0273-2289/1559–0291 (Online)/07/136–140/555–572/$30.00

Evaluation of the pH- and Thermal Stability of the Recombinant Green Fluorescent Protein (GFP) in the Presence of Sodium Chloride

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Abstract

The thermal stability of recombinant green fluorescent protein (GFP) in sodium chloride (NaCl) solutions at different concentrations, pH, and temperatures was evaluated by assaying the loss of fluorescence intensity as a measure of denaturation. GFP, extracted from *Escherichia coli* cells by the three-phase partitioning method and purified through a butyl hydrophobic interaction chromatography (HIC) column, was diluted in water for injection (WFI) (pH 6.0–7.0) and in 10 m*M* buffer solutions (acetate, pH 5.0; phosphate, pH 7.0; and Tris-EDTA, pH 8.0) with 0.9–30% NaCl or without and incubated at 80–95°C. The extent of protein denaturation was expressed as a percentage of the calculated decimal reduction time (*D*-value). In acetate buffer (pH 4.84 ± 0.12), the mean *D*-values for 90% reduction in GFP fluorescence ranged from 2.3 to 3.6 min, independent of NaCl concentration and temperature. GFP thermal stability diluted in WFI (pH 5.94 ± 0.60) was half that observed in phosphate buffer (pH 6.08 ± 0.60); but in both systems, *D*-values decreased linearly with increasing NaCl concentration, with *D*-values (at 80°C) ranging from 3.44, min (WFI) to 6.1 min (phosphate buffer), both with 30% NaCl. However, *D*-values in Tris-EDTA (pH 7.65 ± 0.17) were directly dependent on the NaCl concentration and 5–10 times higher than *D*-values for GFP in WFI at 80°C. GFP pH- and thermal stability can be easily monitored by the convenient measure of fluorescence intensity and potentially be used as an indicator to monitor that processing times and temperatures were attained.

Index Entries: Bioindicator; *D*-value; green fluorescent protein; pH-stability; sodium chloride; thermal stability.

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Introduction

The green fluorescent protein (GFP), is an acidic (p*I* 4.9–5.1) thermostable protein that can be expressed in a wide variety of organisms, in vivo, or in cell cultures. It is used extensively to monitor biological events in a variety of experimental applications as well as a biosensor to monitor industrial and medical processes to ensure product quality or process efficacy. Water activity (a_w), which is defined as the ratio of the vapor pressure of water in a material to the vapor pressure of pure water at the same temperature *(1)*, has become a useful determinant of food stability to measure potential microbial growth and, together with temperature and pH, is one of the major parameters influencing bacterial growth and survival. Water activity is related to solute species and concentration and their effects on microbial growth and protein stability *(1,2)*. Therefore, a_w, which is a measure of the energy status of the water in a system, can be influenced by several factors. Colligative effects of dissolved species (e.g., salt or sugar) interact with water through dipole–dipole, ionic, and hydrogen bonds *(1,2)*. Electrostatic interactions between charged groups on a protein surface are often modified by the presence of salts in the solution. Salt ions are highly mobile and compact units of charge, compared with the amino acid side chains and thus, compete effectively for charged sites on the protein. In this manner, electrostatic interactions among amino acid residues on the protein surface might be shielded by high concentrations of salts and these interactions impact protein stability.

The minimal water activity with glycerol or sodium chloride (NaCl) added to the medium was evaluated for various microorganisms *(3)*. For several microorganisms, glycerol is less inhibitory than NaCl, but in the case of *Staphylococcus aureus*, NaCl is less inhibitory than glycerol. *S. aureus* is known to be the most halo-tolerant nonhalophilic eubacterium that can grow at a_w-values as low as 0.86 (around 20% NaCl) *(4)* owing to its highly effective transport systems. With added sucrose or NaCl, microbial growth is affected not only by the reduction of water activity but also to specific molecular and/or ionic interactions.

The antimicrobial effects of brine solutions on the survival characteristics of nonspore forming bacteria at a_w from 0.90 (15% NaCl) to 0.75 (30% NaCl) were analyzed in other studies. Gram-positive bacteria were less sensitive to lowered a_w than Gram-negative bacteria, as observed: (1) there was a decrease of one log reduction of *Salmonella* sp. after 3 d at 0.75 a_w; (2) 0.14 $\log_{10}$ colony forming unit/g/d decrease at 20°C and 0.90 a_w in salami, and 4.40 $\log_{10}$ colony forming unit/g/d reduction in starch for *Escherichia coli* O157:H7, and, (3) 5 log-cycles reduction in 7 d at 0.91 a_w for *Listeria monocytogenes* cultivated in trypticase soy broth (TSB) yeast extract (YE) and TSB-YE supplemented with NaCl *(3)*.

In the presence of NaCl the thermal stability of thermolysin, a metalloproteinase produced by *Bacillus thermoproteolyticus*, was enhanced, dependent

on the salt concentration. The enzyme's catalytic activity also improved, which can be related to the electrostatic interaction between thermolysin and ions in the medium *(5)*. The activity was enhanced 13–15 times with 4 *M* NaCl (around 25–30% NaCl) at 25°C, pH 7.0 *(6)*. The thermal unfolding of lysozyme, α-chymotrypsinogen A, and yeast alcohol dehydrogenase in aqueous solution showed that water activity (a_w) determined the extent of change in the stability of these proteins and the solvent influenced the direction of the change *(7)*. The effect of unfolding was not evaluated for GFP in this study, but these studies show that solutes and a_w significantly influence protein stability.

The evaluation of GFP thermal stability in brine solutions of up to 30% NaCl allows us to study the behavior of this protein for use as a biosensor in the thermal processing of parenteral solutions (solutions from 0.9 to 20% NaCl or KCl), foods (pasteurization and blanching), and their disposal as effluents into waterways, as well as in the study of halophilic bacteria. GFP thermal stability is dependent on pH and temperature as well as the concentration of ions in the solvent system. The aim of this work was to determine the thermal stability of extracted, purified GFP by heating aqueous solutions with NaCl concentrations ranging from 0.9 (physiological saline) to 30% with a_w ranging from 0.75 to 1.00. This work evaluates the potential utility of GFP as a biosensor for moist-heat treatments, environmental diagnosis, and bioremediation purposes.

Materials and Methods

Green Fluorescent Protein

The expression of recombinant GFP by *E. coli* DH5-α, the extraction and purification of GFP have been outlined in previous experiments *(8–12)*. GFP fluorescence intensity was measured in a spectrofluorometer ($\lambda_{Excitation}$ = 394 nm, $\lambda_{Emission}$ = 509 nm) (RF 5301 PC; Shimadzu Corporation, Kyoto, Japan). Purified recombinant GFP (95% purity, Clontech Laboratories, Palo Alto, CA) was used to generate a standard curve to determine three-phase partitioning-extracted GFP concentration related to fluorescence intensity in Eq. 1:

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

Buffer Solutions

To study GFP pH- and thermal stability, solutions with 0.9 to 30% NaCl (w/v) were prepared in: (1) 10 m*M* sodium acetate/acetic acid buffer (pH 5.0), (2) 10 m*M* potassium phosphate buffer (monobasic/dibasic; pH 7.0), (3) 10 m*M* Tris-EDTA buffer (pH 8.0), and (4) water for injection ("WFI," from the Milli-Q system, Millipore®, Bedford, MA). Buffered solutions and WFI without NaCl were the controls for each system. A defined weight of NaCl (99.5% purity) was diluted in each buffer solution or WFI. After

complete dissolution, the solution was transferred to a 250-mL volumetric flask and the volume was adjusted. The solutions were filter-sterilized (Millipore 0.22 μm membrane), transferred to sterile flasks and stored at 4°C until use. To monitor contamination, 1 mL of each solution, before and after filtration, was plated (plate count agar) and incubated at 35–37°C for 24 h.

Sample Preparation for GFP Stability Determination

To each 4.9 mL of buffered solution or WFI at 25°C, 100 μL three-phase partitioning-extracted GFP (concentration around 400.0 μg/μL) was added to provide a final concentration of 8.0–10.0 μg/mL. GFP fluorescence intensity and pH were measured before heating, immediately after heating, and after storing samples for 24 h at 4°C. Immediately on the addition of GFP into the buffered or WFI solutions, the mixture was gently stirred for 30 s, placed into the cuvet and incubated at a constant temperature of 80, 85, 90, or 95°C. All fluorescence intensities and pH readings of the samples before and after heat treatment were recorded with the solutions at 25°C.

A 2-mL aliquot of sample was transferred to a quartz cuvet (1 cm light path length × 45 mm height) and sealed with a plastic cover. Each cuvet was inserted into an adapter assembly and adjusted in the cell holder. A constant temperature (±0.05°C) was maintained by continuous circulation of water from the water bath to the cell holder and the sample in the cuvet through a circulation pump (Thermo-bath TB-85, P/N 200-65022, Shimadzu Corporation). The moment the sample-filled cuvet was placed in the cell holder and the treatment was initiated, fluorescence readings were recorded at intervals of 5 s with the samples incubated at a constant 80, 85, 90, or 95°C for 1.5 h. With all samples starting at 25°C before heating, the 2 mL sample volume attained the final assay temperatures of 80 or 85°C after 20–30 s, and attained a final 90 or 95°C in 10 s. All samples were tested in triplicate.

Analysis of the Kinetic Parameters

Thermal Treatment

The extent of protein denaturation was evaluated by measuring the loss of fluorescence intensity over time for GFP exposed at temperatures ranging from 80 to 95°C and converted to denatured GFP concentrations (μg/mL). The GFP fluorescence data provided curves that were considered first order models represented by $\mathrm{Log}_{10} I_f = \mathrm{Log}_{10} I_o - (1/D) \times t$ 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 temperature of either 80, 85, 90, or 95°C. The decimal reduction time, (D-value = k/2.303), the interval of time required to reduce one decimal logarithm of the initial fluorescence intensity of GFP at reference temperature, was determined from the negative reciprocal of the slopes of the regression lines, using the linear portions of the inactivation curves ($\log_{10}$ fluorescence

intensity GFP/mL vs incubation time at a constant temperature). The *z*-value may be related to the coefficient Q_{10} of the process by Eq. 2:

$$Q_{10} = \left(10^{10/z}\right) \tag{2}$$

Activation energy (E_a, kcal/mol), represents the energy present in a system, the energy necessary to destabilize a system and can be defined using the Arrhenius equation 3:

$$\mathrm{Log}_{10}k_1 = \mathrm{Log}_{10}k_2 - \left\{\left(E_a / 2.303 \times R\right)\left[(1/T) - (1/T)\right]\right\} \tag{3}$$

where T_1 and T_2 are the incubation temperatures in degrees Kelvin (K), R is the universal gas constant (1.987 cal × mol/K) and k is the inactivation rate constant.

Results and Discussion

The extent of protein denaturation was expressed as a percentage of the calculated *D*-value; the interval of time required for a 90% reduction, or one decimal logarithm of the initial fluorescence intensity of GFP. The denaturation of GFP in NaCl solutions, as measured by the loss of fluorescence intensity, was expressed in the decimal logarithm of the decrease in native GFP concentration vs the incubation time at a constant temperature. To estimate *D*-values at constant heating temperatures and pH, the range of native GFP concentrations evaluated was between 10.0 μg/mL for initial concentration (C_o) to 2.0 μg/mL for final concentrations (C_f), which corresponded to the linear portion of the inactivation curves ($\log_{10}$ fluorescence intensity GFP/mL vs incubation time at a constant temperature). Table 1 shows *D*-values obtained for GFP diluted in WFI, acetate, phosphate, and Tris-EDTA buffered solutions incubated at 80, 85, 90 and 95°C, respectively.

Evaluation of pH for GFP in Sodium Chloride Solutions

The pH of GFP in the NaCl solutions was measured at room temperature (25°C) with mean values: (1) pH 7.65 (±0.17) in Tris-EDTA buffer (pH 8.0); (2) pH 6.08 (±0.60) in phosphate buffer (pH 7.0); and (3) pH 4.84 (±0.12) in acetate buffer (pH 5.0). The mean pH for WFI was 5.94 (±0.60) (Table 1).

Loss of Fluorescence Intensity Before Heating GFP in Solution

The loss of initial GFP fluorescence intensity at 25°C immediately after diluting into the buffered or WFI solutions was dependent on the NaCl concentration and the composition of the solution. The maximum loss of fluorescence was observed for solutions with 30% NaCl in acetate buffer (94.5% loss in fluorescence intensity). Solutions prepared in Tris-EDTA buffer provided optimal conditions for GFP stability, with a maximum loss of 25% fluorescence intensity in solutions with up to 30% NaCl. In WFI or in phosphate buffer with up to 30% NaCl, a 70% drop in fluorescence intensity was

Table 1
D-Values for GFP in NaCl Solutions[a]

			pH		*D*-values (min)			
	NaCl (%)	[b]a_w	Initial	24 h	80°C	85°C	90°C	95°C
Tris-EDTA	0	0.99	7.89	8.07	24.88	8.7	5.02	3.82
	0.9	0.99	7.90	7.90	37.04	9.88	6.12	4.81
	5	0.98	7.73	7.79	40.00	11.61	6.54	4.83
	10	0.918	7.63	7.62	49.26	9.73	7.17	5.14
	15	0.885	7.54	7.54	65.79	14.71	6.75	5.12
	20	0.845	7.50	7.48	52.63	18.12	6.78	5.26
	25	0.795	7.51	7.49	46.95	15.22	7.96	5.45
	30	0.759	7.48	7.48	33.78	13.30	7.26	5.08
	Mean	–	7.65	7.67	–	–	6.94	5.10
	[c]SD	–	0.17	0.23	–	–	0.59	0.23
WFI	0	0.99	6.63	6.47	13.85	4.64	3.46	3.21
	0.9	0.99	6.99	6.72	20.12	8.29	5.42	4.55
	5	0.98	6.12	6.10	8.26	5.67	3.49	3.19
	10	0.918	5.86	5.75	5.74	4.34	2.46	5.48
	15	0.885	5.66	5.63	4.75	3.78	2.76	2.44
	20	0.845	5.53	5.54	4.21	3.56	2.59	2.3
	25	0.795	5.42	5.46	3.58	3.21	2.43	2.14
	30	0.759	5.30	5.40	3.44	2.91	2.32	2.37
	Mean	–	5.94	5.88	–	–	–	–
	SD	–	0.60	0.49	–	–	–	–
Phosphate	0	0.99	7.08	7.05	40.32	9.28	5.52	4.38
	0.9	0.99	6.77	6.81	35.34	10.34	6.15	4.78
	5	0.98	6.33	6.29	21.19	10.39	5.41	4.64
	10	0.918	6.01	6.01	14.25	9.46	5.55	4.82
	15	0.885	5.80	5.81	11.57	8.49	4.87	4.21
	20	0.845	5.6	5.68	8.79	7.45	5.13	4.14
	25	0.795	5.49	5.45	6.99	6.84	4.5	4.02
	30	0.759	5.43	5.38	6.10	6.51	4.49	4.03
	Mean	–	6.08	6.06	–	–	5.16	4.38
	SD	–	0.60	0.61	–	–	0.60	0.36
Acetate	0	0.99	5.05	5.05	4.08	3.12	2.42	2.68
	0.9	0.99	5.00	5.00	3.82	3.44	3.03	3.04
	5	0.98	4.89	4.87	3.72	2.90	2.52	2.11
	10	0.918	4.82	4.80	3.47	3.38	2.12	1.83
	15	0.885	4.80	4.78	3.08	2.50	2.51	2.17
	20	0.845	4.76	4.75	–	–	3.32	3.30
	25	0.795	4.75	4.74	–	–	–	–
	30	0.759	4.71	4.69	–	–	–	–
	Mean	–	4.84	4.83	–	–	–	–
	SD	–	0.12	0.13	–	–	–	–

[a]WFI, water for injection; acetate 10 m*M*; phosphate 10 m*M*; Tris-EDTA 10 m*M* buffers, at 80, 85, 90, and 95°C, respectively.
[b]a_w, water activity.
[c]SD, standard deviation at $p < 0.05$.

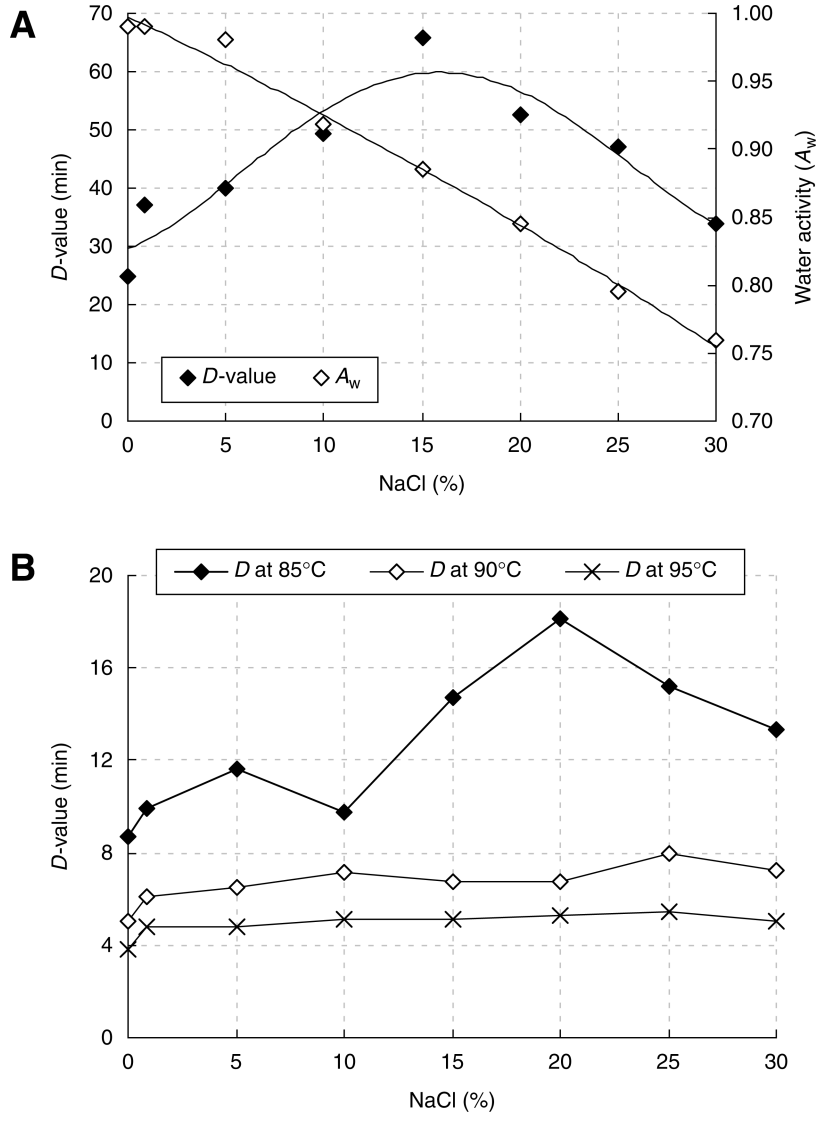

Fig. 1. *D*-value behavior for GFP in Tris-EDTA buffer 10 m*M* at: **(A)** 80°C (bell-shaped, -♦-) and **(B)** 85°C (-♦-), 90°C (-◊-), and 95°C (-×-).

observed. These results confirm that the presence of salts can affect GFP stability, altering either its structure or propensity for self-aggregation, which in these samples did not develop to any visible precipitation.

Thermal Stability of GFP in Tris-EDTA (pH 7.65 ± 0.17) Buffered Solutions

The pH of Tris-EDTA buffered solutions were relatively constant (pH 7.65 ± 0.17) and independent of the solute added (with up to 30% NaCl, Table 1). At 80°C, GFP showed increasing stability in NaCl solutions

(Fig. 1A) with the maximum *D*-value of 65.79 min with 15% NaCl, less than 2- to 2.5-fold greater than the control (*D* = 24.88 min), 0.9% NaCl (37.04 min) and 30% NaCl (33.78 min).

The *D*-value for GFP in Tris-EDTA/0.9% NaCl at 80°C (37.04 min) was comparable to phosphate buffer (pH 6.77 ± 0.01), 1.8-fold greater than in WFI (pH 6.99 ± 0.21) and 10-fold greater than in acetate buffer (pH 5.0; *D*-value = 3.82 min) at the same salt concentration (0.9% NaCl). At 85°C, ≥10% NaCl provided an equivalent thermal stability (mean *D*-value = 10.40 min) relative to the control (8.70 min). The highest *D*-value was attained for 20% NaCl (18.12 min). At 90°C and 95°C, GFP thermal stability was independent of the solute concentrations, but related to temperature, providing mean $D_{90°C}$ = 6.94 (±0.59) min and $D_{95°C}$ = 5.10 (±0.23) min, respectively for the same solution.

GFP thermal stability improved with the addition of 15–20% NaCl, wherein the highest *D*-value was observed at 80°C (65.79 min) and dropped 3.6-fold at 85°C (18.12 min). In contrast, the mean *D*-value decreased 9.5-fold at 90°C (mean 6.94 min) and 13-fold at 95°C (mean 5.10 min), highlighting the dependence at higher temperatures (Fig. 1B). At 90°C and 95°C, GFP showed the same thermal stability in phosphate and Tris-EDTA buffered solution, showing that GFP thermal stability was related to solution composition. In this case, buffer composition was not the main factor involved with thermal stability.

In our previous work, the presence of NaCl into solutions improved GFP thermal stability compared with glucose solutions made with the same buffers used in this work *(11)*. The buffered glucose/NaCl solutions at pH 7.0 provided the highest *D*-values ($D_{90°C}$ = 6.52 min and $D_{95°C}$ = 4.93 min), equivalent for glucose/Tris-EDTA buffered solution (pH 8.0; $D_{90°C}$ = 6.64 min and $D_{95°C}$ = 5.20 min), for the same concentration of glucose, proving that the addition of glucose and NaCl in the same solution affected GFP thermal stability favorably.

Stability of GFP in Phosphate (pH 6.08 ± 0.60) Buffered Solutions

In phosphate buffer at 80°C, the *D*-value for GFP in the control (40.32 min) dropped 14% in 0.9% NaCl, and half in 5% NaCl (21.19 min). The *D*-value varied linearly with the pH of the phosphate-buffered solutions, according to the Eq. 4:

$$D\text{-value} = 21.578 \times \text{pH} - 113.05 \quad (R^2 = 0.98) \tag{4}$$

For solutions with 0.9% NaCl in phosphate buffer (pH 6.77) and Tris-EDTA (pH 7.90), the *D*-values at 80°C were similar, 35.34 and 37.04 min, respectively. At 80°C, *D*-values changed exponentially with NaCl concentration, and were related by the Eq. 5:

$$\text{Log}_{10}\ D\text{-values} = -0.0271 \times [\text{NaCl}(\%)] + 1.5172 \ \left(R^2 = 0.95\right) \quad (5)$$

This exponential relation between *D*-value and NaCl concentration shows that for every interval of NaCl concentration, the *D*-value will change 10-fold. For example, the addition of 30% NaCl lowered the a_w from 1.00 to 0.759, which shows less available water at the GFP surface, promoting aggregation of the protein and the loss in fluorescence intensity from both aggregation and denaturation. At 85°C and 90°C, *D*-values related directly with NaCl concentration, by the Eqs. 6 and 7:

$$\text{Log}_{10}\ D\text{-values}\ (85°\text{C}) = -0.1617 \times [\text{NaCl}(\%)] + 11.02 \ \left(R^2 = 0.97\right) \quad (6)$$

$$\text{Log}_{10}\ D\text{-values}\ (90°\text{C}) = -0.0523 \times [\text{NaCl}(\%)] + 5.95 \ \left(R^2 = 0.94\right) \quad (7)$$

For every 5% increase in NaCl, the *D*-value dropped 10% at 85°C and 5% at 90°C. At 90°C and 95°C, the mean *D*-values were respectively 5.16 ± 0.60) min and 4.38 (±0.36) min, both independent of NaCl concentration and pH. The mean *D*-values at 90°C for GFP in phosphate-buffered solutions (pH = 6.08 ± 0.60) were comparable to the mean *D*-value (5.10 ± 0.23 min) in Tris-EDTA (pH 7.65 ± 0.17). In phosphate buffer (pH 6.08 ± 0.60), although pH decreased with increasing NaCl concentration, this buffer system favored GFP thermal stability compared with WFI solutions, whereby the drop in *D*-values were at least double at comparable pH (Fig. 2).

Stability of GFP in WFI (pH 5.88 ± 0.49)

The initial pH of the WFI solutions dropped with up to 30% NaCl, from pH 6.99 to 5.30, for a mean of pH 5.94 (±0.60), but was stable around a pH 5.88 (±0.49) after 24 h storage at 4°C. Similar changes in pH were observed in the phosphate-buffered solutions, reinforcing the observation that buffer composition was not the main factor responsible for the drop in pH with increasing NaCl concentration. The *D*-values for GFP at 80°C can be considered linearly dependent on the pH by the Eq. 8:

$$D\text{-values} = 9.6865 \times \text{pH} - 49.52 \ \left(R^2 = 0.95\right) \quad (8)$$

Through the analysis of the angular coefficients (θ), the dependence of WFI solutions on pH was observed to be twice lower than that determined (θ = 21.578, Eq. 2) for GFP in phosphate-buffered solutions with up to 30% NaCl. *D*-values were also shown to decrease exponentially with increasing NaCl of up to 30% in WFI, owing to the drop in pH in these solutions. The remarkable influence of pH on GPF thermal stability at 80°C can be represented by the Eq. 9:

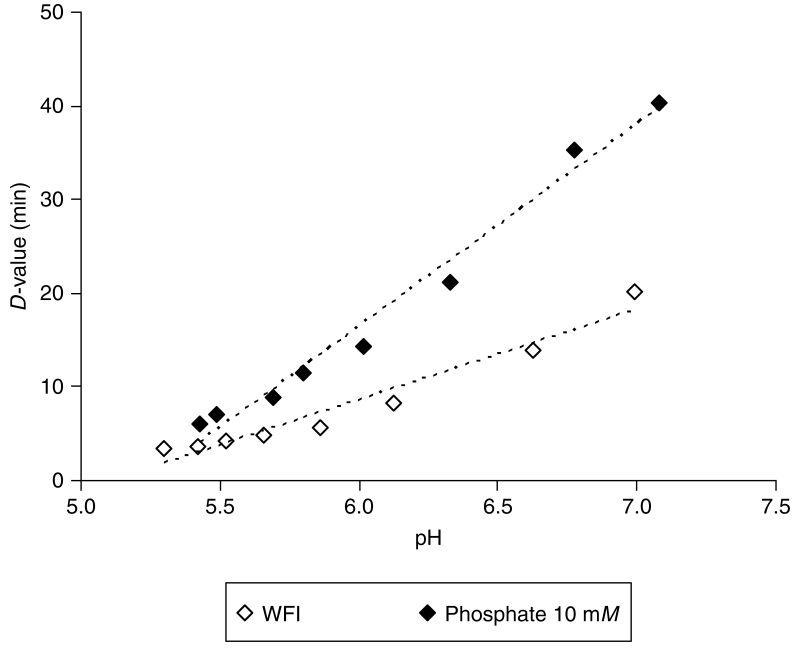

Fig. 2. *D*-value for GFP related to the pH solution in WFI (-◊-), (*D*-value is 9.6865 × pH –49.52, R^2 is 0.95) and phosphate 10 m*M* (-♦-), (*D*-value is 21.578 × pH –113.05, R^2 is 0.98), at 80°C.

$$\mathrm{Log}_{10} D = 0.147 \times \mathrm{pH} - 1.96 \left(R^2 = 0.997\right) \quad (9)$$

The *D*-value for the control (13.85 min) was close to 0.9% NaCl (20.12 min), fell by half with 5% NaCl (8.26 min) and fivefold in the presence of 25–30% NaCl in WFI ($\mathrm{mean}_{25–30\%}$ = 3.51 min; Table 1). At 85, 90, and 95°C, the presence of 0.9% NaCl in WFI favored GFP thermal stability, with the *D*-values of 8.29, 5.42, and 4.55 min, respectively. The *D*-values for GFP for concentrations ≥10% of NaCl were comparable to the control.

Stability of GFP in Acetate-Buffered Solutions (pH 4.84 ± 0.12)

In acetate-buffered solutions the mean pH of 4.84 ± 0.12 was close to the p*I* for GFP (p*I* = 4.9-5.1) and remarkably, lowered GFP thermal stability independent of either NaCl concentration or temperature. In this case, the addition of solutes did not influence GFP thermal stability compared with the control for every temperature studied, up to 15% NaCl between 80–85°C and up to 20% NaCl between 90–95°C. There was a slight tendency for *D*-values to drop at 80–85°C from the control and up to 15% NaCl (at 80°C, D_{control} = 4.08 min; $D_{15\%}$ = 3.08 min and at 85°C, D_{control} = 3.12 min; $D_{15\%}$ = 2.5 min). However, between 90 and 95°C, a drop in *D*-values from 0.9% NaCl (3.03 and 3.04 min, respectively) to 10% NaCl (2.12 and 1.83 min) increased to a mean *D* = 3.3 min in up to 20% NaCl, corresponding to an a_w = 0.845 (Fig. 3).

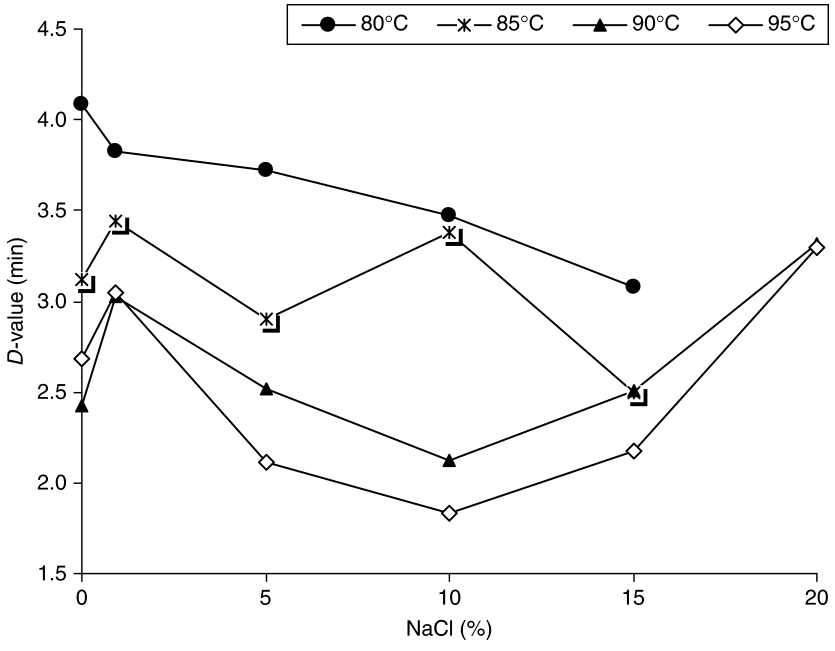

Fig. 3. *D*-value behavior for GFP in acetate buffer 10 m*M* at 80°C (-●-), 85°C (-⁎-), 90°C (-▲-), and 95°C (-◊-).

Kinetic Parameters z-Value, Q_{10} Coefficients, and Activation Energy

The kinetic parameters, *z*-value and the related Q_{10} coefficient ($Q_{10} = 10^{10/z}$), reflect the temperature dependence of GFP denaturation (as measured by fluorescence intensity decrease) subjected to the heating in solutions of different composition (Fig. 4A). The greater the *z*-value, the more stable GFP is in the system.

z-Value

For the range of temperatures from 80 to 95°C for the controls, solutions without NaCl, the *z*-values varied from: (1) z = 24.63°C in WFI; (2) z = 75.76°C in acetate buffer; (3) z = 16.03°C in phosphate buffer, and (4) z = 18.66°C in Tris-EDTA. For the same range of temperatures and the interval of NaCl concentrations, between 0.9 and 30% NaCl, *z*-values varied from: (1) z = 23.58–85.47°C for solutions in WFI; (2) z = 142.86–109.89°C in acetate buffer; (3) z = 17.67–71.43°C in phosphate buffer, and (4) z = 17.45–18.32°C in Tris-EDTA solutions.

z-Values of the systems delineated GFP behavior in the temperature interval from 80 to 95°C. Tris-EDTA influenced the system the least with the addition of NaCl, with *z*-value (mean = 16.16 ± 1.6°C) unchanged with up to 30% NaCl. WFI and phosphate-buffered systems exhibited the positive influence of NaCl on GFP thermal stability. The *z*-values were linearly

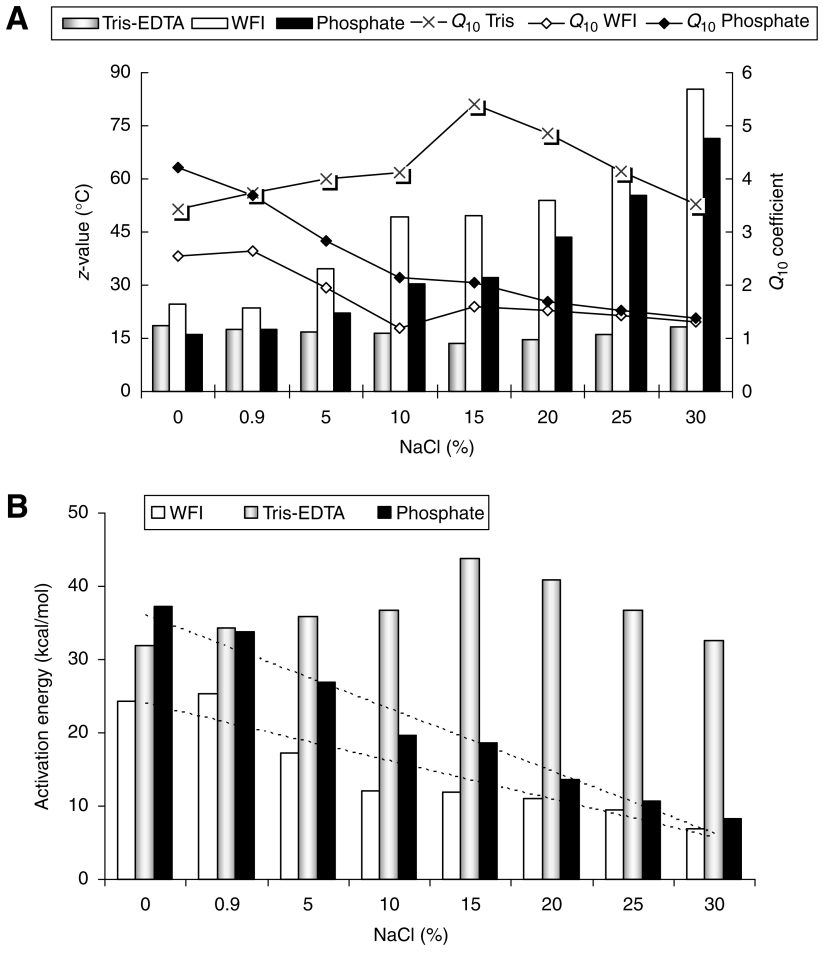

Fig. 4. Kinetic parameters for GFP in WFI, phosphate buffer 10 m*M*, and Tris-EDTA buffer 10 m*M* systems at the interval from 80 to 95°C: **(A)** *z*-value (°C) and **(B)** E_a (kcal/mol) and Q_{10} coefficient.

dependent with increasing NaCl concentration in WFI and phosphate-buffered solutions by the relations: (1) *z*-value (°C) = 1.7962 × (NaCl, %) = 24.288, $R^2 = 0.93$ and (2) *z*-value (°C) = 1.7028 × (NaCl, %) + 13.535, $R^2 = 0.95$, respectively.

Q_{10}-Coefficient

For the range of temperatures from 80 to 95°C, the GFP Q_{10} *coefficients* shifted from: $Q_{10} = 2.55$, $Q_{10} = 1.36$, $Q_{10} = 4.21$, and $Q_{10} = 3.44$ in WFI, acetate, phosphate, and Tris-EDTA buffered control solutions, respectively. With the addition of NaCl and GFP, acetate-buffered solutions exhibited less influence from external conditions, a stable system with Q_{10} varying from 1.36 to 1.23 for the interval up to 15% of NaCl. The Tris-EDTA buffered system provided optimal conditions for GFP stability for the range of

increasing temperatures with Q_{10} coefficients ranging from 3.44 to 5.41, for the control and 15% NaCl. Comparing *z*-values, acetate buffer provided the most uniform system for GFP ($Q_{10} < 1.5$), as well as WFI systems with more than 10% NaCl, and phosphate-buffered solutions with more than 20% NaCl. WFI and phosphate buffer (pH 7.0) up to 5% and between 5 and 15% NaCl respectively, provided intermediate stability ($1.5 < Q_{10} < 3.5$). Tris-EDTA buffered solutions (pH 8.0; $3.5 < Q_{10} < 6.0$) showed that GFP can be less affected by external influences.

Activation Energy

The parameter E_a is related to the intrinsic energy of the system and is correlated with the stability of the system during heating (Fig. 4B). Therefore, stable systems have lower energy than unstable ones. Consequently, systems that exhibit small E_a are less influenced by temperature, as shown in Table 2. Differences in the kinetics of heat activation of GFP result from different solution composition. It is well-known that the water content in the media is an important parameter for the activity of enzymes, and enzyme activity is better quantified in terms of water activity. Water is believed to increase the internal flexibility of the enzyme and thereby increase catalytic activity. a_w is important for protein stability because water is involved in many of the mechanisms influencing protein denaturation *(13)*.

Between 80°C and 95°C, GFP thermal stability (E_a) was highest in acetate-buffered solutions (pH 5.0) owing to aggregation of the protein, promoted by the low pH close to the p*I*. At pH 4.0–5.0, sodium acetate is totally dissociated, the ions compete for water molecules, which have preferential interaction with the ions in the solution, promoting a tendency for GFP to aggregate, which enhanced GFP thermal stability. The acetate-buffered system with the lowest intrinsic energy for spontaneous transformation was independent of either the temperature or NaCl concentrations. Citric and phosphoric acids are commonly used in processed foods to control pH. Sodium chloride can have a huge effect on the environmental pH because of the changes in the ionized state of the food product *(14)*. The addition of sucrose can change the pK_a of the buffer solution, shifting the buffer equilibrium. Citrate buffer was shown to be more resistant to pH changes from added polyols than phosphate buffer *(15)*.

The thermal inactivation of polyphenoloxidase in pineapple puree when heated from 70°C to 90°C, with *D*-values varying from 91.3 and 11.4 min, corresponded to a *z*-value of 21.5°C and E_a of 82.8 kJ/mol (1 cal = 4.18 J; 19.81 kcal/mol) *(16)*. GFP showed greater stability than polyphenoloxidase in the systems composed with water (>5% NaCl), or with phosphate (>10% NaCl) and acetate-buffered solutions (up to 30% NaCl). The E_a for thermolysin was observed to increase up to 30–33 kcal/mol by addition of 0.5–1.5 *M* NaCl (5–15% NaCl). Further increases in NaCl concentration were verified to decrease the E_a to 15 kcal/mol in 4 *M* NaCl (around 25%

Table 2
z-Values, Q_{10} Coefficient, and E_a of Thermal Stability for GFP in NaCl Systems[a]

	Tris-EDTA			WFI			Acetate			Phosphate		
Nacl (%)	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}
0.0	31.97	18.66	3.44	24.31	24.63	2.55	7.87	`75.76	1.36	37.24	16.03	4.21
0.9	34.23	17.45	3.74	25.32	23.58	2.65	4.2	142.86	1.17	33.79	17.67	3.68
5.0	35.84	16.64	3.99	17.28	34.48	1.95	9.52	62.5	1.45	26.97	22.12	2.83
10.0	36.77	16.26	4.12	12.15	49.26	1.2	12.3	48.31	1.61	19.58	30.4	2.13
15.0	43.74	13.64	5.41	11.96	49.75	1.59	5.41	109.89	1.23	18.55	32.05	2.05
20.0	40.86	14.58	4.85	11.01	54.05	1.53	–	–	–	13.58	43.67	1.69
25.0	36.81	16.21	4.14	9.40	63.29	1.44	–	–	–	10.72	55.25	1.52
30.0	32.55	18.32	3.52	6.97	85.47	1.31	–	–	–	8.31	71.43	1.38

WFI (pH 6.0–7.0); 10 m*M* acetate buffer; 10 m*M* phosphate buffer; 10 m*M* Tris-EDTA buffer.

NaCl). In this study, it was observed that the E_a value for GFP also decreased in the presence of increasing concentrations of up to 30% NaCl in WFI, acetate, and phosphate-buffered systems. However, for GFP in Tris-EDTA-buffered system with 30% NaCl, the E_a value (32.55 kcal/mol) was almost the same as that measured for GFP in the control solution, without NaCl (E_a = 31.97 kcal/mol), similar to behavior observed for thermolysin in no salt and 4 *M* NaCl, at pH 7.0 *(6)*.

The stability of scallop transglutaminases (TGase) in the presence of neutral salts (around pH 7.0) showed that NaCl was an effective enhancer of TGase activity, which occurs instantly and reversibly, suggesting small conformational changes of the TGase *(17)*. In this and prior results, GFP did not show fluorescence recovery (renaturation) in all conditions studied. The solubility of lysozyme in aqueous NaCl solutions (pH 4.5 and 7.0), in aqueous sodium acetate (pH 8.3), and in aqueous magnesium chloride solutions (pH 4.1), was observed and related with a preferential solvation of protein in a binary aqueous solution and its solubility *(18)*. Considering the systems wherein GFP thermal stability was evaluated, the activation energies (E_a) are as follows (Table 2):

1. In WFI, the E_a of 24.31 kcal/mol for GFP in the control increased to 25.32 kcal/mol with 0.9% NaCl, decreasing up to 6.97 kcal/mol with 30% NaCl.
2. In acetate buffer (pH 5.0), the smallest E_a value of 4.20 kcal/mol was observed for GFP in 0.9% NaCl solutions, lower than 7.87 kcal/mol for the control, increasing to 12.30 kcal/mol with 10% NaCl.
3. In phosphate buffer (pH 7.0), the E_a range decreased from 33.79 to 8.31 kcal/mol for NaCl concentrations between 0.9 and 30%, showing that the addition of 30% NaCl into solution reduced E_a up to 4.5-fold compared with the control solution, E_a = 37.24 kcal/mol.
4. In Tris-EDTA buffered solutions, the intrinsic energy and change in GFP stability was higher than in WFI, with E_a of 31.97 kcal/mol (control), increasing to 34.23 kcal/mol and attaining the maximum value (E_a = 43.74 kcal/mol) in 15% NaCl, equivalent to results for GFP in 5% glucose solutions (E_a = 43.81 kcal/mol) obtained in prior work (*11*), dropping to 32.55 kcal/mol (30% NaCl), at the interval ranging from 0.9 to 30% NaCl.

As observed in previous work, GFP thermal stability, in terms of *D*-value, was dependent on the pH of the solutions with: *D*-values in acetate buffer < WFI < phosphate buffer < Tris-EDTA buffer and NaCl concentration. Between 80°C and 95°C, the amount of activation energy to destabilize the molecule was dependent on the buffer pH: E_a in acetate buffer < phosphate buffer < WFI < Tris-EDTA. From pH 7.0 to 8.0, GFP is in its native conformation, unfolding in these systems at 80–95°C requires greater energy than that required in WFI. NaCl added to phosphate-buffered solutions and WFI exhibited equal changes in pH, but these

systems influenced GFP thermal stability differently. In acetate buffer (pH 5.0) GFP has a tendency to aggregate, the pH of the solutions is close to the p*I* for the protein, between 4.9 and 5.1. Aggregation promotes an increase in the thermal stability of the protein in this system. The presence of NaCl associated to the ions of the buffer systems provided a positive influence on the stability of the molecule for the systems and range of temperatures studied.

Conclusion

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, and in disinfection and pasteurization processes. GFP was shown to be an efficient marker in a system with up to 30% NaCl when exposed to the typical temperature range used for decontamination processing of solutions with added NaCl. This indicates the high stability of GFP under these conditions, dependent on the processing temperature, as observed in the evaluation of the *z*-value, Q_{10} coefficient, and free energy of these systems.

Acknowledgments

We thank our personal assistants for providing technical support Irene A. Machoshvili and Ricardo Alves Silva. This study was made possible by financial support provided by the Brazilian Committees for Scientific Technology Research (Conselho Nacional de Pesquisa e Desenvolvimento (CNPq), and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

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