EXCLI Journal 2009;8:89-96 – ISSN 1611-2156
Received: April 15, 2009, accepted: May 03, 2009, published: May 05, 2009

Original article:

INSTANTANEOUS MONITORING OF HYDROXYL RADICAL-MEDIATED PROTEIN ALTERATIONS BY GREEN FLUORESCENT PROTEIN

Chartchalerm Isarankura-Na-Ayudhya, Nanchaya Seesai, Sittichai Thinjom, Virapong Prachayasittikul*

Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok 10700, Thailand
* Corresponding author: E-mail: mtvpr@mahidol.ac.th; Telephone: 662-441-4376, Fax: 662-441-4380

ABSTRACT

In the present study, green fluorescent protein (GFP) is successfully applied for instantaneous monitoring of hydroxyl radical-mediated protein alterations. Hydroxyl radical generated from metal-mediated Fenton's reaction (in the presence of 50 µM copper ions, 10 mM ascorbic acid and 1.05 % hydrogen peroxide) rapidly suppressed the fluorescent emission of 60 % in a few seconds followed by a gradual decrease up to 75 % maximum inactivation was reached. The production of hydroxyl radical was experimentally proven to be specifically derived from copper-catalyzed Fenton's reaction in which other divalent cations (e. g. Zn^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+}) exerted no inhibitory interaction. Supplementation of oxidative scavengers and metal chelators into the assay reaction provided protective effects on the fluorescent intensity. The degree of protection was in the order of EDTA > histidine >>> glutathione ~ sodium azide > thiourea ~ mannitol. The findings herein gain insights not only into the deleterious effect of reactive oxygen species on biological macromolecules but also the potential applicability as a versatile antioxidant screening assay.

Keywords: Green Fluorescent Protein (GFP), Cadmium-binding peptide (CdBP), Antioxidant screening, Fenton's reaction, Hydroxyl radical

INTRODUCTION

Reactive oxygen species (ROS) are highly reactive molecules owing to the presence of unpaired electrons at the valence shell. The ROS are implicated in cellular activities such as inflammatory responses, cellular dysfunction and apoptosis. Such toxic effects involved in the damage of DNA, lipid peroxidation, oxidations of amino acid, and inactivation of enzymes participated in cellular metabolism (Sorg, 2004; Valko et al., 2007). Direct detection of ROS can be carried out by using electron spin resonance (ESR) or electron paramagnetic resonance (EPR) spectroscopy. However, applications of the technique become limited due to the following reasons: i) requirement of sophisticated instrumentation, ii) unstability of the ROS for the measurements and iii) interference of electron trapping in the liquid phase (Floyd, 2009; Kopani et al., 2006; Swartz et al., 2007). Therefore, several indirect methods e. g. detection of oxidative products, analysis of antioxidative levels and other measurement systems have continuously been developed (Cao et al., 1993; Giammarioli et al., 1999; Mordente et al., 1987; Tangkosakul et al., 2009).

Green fluorescent protein (GFP) is an auto-fluorescent protein isolated from Pa-

EXCLI Journal 2009;8:89-96 – ISSN 1611-2156
Received: April 15, 2009, accepted: May 03, 2009, published: May 05, 2009

cific Northwest jellyfish, *Aequorea victoria* (Tsien, 1998). It has extensively been applied as reporter of gene expression and protein localization, as immunological diagnostic tools, and as biological sensing molecules for metal and chemical toxic compounds (Hutter, 2006; Li et al., 2008; Suwanwong et al., 2006; Tansila et al., 2007, 2008). Its fluorescent emission is generated by specific cyclization reactions in the tripeptide Ser65–Tyr66–Gly67 and further oxidation in the chromophore (Tsien, 1998). Although the GFP is accepted to be very stable in various hazardous conditions, it has been believed that the GFP is damaged by singlet oxygen ($^1O_2$) formed in the chromophore upon fluorescent excitation, known as photobleaching (Greenbaum et al., 2000). In addition, exposure to UV-photolysis of hydrogen peroxide results in the loss of its autofluorescence, possibly by oxyradical-induced GFP denaturation (Alnuami et al., 2008). However, the underlying mechanisms still remain not fully understood.

Under the oxidative stress, it is well established that proteins are a major target of oxidative-induced protein dysfunction and degradation (Lushchak, 2007). In the present study, the GFP has been used as a reporter molecule for real-time monitoring of hydroxyl radical derived from Fenton's reaction (as shown in Figure 1). The metal-catalyzed Fenton's reaction has been selected since i) it is particularly important as invasive reaction associated with biological systems, ii) the internal effect of UV-induced GFP denaturation as abovementioned can be ruled out and iii) the reaction can be completed within a few seconds in which high-throughput screening of oxidative scavengers can be developed. Therefore, three of the components including copper ions, ascorbic acid and hydrogen peroxide required in the reaction have been optimized for further experiments. Detection of fluorescent emission as a consequence of radical inactivation can kinetically be performed without any additional substrates or cofactors. Protective roles of antioxidants and metal chelators against hydroxyl radical have been investigated.

MATERIALS AND METHODS

Materials

Escherichia coli (*E. coli*) strain TG1 (lac-pro), *Sup* E, *thi* 1, *hsd* D5/F' *tra* D36, pro A^+ B^+, *lac*I, *lac*Z, M15; (*ung*$^+$, *dut*$^+$) harboring pGFPuv (Clontech Laboratories, USA) or pCdBP$_4$GFP (Prachayasittikul et al., 2001) was used for gene expression. The GFP carrying four-repetitive sequences of cadmium-binding peptide (CdBP$_4$GFP) has previously been engineered and applied as potential tools for cell- and membrane-based metal sensor (Prachayasittikul et al., 2001, 2004, 2005). Chelating Sepharose Fast Flow was purchased from Pharmacia Biotech, Sweden. All other chemicals were of analytical grade and commercially available.

Protein expression

Cells were subcultured on LB (Luria-Bertani) agars supplemented with 100 mg/L ampicillin at 37 °C for overnight. Colonies possessing green fluorescence were selected under UV irradiation and further inoculated into 5 ml LB/Amp broths. Cultivation was performed at 37 °C for 6 hrs to reach mid-exponential phase. Cells were subsequently diluted at 1:100 in 500 ml LB/Amp and grown at 37 °C for 2-3 hrs with shaking (150 rpm). Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM at OD_{600} = 0.5.

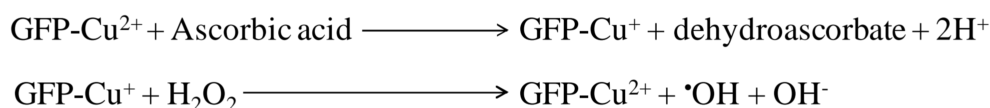

Figure 1: Inactivation of green fluorescent protein by copper-mediated Fenton's reaction

EXCLI Journal 2009;8:89-96 – ISSN 1611-2156
Received: April 15, 2009, accepted: May 03, 2009, published: May 05, 2009

Cells were further incubated until 16-18 hrs and collected by centrifugation at 6,000 rpm for 15 min at 4 °C. Cell pellet was resuspended in 50 mM Na_2HPO_4, 0.3 M NaCl, pH 7.4 (PBS). Cell lysis was performed by sonic disintegration. Cell debris was removed by centrifugation at 10,000 rpm for 15 min at 4 °C and the crude supernatant containing either native GFPuv or $CdBP_4GFP$ was collected.

Purification of native GFPuv by organic solvent extraction

Purification of GFPuv was performed by organic solvent extraction (Yakhnin et al., 1998) with minor modifications. Initially, ammonium sulfate and Triethanolamine base (TEA) were added into the crude extract of GFPuv to yield the final concentrations of 1.6 M and 100 mM, respectively. The solution was laid on ice for 1 h to precipitate other host proteins. These precipitants were discarded by centrifugation at 6,000 rpm, 4 °C for 20 min. Then, ammonium sulfate was slowly added to the supernatant until the final concentration of 2.8 M was reached. Precipitated GFPuv portion was collected by centrifugation as mentioned above.

As schematically depicted in Figure 2A, the pellet of GFPuv was mixed with absolute ethanol at 1:4 (ethanol:pellet) and further centrifuged at 4,000 rpm for 5 min at room temperature. At this step, the GFPuv was dissolved in the upper (organic) phase. Then, *n*-butanol was added at 1:4 (butanol:upper fraction) and the solution was spun. The GFPuv dissolved in the butanol phase was collected and subsequently mixed with an equal volume of chloroform. The aqueous phase containing GFPuv was isolated.

To further separate the GFPuv from other biological macromolecules, the purified fraction was loaded onto a Phenyl Sepharose column pre-equilibrated with 20 mM Tris–HCl, pH 8.0, 1 mM EDTA containing 20 % $(NH_4)_2SO_4$. The column was then washed with the same buffer. Elution of GFPuv was performed by supplementation with buffer containing 0 % $(NH_4)_2SO_4$. Eluate fraction was further dialyzed in PBS and stored until used.

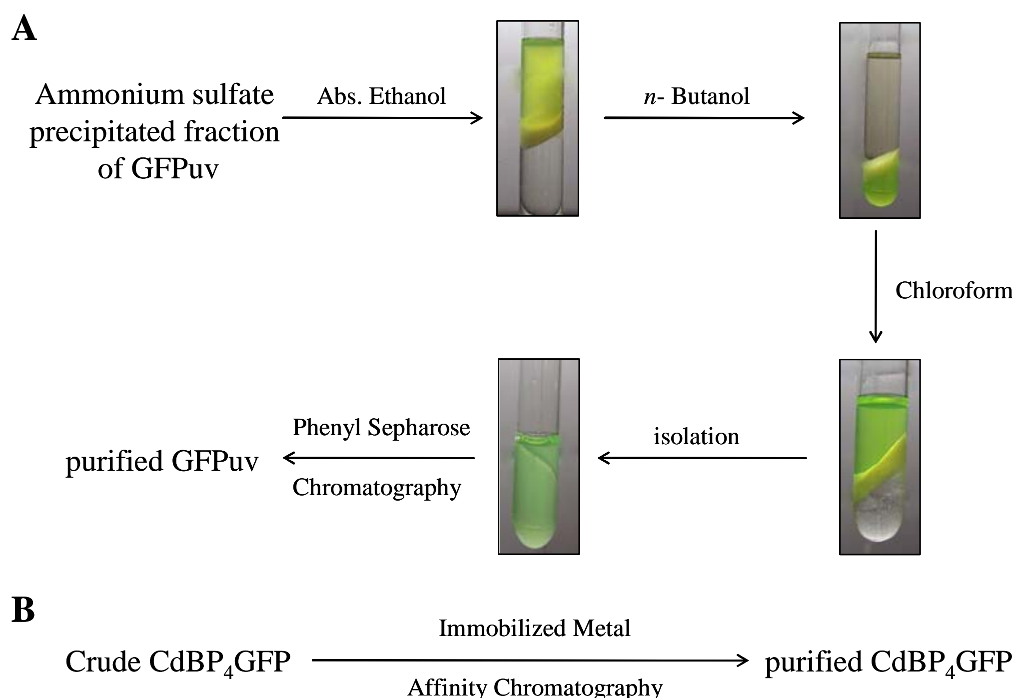

Figure 2: Purification of native GFPuv by organic extraction (A) and $CdBP_4GFP$ by immobilized metal affinity chromatography (B)

EXCLI Journal 2009;8:89-96 – ISSN 1611-2156
Received: April 15, 2009, accepted: May 03, 2009, published: May 05, 2009

Purification of chimeric $CdBP_4GFP$ using immobilized metal affinity chromatography

Purification of chimeric $CdBP_4GFP$ was performed in one-step affinity chromatography (Figure 2B). Briefly, the crude extract of $CdBP_4GFP$ was filtered through the millipore filter (0.45 µm), then subsequently applied to a Metal Chelating Sepharose 6B column (0.7 × 8 cm) charged with 20 mM $ZnSO_4$. Unbound proteins were removed by washing with at least 10 column volumes of PBS. Elution of the chimeric $CdBP_4GFP$ from the column was carried out using PBS supplemented with 20 mM EDTA. The eluted fractions were collected and the fluorescent intensity and optical density of sample at 280 nm were recorded for fraction selection. Fractions were pooled, dialyzed in PBS, and stored at -20 °C until used.

Determination of protein concentration and fluorescent intensity

Protein concentration was determined by using dye binding assay (Bio-rad Laboratories, USA). Bovine serum albumin was used as a standard. Fluorescence was assayed by excitation of the purified GFPs at 395 nm and subsequent emission of photons at 509 nm was recorded via fluorescence multi-well plate readers (FLx800, BIOTEK, USA).

Inactivation of auto-fluorescence of GFPs by Reactive Oxygen Species (ROS) generated from Fenton's reaction

Effect of ROS generated from Fenton's reaction (Feldberg et al., 1985) on GFPs was evaluated by incubating 50 µM GFPuv or $CdBP_4GFP$ with various concentrations of Cu^{2+} (50 nM to 0.5 mM), ascorbic acid (0.625 to 10 mM) and H_2O_2 (0.131 % to 1.05 %) for 1 min prior to investigation of fluorescent intensity. Optimized concentrations of copper (50 µM), ascorbic acid (10 mM) and hydrogen peroxide (1.05 %) were selected for further investigations.

To further investigate whether copper ions play important roles on ROS production and inactivation of fluorescent property, other kinds of divalent cations (e. g. Zn^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+}) at 50 µM were incubated with the protein solutions in the presence of 10 mM ascorbic acid and 1.05 % H_2O_2 for comparison.

Protective activity of antioxidants

To test for the efficiency of antioxidants in protecting the effect of ROS on GFPs, various antioxidants were individually incubated with protein solution for 1 min and the Fenton's reaction was subsequently applied. In some circumstances, metal chelators were then added for comparison.

RESULTS AND DISCUSSION

Deterioration of auto-fluorescence of GFPs by metal-mediated Fenton's reaction

Generation of ROS (in particular hydroxyl radical) by metal-mediated Fenton's reaction has successfully been monitored in real-time by determining the decrease of fluorescent emission of GFPs. The reaction requires optimum concentrations of three components: copper ions (50 µM), ascorbic acid (10 mM) and hydrogen peroxide (1.05 %). As represented in Figure 3, a rapid declining (rate ~3 %/sec) of fluorescence up to 60 % was observed within 10 sec followed by a gradual decrease (rate ~0.02 %/sec) until the fluorescence remained at approximately 25 % (10 min). A combination of copper ions and hydrogen peroxide also gave rise to the production of ROS (Tangkosakul et al., 2009), in which a slower rate of fluorescence reduction (~0.07 %/sec) was continuously found. Neither one nor two of other components exerted any suppressing effect on the fluorescent intensity. More importantly, such effect on the deterioration of auto-fluorescence of GFPs by hydroxyl radical was found to be attributable to protein degradation (data not shown). Supportive evidences on the degradation of lactate dehydrogenase enzyme and bovine serum albumin by hydroxyl radical have also been documented (Tangkosakul et al., 2009). These are as well correlated with the find-

EXCLI Journal 2009;8:89-96 – ISSN 1611-2156
Received: April 15, 2009, accepted: May 03, 2009, published: May 05, 2009

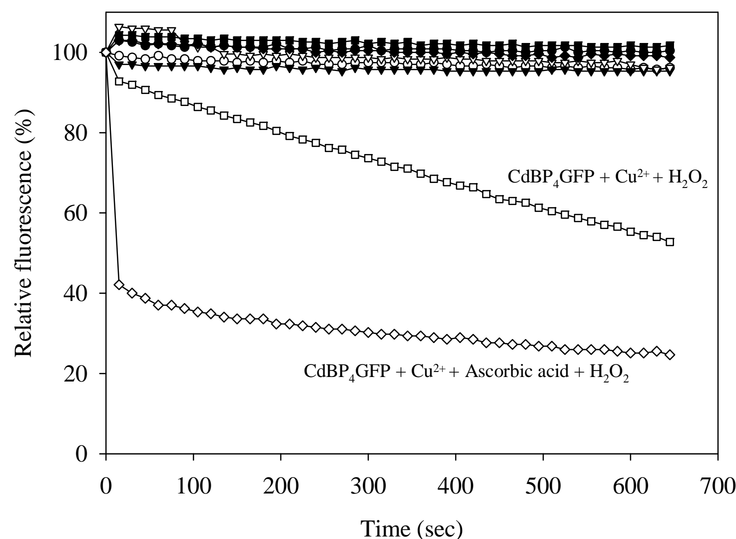

Figure 3: Effects of copper ions, ascorbic acid and hydrogen peroxide on fluorescent emission of $CdBP_4GFP$. Symbols represented as control $CdBP_4GFP$ (closed circle), $CdBP_4GFP$ + 50 μM Cu^{2+} (opened circle), $CdBP_4GFP$ + 10 mM ascorbic acid (closed triangle), $CdBP_4GFP$ + 1.05 % H_2O_2 (opened triangle), $CdBP_4GFP$ + Cu^{2+} + ascorbic acid (closed square), $CdBP_4GFP$ + Cu^{2+} + H_2O_2 (opened square), $CdBP_4GFP$ + ascorbic acid + H_2O_2 (closed diamond), $CdBP_4GFP$ + Cu^{2+} + ascorbic acid + H_2O_2 (opened diamond).

ings that the loss of GFP fluorescence led to the increase of protein carbonyl formation, an indicator of protein damage (Alnuami et al., 2008).

To further confirm that production of hydroxyl radical is specifically mediated by metal-catalyzed Fenton's reaction, other divalent cations were added instead of copper. Figure 4 demonstrates that the presence of zinc ions in the reaction resulted in no significant difference in fluorescent emission as compared to the control. Addition of other metal ions (Cd^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+}) also exhibited the same phenomenon (data not shown).

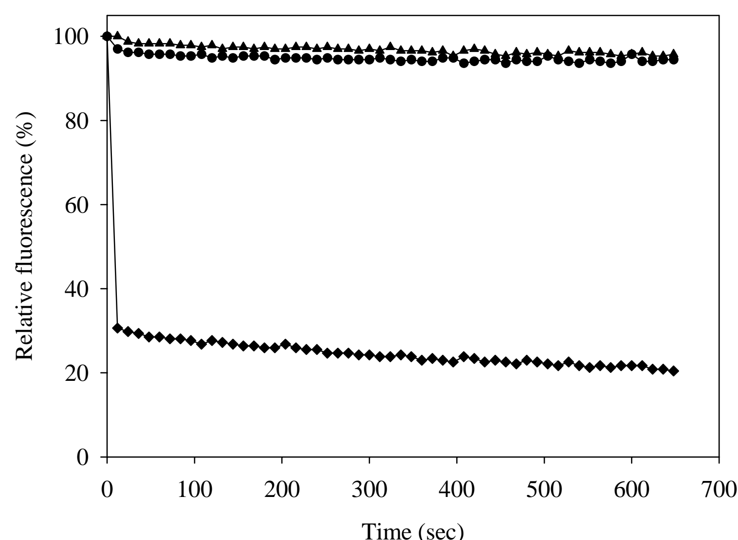

Figure 4: Effects of divalent cations on metal-mediated Fenton's reaction and fluorescent emission of $CdBP_4GFP$. Symbols represented as control $CdBP_4GFP$ (circle), $CdBP_4GFP$ + Cu^{2+} + ascorbic acid + H_2O_2 (diamond), $CdBP_4GFP$ + Zn^{2+} + ascorbic acid + H_2O_2 (triangle).

EXCLI Journal 2009;8:89-96 – ISSN 1611-2156
Received: April 15, 2009, accepted: May 03, 2009, published: May 05, 2009

Figure 5 illustrates the effect of hydroxyl radical on fluorescence of native GFPuv and $CdBP_4GFP$. A similar pattern of fluorescence deterioration was found in both cases. Notification has to be made that the GFPuv was a bit more sensitive to hydroxyl radical than the $CdBP_4GFP$. Explanation can be drawn on the protective role of Cd-binding peptide against metal-mediated Fenton's reaction (Tangkosakul et al., 2009). Although, it seems that such protection is in a lesser extent than the previous findings using Cd-binding lactate dehydrogenase. However, this is not surprising since the presence of Cd-binding peptide can not protect enzyme inactivation from hydroxyl radical in the presence of 5 mM ascorbic acid or 1 mM $CuSO_4$ (Tangkosakul et al., 2009).

In addition, the endogenous radical quenching capability of GFP may also be accounted for the remaining fluorescence of approximately 20-25 % (Bou-Abdallah et al., 2006; Alnuami et al., 2008).

Protective activity of antioxidants

Table 1 shows the protective effects of antioxidants and metal chelators on hydroxyl radical-treated $CdBP_4GFP$. Results revealed that the presence of antioxidants (e. g. thiourea, mannitol, sodium azide and glutathione) and metal chelators (e. g. histidine and EDTA) at 10 mM resulted in minute effect (~5-10 %) on fluorescent emission. Upon exposure to Fenton's reaction, the degree of protection against oxidative stress was in the order of EDTA > histidine >>> glutathione ~ sodium azide > thiourea ~ mannitol. Such protective activities of EDTA and histidine were more pronounced, possibly by disruption of Fenton's reaction upon Cu^{2+} chelation.

CONCLUSION

Assessment of the harmful effects of hydroxyl radical on biological macromolecules can successfully be performed by taking advantages of auto-fluorescent protein. Monitoring of fluorescent responses upon exposure to hydroxyl radical can be monitored in real-time without any additional substrates or cofactors as compared to the other systems (Mordente et al., 1987; Tangkosakul et al., 2009). Supplementation of antioxidants helps to prevent the radical-induced protein dysfunction and denaturation. Therefore, our findings presented herein open up a high possibility to further apply the GFP-based assay as robust and versatile tools for high-throughput screening of antioxidants in the future.

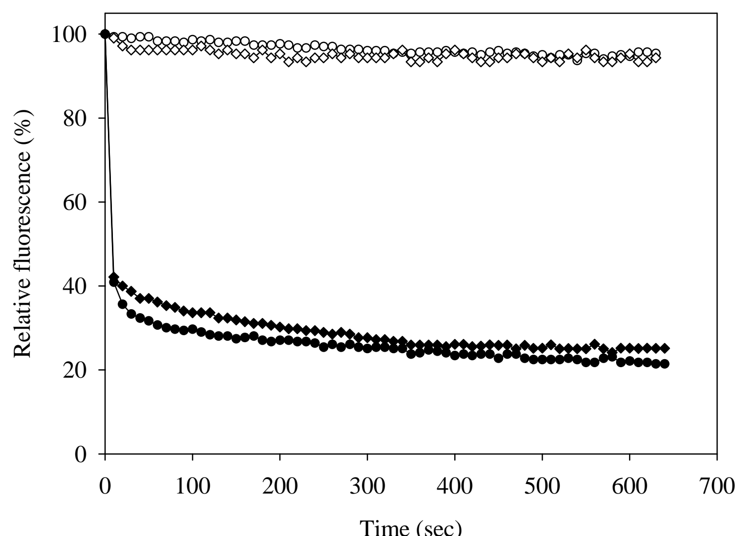

Figure 5: Effects of copper-mediated Fenton's reaction on fluorescent emission of GFPs. Symbols represented as *control systems*: GFPuv (opened circle) and $CdBP_4GFP$ (opened diamond); *tested systems*: GFPuv (closed circle) and $CdBP_4GFP$ (closed diamond).

Table 1: Effects of antioxidants and metal chelators on fluorescent emission of $CdBP_4GFP$ in the absence and presence of copper-mediated Fenton's reaction.

Testing conditions	Relative fluorescence (%)
None	100
$CdBP_4GFP$ + Cu^{2+} (50 µM) + Ascorbic acid (10 mM) + Hydrogen peroxide (1.05 %)	24
$CdBP_4GFP$ + Thiourea (10 mM)	91
$CdBP_4GFP$ + Mannitol (10 mM)	97
$CdBP_4GFP$ + Sodium azide (10 mM)	91
$CdBP_4GFP$ + Glutathione (10 mM)	88
$CdBP_4GFP$ + Histidine (10 mM)	91
$CdBP_4GFP$ + EDTA (10 mM)	92
$CdBP_4GFP$ + Cu^{2+} (50 µM) + Ascorbic acid (10 mM) + Thiourea (10 mM) + Hydrogen peroxide (1.05 %)	28
$CdBP_4GFP$ + Cu^{2+} (50 µM) + Ascorbic acid (10 mM) + Mannitol (10 mM) + Hydrogen peroxide (1.05 %)	28
$CdBP_4GFP$ + Cu^{2+} (50 µM) + Ascorbic acid (10 mM) + Sodium azide (10 mM) + Hydrogen peroxide (1.05 %)	37
$CdBP_4GFP$ + Cu^{2+} (50 µM) + Ascorbic acid (10 mM) + Glutathione (10 mM) + Hydrogen peroxide (1.05 %)	37
$CdBP_4GFP$ + Cu^{2+} (50 µM) + Ascorbic acid (10 mM) + Histidine (10 mM) + Hydrogen peroxide (1.05 %)	81
$CdBP_4GFP$ + Cu^{2+} (50 µM) + Ascorbic acid (10 mM) + EDTA (10 mM) + Hydrogen peroxide (1.05 %)	86

ACKNOWLEDGEMENTS

We thank Mahidol University for the annual budget grant (B.E. 2551-2555).

REFERENCES

Alnuami AA, Zeedi B, Qadri SM, Ashraf SS. Oxyradical-induced GFP damage and loss of fluorescence. Int J Biol Macromol 2008;43:182-6.

Bou-Abdallah F, Chasteen ND, Lesser MP. Quenching of superoxide radicals by green fluorescent protein. Biochim Biophys Acta 2006;1760:1690-5.

Cao G, Alessio HM, Cutler RG. Oxygen-radical absorbance capacity assay for antioxidants. Free Radic Biol Med 1993;14:303-11.

Feldberg RS, Carew JA, Paradise R. Probing Cu(II)/H_2O_2 damage in DNA with a damage-specific DNA binding protein. J Free Radic Biol Med 1985;1:459-66.

Floyd RA. Serendipitous findings while researching oxygen free radicals. Free Radic Biol Med 2009;46:1004-13.

Giammarioli S, Filesi C, Sanzini E. Oxidative stress markers: specificity and measurement techniques. Ann Ist Super Sanita 1999;35:563-76.

Greenbaum L, Rothmann C, Lavie R, Malik Z. Green fluorescent protein photobleaching: a model for protein damage by endogenous and exogenous singlet oxygen. Biol Chem 2000;381:1251-8.

Hutter H. Fluorescent reporter methods. Methods Mol Biol 2006;351:155-73.

Kopani M, Celec P, Danisovic L, Michalka P, Biro C. Oxidative stress and electron spin resonance. Clin Chim Acta 2006;364: 61-6.

Li YF, Li FY, Ho CL, Liao VH. Construction and comparison of fluorescence and bioluminescence bacterial biosensors for the detection of bioavailable toluene and related compounds. Environ Pollut 2008; 152:123-9.

Lushchak VI. Free radical oxidation of proteins and its relationship with functional state of organisms. Biochemistry (Moscow) 2007;72:809-27.

Mordente A, Miggiano GA, Martorana GE, Meucci E, Santini SA, Castelli A. Alkaline phosphatase inactivation by mixed function oxidation systems. Arch Biochem Biophys 1987;258:176-85.

Prachayasittikul V, Isarankura-Na-Ayudhya C, Bulow L. Lighting *E. coli* cells as biological sensors for Cd^{2+}. Biotechnol Lett 2001;23:1285-91.

Prachayasittikul V, Isarankura-Na-Ayudhya C, Boonpangrak S, Galla HJ. Lipid-membrane affinity of chimeric metal-binding green fluorescent protein. J Membr Biol 2004;200:47-56.

Prachayasittikul V, Isarankura-Na-Ayudhya C, Hilterhaus L, Hinz A, Tantimongcolwat T, Galla HJ. Interaction analysis of chimeric metal-binding green fluorescent protein and artificial solid-supported lipid membrane by quartz crystal microbalance and atomic force microscopy. Biochem Biophys Res Comm 2005;327:174-82.

Sorg O. Oxidative stress: a theoretical model or a biological reality? C R Biol 2004;327:649-62.

Suwanwong Y, Isarankura-Na-Ayudhya C, Bulow L, Prachayasittikul V. Insights into the genetic re-engineering of chimeric antibody-binding green fluorescent proteins for immunological taggers. EXCLI J 2006;5: 164-78.

Swartz HM, Khan N, Khramtsov VV. Use of electron paramagnetic resonance spectroscopy to evaluate the redox state *in vivo*. Antioxid Redox Signal 2007;9:1757-71.

Tangkosakul T, Tantimongcolwat T, Isarankura-Na-Ayudhya C, Majare M, Bulow L, Prachayasittikul V. Native and chimeric metal-binding lactate dehydrogenase as detection and protection tools for oxidative stress induced by Fenton's reaction. EXCLI J 2009;8:1-11.

Tansila N, Tantimongcolwat T, Isarankura-Na-Ayudhya C, Nantasenamat C, Prachayasittikul V. Rational design of analyte channels of the green fluorescent protein for biosensor applications. Int J Biol Sci 2007;3:463-70.

Tansila N, Becker K, Isarankura-Na-Ayudhya C, Prachayasittikul V, Bulow L. Metal ion accessibility of histidine-modified superfolder green fluorescent protein expressed in *Escherichia coli*. Biotechnol Lett 2008;30:1391-6.

Tsien RY. The green fluorescent protein. Annu Rev Biochem 1998;67:509-44.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39:44-84.

Yakhnin AV, Vinokurov LM, Surin AK, Alakhov YB. Green fluorescent protein purification by organic extraction. Protein Expr Purif 1998;14:382-6.