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Stability of Recombinant Green Fluorescent Protein (GFPuv) in Glucose Solutions at Different Concentrations and pH Values

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Abstract

The stability at room temperature (25°C) of recombinant green fluorescent protein (GFPuv), expressed by *Escherichia coli* cells and isolated by three-phase partitioning extraction with hydrophobic interaction column, was studied. The GFPuv was diluted in buffered (each 10 m*M*: Tris-HCl, pH 8.0; phosphate, pH 6.0 and 7.0 and acetate, pH 5.0) and in unbuffered (water for injection [WFI]; pH 6.70 ± 0.40) glucose solutions (from 1.5 to 50%). By assaying the loss of fluorescence intensity as a measure of denaturation, the stability of GFPuv in these solutions was evaluated relative to glucose concentration, pH, osmolarity, density, conductivity, and viscosity. The extent of protein denaturation (loss of fluorescence intensity) was expressed in decimal reduction time (*D*-value), the time required to reduce 90% of the initial fluorescence intensity of GFPuv. The *D*-value between 56 and 83 h of GFPuv at 1.5–15% glucose in WFI was equivalent to 20–30% glucose in a phosphate. The stability of GFPuv in 50% glucose was similar for all buffers studied and four times higher than in WFI. By the convenient measure of fluorescence intensity, GFPuv can be used as an indicator to report the extent of denaturation rates of other proteins in glucose solutions.

Index Entries: Protein stability; decimal reduction time; green fluorescent protein; glucose solution; denaturation; conductivity.

Introduction

Protein stability refers to the maintenance of structural integrity and biologic function during changes in environmental conditions (pH and temperature) or exposure to other agents, such as organic solvents, salts, and inert polymers. Because of their unique functional attributes, proteins can be used as an indicator of environmental conditions during processing or storage.

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Among the functional properties of proteins, solubility is of primary importance owing to its significant influence on other functional properties of proteins. The hydrodynamic properties of proteins influence viscosity and aggregation. The solubility of a protein relates to surface hydrophobic (protein-protein) and hydrophilic (protein-solvent) interactions with water. The relative proportion of surface hydrophobic and hydrophilic groups dictates the degree of solvation by water. Therefore, a given set of solution conditions at equilibrium between the hydrophilic and hydrophobic interactions can be expressed as protein-solvent = protein-protein + solvent-solvent.

The solubility of proteins, under defined conditions, has been recognized as a crucial limitation in their use as a biologic reference substance in either drug or food processing, or as a biologic indicator to provide a detectable signal to deleterious environmental changes in the system *(1)*.

In general, to minimize the free energy of stabilization thermodynamically, the hydrophilic amino acids are oriented to the outside of the molecule and the hydrophobic amino acids residues are buried in the interior of the molecule. Thus, the proportion and distribution of surface hydrophobic pockets are the main factors in determining the degree of solubility of protein, rather than total hydrophobicity and charge density based on amino acid composition.

The surface characteristics of a protein are greatly affected by environmental conditions that influence the interrelations among physical factors, such as pH, ionic strength, viscosity, temperature, solvent component, and other components. Protein conformation is dictated by the environment for solvation *(2–4)*.

Carbohydrates, such as sucrose, glucose, mannose, and trehalose, and polyhydric alcohols (glycerol, sorbitol, and mannitol), have frequently been used to enhance the solubility of proteins *(5)*. Because little effect on a protein structure and activity is usually seen in the presence of high concentrations of polyols, especially carbohydrates, the excellent biocompatibility of these compounds makes them an acceptable choice for general use *(1)*.

Low molecular weight sugars have been the most abundant additives used in a variety of different systems, and for many years as stabilizing agents for the maintenance of the biologic activity of macromolecules *(6,7)*. The carbohydrate selected and the concentration are of fundamental importance to the quality of products through their effect on properties in an aqueous phase and on proteins. Sugars have different effects on protein interactions: inter- and intramolecular protein-protein, protein-sugar-protein, and small molecule–protein surfactants.

Osmolytes are small organic solutes such as sugars, methylamines, and amino acids that are found in certain osmotically stressed and freeze-resistant organisms, as well as in the medulla of the mammalian kidney. These compounds are known to inhibit the large-scale conformational changes

associated with protein denaturation. Osmolytes stabilize the native state because they are preferentially excluded from the protein's surface. Preferential exclusion increases the chemical potential of the protein, in proportion to its solvent-exposed surface area. Osmolytes favor a more compact native state over the structurally expanded denatured state *(8).* Osmolytes are uncharged and affect solvent viscosity, surface tension *(9)*, and thermodynamic affinity of protein molecules for the solvent *(10)*.

The interactions of protein molecules (1) with each other, (2) with water molecules, (3) with dissolved ions, and (4) with other high and low molecular weight components in a system form the basis for a variety of their properties, such as the stability of a protein in solution at room temperature *(11).*

Glucose-based parenteral solutions are a major nutritive source and drug delivery system. Dextrose (5%) is the most frequently used iv fluid, either for nutrition or for fluid replacement. The pH range of dextrose solution (5%) can vary from 3.5 to 6.5. The wide range is owing to free sugar acids present and formed during processing and storage *(12–16)*.

The functional attributes of a particular product after processing and storage can be monitored using the structure, environment, and thermal history of the appropriate detector or indicator protein (biologic indicator [BI]).

Green fluorescent protein (GFPuv) is an attractive marker system to monitor bacterial cells in the environment, and its detection does not require any exogenous substrate, complex medium, or expensive equipment *(17)*. It can be used as a reliable BI easily detected and quantified to determine the effectiveness of a thermal process. GFPuv is a 27 to 29-kDa protein that provides a unique environment for three residues in its primary sequence to act as a fluorophore. The protein must be in the proper conformation to provide fluorescence—but once properly formed, it requires no further modification or cofactor to emit light. GFPuv is a thermal stable molecule *(18)* in a broad range of pH from 5.0 to 12.0. When conformational stability limits are exceeded, GFPuv loses its fluorescence. In its natural source, the light emitted from the interaction of apoaequorin with coelenterazine (bioluminescence) provides the excitation light for GFPuv. To emit light, GFPuv undergoes no conformational change, neither an enzymatic modification nor ligand binding—it only needs to be exposed to light of the appropriate wavelengths *(19)*. With the excitation maxima at 395 and 509 nm for emission, GFPuv can be quantified *in situ* using a variety of techniques such as fluorescence microscopy, flow cytometry, and spectrofluorometry.

Optimization of process design and operation used in manufacturing food and drugs requires a thorough understanding of the influence on these processing conditions and formulation on the detector protein's properties. Understanding the molecular basis of protein function under a variety of conditions is challenging because of the compositional, structural, and dynamic complexity of various drug and food products and because of the

wide range of physical and mechanical stress that materials undergo during processing, storage, and use *(20)*.

The objective of the present work was to study the stability of GFPuv in aqueous glucose solutions at different concentrations and varying pH values, at room temperature, before assaying its thermal stability in the same solutions. This study is part of ongoing studies to evaluate its potential use as a bioindicator in the pasteurization process. The effect of glucose on the stability of GFPuv is related to the extent of the exclusion from or binding to the two end states of the protein. The thermodynamic stability of the system will be measured by the decrease in fluorescence intensity denoting unfolding to a fully denatured nonfluorescent form.

Materials and Methods

Expression of GFPuv

Escherichia coli DH5-α cells transformed (pGFPuv; Clontech, Palo Alto, CA), by the standard calcium chloride method *(21)* to express the recombinant GFPuv (excitation/emission maxima at 394/509 nm) were grown (37°C at 100 rpm for 24 h) in Luria-Bertani (LB) broth supplemented with 100 μg/mL of ampicillin (amp). An aliquot of this culture was transferred onto the surface of LB-amp agar with isopropyl-β-D-thiogalactopyranoside (IPTG) added to a final concentration of 0.5 m*M* and incubated at 37°C. After 24 h, the agar surface was illuminated with a handheld ultraviolet lamp at 394 nm (Model UVL 4; UVP, Upland, CA), and isolated, brightly fluorescent colonies were picked and transferred into 25 mL of LB-amp broth ("preinoculum") and incubated (37°C at 100 rpm) up to $OD_{660} = 0.01$ (10^4 CFU/mL). A 1-mL aliquot of this preinoculum was transferred into 25 mL of LB-amp broth, and the 250-mL flasks were shaken (37°C at 100 rpm) until the broth cultures attained $OD_{660} = 0.1$ (10^7 CFU/mL). IPTG was then added to a final concentration of 0.5 m*M*. After 24 h (37°C at 100 rpm), the expression of GFPuv by induced cells was detected by illuminating the broth at 394 nm and visually inspecting for intense green fluorescence. Cells were harvested by centrifugation (4°C at 6000*g* for 30 min). The supernatant was decanted and the cell pellet was resuspended in 1 mL of chilled (4°C) extraction buffer (XE: 25 m*M* Tris-HCl, pH 8.0; 1 m*M* β-mercaptoethanol; 0.1 m*M* phenylmethylsulfonyl fluoride,) prior to subjecting the cells to the three-phase partitioning (TPP) extraction method.

TPP Extraction Method (22) and Hydrophobic Interaction Column Purification (23)

In a 15-mL centrifuge tube, to each aliquot of 4 mL of resuspended cells, 3 mL of 4 *M* $(NH_4)_2SO_4$ and 7 mL of *t*-butanol were added. The mixture was stirred for 8 min at room temperature, centrifuged, and the three phases formed were collected separately. The *t*-butanol upper phase and the white interfacial precipitate were removed and discarded. A second

aliquot of 7 mL of *t*-butanol was mixed into the lower aqueous phase. The mixture was allowed to settle to a visible phase separation and centrifuged. At the separation of the three phases, the upper phase was discarded. The interfacial green phase was collected and dissolved in 1 mL of XE. While the lower phase was still fluorescent, it was subjected to repeated TPP extraction. An aliquot of protein extract (about 50 mL) was mixed with an equal volume of 4 *M* $(NH_4)_2SO_4$, and this mixture was transferred to the top of a butyl support hydrophobic interaction column (HIC), fast flow, coupled to a peristaltic pump for final purification. The HIC was previously equilibrated with 2 *M* $(NH_4)_2SO_4$. The loaded column was first washed with 20 mL of 1.3 *M* $(NH_4)_2SO_4$ to elute proteins that bind with low affinity to the butylated resin. GFPuv was eluted with 50 mL of buffer solution (10 m*M* Tris-HCl; 10 m*M* EDTA, pH 8.0) and stored at 4°C.

Buffer Solutions

To study the stability of GFPuv, buffers at various pH ranges and water were prepared: (1) 10 m*M* sodium acetate/acetic acid (pH 5.0), (2) 10 m*M* potassium phosphate (monobasic/dibasic; pH 6.0 and 7.0), (3) 10 m*M* Tris-HCl (pH 8.0), and (4) water for injection (WFI)(pH 7.0 ± 0.3) (from the Milli-Q system; Millipore®, Bedford, MA).

Glucose Solutions

Glucose solutions (99.7% purified) were prepared at concentrations of 1.5, 5, 7, 10, 15, 20, 25, 30, 35, 40, 45, and 50% (w/v). A defined weight of glucose was diluted in 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 45-µm membrane), transferred to sterilized flasks, and stored at 4°C until used. To monitor contamination, 1 mL of solutions 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.

Determination of Physical Properties of Glucose Solutions

pH and Conductivity

pH and conductivity were measured (Accumet pH meter AR20, pH/mV/conductivity meter; Fisher, Fairview, NJ) at 25°C. pH was measured immediately after solution preparation and 24 h later (solution stored at 4°C). Conductivity was measured after 24 h of solution preparation. Standard buffers at pH 4.0, 7.0, and 9.0 were used to calibrate the pH meter. The conductivity meter was calibrated with a solution of potassium chloride (containing silver chloride) at 146.9-µS conductivity at 25°C.

Osmolarity

The osmolarity of glucose solutions was determined at 25°C after 24 h of solution preparation with an osmometer (Osmomat 030; Gonotec,

Berlin, Germany). The osmolarity of 50 µL of each solution was determined by comparing the freezing point relative to the freezing point of pure water. Pure water has a freezing point of 0°C; a solution with a salt concentration of 1 osmol/kg has a freezing point of –1.858°C. An NaCl solution of 300 osmol/kg of H_2O (9.463 g of NaCl/kg of H_2O) was used to calibrate the equipment.

Viscosity

The viscosity of glucose solutions was determined after 24 h of solution preparation using a viscometer (REOTHEST® LK 2.2 capillary viscometer; Ottendorf-Okrilla, Dresden, Germany). The capillary was numbered 1/2C, which denotes that capillary 1 is calibrated with two constants, and adjusted to measure the viscosity range between 1 and 20 mPa · s (mPa · s = 1 cp). The viscometer executed five readings for each 25-mL sample volume and showed the average of these values converted to 25°C. The conversion was calculated from the coefficients of temperature (TK) by the following equation (*24*):

$$TK = [\ln(\eta_{-\Delta T}) - \ln(\eta_{+\Delta T})]/(2 \times \Delta T)$$

in which at $T_{-\Delta T}$ = 20°C, the viscosity was $\eta_{-\Delta T}$ = 1.002 cp; at $T_{\Delta T}$ = 25°C, the viscosity was $\eta_{-\Delta T}$ = 0.8904 cp; at $T_{+\Delta T}$ = 30°C, the viscosity was $\eta_{+\Delta T}$ = 0.7975 cp; ΔT = temperature gradient in relation to the reference temperature of 25°C, ΔT = (30–25)°C = (25–20)°C = 5°C. By replacing the viscosity values in the equation, $TK = [\ln(1.002) - \ln(0.7975)]/(2 \times 5.0)$, TK = 0.02283.

Density

Densities of glucose solutions were determined in a pycnometer with a bulb capacity of 50 mL at 25°C (±0.1°C). The density of each glucose solution (ρ) was calculated by the following equation:

$$\rho = (m_2 - m_1)/V$$

in which V is the 50-mL volume of the pycnometer; m_1 is the full weight of the dry, clean pycnometer (including the stopper); and m_2 is the weight of the pycnometer plus the glucose solution.

Fluorescence Intensity of GFPuv

The fluorescence intensity of GFPuv was measured in a spectrofluorometer (excitation = 394 nm, emission = 509 nm; RF 5301 PC; Shimadzu, Kyoto, Japan). Purified recombinant GFPuv (>95% purified, Clontech) was used to generate a standard curve to determine TPP-extracted GFPuv concentration and provide an experimental comparison for stability. The fluorescence intensity of the TPP-extracted GFPuv samples was compared with the standard calibration curve: fluorescence intensity *(I)* = 134.64 + 103.61 × (GFPuv µg/mL); (R^2 = 0.985).

Sample Preparation for Determination of GFP Stability

To each 4.9 mL of buffered glucose solution, 100 µL of TPP-extracted GFPuv (at an initial concentration of about 400 µg of GFPuv/mL) was mixed to provide a final concentration of about 8 µg/mL. GFPuv fluorescence intensity and solution pH were measured immediately and 24 h later.

Samples of TPP-extracted GFPuv were diluted in different concentrations of glucose in pH buffers ranging from pH 5.0 ± 0.2 to 8.0 ± 0.2: (1) 10 m*M* acetate (pH 5.0), (2) 10 m*M* phosphate (pH 6.0 and 7.0), and (3) 10 m*M* Tris-HCl (pH 8.0) before exposure to 25°C.

One aliquot sample of 2 mL was transferred to a quartz cuvet ([10 × 10 mm] 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 provided by continuous circulation of water from the water bath to the cell holder and the sample in the cuvet via a circulation pump (Thermo-bath TB-85, P/N 200-65022; Shimadzu). The moment the sample-filled cuvet was placed in the cell holder and the treatment was initiated, fluorescence readings were taken at intervals of 60 s at 25°C during a period of 3 h.

Analysis of Kinetic Parameters

The stability of GFPuv curves was considered first-order models represented by

$$\mathrm{Log}_{10}\, I_f = \mathrm{Log}_{10}\, I_0 - (1/D) \times t = \mathrm{Log}_{10}\, C_0 - (k/2.303) \times t$$

in which I_0 is the initial fluorescence intensity of native GFPuv, and I_f is the final fluorescence intensity of the remaining native GFPuv, after the exposure time (t, h at 25°C), D-value, decimal reduction time (D-value, h), and inactivation rate constant (k, h^{-1}).

The decimal reduction time (D-value = 2.303/k), the interval of time required to reduce one decimal logarithm of the initial fluorescence intensity of GFPuv at reference temperature, was determined from the negative reciprocal of the slopes of the regression lines, using the linear portions of the inactivation curves ($\log_{10}$ µg of GFPuv/mL vs time of exposure at a constant temperature).

The energy of activation (E_a = kcal/mol) represents the energy present in a system. The E_a energy necessary for the destabilization of a system, can be defined for a given temperature of treatment by the relation

$$\mathrm{Log}_{10}\, k = [E_a/(2.303 \times R \times T)] + C$$

in which k is the inactivation rate constant (h^{-1}), R is the gas constant = 1.987 cal/(mol · K), and T is the absolute temperature (K).

Gibbs free energy, ΔG (chemical potential of a substance), is an intrinsic property of the system, a comparison of the changes of enthalpy (ΔH) and entropy (ΔS) before and after changes in the system, which shows the

tendency of the measured transformation (decreases, $\Delta G < 0$; increases, $\Delta G > 0$; or does not change, $\Delta G = 0$) in a process at a constant temperature and pressure.

The tendency of the free energy is a spontaneous decrease in the transformation toward the equilibrium when $\Delta G = 0$ and can be represented by the equation:

$$\Delta G = \Delta H - T \times \Delta S \quad \text{and} \quad \Delta G = R \times T \times \ln(k \times h/B \times T)$$

in which k is the Boltzmann's constant = 1.38×10^{-23} J/K = 5.77×10^{-23} cal/mol; h is the Planck's constant = 3.987×10^{-32} J/min = 16.68×10^{-32} cal/mol; R is the gas constant = 1.987 cal/(mol · K); and T is the absolute temperature (K).

A decrease in the Gibbs free energy (G) associated with a change in state is equal to the maximum quantity of work that can be obtained in the transformation. When $\Delta G < 0$, the transformation can occur spontaneously; when $\Delta G = 0$, the system is in equilibrium relative to this transformation; and when $\Delta G > 0$, the reaction or transformation is not spontaneous. The term *spontaneous* only denotes that the reaction may be possible. In a natural transformation, the temperature and the pressure are constant; ΔG is negative. When ΔG is negative, ΔH is negative, and ΔS is positive, the transformation is exothermic. In a natural and spontaneous transformation, the system adjusts to attain minimum enthalpy (minimum internal energy) and maximum entropy (maximum disorder). The enthalpy was calculated by $\Delta H = \Delta E_a - R \times T$.

Entropy is a measure of disorder of the system. Disorder occurs spontaneously and thus entropy tends to increase. If the number of molecules is unchanged by the transformation, a small change in entropy (positive or negative) is expected. Entropy was calculated by $\Delta S = (\Delta H - \Delta G)/T$.

Results and Discussion

By assaying the loss of fluorescence intensity as a measure of denaturation, the stability of extracted recombinant GFPuv in buffered glucose solutions at various pHs, osmolarities, conductivities, densities, and viscosities was evaluated. Before studying GFPuv as a BI for low-temperature sterilization processes, the influence of glucose on GFPuv denaturation was examined in various buffer solutions and in water, to determine the potential application of GFPuv as an indicator to report the extent of denaturation of other proteins in glucose solutions by the convenient measure of GFPuv fluorescence.

Glucose solutions from 1.5 (0.08 *M*) to 50% (2.78 *M*) are commonly used in health care *(14)*. The preparation of these solutions in WFI (pH 6.0 to 7.0; 1.84 µs/cm; 0.95 g/cm^3; 1.76 mPa · s) exhibited osmolarity ranging from 0.06 to ≥2.28 mØsmol/kg (≥30% [w/v]), conductivity from 1760 to 314 µs/cm, density from 0.937 to 1.12 g/cm^{3,} and viscosity from 1.66 to 5.47 mPa · s.

The addition of glucose to WFI (pH 6.70 ± 0.40) caused a reduction in pH from 6.85 at the dissolution stage (50% glucose) to 6.05 after 24 h at 4°C; and with GFPuv added, the pH varied from 6.54 to 5.21, respectively (Table 1).

GFPuv has a maximum stability, as measured by fluorescence, between pH 6.0 and 8.0, with an isoelectric point (p*I*) between 4.9 and 5.1 *(25,26)*. To minimize the influence of pH on the stability of the protein, the glucose solutions were also prepared in 10 m*M* buffer solutions of acetate (pH 5.0), phosphate (pH 6.0 and 7.0), and Tris-HCl (pH 8.0). The buffered systems allowed examination of the influence of the glucose concentrations on GFPuv fluorescence intensity for a set pH. WFI was a solvent used as a standard.

The buffers prevented fluctuations in pH in the glucose solutions with and without GFPuv. The 24-h buffered glucose solutions did not undergo any significant variation in pH when compared with the solutions in water (pH 5.94–7.78). The pH ranged from 5.08 to 5.27 for acetate buffer (pH 5.0), from 5.70 to 6.10 for phosphate buffer (pH 6.0), from 6.63 to 7.04 for phosphate buffer (pH 7.0), and from 7.50 to 8.22 for Tris-HCl buffer (pH 8.0). The lowest pH was derived from 50% (w/v) glucose, in which the pH was further lowered by the addition of GFPuv to 5.21 in WFI, 5.97 in phosphate buffer (pH 7.0), and 5.80 in Tris-HCl. In acetate (pH 5.0) and in phosphate (pH 6.0) buffers, the pH in the 50% glucose solutions was maintained closer to the buffer's pH, 5.14 in acetate and 5.71 in phosphate.

Although an increase in glucose concentration with added GFPuv (8.0 ± 0.1 μg/mL) lowered the pH, the variation was greater or equivalent for the systems formed in WFI (pH 6.18 ± 0.11), phosphate (pH 5.90 ± 0.03 and 6.45 ± 0.07), and Tris-HCl (pH 6.64 ± 0.10). For the acetate buffer (pH 5.24 ± 0.02), the pH interval was constant.

Table 2 outlines the conductivity of all solvents used in the preparation of the glucose solutions. WFI had the smallest conductance owing to the lack of impurities or ions, and conductivity at 1.84 μs/cm establishes the quality of water used in these experiments. Acetate buffer, composed of acetic acid, a weak acid, showed a lower conductivity when compared with the Tris-HCl solution, which even though partly organic (Tris), has two dissociable ions. Phosphate buffers at pH 6.0 and 7.0 showed intermediate values. The addition of glucose in WFI causes an increase in conductivity. Elevation of conductivity was observed for solutions in water with up to 10% glucose. There was greater variation in conductivity for the interval of concentration from 15 to 30% glucose. For glucose concentrations >30%, the variation in conductivity relative to glucose concentration was less measurable than for concentrations <30% glucose, demonstrating that the quantity of glucose interferes with solution conductivity.

In acetate buffer, conductivity was not affected by the addition of glucose up to a concentration of 7%. Regarding the relationship between glucose concentration and conductivity, a distict behavior was observed for concentrations above 25%, at which the variation in conductivity

Table 1
pH of α-D-Glucose in WFI, Acetate Buffer (pH 5.0), Phosphate Buffers (pH 6.0 and 7.0), and Tris-HCl Buffer (pH 8.0) Before Addition of GFPuv and After Addition of GFPuv and Storage of Solutions at 4°C for 24 h

Glucose		WFI		Acetate buffer (pH 5.0)		Phosphate (pH 6.0)		Phosphate (pH 7.0)		Tris-HCl (pH 8.0)	
(C6H12O6) (%w/v)a	Molarity (M)	pH	pH + GFP (24 h)	pH	pH + GFP (24 h)	pH	pH + GFP (24 h)	pH	pH + GFP (24 h)	pH	pH + GFP (24 h)
0		6.72	6.69	5.18	5.22	6.17	6.13	7.07	7.01	8.10	8.04
1.5	0.08	6.59	6.75	5.13	5.13	6.10	6.07	7.02	6.85	8.22	7.16
5	0.28	7.38	6.79	5.12	5.14	6.05	6.07	6.97	6.80	8.22	7.16
7	0.39	5.94	6.53	5.27	5.29	6.00	6.04	7.04	6.81	8.01	7.14
10	0.56	6.91	6.76	5.26	5.22	5.96	5.99	6.98	6.72	8.02	7.05
15	0.83	7.78	6.62	5.25	5.24	5.92	5.98	6.95	6.67	8.05	6.98
20	1.11	7.76	6.29	5.23	5.24	5.89	5.90	6.87	6.68	7.99	6.89
25	1.39	7.23	6.12	5.23	5.21	5.86	5.83	6.82	6.39	7.88	6.54
30	1.67	7.77	5.70	5.24	5.38	5.79	5.83	6.80	6.25	7.88	6.65
35	1.94	7.30	6.00	5.20	5.32	5.78	5.84	6.78	6.18	8.00	6.30
40	2.22	7.41	5.91	5.20	5.28	5.72	5.75	6.74	6.06	7.95	6.06
45	2.50	6.16	5.42	5.15	5.30	5.70	5.74	6.69	6.04	7.91	5.98
50	2.78	6.05	5.21	5.08	5.14	5.67	5.71	6.63	5.97	7.50	5.80
Mean		7.02	6.18	5.20	5.24	5.87	5.90	6.86	6.45	7.97	6.64
SD		0.68	0.54	0.06	0.08	0.14	0.13	0.13	0.34	0.19	0.50
CI		0.13	0.11	0.01	0.02	0.03	0.03	0.03	0.07	0.04	0.10

[a]SD, standard deviation; CI, confidence interval.

Table 2
Physical Properties of Glucose Solutions at Concentrations Between 1.5 and 50% (w/v) Measured at 25°C (±0.1°C)

Glucose (w/v [%])	Molarity (*M*)	Density (g/cm^3) SD = 0.01	Viscosity (mPa · s) SD = 0.10[a]	Conductivity (µS/cm)					Osmolarity (mosmol/kg)[b]				
				WFI (pH 6.0–7.0)	Acetate (pH 5.0)	Phosphate (pH 6.0)	Phosphate (pH 7.0)	Tris-HCl (pH 8.0)	WFI (pH 6.0–7.0)	Acetate (pH 5.0)	Phosphate (pH 6.0)	Phosphate (pH 7.0)	Tris-HCl (pH 8.0)
0.0	—	0.95	1.76	1.84	656	1210	1940	4460	—	—	—	—	—
1.5	0.08	0.95	1.75	1760	728	1190	1860	2360	0.06	0.13	0.13	0.08	0.13
5.0	0.28	0.96	1.83	1324	664	1070	1710	2090	0.29	0.36	0.36	0.27	0.33
7.0	0.39	0.97	1.88	2210	667	1000	1610	1930	0.37	0.50	0.50	0.40	0.39
10.0	0.56	0.98	1.97	2370	598	892	1400	1760	0.56	0.75	0.75	0.57	0.62
15.0	0.83	1.00	2.15	1085	513	778	1240	1480	0.90	1.18	1.14	0.88	—
20.0	1.11	1.01	2.38	1000	403	662	1010	1250	1.29	1.70	1.54	1.29	1.33
25.0	1.39	1.03	2.64	666	346	563	851	968	1.77	NC	1.98	1.68	NC
30.0	1.67	1.05	3.02	490	292	471	702	865	2.28	NC	NC	NC	NC
35.0	1.94	1.07	3.48	658	254	404	579	205	NC	NC	NC	NC	NC
40.0	2.22	1.09	4.08	466	210	328	487	165	NC	NC	NC	NC	NC
45.0	2.5	1.10	4.83	364	162	260	386	137	NC	NC	NC	NC	NC
50.0	2.78	1.12	5.90	314	130	205	308	339	NC	NC	NC	NC	NC

[a]Viscosity range between 1 and 20 mPa·s.
[b]NC, no crystallization.

was lower and the glucose directly influenced the conductivity up to 50% glucose.

For phosphate buffers (pH 6.0 and 7.0) and Tris-HCl buffer (pH 8.0), the addition of glucose caused a drop in conductivity, with a much greater drop for Tris-HCl, representing a decrease of 47.5% in the conductivity for a 15% increase in glucose.

The conductivity of the glucose solutions, when compared within the same solvent without glucose, was inversely proportional to glucose concentration. Comparison of the conductivity of the glucose solutions in diverse buffers (acetate, phosphate, and Tris-HCl) and the differences in behavior revealed that there are three distinct ranges of conductivity (the lines of inclination are distinct) where the influence of glucose concentration is in the middle: (1) the area above 1000 μS/cm, up to 7% glucose in phosphate buffer (pH 6.0), 20% glucose in phosphate buffer (pH 7.0), and in Tris-HCl buffer (pH 8.0); (2) the range between 1000 and 400 μS/cm, up to 15% glucose in acetate buffer (pH 5.0), between 10 and 30% glucose in phosphate buffer (pH 6.0), between 25 and 50% glucose in phosphate buffer (pH 7.0), and between 25 and 30% glucose in Tris-HCl (pH 8.0); and (3) the area below 400 μS/cm, between 20 and 50% glucose in acetate buffer, between 35 and 50% glucose in phosphate buffer (pH 6.0), and between 35 and 50% glucose in Tris-HCl buffer.

Kinetic Parameters for GFPuv Denaturation in Glucose Solutions at 25°C

The stability of protein was studied through the inactivation rate constant (k, h^{-1}), decimal reduction time (D-value, h), energy of activation (E_a, kcal/mol), Gibbs free energy (ΔG, kcal/mol), and entropy (ΔS, kcal/mol).

Denaturation was monitored by measuring the loss of fluorescence intensity and expressed in the decimal logarithm of the decrease in native GFPuv concentration vs exposure time. The variation in fluorescence intensity of GFPuv diluted in water and buffers at a constant temperature (25°C) was measured spectrofluorometrically and converted to native GFPuv concentrations using the standard curve. The decimal reduction time (D-value = 2.303/k) is the time interval required to reduce 90% of initial GFPuv fluorescence intensity (or 1 Log_{10} μg of GFPuv/mL) at 25°C.

It was observed that GFPuv stability did not have a direct correlation with pH or other physical characteristics of the solutions, which were kept constant after the addition of the protein. Therefore, for each solution examined, the observed variations in D-values (which express the decrease in fluorescene intensity of GFPuv) showed a tendency to decrease with the increase of glucose concentration in solutions.

In WFI, with the D-values outlined in Table 3, for concentrations up to 10% there was a tendency to decrease linearly with an increase in glucose up to 30% (Fig. 1), when the D-value decay corresponded to every 5% increase in glucose added in WFI. For the range from 10 to 30% glucose in

Table 3
D-Values for GFPuv in Various Solutions at 25°C

Glucose (%)	*D*-value (h)			
	Water	Acetate (pH 5.0)	Phosphate (pH 7.0)	Tris-HCl (pH 8.0)
0	69.40	5.55	69.40	69.40
1.5	83.33	2.77	166.67	833.33
5	83.34	5.38	166.67	277.78
7	83.33	17.57	166.67	238.10
10	83.33	12.16	166.67	555.56
15	55.56	13.89	166.67	555.56
20	41.67	23.84	83.33	833.33
25	33.33	22.16	55.56	833.33
30	16.67	33.57	55.56	83.33
35	15.15	28.90	41.67	166.67
40	13.89	41.28	55.56	83.33
45	9.26	46.30	33.33	166.67
50	9.26	39.68	41.67	41.67

WFI, the *D*-value vs concentration was represented by the relation *D*-value = 108.33 − 3.11 × (%glucose), as shown in Fig. 1. The relation between the decrease in pH and the increase in glucose concentration can be represented by the equation (Fig. 1) pH = 6.8673 − 0.0299 × (% glucose).

With up to 30% glucose, the pH was about 6.0, and the stability of GFPuv was independent of pH and directly dependent on glucose concentration, mainly in the range of 10–30%. A decay of 80% in GFPuv stability was a function of the increase in glucose independent of changes in pH. For concentrations between 30 and 50% glucose, the variation in *D*-value was about 45%. From 30 to 50%, the pH dropped to 5.1, and the relation between glucose in WFI and changes in *D*-value was given by the equation *D*-value = 29.414 − 0.414 × (%glucose); for each 5% increase in the glucose concentration, the *D*-value was 20% less. Consequently, there was a loss of 33% fluorescence intensity for up to 15% glucose at pH ≥ 6.5; a loss of 50–60% intensity for 20–25% glucose at pH ≥ 6.0; with the greatest loss, 80% intensity, for an increase of 5% glucose up to 30% glucose in water for pH ≥ 5.5; and a loss of 89% intensity for an increase of 20% glucose up to 50% at pH 5.21. The most significant score in the instability of the protein was verified for concentrations ≥30% glucose in WFI, considered a transition zone when the fluorescence intensity of the protein was strongly influenced by the glucose concentration.

For concentrations >30% glucose in WFI, no crystallization was detected (Table 2) and the freezing point of the solution was not observed. The 30% glucose solution turns amorphous, depending on the viscosity of the system, which doubled at 35% glucose and tripled at 45–50% glucose,

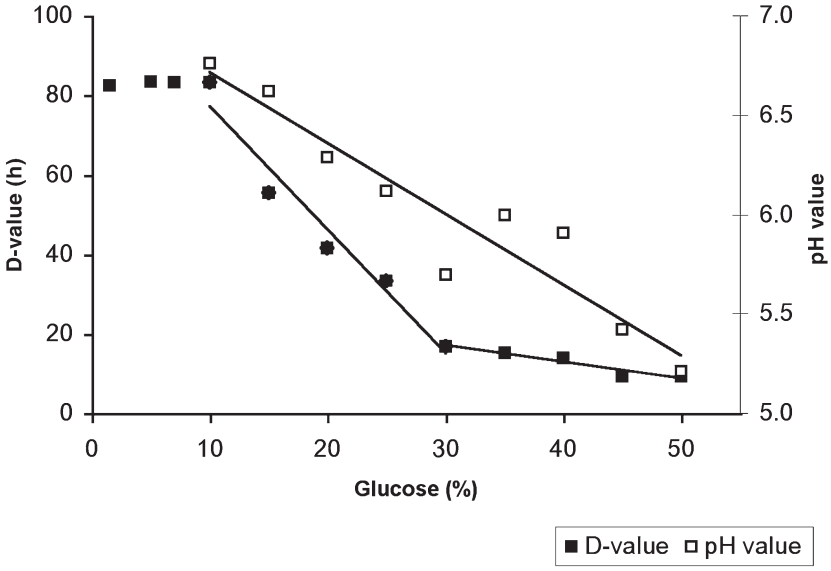

Fig. 1. Variation in *D*-values for GFPuv in WFI (pH 6.18 ± 0.11) with glucose added.

respectively. At a 30% glucose concentration, the physical properties of the solutions changed and decreased GFPuv stability at 25°C.

The lowest *D*-value (9.26 h) corresponded to 45 and 50% glucose in WFI, representing a stability decay of 89 and 33%, respectively, in relation to 10 and 40% glucose in WFI. There was a loss of 90% fluorescence intensity for a range of glucose concentration of 1.5–50% in WFI.

In the commercial manufacture of glucose solutions, the pH is usually at an interval from 4.5 to 6.0 *(14).* To study the influence of glucose concentrations at constant pH over GFPuv stability, the *D*-value of the protein was correlated to the equilibrated pH of the buffer solution.

The lowest stability for GFPuv was found in acetate buffer (pH 5.0) without glucose added, when the *D*-value was 12 times less than the *D*-values for WFI, phosphate buffers (pH 6.0 and pH 7.0), and Tris-HCl (pH 8.0) (Table 3, Fig. 2). The instability of GFPuv in acetate buffer is owing to its proximity to the p*I* of the protein, confirmed by a 90% decrease in fluorescence intensity during 180 min of exposure at 25°C without the presence of glucose (Fig. 2).

In addition, in the glucose-acetate buffers, there was an initial drop in GFPuv fluorescence intensity (loss of about 20% up to 40% glucose, and a loss of 30 and 50% intensity for 45 and 50% glucose concentrations, respectively) after GFPuv was added to the solution. After the first 20 min, the protein was subjected to the glucose-acetate system at 25°C. There was an equivalent decline of about 60–100 relative fluorescence intensity units (RFU), equivalent to 1.9–2.2 µg of GFPuv/mL and corresponding to a *D*-value between 4.62 and 5.55 h. This decay in GFPuv fluorescence was

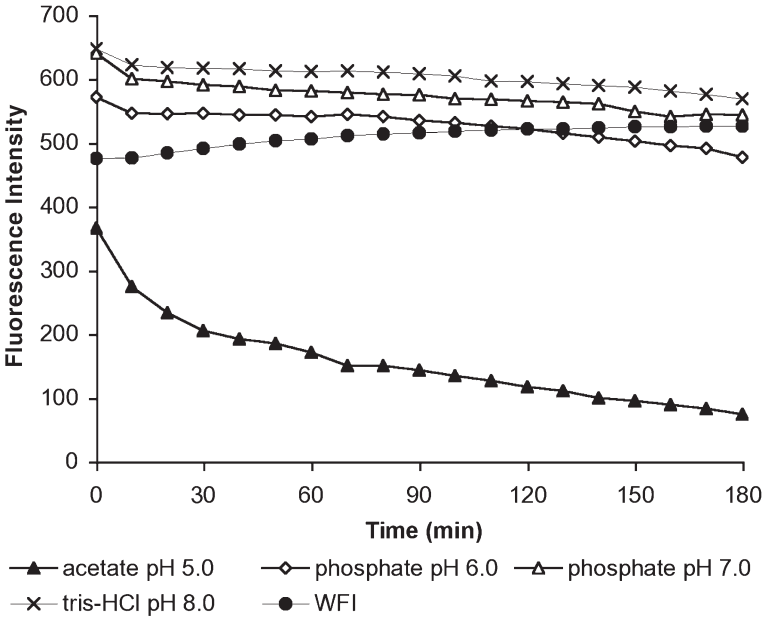

Fig. 2. Loss of fluorescence intensity of GFPuv after 180 min of exposure in WFI and buffer solutions without glucose added.

owing to the acetate buffer, independent of glucose concentration. This period was not considered in the calculated effects on *D*-values. This fact reinforced the observation that the pH values of the glucose solutions were very close to the values of the p*I* of the protein (pH 5.36–5.13) *(26)*. Therefore, in acetate buffers, the mean pH (5.24 ± 0.02) was kept constant for the glucose concentrations evaluated, and the *D*-values obtained for GFPuv (Table 3) showed a linearity for lower glucose concentrations.

At higher glucose concentrations of 45 and 50%, there was also linearity between the fluorescence decay and the time that the protein was subjected to the glucose-acetate system at 25°C. The fluorescence intensity at 25°C did not demonstrate linearity to 20, 25, 30, and 40% glucose concentrations. For this specific range of glucose concentration in the acetate system, the variation in *D*-value was calculated from the relation $t = n \times D$-value or D-value $= t/n$, in which $t = 180$ min of exposure of GFPuv at 25°C, and n = logarithmic cycles of decay in the GFPuv fluorescence intensity.

Therefore, the direct linearity between *D*-values and the increase in glucose concentrations in acetate buffer (pH 5.0) was given by the relation D-value $= 4.682 + 0.819 \times$ (% glucose). Actually, for pH < p*I*, where most likely hydrogen bonding between sugars and more protonated protein molecules becomes weaker, sugars primarily play the role of competitor to protein for water molecules, which leads to protein dehydration followed by extensive protein aggregation *(11)*. This phenomenon reinforces the stability of the native configuration of the protein at high sugar concentrations. The

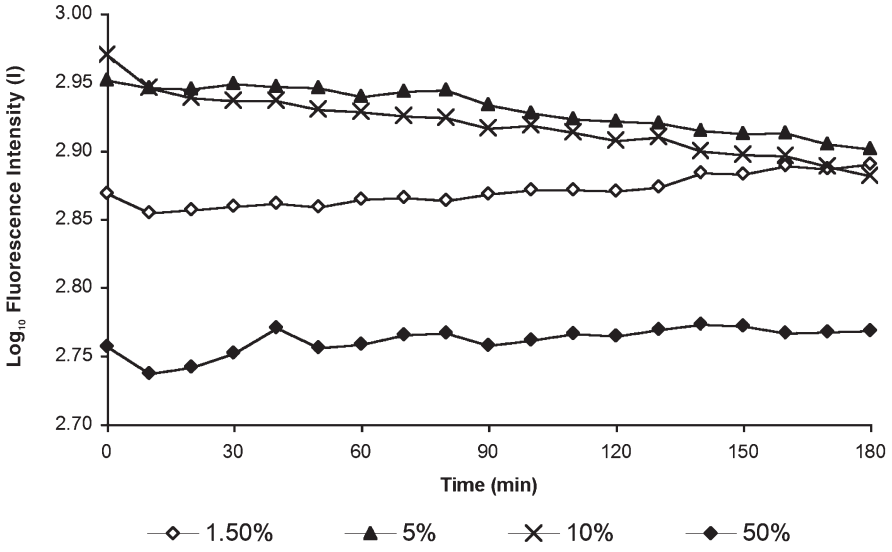

Fig. 3. Fluorescence intensity decay for glucose solutions of 1.5, 5.0, 7.0, and 10.0% in phosphate buffer (pH 5.90 ± 0.03).

behavior of GFPuv in the acetate system was inverse to that observed for the protein in WFI, where the stability of GFPuv decreased proportionally with the increase in high glucose concentrations and decline in pH.

In the phosphate buffer (pH 6.0), the pH of the glucose solutions was kept constant (5.90 ± 0.13) and close to 6.0. On the addition of GFPuv, an increase of 31% in fluorescence intensity was observed in solutions up to 10% glucose, an increase of about 1–5% in solutions up to 40% glucose, and an 8% reduction in fluorescence in 50% glucose (Fig. 3). Optimum GFPuv stability was in phosphate buffer (pH 6.0) in all concentrations of glucose studied. GFPuv instability in this buffer without glucose resulted in a *D*-value of 55.6 h (90% loss of fluorescence intensity every 55.6 h at 25°C) for 5 and 10% glucose in buffer. With higher glucose concentrations, the fluorescence intensity of GFPuv exhibited a slight decline (10–80 RFU) at the first 20 min at 25°C, which also occurred with GFPuv in acetate buffer. The fluorescence intensity promptly recovered and increased by 8–10% in relation to the initial concentration, relating to an increment of 50–80 RFU after exposure to 180 min at 25°C. Except for glucose at 5 and at 10%, there was a decline in fluorescence intensity of 100–150 RFU, equivalent to a loss of about 2.5 μg of GFPuv/mL. The phosphate buffer (pH 6.0) was shown to be the ideal system (Fig. 3) to maintain GFPuv conformation, by neutralizing the deleterious effect of glucose, especially at the higher concentrations up to 50%.

In phosphate buffer (pH 7.0), the mean pH of 6.45 ± 0.07 decreased with increasing glucose concentration (Table 1, Fig. 4). The native GFPuv concentrations dropped for both GFPuv in WFI and in phosphate buffer, respectively, when added to the system. Similarly, there was a loss of about

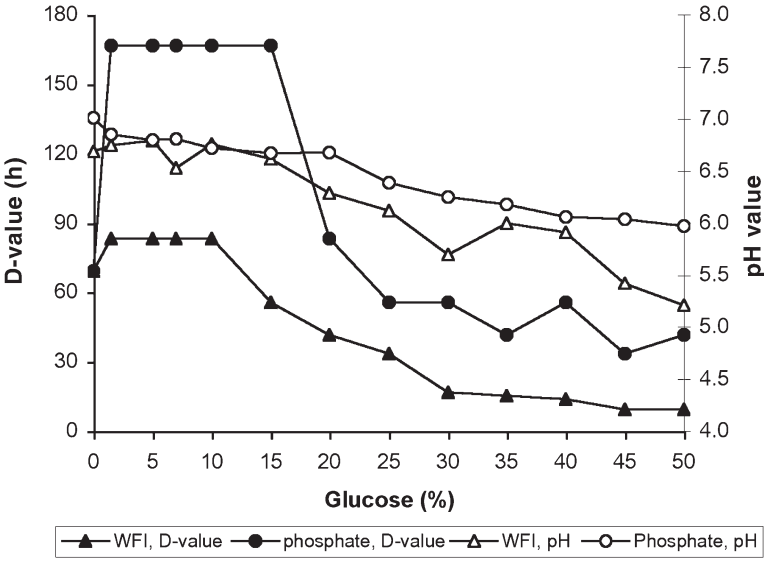

Fig. 4. Comparison of *D*-values for GFPuv in WFI (pH 6.18 ± 0.11) and phosphate (6.45 ± 0.07) buffer systems with glucose.

5% native GFPuv concentration (24 h at 4°C) in up to 35% glucose-phosphate buffer and a maximum 12% loss for 40–50% glucose-phosphate buffer. Therefore, the stability of the protein in the phosphate buffer (pH 7.0) for glucose concentrations up to 15% was unchanged for at least 167 h (*D*-value) at 25°C, twice the *D*-value characterized for GFPuv in WFI with up to 10% glucose. However, with solutions (pH 6.68) of 20% glucose in phosphate buffer, GFPuv stability declined by half, considered a transition zone for concentrations ≥20% glucose where GFPuv fluorescence intensity was strongly influenced by glucose concentration.

The stability, in terms of *D*-value, was reduced by 33% for GFPuv in the 25–45% buffered glucose system, and it was similar to GFPuv in 15–20% glucose in WFI. The mean *D*-value dropped to 33 h for GFPuv in the 50% buffered glucose system, similar to that for GFPuv in WFI with half the glucose (25%) and a 72% higher *D*-value for GFPuv in 50% glucose in WFI. Therefore, the phosphate buffer pH of 6.45 ± 0.07 was confirmed to at least double the protein stability compared with WFI (pH 6.18 ± 0.11) with glucose.

The stability of GFPuv in Tris-HCl buffer (pH 8.0) was shown to be 10 and 5 times greater than the stability determined in WFI and phosphate (pH 7.0) systems, respectively, for concentrations up to 25% glucose (Table 3). The performance of GFPuv in Tris-HCl buffer with concentrations between 30 and 45% glucose was similar to that in phosphate (pH 7.0) for concentrations ≤20% glucose with a similar pH variation in the solutions (Table 1). For a 50% glucose concentration, the fluorescence intensity decreased and showed similar behavior for the systems of Tris-HCl buffer

and phosphate (pH 7.0), confirming that a high concentration of glucose has a strong influence on protein stability.

As an added insight, the pH of the glucose solutions was about 6.0 ± 0.5, indicating that the phosphate buffer (pH 6.0) is the optimal system for future studies of GFPuv stability at higher temperatures (~100°C) in glucose solutions.

The energy of activation (E_a) represents the energy present in a system. The greater the E_a, the easier the system is destabilized because the disorder of the molecules is greater.

Considering the systems of WFI and phosphate buffer (pH 7.0), the highest values of E_a were about 633 kcal/mol for concentrations up to 10% glucose in water and 20% glucose in buffer, and 756 kcal/mol for concentrations up to 15% glucose in buffer, reflecting the greater disorder of the molecules of glucose and protein in the systems (Table 4). 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.

Compared to phosphate buffer (pH 7.0), the decay of E_a between 472 and 512 kcal/mol for the systems with ≥35% glucose represented half the E_a determined for concentrations up to 15% glucose in buffer and twice the E_a found in 45–50% glucose in WFI. The E_a for 20–25% glucose in WFI was equivalent to that for concentrations between 35 and 50% glucose in buffer. The phosphate buffer (pH 7.0) exhibited an ability to keep constant the level of the E_a of the system and maintain twice the stability of the protein as for WFI. The phosphate system also presented the ability to release the protein from a tight immobilized arrangement with glucose and water molecules, when compared with concentrations of >25% glucose in WFI.

The study of GFPuv in WFI was necessary to determine what effect glucose has on proteins in a solution without buffers, compared with buffered glucose solutions, as measured by the stability of fluorescence intensity from native GFPuv. The decline in the E_a of the system with an increase in glucose concentration in water was the most linear compared to the other solutions studied (Fig. 5).

Within the buffer solutions studied, the higher glucose concentrations in acetate (pH 5.0) provided a higher stability of GFPuv by an increase in the E_a for the GFPuv in the system (Table 4). The E_a increased 15 times with the glucose concentration (1.5–50% glucose). The stability of GFPuv in acetate buffer was reduced four times with the addition of 1.5% glucose, but it increased to the same level with 5% glucose. However, 7–10% glucose in the system doubled GFPuv stability. E_a values increased by 70–80% for glucose concentrations >10%. The E_a for 40–50% glucose in acetate was similar to

Table 4
Energy of Activation for Systems with GFPuv in Buffered or Unbuffered (WFI) Glucose Solutions

Glucose	WFI		Acetate (pH 5.0)		Phosphate (pH 7.0)		Tris-HCl (pH 8.0)	
(%)	k (h^{-1})[a]	E_a (kcal/mol)[b]	k (h^{-1})[a]	E_a (kcal/mol)[b]	k (h^{-1})[a]	E_a (kcal/mol)[b]	k (h^{-1})[a]	E_a (kcal/mol)[b]
0	0.033	633.33	0.415	155.23	0.033	601.05	nd	nd
1.5	0.028	633.33	0.831	32.59	0.014	755.68	0.003	1039.71
5	0.028	755.68	0.428	149.74	0.014	755.68	0.008	845.83
7	0.028	633.33	0.131	358.61	0.014	755.68	0.010	818.62
10	0.028	633.33	0.189	293.66	0.014	755.68	0.004	968.16
15	0.041	561.80	0.166	317.14	0.014	755.68	0.004	968.16
20	0.055	511.03	0.097	412.47	0.028	633.33	0.003	1039.71
25	0.069	471.61	0.104	399.58	0.041	561.80	0.003	1039.71
30	0.138	349.33	0.069	472.88	0.041	561.80	0.028	633.33
35	0.152	332.46	0.080	446.44	0.055	511.03	0.014	755.68
40	0.166	317.14	0.056	509.37	0.041	561.80	0.028	633.33
45	0.249	245.58	0.050	529.61	0.069	471.61	0.014	755.68
50	0.249	245.58	0.058	502.40	0.055	511.03	0.055	511.03

[a] $k = 2.303/D$

[b] $Log\ k = [E_a/(2.303 \times R \times T)] + C$, in which D is the decimal reduction time (h), R is the gas constant = 1.987 cal mol^{-1} K^{-1}, and T is the absolute temperature (K).

nd, not determined.

Table 5
Gibbs Free Energy (DG) and Entropy (DS) Determined for Systems Studied

Glucose (%)	ΔG (kcal/mol) [a] WFI	Acetate (pH 5.0)	Phosphate (pH 7.0)	Tris-HCl (pH 8.0)	ΔS (kcal/mol) [b] WFI	Acetate (pH 5.0)	Phosphate (pH 7.0)	Tris-HCl (pH 8.0)
0	–24.31	–22.81	–24.31	—	2.21	0.60	2.10	—
1.5	–24.42	–22.40	–24.83	–25.78	2.21	0.18	2.62	3.58
5	–24.42	–22.79	–24.83	–25.13	0.69	0.58	2.62	2.92
7	–24.42	–23.50	–24.83	–25.04	0.59	1.28	2.62	2.83
10	–24.42	–23.28	–24.83	–25.54	0.59	1.06	2.62	3.33
15	–24.18	–23.36	–24.83	–25.54	0.53	1.14	2.62	3.33
20	–24.01	–23.68	–24.42	–25.78	0.49	1.46	2.21	3.58
25	–23.87	–23.63	–24.18	–25.78	0.46	1.42	1.97	3.58
30	–23.46	–23.88	–24.18	–24.42	0.36	1.67	1.97	2.21
35	–23.41	–23.79	–24.01	–24.83	0.34	1.58	1.79	2.62
40	–23.36	–24.00	–24.18	–24.42	0.33	1.79	1.97	2.21
45	–23.12	–24.07	–23.87	–24.83	0.27	1.86	1.66	2.62
50	–23.12	–23.98	–24.01	–24.01	0.27	1.77	1.79	1.79

[a] $$\Delta G = R \times T \times \ln\left(\frac{k \times h}{B \times T}\right)$$

$$\Delta H = \Delta E_a - R \times T$$

[b] $$\Delta S = \frac{(\Delta H - \Delta G)}{T}$$

in which (Δ*G*) is Gibbs free energy; (Δ*S*) is entropy; (Δ*H*) is enthalpy; *k* is Boltzmann's constant = 1.38 × 10^{-23} J K^{-1} = 5.77 × 10^{-23} cal/mol; *h* is Planck's constant = 3.987 × 10^{-32} J min = 16.68 × 10^{-32} cal/mol; *R* is gas constant = 1.987 cal/mol^{-1} K^{-1}; and *T* is absolute temperature (K).

those found for 35–50% glucose in phosphate (pH 7.0) and for 15–20% glucose in WFI (Fig. 5). Opposite to the other systems, in concentrations >7% glucose in acetate, the addition of glucose showed a tendency to favor GFPuv stability, leading to a direct relation between the E_a values and glucose concentration, represented by the equation E_a (kcal/mol) = 283 + 5.10% glucose (Fig. 5).

The glucose-phosphate solutions (pH 6.0; pH average of 5.87) provided the greatest stability of GFPuv in the system when compared with the WFI and the buffer systems, even the phosphate buffer (pH 7.0).

Where the pH range is considered optimal for maintaining protein stability, near pH 8.0 in the Tris-HCl system, GFPuv showed instability with an increase in glucose, leading to a reduction to half the E_a, varying from 1040 to 511 kcal/mol, for concentrations from 25 to 50% glucose, respectively (Table 4). The elevated E_a, especially for low glucose concentrations, shows high instability in the system that can be influenced by a minimum external

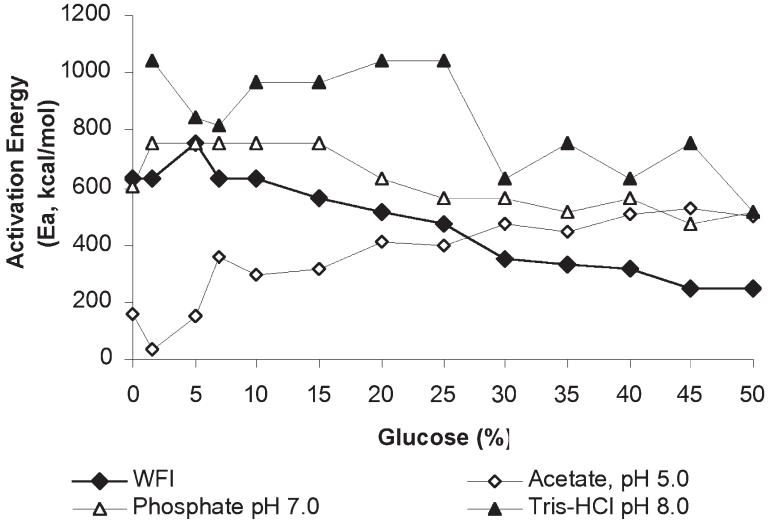

Fig. 5. E_a (kcal/mol) for WFI, acetate, phosphate, and Tris-HCl systems with GFPuv and glucose added.

interference in the solution. In the Tris-HCl system (concentrations ≥30% glucose), the stability of GFPuv was equivalent in phosphate (pH 7.0) for >20% glucose concentrations (Fig. 5), in WFI for concentrations up to 20% glucose, and in acetate buffer for concentrations ≥40% glucose.

The performance of GFPuv showed twice the stability in buffer systems than in WFI, specifically when the average E_a, value of 508 kcal/mol became equivalent for the buffers (acetate, phosphate, and Tris-HCl) with 50% glucose added in relation to the concentration of 25% glucose dissolved in the WFI (Fig. 5).

The Gibbs free energy (ΔG) measures the tendency of a transformation to occur. It is the parameter that indicates the spontaneity of the protein to adjust to the system and maintain structural stability. A decrease in ΔG shows the spontaneous and natural stability of the protein in the system considered.

The ΔG for GFPuv in WFI that increased with increasing glucose concentration exhibited two levels: $\Delta G = -24.31$ kcal/mol for concentrations up to 20% glucose and $\Delta G = -23.39$ kcal/mol for concentrations between 25 and 50% glucose. The same tendency was seen for GFPuv in Tris-HCl, with $\Delta G = -25.51$ kcal/mol for concentrations up to 25% glucose and $\Delta G = -24.72$ kcal/mol for concentrations between 30 and 50% glucose (Table 5).

In phosphate buffer (pH 7.0), the stability of GFPuv was equivalent to $\Delta G = -24.41$ kcal/mol, when taking into consideration every glucose concentration studied, including the buffer solution without glucose. The phosphate system at pH 7.0 maintained GFPuv structure and was not influenced by glucose.

GFPuv stability was the lowest in the acetate system for concentrations up to 5% glucose, increasing for concentrations from 7 to 35% glucose, similar to that found in phosphate buffer (pH 7.0) and Tris-HCl with 40, 45, and 50% glucose.

When 50% glucose was added to acetate, phosphate, and Tris-HCl buffers, GFPuv showed similar averages of ΔG, confirming equivalence in stability for the buffer systems studied. WFI provided the worst stability for GFPuv for concentrations >20% glucose dissolved.

Comparison of the systems studied in terms of entropy (excluding the phosphate buffer [pH 6.0], considered the ideal system to maintain GFPuv stability), showed that the system formed by glucose in WFI was the most organized for concentrations >5% glucose in WFI, directly decreasing with increasing glucose concentration. The dissolution of 1.5% glucose in WFI did not influence the organization of the WFI system, and GFPuv stability was observed to be equal for both solutions: WFI and WFI plus 1.5% glucose. For 45–50% glucose in WFI, entropy dropped 87%, from 2.21 (WFI) to 0.27 kcal/mol. The system became eight times more organized with increasing concentration up to 50% glucose in WFI. Entropy for both WFI without glucose and phosphate (pH 7.0) up to 20% glucose showed a tendency to maintain the disorganization of the systems, considering GFPuv stability. However, the addition of concentrations >20% glucose showed a slight tendency to decrease the entropy 20% to a lower level of 1.79 kcal/mol equivalent to the systems of GFPuv in buffer solutions (pH 5.0, 7.0, and 8.0) up to 50% glucose.

The systems formed with glucose in Tris-HCl and in phosphate (pH 7.0) were observed to subject the protein to a vulnerable state, in comparison with WFI plus glucose. The entropy slightly decreased 15%, from 2.10 to 1.79 kcal/mol in phosphate (pH 7.0), and dropped 50%, from 3.58 to 1.79 kcal/mol, in Tris-HCl (pH 8.0), considering glucose concentrations between 1.5 and 50%, respectively. GFPuv stability in phosphate (pH 7.0) was maintained with greater constancy.

The Tris-HCl system was observed to attain the highest disorganization for the range of 1.5–25% glucose with a reduction of 27% up to 45% glucose and failing to a sharp drop of 30% between 45 and 50% glucose. GFPuv stability was equivalent at entropy of 2.21 kcal/mol, with a favored level of disorganization in WFI with 1.5% glucose, phosphate buffer (pH 7.0) with 20% glucose, and Tris-HCl buffer (pH 8.0) with 30 and 40% glucose. The E_a confirmed the tendency of these systems to maintain GFPuv stability in the same level of E_a (633 kcal/mol), providing sufficient energy to keep the disorganizing system of the protein in glucose solutions. Tris-HCl solutions up to 25% glucose exhibited the highest entropy; phosphate buffer (pH 7.0) supported the best system for the protein in all the glucose concentrations studied.

On the other hand, the entropy of solutions in acetate (pH 5.0) increased directly with the glucose concentration, from 0.18 (1.5% glucose)

to 1.86 kcal/mol (45% glucose), resulting in the highest increment in the disorganization of the system (90%), corresponding to a 16-fold increase (94%) in the E_a (from 33 to 530 kcal/mol) of the system, contrary to what was observed in WFI.

Entropy in acetate (pH 5.0) with 1.5% glucose corresponded to 70% of the entropy found for 45–50% glucose in WFI, and the systems provided similar stability to the protein. The presence of 10 m*M* acetate at pH 5.0 destabilized the arrangement among molecules in WFI, leading to a level of disorganization for the protein equivalent to that found in phosphate (pH 7.0) for glucose concentrations >20% *(28)*.

The same level of stability was attained for 50% glucose dissolved in acetate (pH 5.0), phosphate (pH 7.0), and Tris-HCl (pH 8.0) buffers, denoting a tendency of organization of the system where the protein is less susceptible to adverse conditions. GFPuv stability in phosphate buffers was kept in a lower range of 20% variation, assuring protection to the protein from external influence of the solutions. The vulnerability for GFPuv in Tris-HCl buffer plus glucose, up to 45%, was the highest.

Appendix: Theory of Gibbs Free Energy (DG), Enthalpy (DH), and Entropy (DS) (1,5)

Solvent additives can affect macromolecular structure by direct interaction with the macromolecule and by indirect action through effects on the structure and properties of the solvent or by a combination of both of these mechanisms *(7)*. Studies *(5,6,7,11)* suggest that the cohesive force of sugars responsible for the increase in the surface tension of water is a very important factor governing the preferential interaction of protein with solvent components in an aqueous sugar system.

Glucose is preferentially excluded from protein surfaces by the solvophilic effect of the contact between the polar regions of proteins and the glucose-water mixture, i.e., entropically even more unfavorable than contact with water.

At relatively high concentrations (≥1 *M* glucose in aqueous solution), the cosolvent interferes with the stability of the protein and with binding characteristics of the system. Therefore, concentrations ≥20% glucose in buffer stimulated the association of protein molecules with cosolvent and water molecules. These effects are attributed to differences in the preferential interactions of a protein molecule with cosolvent and water molecules in its immediate vicinity. When the cosolvent concentration in the local domain surrounding a protein is lower than in the bulk solution, the cosolvent is said to be preferentially excluded, and the transfer free energy ($\Delta G_{tr} > 0$) associated with moving the protein from pure water to the cosolvent solution is unfavorable (positive). On the other hand, when the cosolvent concentration in the local domain of the protein is greater than in the bulk solution, the cosolvent is said to be

preferentially accumulated, and the transfer free energy ($\Delta G_{tr} < 0$) is favorable (negative) *(5,6,7,11,27).* The environmental conditions are kept constant (pH, temperature, and pressure).

Conductivity in aqueous solutions reflects the concentration, mobility and charge of the ions in solution. Glucose, a nonelectrolyte, is not readily dissociated and, consequently, does not directly interfere with conductivity, but influences the quantity of free water present in the system, so that the other components of the solution can or cannot conduct electricity.

Protein denaturation may be expressed as a simple two-state equilibrium between the native (*N*) and denatured (or unfolded or destabilized, *D*) state: $N \rightarrow D$; this expresses the shift in the equilibrium toward the native or stable form of the protein by the cosolvent additive (*S*). By definition, the equilibrium constant (*K*) of this reaction is a function of the stabilizer concentration, represented by $K(S) = [D]/[N]$. It states that on changing only the *S* concentration, the change in the equilibrium constant is given by the difference in the number of cosolvent molecules bound by the *D* and *N* protein molecules. With stabilization, the reaction is shifted to the *N* form, so the equilibrium constant must decrease with an increase in the concentration of the additive. In other words, there must be less binding of *S* to the *D* form of the protein than to the *N* form. There is an excess of water surrounding the protein, which means that the protein is preferentially hydrated or, as it is often expressed, there is preferential exclusion of the ligand from the protein domain. The exclusion phenomenon is a migration of the solvent molecules away from binding regions of the protein surface into the bulk solvent, leaving a layer enriched with water.

The most convenient way of discussing the solution properties of a substance *i* is in terms of the thermodynamic quantity known as the chemical potential (μ). The μ can be thought of as a quantity that describes the way in which the free energy of an open system is altered when the number of moles of *i* (n_i) changes at constant temperature (*T*) and pressure (*P*) *(1).* As a thermodynamic quantity, binding simply reflects the perturbation of the standard chemical potential ($\Delta\mu°$) of the protein by the cosolvent.

Certain components (glycerol, sucrose, and salts) are used to preserve protein structure and to stabilize biologic activity when added at high concentrations (≥1*M*), such as aqueous glucose solutions at concentrations ≥15% (0.83 *M*). The addition of glucose at concentrations higher than 25% caused a slight increase in the chemical potential of the protein GFPuv, and the free energy of the system was kept negative (Δ*G* from –23.87 up to –23.12 kcal/mol for 50% glucose in WFI). This is a thermodynamically favorable situation.

In these studies, the protein is immersed in an aqueous solution of the cosolvent glucose. Preferential exclusion of the cosolvent can be expressed as a model by a zone of exclusion. Cosolvents at high concentrations are

known to stabilize the native structure of proteins and are excluded from the protein. The mechanism is based on the perturbation of the surface free energy (surface tension) of water by the cosolvent (zone of exclusion). Since the surface of contact between protein and solvent constitutes an interface, there must be in this surface an interfacial (surface) tension.

Additives perturb the cohesive force of water and hence its surface tension. Thus, as shown by the Gibbs adsorption isotherm, these results are in an excess or a deficiency of the additive at the surface layer. It is clear that if a substance increases the surface tension of water, its excess at the surface layer will be negative; that is, it will be preferentially excluded from the surface. This mechanism gives rise to observed preferential exclusion of sugars, amino acids, and structure-stabilizing salts, and it appears to be the most widespread mechanism in protein-stabilizing solvent systems. The mechanism is not determined by chemical interactions between the solvent components and the protein structure; it is a mechanism in which the interactions with proteins are determined strictly by the properties of the solvent—that is, the proteins are basically inert.

The surface of a globular protein at neutral pH can be regarded as a mosaic of charges, and the charge density may be very high even though the net charge may be close to zero. The net effect is a balance between the two types of interactions, with preferential exclusion generally predominating. The net effect between stabilizing cosolvents and native globular proteins is one of preferential exclusion. This does not mean that cosolvent molecules are totally excluded from contact with proteins. In fact, they can penetrate into the zone of preferential hydration and bind proteins at specific sites. The net observation is the balance between binding and exclusion, which, for stabilization, favors the latter. Therefore, when the interactions are defined by the properties of the solvent system, the protein being essentially an inert component, the extent of exclusion should be determined by the surface contact with the solvent. This surface increases on denaturation, so preferential exclusion should increase. This leads to protein structure stabilization. If it is accepted that preferential exclusion of the cosolvent varies approximately as does the total surface of contact with the protein, then preferential exclusion from the unfolded (*D*) form must be greater than from the native (*N*) form. Thus, the equilibrium must shift to the native form; that is, the protein is stabilized in the folded form (*N*) *(5)*.

The solubility of proteins (to be used as a parameter of some physical or chemical effect in a complex system in which maximum stability is required) is determined in either salt-free water or water containing a specific salt buffered at a constant pH and ionic strength. The choice of a buffer has many important implications besides the creation of a stable pH environment. Thus, the lowest possible concentration of buffer required for maintaining the pH is used to minimize the nonspecific ionic strength

effect. In most cases in which the protein is easily dissolved, a buffer concentration of 20–100 m*M* is used, depending on the pH range *(2)*. In the present study, 10 m*M* buffer concentrations were used to stabilize the pH of proteins in aqueous glucose solutions.

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