	—	—	—
Freeze/	First	30.99	304.07	9.81	21.63
thaw/	Second	41.89	479.47	11.44	34.12
sonication[c]	Third	35.26	314.27	8.913	22.36
	Fourth	15.31	212.47	13.88	15.12
	Fifth	69.67	95.17	1.366	6.77
	Sixth	—	—	—	—
Total					100.00

Experiment 4

Cycle		Total protein (mg/mL)	gfp_{uv} (μg/mL)	Specific gfp_{uv} (μg/mg)[a]	Yield (% [w/w])[b]
Freeze	(–70°C)	—	—	—	—
Freeze/	First	51.01	633.34	12.415	8.53
thaw/	Second	67.16	634.87	9.453	8.55
sonication[c]	Third	55.12	667.57	12.11	8.98
	Fourth	49.42	799.74	16.18	10.77
	Fifth	116.36	1549.88	13.32	20.87
	Sixth	171.82	3142.16	18.29	42.30
Total					100.00

[a]Specific gfp_{uv} (μg gfp_{uv}/mL)/(mg total protein/mL)

[b]Yield fluorescence (% [w/w]).

[c]Freeze (–20°C)/thaw (0.83°C/min)/sonication (3 pulses/23 s).

Table 1 shows the results of experiment 1, which corresponded to the application of four repeated freeze/thaw cycles to the cell pellets. Freezing (at –70°C)/thawing was observed to have the same effect on releasing the equivalent yield of gfp_{uv} (6.98%) as the first freeze (–20°C)/thaw cycle (6.18%). This yield decreased to 5.26, 5.77, and 4.50%, respectively, for the second, third, and fourth cycles. However, the concentration of protein liberated from freezing (at –70°C) was 13.29 μg/mL, similar to those of 11.78, 10.01, 10.99, and 8.56 μg/mL obtained from the first, second, third and fourth freeze/thaw cycles, respectively.

The specific gfp_{uv} contents released from the permeabilized cells were shown to be higher (3.36 μg of gfp_{uv}/mg of total protein) for –70°C than for freezing (–20°C)/thawing along with the first, second, third and fourth cycles, when the specific gfp_{uv} contents decreased to 1.55, 0.68, 2.11, and 2.25 μg of gfp_{uv}/mg of total protein, respectively. However, the specific gfp_{uv} was about 10 times lower (2.25 μg of gfp_{uv}/mg of total protein) after the fourth freeze/thaw cycle than it was after four cycles of sonication, when the specific content increased to 21.22 μg of gfp_{uv}/mg of total protein. This evidence meant an increase in gfp_{uv} yield from an average of 6.0–71.3%. Neither procedure (freeze/thaw and sonication) provoked the complete release of cell content. The bulk of the endogenous cellular material was easily removed by centrifugation of the permeating cells, and the supernatants remained extremely clear for further readings in the spectrophotometer and fluorometer, without any interference of debris or opaque viscosity.

In Fig. 1, at the end of physical treatments, the cells showed a faded fluorescence from a gfp_{uv} remaining inside, without distinguishable diffusion. At that stage, further freeze/thaw or sonication applications could not provoke continuous permeation of the cells. These results are in agreement with those of other investigators *(7–9)*, who stated that the protein released just by the freezing and thawing method is usually minimal, in the order of 10%, so further disruption of bacterial cells is usually necessary *(7)*.

Five repeated cycles of monitored slow freezing and thawing interlaid by gentle sonication were applied to the cell pellets of experiments 2, 3, and 4 (Table 2), with the aim of permeating gfp_{uv} with cytoplasm lysate from cell membrane (Fig. 1), without breaking it completely.

The freeze/thaw/sonication procedure was mild and provided an increasing yield of gfp_{uv} diffused to the medium, as shown in Table 2. The second group of experiments characterized the best stages of gfp_{uv} permeation in relation to the total protein liberated. Damage to the membrane caused by this association was able to permeate about 1.0–5.0 mg of protein/mL of lysate. Comparison of the data of experiments 1 (Table 1) and 2 (Table 2), reveals that the yields of 6.98 and 7.76% of gfp_{uv}, respectively, released by freezing at –70°C were close; however, the content of 32.95 μg/mL in experiment 2 was more than double that in experiment 1 (13.29 μg/mL), and the specific gfp_{uv} was about three-fold (9.21 μg/mg) higher than that observed for experiment 1 (3.36 μg/mg), for a range of

3.58–3.95 mg of total protein liberated. For the same growth conditions (experiments 1 and 2), *E. coli* cells overexpressed different specific contents of gfp_{uv}.

Therefore, in experiment 2, the specific content of gfp_{uv} increased about 100 times, to 150.01 and 114.82 μg/mg, respectively, for the first and second freeze/thaw/sonication cycles, and about four times (9.94 and 9.44 μg/mg), respectively, for the third and fourth cycles in relation to the corresponding freeze/thaw cycles in experiment 1. The first and second freeze/thaw/sonication cycles released at least five times more specific gfp_{uv} in experiment 2 than the sonication cycles separately applied to the cell pellets in experiment 1 (21.22 μg/mg). It was clearly observed that the yield of gfp_{uv} isolated in experiment 2 gradually decreased for subsequent freeze/thaw/sonication applications, about three times from the second to the third and from third to fourth cycles, the gfp_{uv} yield of which was similar to that released by the fourth freeze/thaw cycle in experiment 1. In Fig. 1D, the fourth cycle was characterized by small fading fluorescent permeable cells and a lack of gfp_{uv} diffused in the medium.

In experiment 3 (Table 2), for the four freeze/thaw/sonication cycles, the content of gfp_{uv}(μg/mL) lysate increased 2.07, 2.87, 5.74, and 9.30 times respectively, contrary to experiment 2, when the release of gfp_{uv} decreased from the first to the fourth cycle. However, the specific content of gfp_{uv} (μg/mg) carried with total protein was 15 and 10 times lower for the first and second cycles (from 150.01 to 9.81 μg/mg and from 114.82 to 11.44 μg/mg), respectively, which means that experiment 3 released a greater amount of total protein with a lower content of gfp_{uv} in it. For the third and fouth cycles, the specific gfp_{uv} was close for both experiments 2 and 3; although the yield was two- and three-fold higher, respectively, in experiment 3 than in experiment 2. For the fifth cycle, the increase of five times in the total protein carried twofold lower gfp_{uv} content and yield, and 10 times lower specific gfp_{uv} than the fouth cycle, signifying that the increment in total protein is not proportional in gfp_{uv} on the addition of repeated cycles.

On analyzing the fluorescence values in Table 2, the best yield was obtained in the second freeze/thaw/sonication cycle, 39.38 and 34.12%, respectively, for experiments 2 and 3. In the third and fourth cycles, the yields of experiment 2 of 12.90 and 5.38% were half those of experiment 3 of 22.36 and 15.12%. The sharp decrease in gfp_{uv} yields to 5.38 and 6.77% obtained from the fourth and fifth cycles, respectively, for experiments 2 and 3, and also 10 times lower specific gfp_{uv} released in the fifth cycle, justified the termination of the freeze/thaw/sonication in the fourth cycle application of the pelleted cells.

The culture of cells of experiment 4 expressed and released an average of two- to threefold higher gfp_{uv} contents/mL of lysate from the first cycle (304.07–633.34 μg/mL) to the fourth cycle (212.47–799.74 μg/mL) than in experiment 3, although the yield of gfp_{uv} ranged from 10 to 30% for both experiments. However, the yield jumped to 20.87 and 42.30% in experi-

ment 4, corresponding to gfp_{uv} contents of 1549.88 and 3142.16 μg/mL of lysate, and also two times higher from the fourth to fifth cycle and from the fifth to sixth cycle, for a specific gfp_{uv} average of 16 μg of gfp_{uv}/mg of total protein. For increasing amounts of expressed gfp_{uv} in cells, permeation was proportional to the release of greater yields of gfp_{uv} /mL of lysate and was in the range of 10–40% gfp_{uv}.

The results shown in Tables 1 and 2 are in agreement with data of other investigators *(6–9)*, who stated that by selective permeation the yield of 10% of the protein of interest is usually the average obtained. Extractions of the enzyme xylanase performed by aqueous two-phase and reverse micellar systems provided different recovery yields. While the liquid-liquid extraction by reverse micelles yielded approx 10% xylanase *(11)*, the aqueous two-phase extraction recovered 80% of the same enzyme *(12)*.

The freeze/thaw method has been shown to be effective in the release of proteins from 8.5 to 29 kDa, in both monomeric and dimeric forms *(1,6)*. Slow freezing and thawing cycles were applied to the samples with a view to forming large ice crystals. During lowering of the temperature, the sharp point of the crystals broke the cellular wall during the thawing operation. To assist the permeabilization of the cells, the cycles lowering and increasing the temperature were interposed by application of ultrasounds to the samples, thus making the cellular wall more vulnerable when subjected to oscillations in temperature and to the action of the ice crystals. In this work, during the freezing operation, the phenomenon of superfusion was observed (Fig. 2) when samples were supercooled down from –11°C to –14°C, before freezing set in. The water molecules distributed their structures best at this point and released the excess temperature to the medium. This explains the increase in temperature observed in Fig. 2. It was during this phase, after molecular adjustment, that the samples were fully frozen. Following the supercooling step, a sudden rise in temperature to about 3°C (average value) occurred, when the temperature dropped gradually (0.83°C/min) until the samples reached an average temperature of –20°C, and subsequently began linearly increasing the temperature until it reached 20°C in a continuous process. Care should be taken when freezing the cells, ensuring that they really attain the desired temperature.

The gfp_{uv} must be in an oxidized state to fluoresce, the chromophore formation being dependent on oxidation of Tyr^{66}, and the fluorescence form proportional to atmospheric oxygen *(9)*. Nascent gfp_{uv} is not fluorescent. The chromophore is formed by a cyclization reaction and an oxidation step that requires molecular oxygen *(13–15)*. For experiments 3 and 4, the dissolved oxygen was increased in the culture broth, improving *E. coli* growth and consequently gfp_{uv} expression, resulting in an increase in the intensity of fluorescence, as shown in Table 2. A simple twofold gauze cover on the flask mouth allowed maintenance of the dissolved oxygen (0.15 mg of O_2/L), maintaining the same conditions of culture imposed in all experiments.

Through fluorescence intensity readings, a remarkable increase was observed during the freeze/thaw/sonication procedures, and a significant

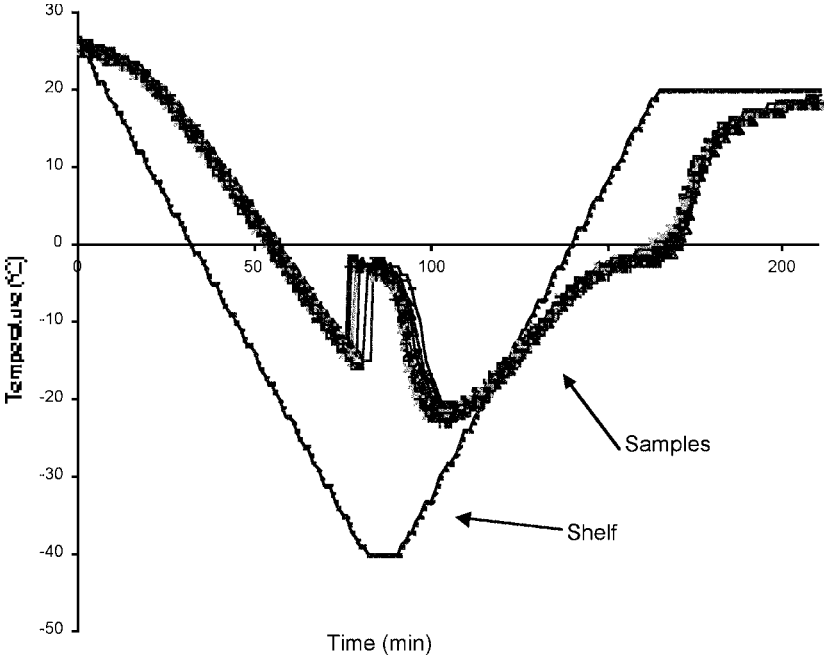

Fig. 2. Freeze/thaw cycles applied to *E. coli* pellet cells.

($p < 0.05$) drop in the yields was also emphasized after four successively repeated cycles. When the sediments were examined under UV light (λ = 395 nm) after every permeabilization cycle, it was noted that they were still fluorescent (Fig. 1). However, the intensity of fluorescence decreased, and the resulting yields did not justify continuation of the repeated extraction cycles, since a greater amount of gfp_{uv} had already been released with total protein by the four freeze/thaw/sonication cycles.

In conclusion, the beneficial association of four repeated freeze/thaw/sonication cycles was selected to release gfp_{uv} from the *E. coli* cytoplasm cells, concentrated in clear and translucent supernatants.

Acknowledgments

We thank Dr. Rogério Meneghini for his orientation, and Norah Duncan for English revision of the manuscript. This study was made possible by financial support provided by Brazilian Committees for Scientific Technology Research (Conselho Nacional de Desenvolvimento Científico e Tecnológico and Fundação de Amparo à Pesquisa do Estado de São Paulo).

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