STUDIA UBB CHEMIA, LIX, 1, 2014 (p. 135-147)
(RECOMMENDED CITATION)

COMPARATIVE STUDY OF GREEN FLUORESCENT PROTEIN MUTANTS

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ABSTRACT. Two histidine - substituted mutants of Enhanced Green Fluorescent Protein (S202H-EGFP and S202H/Q204H-EGFP) was realized and the effect of mutation on the spectral properties of mutant proteins was examined. The denaturation of the wild type and mutant proteins in guanidine hydrochloride solution was also studied. The structural stability analysis revealed that the global structure of the protein is partially destabilized due to the performed mutations. Meanwhile the sensitivity of mutant proteins to the solvent acidity increased. It was found that with increase of proton concentration in the solvent the absorbance and the fluorescence intensity of proteins decreased.

Keywords: *green fluorescent protein, histidine, fluorescence, absorbance, chromophore*

INTRODUCTION

The green fluorescent protein is an autofluorescent protein that emits green light, upon exciting with ultraviolet light, without the addition of any exogenous substrate [1]. This protein has a characteristic structure that consists of a small, compact β-barrel. The beta-barrel is constructed of eleven beta-strands and an alpha-helix runs through the centre of the barrel. The protein's chromophore is located in the centre of the barrel structure. The compact "*beta*-can" structure assures high level of stability to the protein and also protects the chromophore group from the destructive effects of the bulk solvent [2].

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It is shown that proteins with beta barrel structure are more stable than globular or other proteins; also the energy barrier of unfolding is higher at this type of proteins [14]. Although GFP, its mutants and some other fluorescent protein (DsRed, zFP506) have very similar structure, still it was shown that their structural stability may differ significantly [18]. Several studies exist in which the structural stability of GFP and its mutant forms were investigated. These proteins proved to be more resistant against the denaturizing effects of chaothropic agents (GuHCl, SDS) and also had higher thermal stability like other proteins [14,15,16, 17].

The protein's color originates from a conjugated pi electron system that belongs to the chromophore group. The chromophore group is generated in the protein's interior by cyclization of three consecutive amino-acid residues (Ser65, Tyr66 and Gly67) in a self-catalyzed intramolecular rearrangement of this tripeptide sequence [3]. Fluorescence emission occurs when the chromophore group of the protein is raised to an excited state as a result of electron transfer from a ground state to a higher energy level, as the electrons drop back to the ground state, the chromophore group emits energy in the form of green light [4].

The native green fluorescent protein's fluorophore exists in two conformations in ground state. A protonated form, the predominant state, which has an excitation maximum at 395 nanometers and a less prevalent, unprotonated form that absorbs at approximately 475 nanometers [5]. The relative quantity of these two forms is affected by the proton concentration of the environment. Protons from the solvent could enter into the protein’s interior and alter the ionization state of the chromophore [9]. The pH sensitivity of the chromophore is determined by the proton transfer from the protein surface to the interior of the protein. The crystal structure studies at different pH revealed that some of the side chains of the beta barrel could change their orientation in the function of medium’s pH [10]. The amino acid residues in the proximity of the chromophore group and its hydrogen bond network, through their orientation could promote either the protonation or deprotonation of the hydroxyl group of Tyr66, but both forms are always present [9]. At wtGFP the phenol group of tyrosine 66 is predominantly uncharged, while at EGFP the charged Tyr is the dominant form [9]. The emission wavelengths of these forms of the chromophore are similar, but their fluorescence lifetimes differ. [13]

The wild type GFP was subjected to several mutations to improve one or more characteristics of the protein [6]. The mutational investigations have revealed that the protein's fluorescence is linked to its structural integrity [7, 23]. As the protein is denaturated, the protection of the chromophore is loosed and it is turned to a nonfluorescent state [8]. The EGFP (Enhanced

Green Fluorescent Protein) is a mutant form of GFP with two mutations (F64L, S65T) in the chromophore region and have a single excitation peak at 488 nm. These modifications resulted in a protein with greater fluorescence intensity [3].

GFP and it mutant forms are widely used as fluorescent markers in cell biology and biotechnology. By modification of fluorescent proteins many research groups tried to develop biosensors like pH sensors, metal sensors, chloride ion sensors or intracellular red-ox sensors [6].

Two histidine-substituted mutants of EGFP were created, namely the S202H-EGFP and the S202H/Q204H-EGFP mutant protein, as described in a previous study. This work's aim was to obtain metal binding mutants of EGFP, which can be further used as metal sensor [12]. In the present study the effect of mutation was examined on the spectral properties and structural stability of mutant proteins. The structural model of mutant proteins was prepared *in silico*. The results of the spectral studies and the structural stability analysis show that these modifications did not have significant effect on the global structure of the protein.

RESULTS AND DISCUSSION

Absorption spectra analysis

EGFP has an excitation maximum at 487 nm. The absorption spectrum of the wild type EGFP and its mutants was determined in mediums with different pH values (pH 4.6-8), to investigate the effect of mutations to the spectral properties of the proteins.

The phenol group of Tyr66, which is part of the protein's chromophore group, has pH dependent ionization states, a phenolate form and an uncharged phenol form [10]. The absorption characteristics of the two forms of the chromophore are different, the protonated form has an absorption maximum at 395 nm, while the unprotonated form absorbs at 485 nm, as shown in the Figure 1.

At neutral pH the absorption spectra of mutant proteins were similar with the spectrum of the wild type protein (WT-EGFP). Differences appear only in the minor peaks sizes. At 395 nm the S202H-EGFP and the WT-EGFP show greater absorption than the S202H/Q204H-EGFP mutant protein. So at these two proteins (S202H-EGFP and the WT-EGFP) the ratio of the protonated and unprotonated form of the chromophore is bigger than at the S202H/ Q204H-EGFP mutant protein.

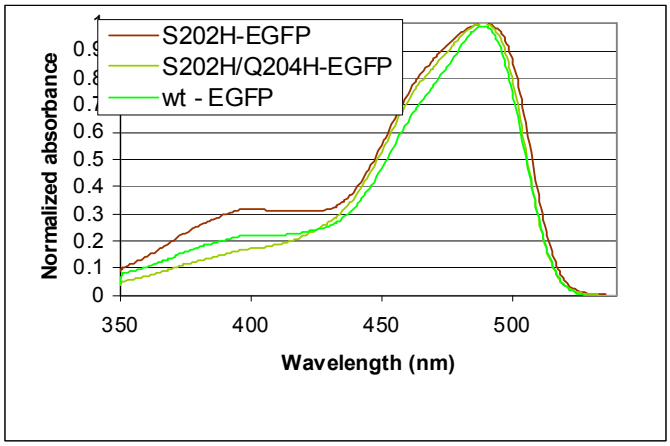

Figure 1. Normalized absorption spectrums of EGFP and its mutants

On the obtained spectrums it can observed two absorption peaks, a minor peak at 395 nm and a major peak at 478 nm. These two absorption maximums can be explained with the simultaneous presence of the protein's chromophore in two ionization forms (protonated and unprotonated).

As can be observed on Figure 2. at EGFP the protonation of hydroxyl group of Tyr-66, which is part of the chromophore, induces a decrease of absorption at 488 nm while the absorption at 390 nm increases. By reducing the pH from 8 to 4.6 the protein's absorption decreased at 488 nm and in the same time an increase of absorption at 390 nm was observed. U. Haupts *et al.* also investigated the effect of medium's pH to the absorbance of EGFP, they obtained similar results [9].

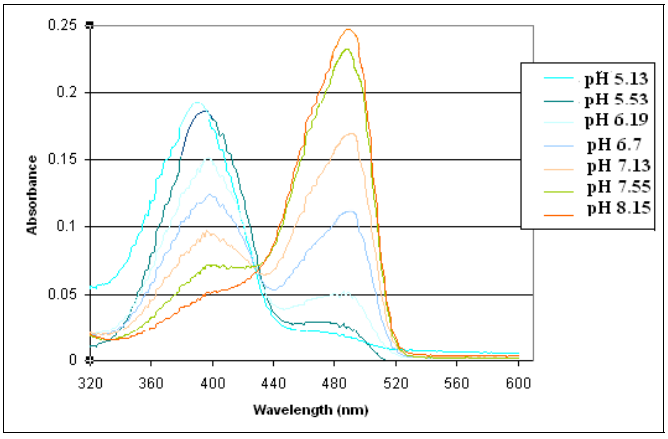

Figure 2. Normalized absorption spectrums of WT-EGFP at different pH

The equilibrium between the two ground state conformations of the GFP is controlled by the proton concentration of the medium [11]. At low proton concentration the conditions favor the formation of the phenolate form of tyrosine, so the majority of the chromophore is unprotonated. At high proton concentration a proton from the bulk solvent protonates the phenol group of chromophore. In this protonated form the protein's chromophore is nonfluorescent [10].

Although a compact beta can structure surrounds the chromophore, it is proposed that the Tyr66 is in direct contact with a buried water molecule. Through this water the molecules of solvent can affect the chromophore's protonation state [5]. These two forms of chromophore can transform to each other in a reversible manner, the process is controlled by the proton concentration of the medium. [10]

Emission spectra analysis

The effect of the medium's pH to the emission spectrums of the wild type EGFP and its mutants has been studied. The emission spectra of proteins in solutions with different pH values was measured. In solutions with neutral pH the emission spectrums of investigated proteins are practically identical. Deviations in the proteins emission spectrums appear in acidic environment.

The investigated three proteins showed different sensitivity towards environmental pH.

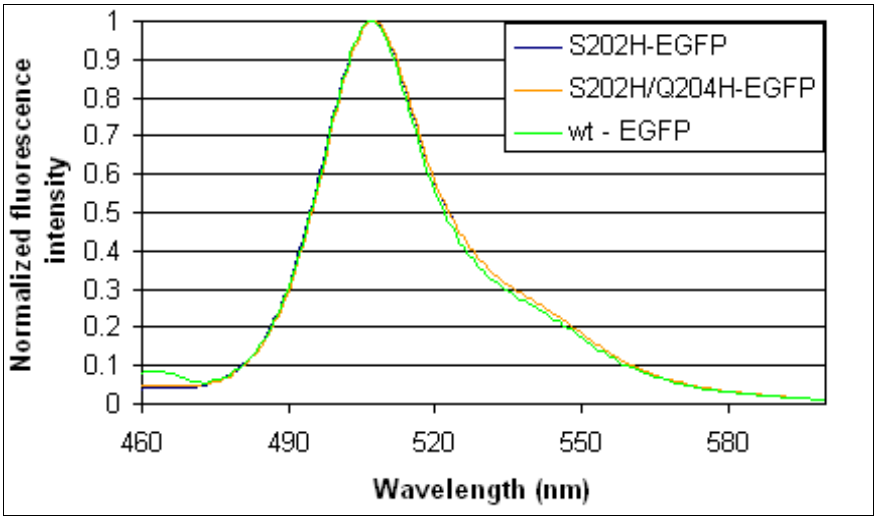

Figure 3. Emission spectra of WT- EGFP and its mutants (Excitation: 400 nm)

It was observed that fluorescence intensity decreased with decreasing of the pH value, on the other hand the positions of the emission maxima of investigated proteins are independent of the environmental pH. Effect of pH on the fluorescence intensity of the S202H/Q204H mutant protein is illustrated in Figure 4.

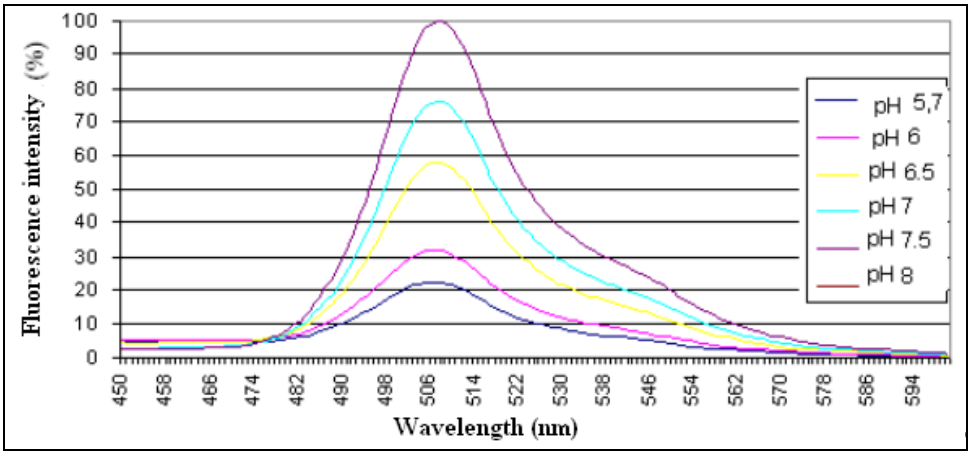

Figure 4. Normalized emission spectrums of S202H/Q204H mutant of EGFP at different pH (Excitation: 400 nm)

All the three investigated proteins showed similar changes in the emission spectra. The lower emissions at lower pH values correlate with the reduced absorption at 488 nm in similar conditions, as described earlier [5]. The obtained fluorescence intensities in the function of the environmental pH are illustrated in Figure 5.

At Figure 5. in acidic medium the chromophore is fully protonated and the protonated form is nonfluorescent. It may be noted that in mediums with low pH values (pH <= 4) the investigated proteins lose their fluorescence. The wild type protein proved to be the most sensitive to the pH of the environment, its fluorescence intensity decreased faster than the mutant protein's. The mutant proteins' pH sensitivity also differs, the S202H mutant protein proved to be more sensitive than S202H/Q204H mutant.

It has been shown that the pH induced changes in fluorescence intensity are completely reversible, in the range of 5-8 pH. In this pH range by using CD spectrometry couldn't be detected any conformational changes in the global structure of the protein. Despite this, it was proposed that some slight structural changes near the chromophore occur that allow the proton transfer form the bulk solvent to the interior of the protein [9].

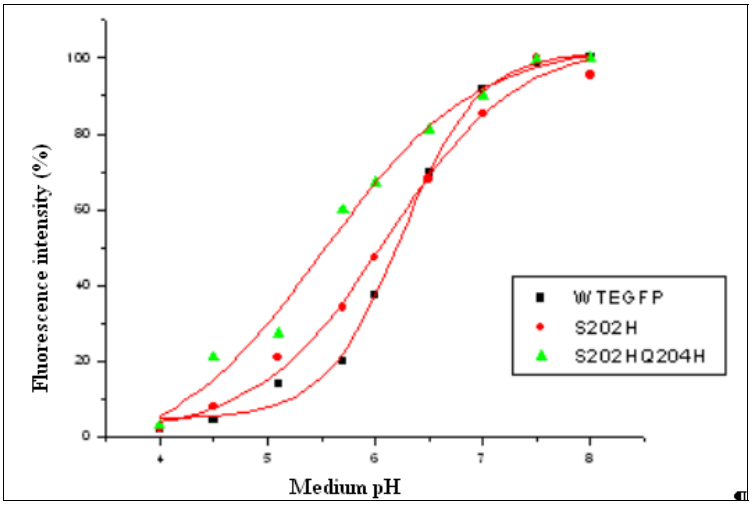

Figure 5. pH sensitivity of EGFP and its mutants

Structural stability analysis of mutant proteins

It is known that the fluorescence of these proteins is closely related to their structural integrity. These proteins can emit fluorescence only in native form, their chromophore group must be protected. The beta can structure assure a high level stability to the protein and at the same time protect the chromophore from the molecule of the solvent [6]. So the unfolding of these proteins results in the loss of their fluorescence. Due to the extremely compact structure of the fluorescent protein, the unfolding is a very slow process [14].

The structural stability of three green fluorescent proteins (EGFP, S202H-EGFP, S202H/Q204H-EGFP) was analysed.

By measuring the fluorescence intensity in the presence of chaotropic agents one can obtain information about the structure destabilization, the protein unfolding. Denaturation of the wild type EGFP and its mutants in guanidine hydrochloride solutions has been studied by measuring the decrease of fluorescence intensity. This type of monitoring of the fluorescent protein's unfolding has been used in several studies [8, 14, 16, 18, 19].

During denaturation the protein's structure is destabilized, beta strands are shifted. This shift generates a gap between the beta strands, thus decreases the protection of chromophore group and it becomes accessible for small molecules. The water molecules get inside the protein and protonate the chromophore. Protonated form of chromophore is non-fluorescent [8]. So the fluorescence intensity of EGFP decreases during protein denaturation.

Denaturation of the proteins was studied in solutions that contained guanidine hydrochloride in different concentration. Figure 6. shows the remaining fluorescence intensity of wt-EGFP and its mutants in denaturing medium, after incubation for 20 hours at room temperature (298 K).

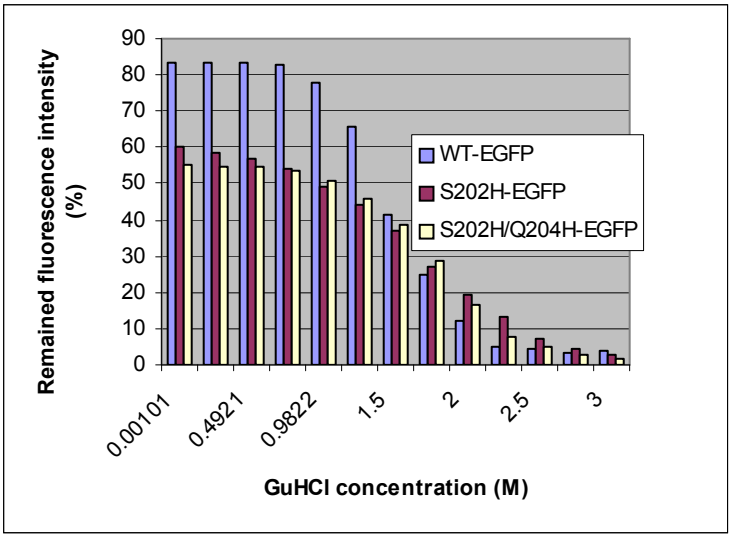

Figure 6. Decrease of fluorescence intensity during denaturation of the proteins

Fluorescence intensity of EGFP increased with 15-20% in the presence of 0.1-0.2 M GuHCl, referred to the initial intensity [14, 18, 19]. In this study it was also observed a similar effect of GuHCl in small concentrations. This increased fluorescence intensity could be due to the altered spatial arrangement of the chromophore [17].

It was proposed that after 20h incubation of the proteins in the presence of GuHCl, its denaturation became into an equilibrium state, where the protein is also present in native and in denaturated forms. The ratio between these two states is the function of GuHCl concentration. This equilibrium is described in the equation 1.

$$Native\ form \leftrightarrow Denaturated\ form \tag{1}$$

The equilibrium constant of the process can be described by the equation 2.

$$K_d = \frac{\mathrm{F}_{\mathrm{denaturated}}}{\mathrm{F}_{\mathrm{native}}} \tag{2}$$

The measure of the native state stability is the difference between the standard free energy (delta G) of the native state and denatured state of the protein, and can be described by the equation 3.

$$\Delta G^0 = -RT * \ln(K_d), \text{ where } R = 1.987\ cal/mol * K \quad (3)$$

Denaturation of the wild type and their mutants is shown in Figure 6. Illustrating the variation of free energy in the function of guanidine concentration can determine the value of free energy of the protein in water. Free energy of the protein in guanidine hydrochloride solutions can be described by the equation 4.

$$\Delta G^{\circ}_{GuHCl} = \Delta G^{\circ}_{H_2O} + m * [GuHCl] \quad (4)$$

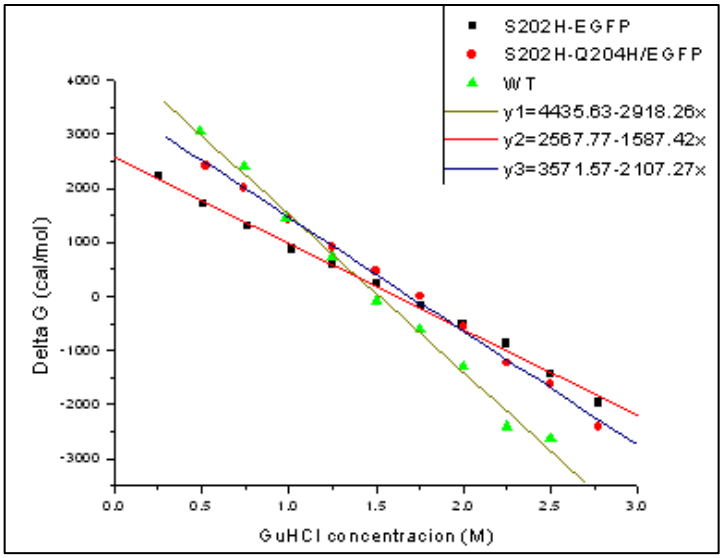

Figure 7. The free energy of proteins in different guanidine hydrochloride solutions

In this study the same method described in [18] was used for determination of the standard free energy of denaturation.

Comparing the values obtained for the free energies of the proteins, as shown in figure 8, it can be observed that wild type protein proved to be the most stable in guanidine solutions. It's free energy in water is 4435.6 cal/mol, Olesia V. Stepanenko et al. obtained similar magnitude for conformational stability of wt EGFP [18]. As it is shown on the Figure 8., the S202H / Q204H mutant is more stable than the S202H mutant. These results suggest that the effectuated substitutions in the protein's beta barrel slightly altered the compact structure of EGFP, causing a partial destabilization in it.

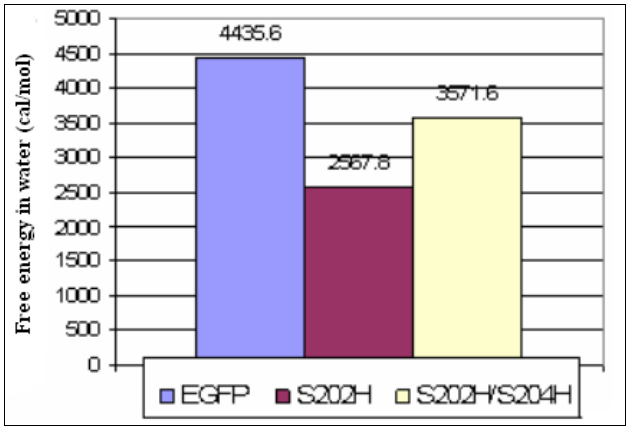

Figure 8. Free energy of proteins in water

CONCLUSIONS

Spectral characteristics of the original protein and their mutants at neutral pH are similar. Spectroscopic studies have shown that both fluorescence and absorption of proteins is influenced by environmental acidity, as described in [7], [11]. In the case of all the three investigated proteins two absorption maximums can be observed in the absorption spectra, one is at 395 nm and another at 485 nm, that corresponds to the two ionization forms of the chromophore.

It was observed that the fluorescence intensity decreased with the decreasing of the medium's pH. At high proton concentration (pH<4) the proteins do not emit fluorescence. It was observed that mutant proteins (S202H/EGFP and S202H/Q204H/EGFP) are less sensitive to environmental acidity than the original protein (WT-EGFP). This unexpected behaviour of mutant proteins apparently contradicts with our results obtained from structural stability analysis, where the original protein proved to be the most stable, and the global structure of mutants is partially destabilized. This anomaly can be explained by the appearance of a local buffer effect due to the imidazole groups of histidines introduced in mutant proteins by directed mutagenesis.

It is known that histidines are potential proton acceptors and, due this fact, it can be proposed that through the protonation of these groups the proton concentration is reduced in the proximity of the chromophore group. It seems that the newly introduced histidine residues (Hys202 and Hys 204) protects the chromophore group from protonation. This effect is pronounced in the range of pH 5-6, where a relatively big difference between fluorescence intensity decrease of mutant proteins and wild type protein.can be seen.

Finally, summarizing all results, it can be concluded that the presence of histidine residue in the proximity of the chromophore reduces protein's sensitivity to environmental acidity. This hypothesis is confirmed by the most reduced sensitivity towards the environmental pH of the S202H/Q204H/EGFP mutant, where two histidines were introduced. But this local buffering effect is limited to a relatively small pH range, it emerges between 5-6.5 pH values.

EXPERIMENTAL SECTION

Protein expression

The mutations were carried out by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), as described in earlier work [12]. The wild type EGFP and its two histidine substituted mutants (S202H/S204H and S202H) were expressed in *E. coli* strain BL21 Star (DE3) cells, by using the pET15b expression vector. The recombinant proteins contained an N-terminal hexa-histidine tag, so the proteins were purified by immobilized metal (Ni^{2+}) affinity chromatography. Purified proteins were dialyzed against 25 mM sodium phosphate buffer, with pH 7.

Absorption spectra measurements

Absorption spectra measurements were made in 2ml quartz cuvettes at room temperature (293K). 1 ml of the purified protein solution (protein dissolved in 20 mM sodium phosphate, 100 mM NaCl, pH = 7) was used to the measurements. Measurements were performed in solutions with different pH values (pH = 4.6 - 8.1), in phosphate buffer. The concentration of proteins was different so to compare the obtained spectrums, the spectrum of the most concentrated protein was normalized.

Emission spectra measurements

Fluorescence intensity measurements were made in 3ml quartz cuvette. The fluorescence spectra of EGFP and its mutants was carried out by a Fluoro Max Spex 320 spectro-fluorimeter, at 298 K. Excitation wavelengths were assayed at 400 and 450 respectively. The emission was detected in the range of 460-600 nm. 2ml samples were used for measurements. The samples containing the protein (His6EGFP) in low μM range in 20 mM Na_2HPO_4, with different pH (pH= 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8). The concentration of proteins was different so to compare the obtained spectrums, the spectrum of the most concentrated protein was normalized.

Analysis of protein denaturation in guanidine solution

Protein denaturation in guanidine solution was analysed. In these experiments different concentrations of guanidine hydrochloride solution was used (0.5-5M). The protein concentration in the denaturing solutions was in low micromolar range. After 16 hours of incubation at room temperature, the fluorescence intensity of denatured EGFP was measured by a FluoStar OPTIMA fluorimeter, excitation wavelength was 485 nm and emission wavelength was 520 nm.

ACKNOWLEDGMENTS

The work has been funded by the Sectoral Operational Programme Human Resources Development 2007-2013 of the Romanian Ministry of Labour, Family and Social Protection through the Financial Agreement POSDRU/6/1.5/S/16.

This study was supported by the Department of Bioengineering of Sapientia Hungarian University of Transylvania.

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