IMPROVEMENTS IN QUALITY OF HISTIDINE-MODIFIED ENHANCED GREEN FLUORESCENT PROTEIN THAT BINDS COPPER IONS WITH NANOMOLAR AFFINITY

Judit PETRES[1], Emese-Éva BÁLINT[2], Mária SZABÓ[3], László SZILÁGYI[4], Beáta ÁBRAHÁM[5]

The object of this research was to determine those experimental conditions which contribute to achieve the EGFP-2H mutant protein's extremely high affinity for copper ions in comparison with the wild-type protein's affinity. Therefore, a comparative study has been performed to determine the factors that contribute in results improving. We have produced a mutant protein substituting the serine at position 202 and the glutamine at position 204 with histidine. Spectral properties and K_d values were examined under different experimental conditions. High binding affinity was achieved in the nanomolar range (K_d = 15nM) using properly purified and stabilized proteins under optimal experimental conditions.

Keywords: Histidine-Modified EGFP mutant, protein cloning, expression and purification, dissociation constant, copper affinity

1. Introduction

The use of EGFP variants revolutionized the life sciences owing to their extensive usability and their unique optical and structural properties. Structural analysis by x-ray crystallography indicated that the GFP, extracted from jellyfish *Aequora Victoria,* consists of a β-barrel in which an oxidized tri-amino acid chromophore is buried [1, 2].

Production and characterization of various GFP mutants has expanded the possible applications of fluorescent proteins. Moreover, the GFPs have been employed as genetically encoded fluorescent probes for cellular applications. The GFP being available in genetically encodable probes can be utilized for the detection of processes taking place in the cells. In this way, can determine the transition metal ions or heavy metal ions quantity, various gradient values, pH values and so on [3-5].

[1] PhD student, University POLITEHNICA of Bucharest, Romania, mail:szindy78@yahoo.com
[2] Researcher, Faculty of Siences, University SAPIENTIA, Cluj-Napoca, Romania
[3] Researcher, Faculty of Siences, University SAPIENTIA, Cluj-Napoca, Romania
[4] Researcher, Faculty of Siences, University SAPIENTIA, Cluj-Napoca, Romania
[5] Head of Department, researcher, Faculty of Siences, University SAPIENTIA, Cluj-Napoca, Romania

The green fluorescent protein and its mutants have been shown to bind copper ions resulting in quenching of its fluorescence. Some of these proteins were found to be highly selective, reversible and sensitive toward copper ions. By utilizing this ability, various biosensing systems for Cu^{2+} determinations have been developed [4, 6, 7].

Cu^{2+} is essential component in several enzymes, participates in several cellular pathways, electron transports and neuronal communications. Important neurological diseases have been linked to defects in copper homoeostasis [8, 9]. Menkes's, Alzheimer's, Huntington's and Wilson's diseases, all neurological disorders, are attributed to the body's inability to metabolize copper effectively. The physiological role of copper ions in blood is beneficial in micromolar amounts [10].

Our aim was to obtain highly sensitive EGFP protein to transition metals, i.e. to achieve the lowest possible value of the dissociation constant. The wild-type EGFP have been re-engineered by introducing two histidines at position 202 and 204 replacing the serine and glutamine for increasing the sensitivity of the protein towards different metal ions. Thus, we created possible metal binding sites group near the chromophore by site-directed mutagenesis [11, 12].

In previous study, this mutant protein's properties were examined and compared with those of wild-type EGFP. At the same time, we determined the optimal temperature and pH values for the fluorescence quenching analysis.[11].

The results showed that we obtained an EGFP mutant which bound nanomolar concentrations of Cu^{2+} in a reversible manner. The detection limit for copper was determined to be 10 nM which is below 1 ppb (1 ppb for Cu^{2+} is 15.7 nM) [11].

The object of this research was to determine those experimental conditions which contribute to achieve the mutant protein's extremely high affinity for copper ions in comparison with the wild-type protein's affinity. Therefore, a comparative study has been performed to determine the factors that contribute in results improving. Spectral properties and K_d values were examined when the conditions of measurement were changed.

2. Experimental

2.1. Construction of expression vectors

Recombinant plasmids, named pET15b-EGFP, a generous gift of L. Radnai (Dept. Of Biochemistry, Eötvös Loránd University, Budapest, Hungary) was used to create the modified EGFP-s. The modified EGFP-s (i.e. S202H and Q204H mutations in protein structure) were achieved by specific site mutagenesis using the Quick Change Site Directed mutagenesis kit (Stratagene), as described earlier [12].

The mutated gene fragments from pET-EGFP were amplified with NdeI and BamHI primers by PCR. The resulting gene fragments were digested with NdeI and BamHI restriction enzymes and then ligated into the pET15b vectors. The mixture contained 25-45 ng of genes of interest and 55-75 ng digested vectors. The success of the operations was verified by agarose gel electrophoresis.

2.2. Expression of EGFP/2H

Recombinant plasmids were introduced by heat-shock transformation into *E. coli* BL21 (DE3) STAR host cells (1uL vector construct was added to 100 uL chemically competent cell).

The expression systems contained no pLys plasmids because the expression should not be induced. Therefore, without induction a weak expression (leaky expression) carried out and well-folded proteins were obtained.

The transformed bacterial colonies were incubated in 250 mL LB supplemented with 100 ug/uL ampicilin, at 37^{o}C, overnight. In order to recover the cells, they were centrifuged with Beckman centrifuge Model, JLA-10500 at 6000 rpm for 20 minutes. The supernatant was discarded, and the pellet was resuspended in about 40 mL of 10 mM MOPS buffer pH 7.5 and incubated at -20^{o}C overnight. Bacterial suspend was lysed by sonication at 4°C using Sonics Vibra-Cell VC 505 sonifier. In order to clarify and separate the soluble proteins from insoluble cell components, the resulting solution was subjected to a new centrifugation with Beckman Model JA-20 at 13000 rpm for 30 minutes.

2.3. Purification procedures

pET15b expression vector offers not only the advantage of high level of expression, but also a very simple and effective way of purifying the expressed protein. Thus, the protein is fused to a region consisting of six histidine residues that confer affinity for Ni^{2+} ions.

The purification of recombinant proteins was performed by an immobilized metal affinity chromatography (IMAC) using nickel charged polymer matrix named Ni-NTA marketed by Novagen. Two methods were evaluated and compared in order to obtain the desired yield and purity of target proteins. Purification with step elution was first performed, which is simpler and does not require sophisticated instrumentation. During the process, 40 mL of protein suspension flowed over the echilibrated chromatography column. Then it was washed out by 80 mL buffer solution (pH 7,5) containing 50 mM of sodium dihydrogen phosphate and 500 mM NaCl. Elution process in single step was effectuated using about 20 mL elution buffer containing 50 mM of sodium dihydrogen phosphate, 500 mM NaCl and 250 mM imidazole (pH 7.5).

In order to separate accurately the proteins remaining in suspension gradient elution may be used instead of step elution. Gradient elution was performed using FPLC instrument from Amersham Biosciences. Using analytical instrument FPLC (Fast Protein Liquid Chromatography) is a favorable alternative to define the eluent flow rate, the gradient elution program to control the imidazole concentration and amount of each fraction. A built-in spectrophotometer instrument set at 280 nm measured the solution absorbance, which is proportional to the concentration of eluted proteins. This signal was represented graphically by the instrument's recorder.

A benefit of this method is the selective elution of the components in the presence of various concentrations of imidazole. The imidazole concentration was increased by adjusting gradients according to the following program:

	Imidazole concentration		Solution volume
⟶	0	30%	4xCV (~40 mL)
⟶	30%	100%	3xCV (~30 mL)
⟶	100%		3xCV (~30 mL)

The flow rate was adjusted to 1.5 ml / min. 5 ml fractions were collected absorbance of which at 280 nm can be read from the chromatogram. Efficiency of the procedure was verified on 13,5% SDS-PAGE.

Purified proteins were dialyzed against 4x2 L 10 mM MOPS buffer (4-morpholinepropane sulfonic acid) pH 7.5 (in both cases 48 hours) in order to remove imidazole. 500 mg/L Amberlite CG50 cation exchange resin was added in the buffer solution to remove residual metal ions remaining in the suspension of protein. The resin is fine-grained and does not sediment under stirred conditions. This method is gentler than the purification of proteins with ion-exchange chromatography.

2.4. Analysis of spectral characteristics

After removing residual impurities from the probes, absorption spectra of purified samples (1 mL) were recorded from 600 nm to 250 nm using Beckman DU650 Spectophotometer. Protein was quantified using $OD_{488\ nm}$ (ε = 55,000 M^{-1} cm^{-1}).

Analysis of fluorescence intensities of target proteins was performed using FluoroMax Spex 320 spectrofluorimeter, with 0.33 min. integration time, under high voltage of 950 V. The solutions in instrument were thermostated and maintained at 20°C with a circulating water bath. The emission spectra were measured from 480 nm to 580 nm wavelength range (λ_{ex} = 470 nm).

The samples of protein were prepared at 0.10, 0.15, 0.20, 0.30, 0.35 and 0,40 μM final concentrations in 10 mM MOPS (pH 7.5). For binding studies these probes were supplimented with 10 uL copper solution with varying concentrations of copper ion between 0,05 uM and 30 μM. For titration experiment $CuSO_4.5H_2O$ was used, which was dissolved in ultra pure water. To stabilize the system, the samples were incubated at 20 ° C for 40 min. The measuring was effectuated with 3.0 mL protein sample in a quvette with 10 mm light path. All samples were run against blanks containing the same concentration of all reactants, excluding protein.

2.5. Data Analysis

The dissociation constants were evaluated according to the method described in the previous study [11]. According to this, the fitting procedure to the values from the fluorescence intensity reduction was performed based on the quadratic ecuation:

$$I_{Cu}/I_0=1-\{K_d+[Cu]+[P]-sqrt(([K_d+[Cu]+[P]))^2-4[Cu]*[P])/2[P]\} \quad (1)$$

where I_{Cu}/I_0 is the relative intensity, K_d is the dissociation constant, [Cu] and [P] are total concentrations of copper ion and protein, respectively.

3. Results and discussion

After expression, the resulting protein consists of 266 amino acids, which has molecular weight as 29873.6 Da.

The target proteins were purified using two methods as described in the Material and Methods. The results of purification with step elution were analyzed by polyacrylamide gel electrophoresis (fig. 1).

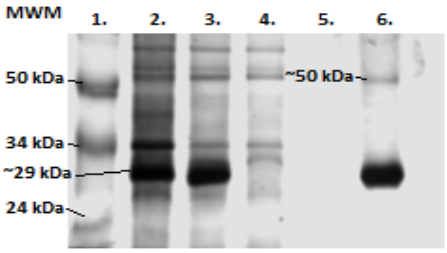

Fig. 1. SDS-PAGE gel illustrates the results of step elution. Lane 1 – protein marker- Amersham MWM. Lane 2 - Unpurified cell suspension. Lanes 3, 4 – flow-through containing cellular components. Lane 5 – sample containing target protein.

Purification by step elution gave satisfactory results, but the image of SDS-PAGE gel reveals that in the sample were remained different kinds of proteins (Lane 6: unspecified 50 kDa residues).

Gradient elution was used in order to achieve more accurate results. It is expected that the remaining impurities had a lower affinity than the His_6-Tag from fluorescent proteins and it could be eliminated with lower concentrations of imidazole.

The elution of EGFP/2H was monitored by measuring its absorbance at 280 nm. After addition of low concentrations of imidazole (about 75 mM, 30%) appeared a single peak on elution spectrogram, which represents the presence of the residual components. Purified EGFP/2H protein fractions were collected after passing through the fast increased concentration of imidazole up to 100%. Using non-linear gradient elution delivers smaller product pool volumes and higher product concentration. Chromatogram of procedure is illustrated on fig. 2.

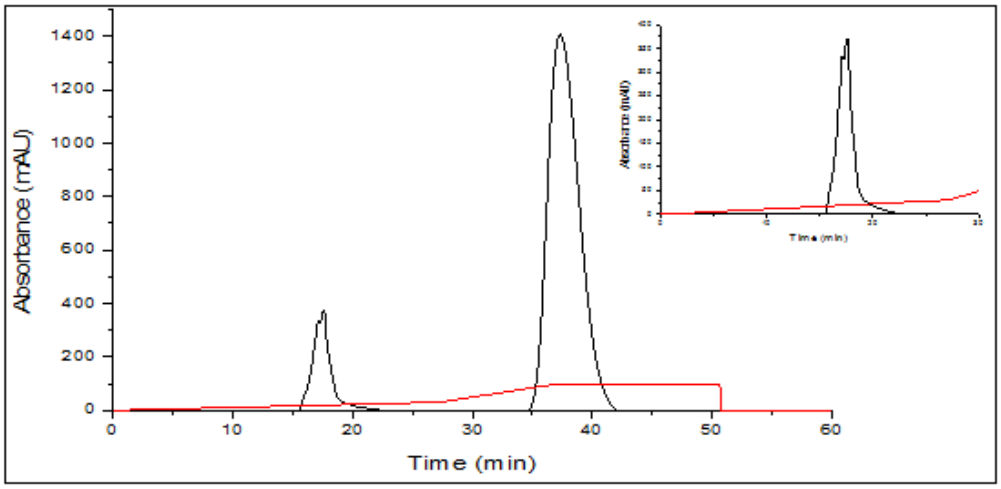

Fig. 2. Elution profil of EGFP/2H. The solid red line represents the imidazole concentration during the elution process. The insert illustrates the zoomed version of peak of contaminants removed at low concentrations of imidazole.

The purity of eluted fractions was checked on SDS–PAGE gel (fig. 3).

In order to achieve higher purity level, the remaining metal ion contaminants was eliminated using cation-exchange resin during dialysis. The process was repeated several times to get more pure samples. This process helps in the removal of metal ions from strongly bound proteins.

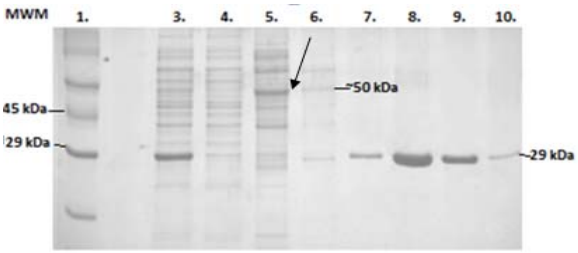

Fig. 3. SDS-PAGE gel analysis of collected fractions purity. Lane 1: protein marker from Amersham MWM. Lane 3: *E. coli* extract before the chromatographic purification, Lane 4, 5, 6: flow-through containing contaminants eluted with about 25-30% or 75 mM imidazole. Lane 7, 8, 9, 10: fractions contained purified EGFP samples.

In order to analyze the quality of samples purified with two different elution process, absorption spectra were monitored. Spectra were normalized to 488 nm and compared with the purpose to illustrate the differences (fig. 4).

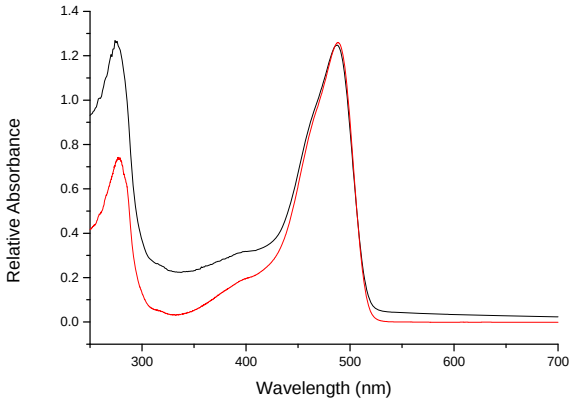

Fig.4. Absorbance spectra of purified EGFP/2H proteins with step elution (solid black line) and gradient elution (solid red line). 1-1 mL of the samples were monitored in the wavelength range from 750 nm to 240 nm with scan rate of 60 min / sec.

Maximum absorption spectrum at 488 nm corresponds to the anionic chromophore and absorption at 280 nm to the aromatic amino acids. Significant differences appear between the absorption spectrum of the purified protein by step elution and the spectrum of the purified protein with gradient elution. The maximum absorption at 280 nm is much lower after gradient elution, which means that the process is able to remove the contaminants. Thus, the purity of the sample is indicated by the peak ratio 488/280. The higher ratio value means more pure protein. For subsequent experiments we used samples whose purity indicated by the ratio 488/280 is greater than 1.5. In the range 600-700 nm slight increase is observed. The slope is the consequence of sample opacity, which disappears in the case of proteins purified with gradient elution.

To further check the effectiveness of purification procedure, we examined the stability of proteins purified in two different ways. The protein is stable if it retains its original structure, i.e. proteolysis does not occur. In order to analyze this, the samples were verified periodically by SDS-PAGE. The proteolysis was indicated by comparison of probes that were treated for SDS-PAGE (boiled in loading buffer at 95^{o}C) at different times. The control sample were treated and stored at -20^{o}C immediately after purification. After one week, the proteins used for measurements and frozen samples were compared using SDS-PAGE.

First, we analyzed the protein stability using samples purified with step elution technique (fig. 6).

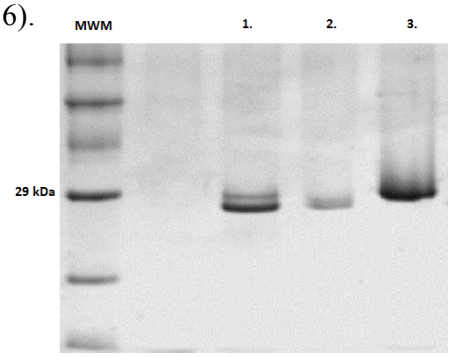

Fig. 6. SDS-PAGE gel. Lane MWM - protein marker, Lane 1. EGFP/2H treated on the day of verification at apr. 28. Lane 2. EGFP/2H treated with loading buffer and stored at -20^{o}C at apr.22. Lane 3. EGFP/2H treated on the day of purification with loading buffer and stored at -20^{o}C at apr.18.

According to SDS-PAGE gel image, proteins begin to decompose slowly a few days after the production. The proteins size is visibly reduced, which means that larger parts are broken down from N- and C-terminal ends of the protein. We assumed that the His-Tag is also breaks off, which may have an influence on dissociation constant value. In order to prove this, the His-Tag was removed from protein by digestion with thrombin (fig. 7).

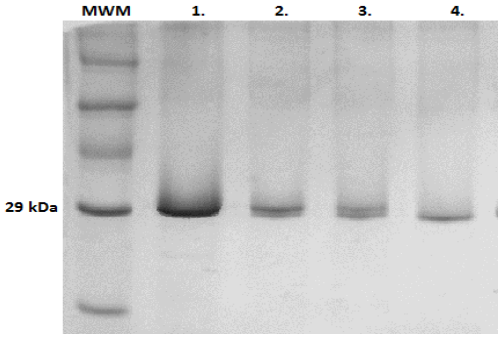

Fig. 7. Lane MWM. Protein marker. Lane 1. Control sample. Lane 2. Proteins treated one week after production. Lane 3. Proteins treated two weeks after production. Lane 4. Proteins treated with thrombin.

The fluorescence emission spectra of recently produced and thrombin-treated protein samples were monitored, which showed emission maxima at 507 nm.

The scanned spectra were normalized at 507 nm, and the changes in fluorescence decay were compared.

Copper binding study was performed by adding different concentrations of copper to the protein solution. The chemical nature of the binding sites has been investigated with the use of titration methods presented in Material and Methods.

Fluorescence intensities decay data were plotted as a function of the copper ion concentration, and descending curves were fitted to these data sets using quadratically derived function (fig. 8.). Fluorescence titration curves were analyzed as follows to derive the dissociation constant (K_d) which defines the complexing capacities of the target proteins.

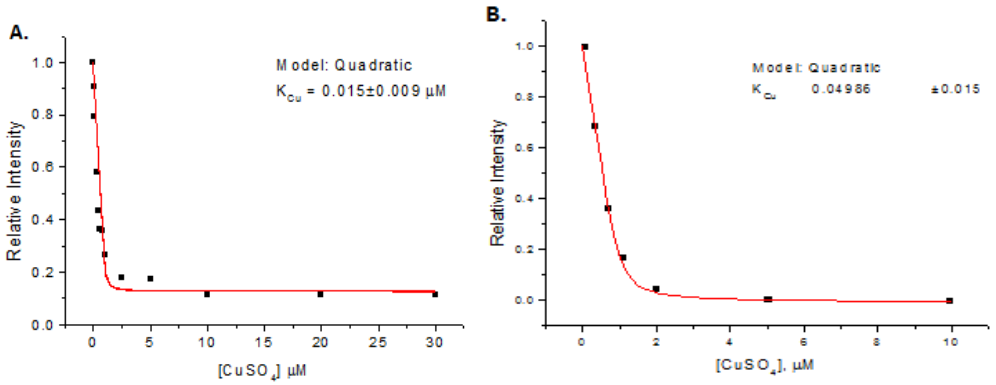

Fig. 8. Fluorescence quenching data evaluation of recently produced EGFP/2H (A) and thrombin-treated EGFP/2H (B).

The dissociation constants calculated with quadratic model for recently produced and thrombin-treated EGFP/2H are K_d=0,015 ± 0.009 uM and K_d = 0.049 ± 0.015 uM, respectively. Thus, the dissociation constant obtained from measurements using thrombin-treated proteins is twice greater (with the exception of experimental errors) than the constant value obtained from analysis using proteins which were not proteolysed.

It was concluded, that the proteolysis of proteins may influence its metal-binding ability. Nevertheless, the protein preserves bright green color over a long period, which means that the β-barrel basic structure remains stable.

The protein stability, after purification with gradient elution, was also examined by SDS-PAGE in the same manner, as described earlier. It was observed that the proteins treated with loading buffer, frozen at -20^{o}C and examined at various times, appeared to be more stabile. Therefore, proteins

treated two weeks after production and treated with thrombin were compared (fig. 9).

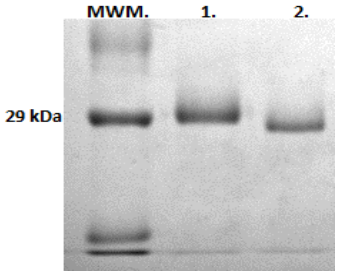

Fig. 9. SDS-PAGE gel. Lane MWM - protein marker, Lane 1. EGFP/2H purified with gradient elution and treated two weeks after production. Lane 2. EGFP/2H treated with thrombin.

The gel image illustrates that the proteins purified with gradient elution, and verified two weeks after purification are smaller than the thrombin-treated proteins. This means that the proteins purified with gradient elution is more stable than those which were purified with step elution. The lower degree of proteolysis is a result of residues removal during the gradient elution, which may contain proteases or non-enzymatic substances that affect the proteins stability.

The proteins fluorescence purified with gradient elution (treated and untreated with thrombin) was also detected in the presence of different concentrations of copper ions. The experiment was carried out under similar conditions so the calculations were comparable with the previous results. We found that the comparison of measurements in which it were used proteins immediately after purification with step elution and stabilized proteins after several weeks of storage, resulted about the same dissociation constant values. However, the dissociation constant of the proteins purified with step elution and stored for a few weeks increased.

It was determined previously that the copper addition causes more than 90% decrease in mutant EGFP proteins fluorescence. It should be noted that the measurements of low concentrations of copper ions are influenced by the protein concentrations. Therefore, it was compared the experimental data that was carried out with different protein concentrations (100 nM, 150 nM, 200 nM, 300 nM, 350 nM and 400 nM) in the presence of low copper ion concentrations (0.05 - 1.5 uM) (fig. 10).

Titration experiments showed that using 100 nM of EGFP/2H for detection of lower concentration of copper ions, showed greater change in fluorescence intensity than changes caused by other protein concentrations. 0,3 uM Cu^{2+} causes 50% decrease in 100 nM protein fluorescence emission intensity.

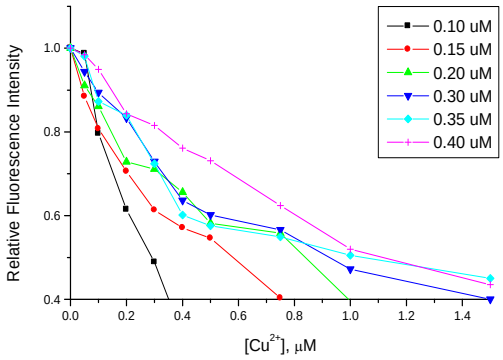

Fig 10. Titration of different concentrations of EGFP/2H protein in the presence of copper ions (Applied protein concentrations: 0.10 uM, 0.15 uM, 0.20 uM, 0.30 uM, 0.35 uM, 0.40 uM and copper ion concentrations: 0.05 uM, 0.1 uM, 0.2 uM, 0.3 uM, 0.4 uM, 0.5 uM, 0.75 uM, 1 uM and 1.5 uM)

The 400 nM protein fluorescence intensity was reduced only by 15% by 0,3 uM Cu^{2+}. During the low copper concentration analysis it was established that threefold molar excess of copper concentration caused minimum 50% quenching in 100 nM protein fluorescence. However, the same extent of quenching in 400 nM protein fluorescence was achieved by addition of 3.75-fold molar excess of copper. It was concluded that the varied concentration of protein are not proportional with the extent of its fluorescence quenching caused by the same concentration of copper.

4. Conclusions

In this paper it has been demonstrated that the measurement conditions affect the metal binding results. It is possible to optimize the protein–metal interactions by taking account the following aspects: it is recommended that the target protein should be purified from other cellular residues and metal ions with gradient elution and with dialysis containing cation exchange resins, respectively. The analysis has to be performed by applying relatively low concentrations of proteins in which the proteolysis has not been done.

As a conclusion, the optimal experimental conditions for copper sensing system have been determined based on the high copper binding ability of the EGFP/2H mutant. The high binding constant for copper may be very useful in determining levels of copper in biological and environmental samples.

REFERENCES

[1] *F. Yang, L. Moss, G.N. Phillips Jr*, The molecular structure of green fluorescent protein. Nat. Biotechnol., 1996. **Vol**. **14**, pp. 1246-1251.

[2]*M. A. Rosenow, H.A.Huffman, M. E. Phail, R. M. Wachter*, The Crystal Structure of the Y66L Variant of Green Fluorescent Protein Supports a Cyclization-Oxidation-Dehydration Mechanism for Chromophore Maturation. Biochemistry, 2004. **Vol. 43**, pp. 4464-4472.

[3] *C. D. Geddes*, Optical halide sensing using fluorescence quenching: theory, simulations and applications—a review. Institute of Physics Publishing, 2001(The Photophysics Group, Department of Physics and Applied Physics, University of Strathclyde, Scotland, UK).

[4] *V. Hsiu-Chuan Liao, Ming-Te Chien, Yuen-Yi Tseng, Kun-Lin Ou,* Assessment of heavy metal bioavailability in contaminated sediments and soils using green fluorescent protein-based bacterial biosensors. Environmental Pollution, 2006. **Vol. 142**, no. 1, pp. 17-23.

[5] *M. Kneen, J.Farinas, Y. Li, A. S. Verkman,* Green Fluorescent Protein as a Noninvasive Intracellular pH Indicator. Biophysical Journal, 1998**. Vol. 74**, pp. 1591-1599.

[6] *J. P. Sumner, N.M.Westerberg, A. K. Stoddard, T. K. Hurst, M. Cramer, R. B. Thompson, C. A. Fierke, R. Kopelman,* DsRed as a highly sensitive, selective, and reversible fluorescence-based biosensor for both Cu+ and Cu2+ ions. Biosensors and Bioelectronics, 2006. **Vol. 21**, no. 7, pp. 1302-1308.

[7] C. *Isarankura-Na-Ayudhya et al.,* Fluorescent Protein-Based Optical Biosensor for Copper Ion Quantitation. Biological Trace Element Research, 2010. **Vol. 134**, no. 3, pp. 352-363.

[8] *V. Desai, S.Kaler*, Role of copper in human neurological disorders. The American Journal of Clinical Nutrition, 2008. **Vol. 88,** no. 3, pp. 855S-858S.

[9] *D. J. Waggoner, T.B.Bartnikas, J. D. Gitlin,* The Role of Copper in Neurodegenerative Disease. Neurobiology of Disease, 1999. **Vol. 6**, no.4, pp. 221-230.

[10] *D.E. Hartter, A.Barnea*, Brain tissue accumulates 67copper by two ligand-dependent saturable processes. A high affinity, low capacity and a low affinity, high capacity process. The Journal of Biological Chemistry, 1988. **Vol. 263,** pp. 799-805.

[11] *E. É. Bálint, J.Petres, M. Szabó, Cs. K. Orbán, L. Szilágyi, B. Ábrahám*, Fluorescence of a Histidine-Modified Enhanced Green Fluorescent Protein (EGFP) Effectively Quenched by Copper(II) Ions. Journal of Fluorescence, 2013. **23**, no. 2, pp. 273-281.

[12] *M. Pálfi, E. Kovács, I. Miklóssy, L. Szilágyi, B. Ábrahám, Sz. Lányi,* Engineered Green Fluorescent Protein as a Potential Metal Sensor. Studia Universitatis Babes-Bolyai Chemia, 2009(Special Issue): pp. 35-45.