DEVELOPMENT OF NOVEL DNA-CUPRAC COLORIMETRIC PROBE FOR DETERMINATION OF HYDROXYL RADICAL DAMAGE AND RELATED ANTIOXIDANT ACTIVITY

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Abstract **-** An unbalance between reactive oxygen species and antioxidants in favour of oxidants may cause health issues. In the study DNA was used as probe material to measure oxidative damage caused by hydroxyl radical ($^{\bullet}OH$) and potential protective effects of certain water soluble antioxidants. $^{\bullet}OH$ was produced by Fenton method and a modified colorimetric CUPRAC method was developed. The absorbance difference originated from the fact DNA degradation products but not the original DNA was responsive to the CUPRAC reagent.

Keywords: *oxidative stress, hydroxyl radical, DNA sensor, antioxidant, CUPRAC method.*

1. INTRODUCTION

Reactive oxygen species (ROS) is a common name of a series of oxygen compounds either radicalic (e.g., hydroxyl ($^{\bullet}OH$), superoxide ($O_2^{\bullet-}$)) or non-radicalic (e.g., ozone (O_3), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2)). Reactive nitrogen species (RNS) depend on the same principle. These compounds may be at different reactivities [1]. Since ($^{\bullet}OH$) is the most reactive species among ROS, it has a special importance. ($^{\bullet}OH$) can react with all biomacromolecules such as DNA, lipids and proteins. Although ROS/RNS have some biologically necessary functions in the human body, their concentrations should be balanced with antioxidants. The oxidative damage of a certain molecule can be delayed, prevented or removed by antioxidants (Aox) [2]. Human body has its own antioxidative defense system consisting of certain enzymes and some low molecular weight compounds [3]. Except endogenous ones, dietary foodstuffs are the other important Aox sources. ROS can most specifically be detected by ESR, or electron spin resonance. This

system needs high-cost instrumentation and qualified operators restricting its common usage. For this reason, indirect determination methods of ROS attract attention. In these kinds of methods, a probe compound is used and the effect that radical causes on the probe material is measured. The most known method in this area is probably the thiobarbituric acid reactive substances (TBARS) assay [4]. The method is mostly used for ROS determination and it is quite open to interferences [5]. A few years ago, Bektaşoğlu, Özyürek, Güçlü and Apak were able to determine $^{\bullet}OH$ by using a salicylate probe in a modified CUPRAC method [6]. In similar other methods for indirect determination of hydroxyl radical, hydroxylation of the aromatic ring is employed, such as aminosalicylate, and 3- (or 4-) hydroxybenzoate [7]. Use of DNA as a probe material has great importance to understand oxidative damage in the body [8]. Guanine is the most easily oxidized base of DNA, and therefore to measure 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) compounds formed by oxidation of guanine and/or 2'-deoxyguanosine is highly common. On the other hand, besides 8-OHdG and 8-oxodG, there are a series of other DNA oxidation products over twenty [9]. So determination of all oxidation products may be much more meaningful to measure total DNA damage.

2. EXPERIMENTAL

2.1. Reagents and solutions

In the study, three different types of DNA were used: herring sperm, fish sperm and calf thymus DNA (hs, fs and ctDNA, respectively). While hs and fsDNA solutions were prepared at 2.0 mg mL^{-}

[1]concentrations, ctDNA was prepared at 4.0 mg mL^{-1} in pH 7 phosphate buffer. ***Aox samples***: L-glutathione reduced, GSH; ascorbic acid, AA; N-acetyl cysteine, NAC; gallic acid, GA; cysteine, CYS; homocysteine, HCYS) were prepared at 10 mM concentrations. For dissolving CYS and HCYS were 0.5 mL of 1.0 M HCl was used and they were completed to volume with ultrapure water (UPW). The other Aox were prepared in UPW directly. To prepare $(NH_4)_2Fe(SO_4)_2.6H_2O$ at 2.0×10^{-2} M, a suitable amount of compound was dissolved by 1.0 mL of 1.0 M HCl and diluted to volume with UPW. To prepare H_2O_2 solution at 1.0×10^{-2} M, 30% (w/v) was diluted at appropriate ratio. To prepare 2-Thiobarbituric acid (TBA) solution at 0.35 M a suitable amount of solid was dissolved in 0.3 M NaOH. The other solutions were as follows 1.0 M CH_3COONH_4 (NH_4Ac) (pH 7.0 buffer), 0.6 M thrichloroacetic acid (TCA), 1.0×10^{-2} M $CuCl_2.2H_2O$, 2.0×10^{-2} M Na_2EDTA, 1.5% (w/v) Na_2CO_3 and 7.5×10^{-3} M neocuproine (Nc). Only Nc was prepared in ethyl alcohol (EtOH) and other mentioned reagents in UPW.

2.2. Formation of $^{\bullet}$OH and oxidation of DNA: 1.5 mL pH 7 phosphate buffer + 0.5 mL DNA (2.0 mg mL^{-1}) + 0.25 mL Na_2EDTA (20 mM) + 0.25 mL $(NH_4)_2Fe(SO_4)_2.6H_2O$ (20 mM) + (2-x) mL UPW + x mL Aox (as radical scavenger) + 0.5 mL 10 mM H_2O_2 were mixed. After the reaction mixture was let to stand for 10 min in a water bath at 37°C, reaction was stopped by adding 1.25 mL of TCA (0.6 M) (V_{final} = 6.25 mL).

2.3. Determination of DNA Oxidation Products

Modified CUPRAC Method: 1.0 mL $CuCl_2$ (1.0×10^{-2} M) + 1.0 mL Nc (7.5×10^{-3} M) + 2.0 NH_4Ac (1.0 M) + 0.5 mL incubated solution of DNA (as described in 2.2) + 0.5 mL Na_2CO_3 (1.5%). After the mixture kept at room temperature for 5 min, absorbance was read at 450 nm against a reagent blank [10].*

Preparation of Reagent Blank: To prepare a reagent blank, 0.5 mL oftheincubation solution (described in 2.2 without DNA or any scavenger Aox) was taken and used as reagent blank.

2.4. Modified TBARS Method: TBARS method originally developed by Halliwell and Gutteridge [4] was modified by Li, Mai, Wang and Han to determine DNA oxidative damage spectrophotometrically [11]. This method was applied as standard method after slight modifications. DNA oxidation was realized as described at 2.2. so as to achieve the same DNA damage. In the procedure, after addition of 1.25 mL TCA only, 0.75 mL of TBA (0.35 M) was added to the reaction medium (V_{Final}= 7.0 mL). Obtained reaction mixture was let to stand for 15 min in a boiling water bath, and the absorbance was read at 530 nm against phosphate buffer.

2.5. Determination of Aox Activity: ToInvestigate protective effect of Aox compounds on DNA damage, a series of water-soluble Aoxs were tested. For this purpose, different volumes of Aox solution between 0.1 – 1.5 mL were added to the Fenton system (as described in 2.2) The concentration of selected Aox were 1.0 mM forCYS, NAC, AA and GA and 5.0 mM for GSH andHCYS. The modified CUPRAC method (as described in 2.3) was applied with or without Aox samples and absorbance differences were calculated in the presence and absence of Aox compounds (ΔA). For each of tested samples, these ΔA values were recorded against Aox concentration and calibration graphs were obtained.

2.6. Investigation of Correlation Between Proposed Modified CUPRAC Method and Reference TBARS Method: For this purpose, a suitable concentration range was determined for both methods. The final concentration interval was chosen as 3.20 – 16.0 mg mL^{-1} and Fenton procedure was applied as described in 2.2.; then modified CUPRAC and TBARS methods were applied separately. The calibration graphs were drawn for the two methods between concentration and absorbance. Finally, the absorbance values of the two methods corresponding to identical concentrations were placed to abscissa and ordinate for investigating the correlation between the two tested methods, namely modified CUPRAC and TBARS.

3. RESULTS AND DISCUSSION

3.1. ***Controls of DNA Samples Before and After Oxidative Damage:*** For this purpose, three kinds of synthetic DNA samples, namely hs, fs and ctDNA, were subjected to Fenton procedure as described earlier (section 2.2) and before and after Fenton reaction, modified CUPRAC and TBARS methods were applied. In the study hs and fs DNA were at

2.0 mg mL^{-1} and ctDNA was 4.0 mg mL^{-1}. 0.5 mL volumes of aliquots were taken, and modified CUPRAC and TBARS methods were applied directly (without Fenton reaction). The modified CUPRAC absorbances measured for hs, fs and CtDNA were 0.0028, 0.0038 and 0.0092, respectively. Likewise, the absorbances were nearly zero for TBARS reaction. To control the oxidatively damaged DNA, same amount of samples was subjected to Fenton reaction and two spectrophotometric methods were applied after Fenton procedure. For three replicates, modified CUPRAC absorbances were as follows: 0.4099±0.13 (for hsDNA), 0.4212 ± 0.14 (for fsDNA) and 0.2696 ± 0.18 (for ctDNA). On the other hand, for TBARS method absorbance were 0.6410 ± 0.11, 0.4661 ± 0.09 and 0.8587 ± 0.10 for hsDNA, fsDNA and ctDNA, respectively. Since modified CUPRAC and TBARS assays had different sensitivities, samples were diluted at different ratios. Final concentrations were 0.016 mg mL^{-1} for hs and fsDNA, 0.032 mg mL^{-1} for ctDNA with modified CUPRAC method, and 0.140 mg mL^{-1} for hs and fsDNA and 0.280 mg mL^{-1} for ctDNA with TBARS method.

3.2. Determination of Aox Activity and Protective Effects of Selected Water-Soluble Aox Compounds:

There are a few commonly used solvents to dissolve Aox and to extract antioxidative compounds in plant matrices such as ethanol, methanol, acetone, acetonitrile and dimethyl sulfoxide. To test the effects of these solvents on the presented methods, 0.5 mL aliquots were taken and diluted at different ratios. Fenton oxidation procedure (section 2.2) followed by modified CUPRAC assay (section 2.3) yielded positive results in the diluted solvents. Therefore, only water-soluble Aox compounds were tested so as to eliminate positive error from solvents. The selected Aox compounds were GSH, AA, NAC, GA, CYS, and HCYS. These compounds were added to the Fenton system and modified CUPRAC method was applied. In the presence of Aox compounds showing radical scavenging effects, obtained modified CUPRAC absorbances decreased as expected. The decrease in the absorbance was named as ΔA. Modified CUPRAC absorbances were recorded in the presence and absence of Aox compounds at different concentrations. For all tested Aox compounds, calibration graphs were drawn between concentration and ΔA. The regression equations of calibration graphs were calculated using Microsoft Excel 2016 (Table 1). A sample graph for GSH is shown in Figure 1.

Table 1. Equations of Linear Calibration Graphs between concentration (C) and absorbance difference (ΔA), and R^2 Values for Tested Aox Compounds according to CUPRAC method for fs DNA

AOx compound	Final concn. range (M)	Linear equations of calibration for tested Aox compounds
CYS	8.0×10^{-7}-1.12×10^{-5}	$\Delta A=3.00\times10^{4}C + 0.02$ (R^2 = 0.9846)
HCYS	8.0×10^{-6}-8.0×10^{-5}	$\Delta A=0.48\times10^{4}C + 0.07$ (R^2 = 0.9905)
NAC	1.6×10^{-6}-1.28×10^{-5}	$\Delta A=3.76\times10^{4}C + 0.06$ (R^2 = 0.9663)
GSH	8.0×10^{-6}-5.6×10^{-5}	$\Delta A=0.85\times10^{4}C + 0.09$ (R^2 = 0.9925)
AA	3.2×10^{-6}-1.6×10^{-5}	$\Delta A=2.35\times10^{4}C + 0.08$ (R^2 = 0.9076)
GA	8.0×10^{-7}-8.0×10^{-6}	$\Delta A=5.06\times10^{4}C + 0.08$ (R^2 = 0.9719)

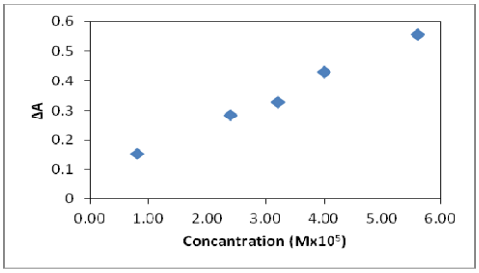

Figure 1. Calibration graph between concentration and absorbance difference (ΔA) in the presence and absence of GSH for modified CUPRAC method.

Aox activity of tested compounds could be sorted according to their slopes as: GA > NAC > CYS > AA >GSH > HCYS. Similar experiments were repeated for TBARS method and activity of tested water-soluble Aox were sorted as: CYS > AA > GSH > NAC > HCYS (Because of the dark color of GA at tested concentration, it could not be examined by TBARS method)

3.3. ***Correlation Between modified CUPRAC and TBARS Methods:*** Experiments were conducted as described at 2.6. When calibration graphs were drawn between Aox concentration and absorbance,

determination coefficients (R^2) were found as 0.999 and 0.9834 for modified CUPRAC and TBARS methods, respectively. Then TBARS absorbances were drawn against CUPRAC absorbances for identical fsDNA samples after subjecting to Fenton procedure. There was a good correlation between the two spectrophotometric methods, the correlation coefficient being 0.9727 (Figure 2). This good correlation between the proposed CUPRAC and reference TBARS methods can be explained by the fact that both methods measure the total oxidative damage on DNA *via* a representative product.

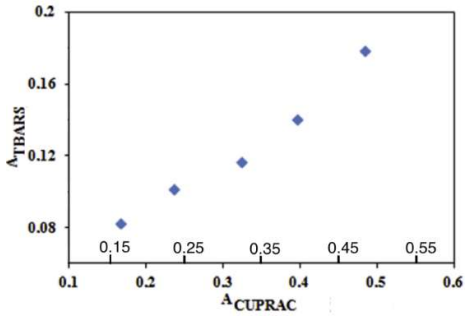

Figure 2. Calibration graph as A_{TBARS} *versus* A_{CUPRAC} for identical DNA solutions Fenton-oxidized

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