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Study on the thermal stability of green fluorescent protein (GFP) in glucose parenteral formulations

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Abstract

Large volume parenteral solutions (LVPS) that are widely used in the healthcare system must be processed by moist-heat treatment to an assured sterility level in which the efficacy is measured by a bioindicator (BI) that provides fast, accurate and reliable results. This study evaluated the thermal stability of green fluorescent protein (GFP) into glucose-based LVPS (1.5–50%) solutions to determine its utility as a BI for thermal processes. GFP, expressed by *Escherichia coli*, isolated/purified by TPP/HIC, was diluted in buffered (each 10 mM: Tris–EDTA, pH 8; phosphate, pH 6 and 7; acetate, pH 5) and in water for injection (WFI; pH 6.70 ± 0.40) glucose solutions (1.5–50%) and exposed to constant temperatures from 80 °C to 95 °C. The thermal stability was expressed in decimal reduction time (*D*-value, time required to reduce 90% of the GFP fluorescence intensity). At 95 °C, the *D*-values for GFP in 1.5–50% glucose were: (i) 1.63 ± 0.23 min (pH 5); (ii) 2.64 ± 0.26 min (WFI); (iii) 2.50 ± 0.18 min (pH 6); (iv) 3.24 ± 0.28 min (pH 7); (v) 2.89 ± 0.44 min (pH 8). By the convenient measure of fluorescence intensity and its thermal stability, GFP has the potential as a BI to assay the efficacy of moist-heat processing of LVPS at temperatures ≤100 °C.

Keywords: Large volume parenteral solutions; Glucose; Green fluorescent protein (GFP); Decimal reduction time (*D*-value); Thermal stability

1. Introduction

Large volume parenteral solutions (LVPS) are the most widely used medications in the healthcare system for the replacement of body fluids, nutrition and as delivery vehicle for the administration of other medications. LVPS are prepared in water for injection (WFI) (US Pharmacopoeia, 2004; British Pharmacopoeia, 2005) packed in single plastic vessels containers, which are terminally sterilized by moist heat (Anvisa, 1997). About 150 million units of LVPS are used in Brazil per year, of which 80% are formulated with sodium chloride and glucose (ABRASP, 2006; Aster, 2006).

Thermal processing is essential for product safety in many areas of health care to guarantee the absence of pathogens or other microorganisms that may develop during the storage at room temperature (Hugbo et al., 1998). LVPS, including the glucose-based (1.5–50%) solutions, must be processed by moist heat treatment to an assured sterility level ($\geq 10^{-6}$) in which the efficacy is measured by an appropriate biological indicator (BI) that provides fast, accurate and reliable results (ISO 11137, 2000; ISO 11138-1, 2000). For terminal autoclaving, the establishment of binomial time and temperature parameters for each kind of product and packaging must be defined. The choice of the bioindicator and the temperature depend on the type of package (Spicher et al., 2002). Polyethylene flasks, made of low density polyethylene, is the packaging most used in Brazil, which requires temperatures between 90 °C and 104 °C by the steam method with the adjustment of respective increase in processing time (Anvisa, 1997; Penna et al., 2002). *Bacillus subtilis* spores, reclassified as *Bacillus atrophaeus* are chosen as BI (Anvisa, 1997; Burke et al., 2004), in which spore survival and growth are dependent on pH and glucose concentration in the LVPS.

Proteins can be used as biological indicators to evaluate the efficacy of temperature upon the product (Albert et al., 1998;

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McCormick et al., 2003). A thorough understanding of the thermal stability of a protein must be determined to evaluate its utility as a biological marker for industrial thermal processing (Miroliaei and Nemat-Gorgani, 2001).

The thermal inactivation of pectin methylesterase (PME) (Laratta et al., 1995; Basak and Ramaswamy, 1996; Tijskens et al., 1999), peroxidase (POD), lipoxygenase (LOX) (Gökmen et al., 2005) and lysozyme (Makki and Durance, 1996) have been used as bioindicators to monitor the thermal treatments of food and beverages to assure both the microorganisms destruction and enzyme inactivation, which can damage the product.

Proteins are amphoteric molecules containing a number of acidic and basic groups situated on the surfaces of water-soluble proteins. The charges on these groups will vary according to their dissociation constants, pH and the distribution of charge on the surface. The variation of activity with pH, within a range of 2–3 units from each side of the p*I*, is normally a reversible process. Extremes of pH will cause a time- and temperature-dependent, essentially irreversible, denaturation. This relationship is further complicated by the effect of pH with both the duration of the heating process and the temperature or temperature–time profile (Timasheff, 1992; Bolen, 2004; Collins, 2004; Bostõm et al., 2005).

GFP is a compact, globular acidic protein (p*I* 4.6–5.4) with one fluorophore consisting of a cyclic tripeptide in the primary protein sequence, a chain of ~238 amino acids. It has shown resistance to heat ($T > 70\,^{\circ}C$) and alkaline pH (between pH 5.5 and 12.0; optimum pH 8.0) (Penna et al., 2004a,b,c). The effects of various environmental parameters on GFP are easily monitored by measuring changes in its fluorescence. Previous studies with GFP held at a constant temperature (25 °C) have shown that the changes in surface charge by pH significantly affect fluorescence emission, as well as the structural stability and solubility of the protein (Penna et al., 2005).

Increasing temperature promotes the unfolding of protein-heat denaturation. The term "thermal stability" (also "thermostability") refers to the resistance of a protein to adverse intrinsic and extrinsic environmental influences, that is, the inherent properties of a protein to resist unfolding and inactivation upon exposure to high temperatures or other deleterious agents. Increasing recombinant green fluorescent protein thermal stability would allow the use of this protein as a bioindicator in moist heat treatment.

One of the most important indices to measure protein stability is the decimal reduction time, *D*-value, the time required to reduce 90% of the initial native protein concentration exposed to the reference temperature (Penna et al., 2002, 2004a,b,c, 2005). In this work, GFP was diluted in buffered and unbuffered glucose solutions (1.5–50% glucose) with pH between 5 (close to the p*I*) and 8 (the optimal pH) and, subjected to temperatures from 80 °C to 95 °C. The evaluation of kinetic parameters, mainly the *D*-value, for protein denaturation will provide information about the influence of increasing glucose concentration, as well as the pH and buffer, on GFP thermal stability. The use of a fluorescent marker designed for a quick and reliable assay, detectable by microscopy, spectrofluorometry or hand-held UV lamp, is examined in this work.

2. Material and methods

The expression of recombinant green fluorescent protein (GFP) by *Escherichia coli*, the extraction and purification of GFP has been outlined in previous experiments (Penna and Ishii, 2002; Penna et al., 2004a,b,c).

GFP fluorescence intensity was measured in a spectrofluorometer (excitation = 394 nm, emission = 509 nm; RF 5301 PC, Shimadzu Corporation, Kyoto, Japan). Purified recombinant GFP (95% purity, Clontech, USA) was used to generate a standard curve to determine TPP-extracted GFP concentration:

$$\text{fluorescence intensity } (I) = 134.64 + 103.61x \text{ (GFP } \mu\text{g/mL)}; \quad R^2 = 0.98. \tag{1}$$

2.1. Buffer solutions

To study GFP stability, buffers at various pH ranges and water were prepared: (i) 10 mM sodium acetate/acetic acid (pH 5); (ii) 10 mM potassium phosphate (monobasic/dibasic; pH 6 and 7); (iii) 10 mM Tris–EDTA (pH 8); (iv) water for injection ("WFI"; pH 7.0 ± 0.3; from the Milli-Q system, Millipore®, Bedford, MA, USA). All controls were GFP in either buffered solutions or WFI, without glucose.

2.2. Buffered glucose solutions

Glucose (99.7% purity) solutions were prepared at concentrations: 1.5, 5, 7, 10, 15, 20, 25, 30, 35, 40, 45 and 50% (w/v). A defined weight of glucose was diluted into each buffer solution or WFI. After complete dissolution, the glucose solution was transferred to a 250 mL volumetric flask and the volume adjusted. The solutions were filter-sterilized (Millipore® 0.22 μm membrane), transferred to sterilized flasks and stored at 4 °C until used. To monitor contamination, 1 mL of each solution, both before and after filtration, were plated (plate count agar) and incubated at 35–37 °C for 24 h. Sugar crystallization in these solutions was not observed.

2.3. Sample preparation for GFP stability determination

To each 4.9 mL of buffered or unbuffered glucose solution at 25 °C, 100 μL TPP-extracted GFP (at initial concentration around 400 μg GFP mL^{-1}) was added to provide a final concentration of 8–10 μg mL^{-1}. GFP fluorescence intensity and solution pH were measured immediately and after heating then storing at 4 °C for 24 h; measurements were taken after heating and 24 h/4 °C storage to assay for any possible renaturation. Immediately after the addition of GFP into the glucose solutions, the mixture was gently stirred for 30 s, placed into the cuvette and subjected to a constant exposure at 80 °C, 85 °C, 90 °C or 95 °C. All fluorescence intensity and pH readings of the samples before and after heat treatment were made with the solutions at 25 °C.

A 2 mL aliquot of sample was transferred to a quartz cuvette (1 cm light path length) and sealed with a plastic cover. Each cuvette was inserted into an adapter assembly and adjusted in

the cell holder. A constant temperature (±0.05 °C) was provided by continuous circulation of water from the waterbath to the cell holder and the sample in the cuvette via a circulation pump (Thermo-bath TB-85, P/N 200-65022, Shimadzu). The moment the sample-filled cuvette was placed in the cell holder and the treatment was initiated, fluorescence readings were taken at intervals of 5 s at 80 °C, 85 °C, 90 °C or 95 °C, for a maximum exposure time of 1.5 h. With all samples starting at 25 °C prior to heating, the 2 mL sample volume attained the final assay temperatures of 80 °C or 85 °C after 20–30 s, and attained a final 90 °C or 95 °C in 10 s.

2.4. Analysis of the kinetic parameters

The GFP fluorescence data plotted provided curves that were considered first order models represented by:

$$\log_{10} I_f = \log_{10} I_o - \left(\frac{1}{D}\right) t = \log_{10} C_o - \left(\frac{k}{2.303}\right) t, \quad (2)$$

where I_o was the "initial fluorescence intensity" of native GFP and I_f was the "final fluorescence intensity" of remaining native GFP, after the exposure time (min), at a constant temperatures of 80 °C, 85 °C, 90 °C or 95 °C.

The decimal reduction time, the interval of time required to reduce one decimal logarithm of the initial fluorescence intensity of GFP at reference temperature (Eq. (3)), was determined from the negative reciprocal of the slopes of the regression lines, using the linear portions of the inactivation curves ($\log_{10}$ μg GFP mL^{-1} versus time of exposure at a constant temperature):

$$D\text{-value} = \frac{2.303}{k}. \quad (3)$$

The z-value may be related to the coefficient Q_{10} of the process by:

$$Q_{10} = 10^{10/z}. \quad (4)$$

Activation energy (E_a, kcal mol^{-1}), which represents the energy present in a system, was estimated by using the Arrhenius equation, which was applied to evaluate the dependence of the inactivation rate constant, k, on the heating temperature:

$$\log_{10} k_1 = \log_{10} k_2 - \left\{\left(\frac{E_a}{2.303R}\right)\left[\left(\frac{1}{T_1}\right) - \left(\frac{1}{T_2}\right)\right]\right\}, \quad (5)$$

where T_1 and T_2 are the heating absolute temperatures (K), and R is the universal gas constant (1.987 cal mol^{-1} K^{-1}).

3. Results

3.1. Evaluation of pH of GFP in buffered and unbuffered glucose solutions

The pH of the GFP/glucose solutions taken at room temperature (25 °C) shortly before heating ranged from 5.14 (±0.11) to 7.82 (±0.12) and, after 24 h/4 °C storage, from 5.13 (±0.11) to 7.85 (±0.13) for the same systems.

The pH of the buffered 1.5–50% glucose solutions were minimally altered, with a maximum standard deviation of 0.18 units: (i) pH 7.82 (±0.12) in Tris–EDTA (pH 8); (ii) pH 6.79 (±0.18) in phosphate (pH 7); (iii) pH 5.97 (±0.16) in phosphate (pH 6); (iv) pH 5.14 (±0.11) in acetate (pH 5). The mean pH for WFI and phosphate buffered (pH 7) solutions were comparable, although for WFI, the mean variation in pH was greater, pH 7.05 (±0.31) than for the phosphate buffered/glucose solutions.

After heating and 24 h storage at 4 °C, the pH did not change significantly and all samples appeared clear, without any precipitation or crystallization visible.

3.2. Thermal stability of GFP in buffered and unbuffered glucose solutions

The amount of native GFP added to the solutions was expressed in relative fluorescence intensity units (ex/em = 394/509 nm) and converted to the corresponding concentration (μg mL^{-1}) from a standard curve of a commercially available purified GFP (Eq. (1)).

After initial preparation, acetate (pH 5) and phosphate (pH 6) buffered solutions exhibited the greatest loss in native GFP concentrations (2–3 μg mL^{-1}) compared to WFI, phosphate (pH 7) and Tris–EDTA solutions (<1 μg mL^{-1}). In phosphate (pH 7) and Tris–EDTA (pH 8), there was a tendency for a gradual drop in GFP concentrations with increasing glucose but there was no significant change in GFP concentration, with or without glucose. In WFI, the amount of GFP added to the control and glucose solutions was relatively unchanged after initial mixing.

After 90 min heat exposure for all temperatures studied and then 24 h/4 °C storage, acetate had the lowest average recovery of fluorescence from GFP renaturation (3.32 ± 0.08–5.31 ± 0.09 μg mL^{-1}), followed by phosphate (pH 6) solutions (6.46 ± 0.10 μg mL^{-1}). Phosphate (pH 7) and Tris–EDTA (pH 8) solutions lost an average about 1 μg mL^{-1} of native GFP and <1 μg mL^{-1} for WFI solutions (Table 1). From all solutions examined, WFI favored renaturation of GFP, followed by Tris–EDTA (pH 8) and phosphate (pH 7) buffered solutions. Acetate buffered solutions provided the least favorable conditions for renaturation.

To estimate D-values (Tables 2 and 3) at constant temperatures and pH, the range of native GFP concentrations evaluated was between <10.0 μg mL^{-1} for initial concentration (C_o) down to 1.00–1.55 μg mL^{-1} for final concentrations (C_f) after 90 min heating, from 80 °C to 95 °C, which corresponded to the linear portion of the inactivation curves ($\log_{10}$ μg GFP mL^{-1} versus time of exposure at a constant temperature; Fig. 1A and B).

3.2.1. GFP in water for injection (WFI) glucose solutions

At 80 °C, the stability of D-values for GFP in WFI was constant for up to 10% glucose (Table 2), comparable to the WFI control. GFP stability reached a maximum plateau between 25% and 35% glucose and the D-values decreased up to 50% glucose, showing two distinct linear regions in the intervals between: (i) 10–35% glucose and (ii) 35–50% glucose.

The conductivity of the solutions was the highest with 10% glucose and dropped from 15% to 50% glucose. For the interval

Table 1
Average amounts of GFP added to assay solutions, average native GFP concentrations remaining after initial preparation prior to heating ("Initial") and, after 90 min of heating and storage for 24 h at 4 °C ("After"), for all temperatures studied

	Native GFP concentration ($\mu g\,mL^{-1}$)				
	Acetate pH 5	Phosphate pH 6	WFI pH 6–7	Phosphate pH 7	Tris–EDTA pH 8
Added to all solutions	8.53 (±0.21)	8.70 (±0.04)	8.87 (±0.51)	8.95 (±0.60)	9.21 (±0.42)
Initial control	6.79 (±0.67)	6.90 (±0.67)	8.59 (±0.91)	8.12 (±0.71)	8.93 (±0.75)
Initial 1.5% glucose	5.82 (±0.21)	6.92	8.59	8.12	8.93
Initial 50% glucose	6.43 (±0.20)	(±0.08)	(±0.91)	(±0.71)	(±0.75)
Initial average	5.46 (±0.38)	6.97 (±0.15)	8.46 (±0.92)	7.97 (±0.64)	8.31 (±0.94)
After 1.5% glucose	3.32 (±0.08)	6.46	8.28	7.89	7.92
After 50% glucose	5.31 (±0.09)	(±0.10)	(±0.61)	(±0.55)	(±0.79)

from 30% to 50% glucose, the viscosity doubled (from 3.02 to 5.9 mPa s; control = 1.76 mPa s) while the conductivity dropped 2.5-fold (from 699.91 to 279.35 mS/cm; control = 1.84 mS/cm), and the *D*-value at 25 °C fell 1.5-fold, from 28.01 to 19.05 min (Penna et al., 2002).

3.2.2. GFP in acetate (pH 5) buffered glucose solutions

At 80 °C, GFP thermal stability in acetate (pH 5) buffered solutions was constant, with an average *D*-value (4.48 ± 0.44 min) independent of increasing glucose up to 35%, and doubled ($D = 8.97 \pm 1.47$ min) for a narrow interval from 40% to 50% glucose.

At 85 °C, the mean *D*-value (2.50 ± 0.90 min) for GFP in buffered 1.5–50% glucose/acetate solutions confirmed a steady influence of this buffer system upon GFP stability although the pH was close to the p*I* (4.92–5.50).

At 90 °C, the average *D*-value (1.73 ± 0.26 min) showed no influence of increasing glucose concentrations up to 40% glu-

Table 2
D-values for GFP in glucose solutions (at 80 °C and 85 °C)

Glucose (%)	*D*-value (min)				
	WFI	10 mM Acetate pH 5	10 mM Phosphate pH 6	10 mM Phosphate pH 7	10 mM Tris–EDTA pH 8
80 °C					
0	13.85	4.08	15.41	40.32	27.03
1.5	13.33	4.79	9.20	29.07	25.25
5	12.58	4.24	8.51	41.32	34.25
7	14.49	4.02	11.95	35.71	38.61
10	12.00	4.64	9.65	36.76	52.08
15	16.03	3.70	8.27	43.67	57.80
20	18.38	4.46	15.34	48.08	67.11
25	27.62	5.15	10.73	52.08	67.57
30	28.01	4.51	13.75	59.88	97.09
35	29.07	5.24	18.48	72.46	86.96
40	19.05	7.29	22.78	74.07	94.34
45	21.65	9.62	28.98	89.29	92.59
50	21.10	10.00	25.64	120.48	107.53
85 °C					
0	2.82	1.94	3.64	10.65	6.71
1.5	5.05	1.85	4.08	9.80	6.66
5	5.24	1.84	3.82	11.15	6.65
7	4.36	1.80	3.98	10.82	6.07
10	5.38	2.51	4.42	10.65	6.88
15	5.76	2.01	5.58	12.63	12.24
20	4.84	2.61	4.30	11.25	12.06
25	5.93	2.13	4.50	19.31	17.73
30	5.45	2.95	4.52	17.27	12.21
35	5.43	2.44	4.98	22.88	17.70
40	5.95	3.60	5.15	22.32	16.02
45	5.11	3.50	6.92	23.92	28.65
50	7.73	3.29	5.46	32.36	33.11

(i) Water for injection, WFI (pH 6–7), (ii) 10 mM acetate buffer (pH 5), (iii) 10 mM phosphate buffer (pH 6 and 7) and (iv) 10 mM Tris–EDTA (pH 8).

Table 3
D-values for GFP in glucose solutions (at 90 °C and 95 °C)

Glucose (%)	*D*-value (min)				
	WFI	10 mM Acetate pH 5	10 mM Phosphate pH 6	10 mM Phosphate pH 7	10 mM Tris–EDTA pH 8
90 °C					
0	2.22	1.41	2.78	3.56	3.30
1.5	3.24	1.56	3.31	3.78	4.05
5	3.35	1.64	2.67	3.84	3.45
7	3.21	1.60	2.82	3.90	3.48
10	3.55	1.61	3.14	4.00	3.83
15	3.68	1.62	2.89	4.15	3.86
20	3.64	1.51	3.01	4.33	5.73
25	4.34	1.81	2.93	5.10	5.30
30	3.88	2.30	3.16	5.12	4.26
35	3.48	2.01	3.21	6.83	4.19
40	4.19	1.97	3.59	7.17	4.53
45	3.79	2.54	4.00	8.70	5.91
50	3.63	2.71	3.71	8.10	6.12
95 °C					
0	2.18	1.65	2.35	2.91	2.37
1.5	2.47	1.56	2.32	3.12	2.50
5	2.59	1.51	2.40	3.12	2.55
7	2.76	1.35	2.38	2.99	2.54
10	2.87	1.54	2.41	3.04	2.51
15	2.67	1.52	2.44	3.04	2.96
20	2.48	1.39	2.44	3.12	2.74
25	2.47	1.46	2.48	3.15	3.07
30	2.30	1.91	2.54	3.34	2.89
35	2.98	1.60	2.74	3.52	2.93
40	2.74	1.63	2.47	4.07	3.10
45	2.81	2.16	2.98	3.65	3.51
50	3.06	1.94	2.61	3.77	3.91

(i) Water for injection, WFI (pH 6–7), (ii) 10 mM acetate buffer (pH 5), (iii) 10 mM phosphate buffer (pH 6 and 7) and (iv) 10 mM Tris–EDTA (pH 8).

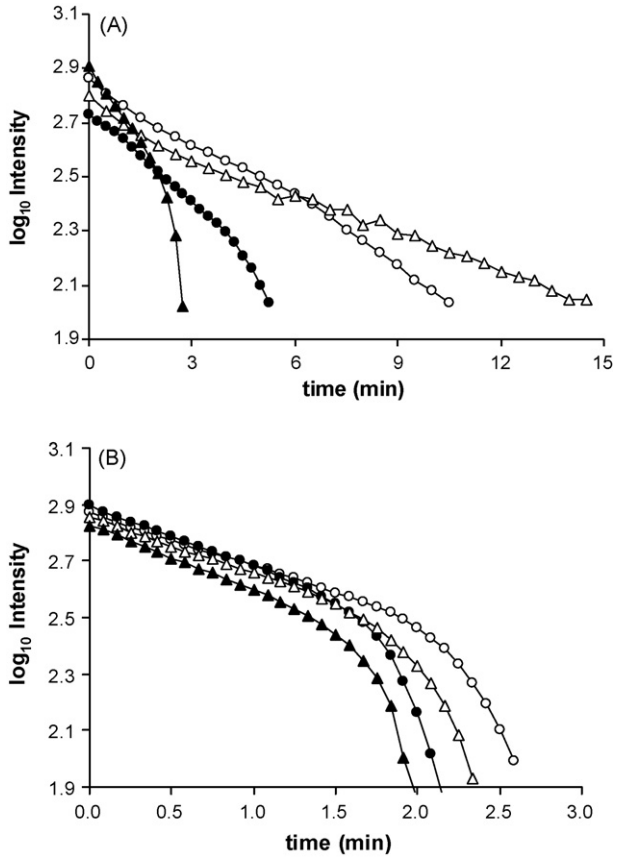

Fig. 1. Inactivation curve for GFP in glucose/WFI solutions: (◯) 1.5% and (△) 50% glucose solutions at 80 °C, and (●) 1.5% and (▲) 50% glucose solutions at 85 °C (A), 90 °C (open symbols) and 95 °C (filled symbols; B). The *D*-values were determined from the reciprocal of the slope of the GFP inactivation curves, considered pseudo first-order models.

cose, when a 1.5-fold increase (*D* average, 2.63 ± 0.12 min) occurred with 45–50% glucose (Table 3).

The independence of GFP stability with glucose concentration in the acetate buffer system was confirmed for the range from 1.5% to 50%, when the mean *D*-value ($D_{90\,^\circ C}$ = 1.87 ± 0.41 min) fell 33% going from 85 °C to 90 °C. This behavior was the same at 95 °C, when an average *D*-value (1.63 ± 0.23 min) was 15% less than that obtained at 90 °C and dropped three-fold going from 80 °C to 95 °C with 1.5–50% glucose (Tables 2 and 3).

3.2.3. GFP in phosphate (pH 6) buffered glucose solutions

At 80 °C, the *D*-value for the control dropped between 1.5% and 15% glucose (9.52 ± 1.47 min) and recovered the average of 14.58 ± 3.23 min between 20% and 35% glucose, showing the influence of glucose over the thermal stability of GFP.

At 85 °C, *D*-values dropped three-fold up to 30% glucose and four-fold between 35% and 50% glucose. With an average *D*-value (4.32 ± 0.57 min) close to the control, the addition of up to 30% glucose showed no influence upon GFP thermal stability and the interval between 35% and 50% glucose provided a gradual 30% increase in *D*-value (5.63 ± 0.88 min).

At 90 °C and 95 °C, there was a 20% difference in thermal stability for up to 50% glucose, for the average *D*-values, $D_{90\,^\circ C}$ = 3.17 (±0.39) min and $D_{95\,^\circ C}$ = 2.50 (±0.28) min.

3.2.4. GFP in phosphate (pH 7) buffered glucose solutions

At 80 °C, GFP thermal stability in phosphate buffered (pH 7) solutions showed increasing stability with increasing glucose concentrations, with *D*-values tripling up to 50% glucose improving the protein stability.

At 85 °C, GFP thermal stability dropped four-fold compared to 80 °C; *D*-values declined from 40.32–120.48 min to 10.65–32.36 min at 85 °C, respectively.

At 90 °C there was a three-fold reduction of GFP thermal stability from control to 40% glucose and a four-fold decrease in 45–50% glucose (8.40 ± 0.42 min) compared to same solutions at 85 °C. However, the maximum stability attained ($D_{45–50\%}$ = 8.40 ± 0.42 min) represents a similar response in 1.5% glucose solution at 85 °C (Tables 2 and 3). With up to 20% glucose the system stabilized GFP consistently with an insignificant increase of 1 min. With concentrations higher than 20% glucose a proportional increase in GFP thermal stability was observed with greater correlation between the inactivation curves.

An average $D_{95\,^\circ C}$ value of 3.22 (±0.28) min confirmed the main influence of temperature exerted upon GFP thermal stability than the glucose concentration at 95 °C, similar to the behavior at 90 °C.

3.2.5. GFP in Tris–EDTA (pH 8) buffered glucose solutions

At 80 °C, compared to the control *D*-value, 7% glucose showed a 20% increase, 10–25% glucose showed a 2–2.5-fold increase, and >30% glucose exhibited a three- to four-fold increase in GFP thermal stability (Table 2).

At 85 °C, the general behavior of the inactivation curve was more pronounced compared to what was observed at 80 °C. However, up to 50% glucose still enhanced the capacity of the protein to resist unfolding; *D*-values increased five-fold, from 6.66 min to 33.11 min for concentrations from 1.5% to 50% of glucose. The *D*-value for the control at 85 °C exhibited a four-fold reduction in GFP thermal stability compared to the control at 80 °C. In concentrations of up to 10% glucose, thermal stability was constant relative to the control, but dropped seven-fold going from 80 °C to 85 °C. From 15% to 40% glucose, *D*-values were 2–2.5-fold greater than the control, repeating the phenomenon observed at 80 °C for 10–25% glucose (Table 2).

At 90 °C, *D*-value doubled between 0% and 50% glucose. Compared with the control, up to 15% glucose had no effect upon GFP thermal stability, as observed for GFP in phosphate pH 7 for the same conditions (Table 3).

At 95 °C, the average *D*-value for GFP of 2.89 (±0.43) min between 0% and 50% glucose, showed similar behavior that was demonstrated in phosphate (pH 7), being independent of glucose concentration, temperature and buffer composition.

At 90 °C and 95 °C, the decrease in GFP fluorescence intensity showed two distinct inclinations of the straight lines for all glucose concentrations. These inclinations were related to *D*-values of the glucose and control solutions (Table 3 and Fig. 1).

Pseudo first order inactivation models were routinely used to characterize the linear decrease for protein denaturation even though the two inclinations were shown to be quite distinct (Penna et al., 2002). Although some solutions seem to have contributed to changes in the shape of the inactivation curve, the mean and general *D*-values were calculated from the slope of the average semi-logarithmic inclination of the straight line.

For evaluation of GFP thermal stability, decimal reduction times were determined with a pseudo first order model or common curve (general *D*-values), although *D*-values were also calculated for the first and for the second inclinations of straight line tendencies (D_1 and D_2) beyond the inflection point, where the change in behavior occurred. Because the inclination of the two distinct lines is similar, the general *D*-values were shown to optimally characterize the exposure time for an inflection point.

The coincidence between the mean *D*-values and the exposure time when the inflection in the curve occurred was remarkable and reinforces the use of the mean *D*-value to study protein thermal stability in Tris–EDTA/glucose solutions. For instance, for 50% glucose the mean *D*-value of 6.12 min is very close to the time when the inflection occurred (6.0 min). Therefore, the mean *D*-values were used to characterize the thermodynamic stability of GFP at 90 °C and 95 °C.

The difference in the inflection points signifies the influence of both the 50% glucose and the temperature upon GFP stability compared with 1.5% glucose at 90 °C and 95 °C. At 90 °C, 50% glucose doubles GFP thermal stability compared to 1.5% glucose. The mean *D*-values increased with increasing amounts of glucose indicating that the protein becomes less temperature sensitive. A higher thermodynamic stability enhanced by glucose is also evident when increasing the temperature.

Similar GFP stability was seen at 95 °C, when the straight line inflection point time and *D*-value for GFP in 50% glucose was double that observed at 1.5%. However, the distinct inclinations characterizing GFP thermal stability change were consistent and the straight lines were parallel, even the 50% glucose favored GFP stability.

3.3. Kinetic parameters: z-value, Q_{10} coefficients and activation energy (E_a)

The kinetic parameters *z*-value, Q_{10} coefficient and activation energy (E_a) reflect the temperature dependence of denaturation, in the case of GFP measured by a decrease in the fluorescence intensity. Related to the intrinsic energy (E_a) and the stability of the system during heat treatment, stable systems have lower energy than unstable ones (Table 4).

Glucose in acetate buffer pH 5 was shown to be the most uniform system for GFP, where the average for *z*-value in up to 35% glucose (34.02 ± 4.51 °C) was the highest, the Q_{10} coefficient was established between 1.0 and 2.5 and the activation energy in acetate (17.84 (±2.26) kcal mol^{-1}) assured low entropy (−0.0009 (±0.0004) kJ mol^{-1}).

For WFI and phosphate (pH 6), the presence of up to 15% glucose provided equal or higher thermal stability compared to the controls. Similar *z*-values for GFP dropped 1.5-fold for the

Table 4
z-Values, Q_{10} coefficient and E_a of thermal stability for GFP in glucose systems

Glucose (%)	WFI			Acetate, pH 5			Phosphate, pH 6			Phosphate, pH 7			Tris–EDTA, pH 8		
	z-Value (°C)	Q_{10}	E_a (kcal/mol)	*z*-Value (°C)	Q_{10}	E_a (kcal/mol)	*z*-Value (°C)	Q_{10}	E_a (kcal/mol)	*z*-Value (°C)	Q_{10}	E_a (kcal/mol)	*z*-Value (°C)	Q_{10}	E_a (kcal/mol)
0	19.90	3.18	30.10	38.17	1.17	15.80	19.49	3.26	30.70	12.82	6.03	46.53	14.37	4.96	41.52
1.5	21.10	2.99	28.50	32.57	1.40	18.39	26.53	2.38	22.48	15.06	4.61	39.61	15.58	4.38	38.51
5	22.20	2.82	26.88	35.59	1.45	16.69	27.70	2.30	21.55	13.05	5.83	45.67	13.74	5.34	43.81
7	21.80	2.87	27.41	34.25	1.49	17.60	22.22	2.82	26.90	13.60	5.43	43.81	13.12	5.78	45.24
10	24.40	2.56	24.39	30.49	1.79	19.46	25.58	2.46	23.33	13.60	5.43	43.80	11.84	6.99	50.22
15	19.90	3.18	30.17	39.68	1.48	14.97	26.67	2.37	22.33	12.64	6.18	47.15	11.44	7.48	52.17
20	18.40	3.50	32.63	28.41	2.37	20.95	19.61	3.24	30.47	12.61	6.21	47.32	11.13	7.92	53.52
25	15.30	4.52	39.15	29.24	1.60	20.46	23.87	2.62	25.00	11.80	7.03	50.40	10.89	8.13	54.23
30	14.70	4.81	40.63	41.15	1.76	14.63	21.23	2.96	28.13	11.65	7.21	51.10	9.90	10.24	60.11
35	15.90	4.27	37.79	30.67	1.28	19.45	18.66	3.44	32.00	11.20	7.82	53.15	9.92	10.19	60.12
40	18.70	3.44	31.95	22.68	2.77	26.39	16.39	4.07	36.43	11.85	6.98	50.24	10.04	9.91	59.62
45	17.80	3.64	33.33	24.10	2.24	24.91	15.63	4.37	38.22	10.66	8.67	55.83	10.10	9.77	58.93
50	17.70	3.68	33.93	22.52	2.29	26.51	15.90	4.26	37.55	9.75	10.62	61.08	9.90	10.24	60.15

(i) WFI (pH 6–7), (ii) 10 mM acetate buffer (pH 5), (iii) 10 mM phosphate buffer (pH 6 and 7), and (iv) 10 mM Tris–EDTA (pH 8).

increase from 20% to 50% glucose, showing the influence of glucose concentrations in the system.

The z-values for GFP in the controls were similar to those in up to 10–20% glucose in WFI, acetate and phosphate (pH 7) buffered solutions, respectively, and up to 7% glucose in Tris–EDTA, in which the contribution of glucose provided a greater change in GFP stability, followed by the phosphate (pH 7) system.

In phosphate (pH 6), the E_a for the control was similar to that for WFI and a decline to 24.58 (±3.94) kcal mol^{-1} for 1.5–25% glucose rose to 37.40 (±0.90) kcal mol^{-1} between 40% and 50% glucose, confirming an increase in the affinity of GFP for glucose, in which their stable association improved the entropy of GFP with each increment of glucose added to the system (−0.0025 (±0.001) kJ mol^{-1}).

A concentration of 50% glucose in acetate provided GFP with the same activation energy required to maintain thermal stability in phosphate (pH 6) with 1.5–30% glucose and, in WFI with 1.5–10% glucose.

In phosphate (pH 7), the average activation energy ($E_a = 44.56 \pm 2.87$ kcal mol^{-1}) up to 20% glucose solutions increased to 52.14 (±2.36) kcal mol^{-1} for the range from 25% to 45% glucose and rose to a maximum of 61.08 kcal mol^{-1} with 50% glucose, corresponding to the entropy of 0.0060 kJ mol^{-1}.

In Tris–EDTA, the activation energy increased 1.5 times up to 30% glucose ($E_a = 60.11$ kcal mol^{-1}), with a stable system up to 50% glucose. With glucose concentrations >25%, Tris–EDTA exhibited the longest range for GFP thermal stability (E_a average = 59.78 (±0.52) kcal mol^{-1}) maintained by a high intrinsic energy in balance with entropy (0.0056 (± 0.0004) kJ mol^{-1}).

For phosphate (pH 7) and Tris–EDTA, the vulnerability of GFP thermal stability between 80 °C and 95 °C was worst in 50% glucose compared to either WFI or phosphate (pH 6), where external influences can easily change the stability of GFP. GFP in phosphate (pH 7) and Tris–EDTA provided similar E_a values for 1.5% and 50% glucose. In Tris–EDTA, the highest activation energy was attained with 30% glucose and was steady up to 50% glucose, while GFP in phosphate (pH 7) attained a maximum with 50% glucose, both two to four times higher than in acetate (pH 5). Unique to all the systems examined, the activation energy in phosphate (pH 7) and in Tris–EDTA exhibited a gradual increase for the increment of up to 25% glucose. The entropy of the GFP in phosphate (pH 7) and Tris–EDTA was the highest and, lowest in acetate (pH 5).

The variation between z-values for the increment of up to 50% glucose was the lowest in WFI (∼3.40 °C), between 5.31 °C and 5.68 °C in phosphate (pH 7) and Tris–EDTA, and the highest in acetate and phosphate (pH 6) (10.05 °C and 10.63 °C), showing that WFI, with and without glucose, provided more uniform thermal stability for GFP from 80 °C to 95 °C. GFP exhibited less energy in acetate during heating than in WFI and phosphate (pH 6), but exhibited higher energy in both phosphate (pH 7) and Tris–EDTA to maintain protein structure. However, the difference in activation energy between 1.5% and 50% glucose was the lowest in WFI ($\Delta E_a = 5.43$ kcal mol^{-1}), tripling for phosphate (pH 7) and Tris–EDTA ($\Delta E_a = 21.55$ kcal mol^{-1}).

4. Discussion

Heat treatment (blanching, pasteurization, disinfection and sterilization) continues to be one of the most widely used means of ensuring product safety and minimum loss of quality. The inactivation of either enzymes or microorganisms by moist heat follows a first order kinetic where thermal stability is described by two parameters: D- and z-values. The interest in developing time–temperature bioindicators for evaluating thermal processes has required more extensive studies for the inactivation of proteins, enzymes, spores or other temperature dependent effects (Penna et al., 2005; Mackey et al., 2006). The measurement of D- and z-values for GFP at variable conditions such as pH, water activity (a_w) and solution composition can demonstrate kinetic models for predicting protein inactivation by measuring fluorescence intensity (Penna et al., 2004c, 2005). Thus, a comparison with some microorganisms or proteins that are commonly used as bioindicators (BI) to thermal resistance of GFP can be established.

Pectinesterase (PME), the enzyme responsible for the hydrolysis of the pectin present in citrus juice (pH 3–4), is more thermal stable than the natural contaminant microorganisms which can survive during storage, and its inactivation is used as an indicator for the adequacy of the pasteurization process (Basak and Ramaswamy, 1996; Collet et al., 2005) found a D-value of 3.80 min ($k_1 = 0.6053$) for PME in pasteurized orange juice (pH 3.87 ± 0.02) at 87.5 °C. This D-value was similar to the D-values for GFP exposed to the temperatures: (i) 90 °C in 5% glucose at pH 7.0, (ii) 10% glucose at pH 8.0, (iii) 30% glucose in WFI and (iv) 45% glucose at pH 6.0. At 95 °C, D-values ranged from 3.50 to 3.91 min for concentrations higher than 40% glucose in phosphate (pH 7.0) and Tris–EDTA (pH 8.0) (Tables 3 and 4). The activity of PME was determined in fruit juices (pH 3–4) (Laratta et al., 1995) with a z-value of 32 °C for an interval between 90 °C and 103 °C, which is close to the mean z-value (34 ± 4 °C) for GFP in acetate (pH 5) with up to 35% glucose, when z-value dropped to 25 ± 1 °C with 40–50% glucose, suggesting that glucose may be an effective additive for the stabilization of GFP in acidic solutions.

Thermal stability of lysozyme was evaluated in aqueous solutions at temperatures (73–100 °C), pH (4.2–9.0), levels of sucrose (0.5% and 15%) and sodium chloride (0.01 M and 1 M) (Makki and Durance, 1996). Lysozyme that is used to inhibit growth of spoilage bacteria, exhibits a relatively high thermal stability that also makes it attractive for use as bioindicator in pasteurized and heat-sterilized products. Experimental D-values for lysozyme showed higher heat stability in acidic solutions (i.e., far from its isoelectric point 10.7) and with increasing concentrations of sucrose up to 15%, in which sugars and polyols were confirmed to stabilize proteins against heat denaturation. In accordance with the authors, stabilization was ascribed to strengthening of the interactions between hydrophobic regions of lysozyme, induced by the effect of sucrose on the structure of water. At 75 °C, at pH from 7.2 and 9.0, the D-values for lysozyme decreased from 800 min to 33.2 min, respectively. Those findings are consistent with differential scanning calorimetry (DSC) studies (Back et al., 1979; Kristjansson

and Kinsella, 1990) in which sugars and polyols were observed to stabilize proteins against heat denaturation.

In this work, *D*-values for GFP in solutions (pH 6–8) far from its isoelectric point (pH 5.1) was observed to increase with glucose concentrations up to 50%. Although, lysozyme in acidic solutions exhibits thermal resistance (75–91 °C) 200 times higher than that shown by GFP in basic solutions at 80–95 °C, GFP can be considered a better marker because it can be easily and rapidly detected.

From the thermal inactivation curves for peroxidase (POD) and lipoxygenase (LOX), both in phosphate buffer (pH 6.0), their *D*-values were estimated, which ranged, respectively from 43.13 min to 188.77 min at 60 °C; from 34.63 min to 31.76 min at 65 °C; from 17.16 min to 4.06 min at 70 °C; from 11.69 min to 1.55 min at 75 °C; from 0.59 min to 1.99 min at 80 °C and from 0.79 min for the peroxidase at 90 °C (Gökmen et al., 2005). In this work, GFP, in phosphate buffer (pH 6), had mean *D*-values higher than those found for peroxidase and similar to those estimated for PME, both used as bioindicator in moist-heat processes. However, the three proteins exhibited thermal resistance greater than those found for microbial pathogens found in processed products. For *E. faecium* a *D*-value of 18 min at 65 °C ($z = 6$ °C) was determined, equivalent to 0.0012 min at 90 °C (Mackey et al., 2006). The exposure time to decay 12 $\log_{10}$ of *E. faecium* would be less than 1.0 min, where GFP as bioindicator might be applied to monitor the thermal treatment.

Enzymes usually require substrates and reaction kinetics to quantify their activities, as used in assays for detection of peroxidase, PME and lysozyme. However, GFP does not require any complex means to quantify its fluorescence intensity because its fluorescence is an intrinsic characteristic easily detectable by a UV lamp or a spectrofluorometer.

Apart from providing a practical means of detection, GFP improves process parameters, preventing overheating of thermal processed solutions, because the similarity of its thermal resistance, which increases with glucose concentrations, to that for *B. atrophaeus*, generally used as standard BI in autoclaving solutions with pH ≥ 5. The *D*-value of 8.0 ± 0.8 min for GFP in phosphate (pH 7.0) with 40%, 45% and 50% glucose at 90 °C (Table 4) showed equivalent thermal stability as the spores of *B. subtilis* (8.0 ± 1 min) in parenteral solutions with up 50% glucose concentration (Penna et al., 2002).

The protective effect of sugars on protein stability against heat denaturation has been well-known for many years. Considering the presence of sucrose and glucose (sugars) and sorbitol and glycerol (polyols) over ovalbumin (pH 7), lysozyme (pH 3 and 7), conalbumin (pH 7), and α-chymotrypsinogen (pH 3), glucose proved to be a better stabilizer than other solutes, in combination with hydrophobic interactions, the main force that influences the three-dimensional structure of proteins, sugar and water molecules, stabilizing the proteins against denaturation. The addition of 50% of glucose into lysozyme and ovalbumin solution (pH 7) increased the change in denaturation temperature (ΔT_m) in between 15.5 °C and 17 °C (Back et al., 1979). For GFP, the addition of 50% glucose into phosphate buffer solution (pH 7) increased *D*-values three-fold, when exposed to 80 °C and 85 °C and twice at 90 °C, maintaining the same thermal stability when exposed to 95 °C (Tables 2 and 3).

Our previous study evaluated the GFP stability at 25 °C in solutions ranging from 1.5% to 50% glucose (Penna et al., 2005). GFP stability was shown to be influenced by glucose concentration, buffer system and pH of the solutions. The activation energies for the systems varied from (E_a) 245.58 kcal mol^{-1} in WFI (pH 6.0–7.0) and doubled for GFP in acetate (pH 5.0; $E_a = 502.40$ kcal mol^{-1}) and in phosphate (pH 6.0; $E_a = 511.03$ kcal mol^{-1}). The greater the concentration of glucose the lower the E_a decreasing for 45–50% glucose concentrations in WFI, representing 60% less energy in the system and a strong immobilized arrangement between the molecules of glucose, water and the protein. The lowest E_a and highest stability of the protein in 50% glucose/WFI solution is dependent on the favorable immobilized arrangement among molecules, with the protein exposed to external physical stresses.

By the convenient, easy and rapid measure of fluorescence intensity, GFP can be used as an indicator in glucose solutions to report that processing times and temperatures have been attained.

The high thermal stability of GFP in glucose solutions make it attractive for use in thermal processes, optimizing the relation time–temperature of product exposure, and presenting an innovate alternative to certify the operation. The main advantage in using GFP, the optimization of the process will reduce costs and ensure the integrity of products.

The use of alternative methods to determine the quality of processes is becoming commercially attractive due to the fast results and the widely used concept of parametric release.

The thermostability of GFP provides the basis for its potential utility as a fluorescent biological indicator to assay the efficacy of moist-heat treatments at temperatures lower than 100 °C.

5. Conclusion

The evaluation of GFP thermal stability in buffered and unbuffered solutions with increasing glucose concentrations favored a more thorough analysis of the behavior of the protein exposed from 80 °C to 95 °C. Increasing glucose concentrations promoted GFP thermal stability, with the greatest influence observed between 20% and 25% glucose; thermal resistance increased linearly with the increasing glucose concentrations.

Exposure to 90 °C and 95 °C showed a remarkable and a more predominant influence of temperature over GFP stability than the amount of glucose for most of the systems studied, except for WFI; GFP stability was shown to be independent of glucose concentration in this system.

The system with the lowest intrinsic energy for spontaneous transformation independent of temperature was the acetate (pH 5) with glucose between 1.5% and 35% glucose. The most stable systems for the protection of native GFP structure followed in decreasing order of deactivation free energy: Tris–EDTA (pH 8), phosphate (pH 7), WFI (pH 6), phosphate (pH 6), and acetate (pH 5).

The performance of GFP under these conditions confirmed its potential utility as a biological indicator for use in a variety

of applications: in the decontamination of parenteral solutions and WFI. Heat treatment below 100 °C is routinely used in the processing of a wide variety of food products, sera, plasma and other blood products, enzymes, growth factors, vaccines and bioprostheses. Further studies may determine if GFP may also be useful in monitoring disinfection and pasteurization processes for other, more complex solutions as well.

GFP was shown to be an efficient marker as applied in the heat processing of solutions with added glucose, similar to parenteral solutions, where the small variation of z-values with increasing glucose concentration indicates the high stability of GFP, independent of the glucose concentration, but dependent only on processing temperature, as observed in the evaluation of the free energy, enthalpy and entropy of these systems.

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