POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES

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Pol. J. Food Nutr. Sci.
2009, Vol. 59, No. 2, pp. 123-127

EVALUATION OF ANTIOXIDANT ACTIVITY OF ALPHA-TOCOPHEROL AND QUERCETIN DURING OXIDATION OF PHOSPHATIDYLCHOLINE USING CHEMILUMINESCENT DETECTION OF LIPID HYDROPEROXIDES

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Key words: luminol chemiluminescence, phosphatidylcholine, antioxidant activity, tocopherol, quercetin

Lipid hydroperoxides are relatively stable intermediates during oxidation of fats and lipids. Lipid hydroperoxide formation during phosphatidylcholine thermal autoxidation was measured with the luminol-enhanced chemiluminescent (LCL) method. The method optimized for best noise/signal ratio provided a reliable and sensitive data up to 50 pmol/L of detected hydroperoxide. The observed chemiluminescence was calibrated with (9Z,11E,13S)-hydroperoxyoctadeca-9,11-dienoic acid (13-HPODE) as an external standard. To validate this method the spectrophotometric assay at 234 nm was compared with LCL results and high correlation was found. The presence of antioxidants inhibited chemiluminescence in a dose--dependent manner. Quercetin at low concentration, up to 2 μmol/L, whereas α-T above 20 μmol/L exhibited prooxidative properties. Possible mechanisms were discussed. The described LCL method is sensitive, fast and simple, is not specific to the lipids but may be used to determine the presence of the other types of peroxides.

INTRODUCTION

Lipid oxidation is considered as a free radical chain reaction with the first step as hydrogen abstraction followed usually by lipid hydroperoxide formation. Hydroperoxides exhibit positional and geometrical distributions of isomers, which depends on fatty acid and oxidation environment [Porter *et al.*, 1995]. Despite the fact that the formed lipid free radicals may undergo many reactions, the hydroperoxide intermediates are the key molecules showing the progress of lipid oxidation. In the absence of decomposing factors as redox-active metals, heat, ultraviolet light or antioxidants, hydroperoxides are relatively stable products of lipid oxidation. In such a case, the formation of hydroperoxides is a good measure to detect early stages of lipid oxidation, however, the reaction condition and solvent should be taken into account.

There are ample analytical methods available to measure oxidation of lipids. The choice of the method depends on what parameter is measured and which phase of oxidation we want to monitor. To measure total hydroperoxide content during oxidation the peroxide value (PV) has been introduced. This value was measured by a variety of methods including iodometric titration [Simic *et al.*, 1992], colorimetric methods measuring the ability of lipid hydroperoxides to oxidize ferrous ions to ferric ions, which are complexed by dyes as for example by xylenol orange, and in this case called the FOX method [Frankel, 1991], FTIR spectroscopy [Dauben *et al.*, 1969], conjugated dienes and trienes [Waters, 1971]. Chemiluminescence (CL) method has frequently been used to monitor on-line detection for lipid hydroperoxides in connection with HPLC [Ohba *et al.*, 2002]. The use of the CL method for analyzing lipid peroxidation was reported by Vladimirov & Petrenko [1976]. Since then, the method has been modified to avoid some drawbacks. Application of luminol-enhanced CL shifted the detection limits of hydroperoxide to the level of single picomoles, the sensitivity unreachable by the other analytical methods [Navas & Jimenez, 1996].

The objective of this study was to report a new procedure of luminol-enhanced CL for determining the antioxidant potential of α-tocopherol and quercetin during phospholipids oxidation in liposomes. During this reaction, luminol is oxidized by free radicals formed during hemin-catalysed disruption of lipid hydroperoxides as well as by hemin itself. The process of luminol oxidation is accompanied by emission of light with the maximum at 430 nm. The CL intensity is proportional and increases with an increasing concentration of peroxides. Addition of α-tocopherol and quercetin, free radical scavengers, to a solution leads to a decrease in CL intensity, which may be used as a measure of their antioxidant activity. For our studies, we have modified the method of Vladimirov and calibrated the chemiluminescence scale with (9*Z*,11*E*,13*S*)--hydroperoxyoctadeca-9,11-dienoic acid as an external standard.

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MATERIALS AND METHODS

MATERIALS

Luminol, TRITON X-100, buffer CAPS, methanol, hemin, α-tocopherol, quercetin and phosphatidylcholine were purchased from SIGMA, doubly distilled water and the other reagents used were of HPLC grade. Pure 13-HPODE needed for chemiluminescence calibration was obtained by enzymatic oxygenation of linoleic acid, followed by HPLC purification of the major oxygenation products according to Spiteller *et al.* [2001].

Lecithin is usually used as a synonym for phosphatidylcholine (PC) which is the major component of the phosphatide fraction isolated from either egg yolk or soy beans. PC is a mixture of differently substituted *sn*-glycerol-3-phosphatidylcholine backbones. In PC from egg yolk in *sn*-1-position saturated acyl groups, and in *sn*-2-position, unsaturated fatty acids are more common. The applied lecithin from egg yolk is composed approximately of: 33% palmitic acid (16:0), 13% stearic acid (18:0), 31% oleic acid (18:1), 15% linoleic acid (18:2), other fatty acids being minor contributors, with average molecular weight of 768 g, which indicates that at least in 50% PC bilayer is composed of unsaturated fatty acids.

METHODS

Preparation of linoleic acid hydroperoxide (13-hydroperoxyoctadeca-9,11-dienoic acid) standard

The synthesis of free fatty acid hydroperoxides (FAOOHs) standards was carried out as described by Spiteller & Spiteller [1997] and Spiteller *et al.* [2001], but their procedures were modified for the synthesis of 13-hydroperoxyoctadeca-9,11--dienoic acid (13-HPODE) standards [Nogala-Kalucka *et al.*, 2007].

Oxidation of samples

Samples of 2 mg/mL of PC were dried in a stream of nitrogen and resuspended in 5 mL PBS containing 0.05% SDS and sonicated for 10 min in ice-cold water. After sonication, the tested antioxidants were added in 1, 5, 10, 20, 40 μmol/L concentrations. The incubation was carried out at 60±1°C for max. 24 h without light, and the hydroperoxides were determined periodically.

Chemiluminescence measurements

In order to obtain the highest sensitivity to detect hydroperoxides, reproducibility and low background some experiments have been carried out with changing luminol and hemin concentration. Finally, the chemiluminescent solution consisted of: luminol 0.1 mmol/L, 5% methanol, 0.025% TRITON X-100, buffer CAPS 0.1 mol/L pH 10, and hemin $5x10^{-6}$ mol/L. The chemiluminescent reaction was started by addition of hemin to the mixture containing thermally-oxidized lipid emulsion. A 10-mL cuvette was placed directly before an photocathode of an RCA EMI 9558 QB photomultiplier connected to data collecting board in a computer. The area under registered signal was used as a measure of CL. In order to obtain a blank level of CL emission, the registration started before any component was added to the cuvette.

Reagent composition studies

The most pronounced changes of LCL are connected with pH of the solution. Increasing pH from 9 to 11 causes 5 times increase of CL intensity. However, higher pH increases the rate of hydroperoxide decomposition and background. The chosen pH values are a compromise between those important factors affecting sensitivity and reproducibility of the method. Hemin concentration is another factor influencing the shape of CL. Its increasing concentration is narrowing the shape and at higher concentration it inhibits the CL. The effect of luminol concentration on CL signal exhibits the maximum at 40 μmol/L and was used at even higher concentration in order to increase oxidative action of the substrate towards luminol. Detergent, below its critical micelle concentration (cmc), was used in order to assure better dispersion and maintain stability of the emulsion. Detergent concentration above its cmc, due to micelle formation and diluting substrate, produced a very broad peak with slowly decaying CL.

RESULTS AND DISCUSSION

Chemiluminescence calibration

Detection of hydroperoxides in the sample occurs due to the fact that hemin catalyzes the oxidation of luminol to an electronically-excited form of 4-aminophthalate, which emits a photon upon radiative deexcitation to ground state. To obtain acceptable signal/noise ratio of the CL detection system some parameters were optimized, like concentrations of hemin, luminol and theirs ratios, the presence of SDS and methanol. The kinetics of the optimized chemiluminescent reaction is presented in Figure 1. It shows the maximum a few seconds after addition of hemin, then the signal decays exponentially until LCL intensity reaches about 10% of the maximal value, which takes about 2 minutes. In the absence of luminol a measurable ultraweak CL arising from the other free radicals reactions could be recorded but the intensity was at lest one order of magnitude lower than with luminol.

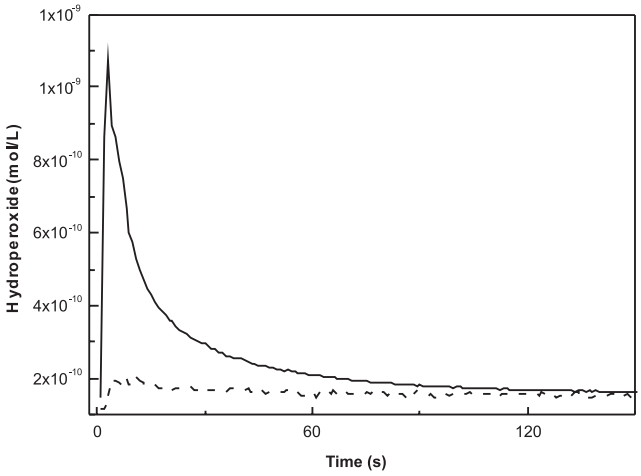

FIGURE 1. Typical LCL signal obtained during measurement. The reaction mixture contained in final volume of 3 mL: 100 pmol/L of LOOH, luminol 0.1 mmol/L, 5% methanol, 0.025% TRITON X-100, buffer CAPS 0.1 mol/L pH 10. The reaction was started by addition of hemin 510^{-6} mol/L 30 s after the beginning of data acquisition. The lower curve shows emission in the absence of luminol.

The background emission, *i.e.* in the absence of luminol, is also shown in Figure 1.

In order to estimate the sensitivity and feasibility of the LCL-method the external lipid hydroperoxide was used. The LCL intensity scale was calibrated using 13-HPODE as an external standard.

The calibration curve of integrated area of LCL emission *versus* lipid hydroperoxide standard concentration is presented in Figure 2. It shows linear dependence between the amount of hydroperoxide and LCL intensity. Background LCL due to hemin catalyzed decomposition of lipid hydroperoxides and hemin itself was subtracted for the calculation of the calibration curve. The background value determined the minimal detectable amount of lipid hydroperoxides and the sensitivity was estimated at about 50 pmol/L.

Thermal oxidation of PC liposomes

During feasibility studies of the LCL method, the amount of lipid hydroperoxide formed during thermal oxidation of PC membrane at 50, 60 and 70°C was measured. Next, the antioxidant activity of α-tocopherol and quercetin during oxidation of PC in emulsion was calculated with the LCL method. The results of heating phosphatidylcholine at different temperatures are presented in Figure 3. It shows that increasing temperature increases LCL intensity and, as expected, indicates the higher amount of hydroperoxides formed. At a given temperature, the prolonged time of oxidation leads to the higher quantities of the hydroperoxides formed. The increasing amount of hydroperoxides has been confirmed by HPLC and conjugated dienes methods (data not shown).

Antioxidant activity determination

In order to determine the antioxidant activity (AA) of tocopherol and quercetin with the LCL method against PC oxidation the temporal and concentration studies have been carried out. During thermal oxidation of PC membrane, the presence of the antioxidants, α-tocopherol and quercetin, decreased the LCL intensity (Figures 4 and 5). Both antioxidants are considered as inhibitors of primary products formed during oxidation of fatty acids and lipids. The mechanism of the observed effect is connected with the ability to scavenge the hydroperoxide radicals in the sample, thus the formation of hydroperoxides is diminished and finally less LCL is produced.

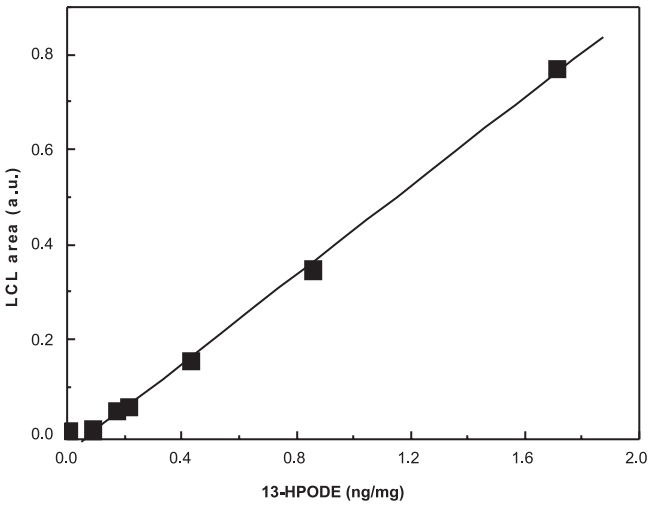

FIGURE 2. Calibration curve obtained as a plot of integrated LCL signal *versus* standard 13-HPODE concentration. The other concentrations of mixture as given in the Legend to Figure 1.

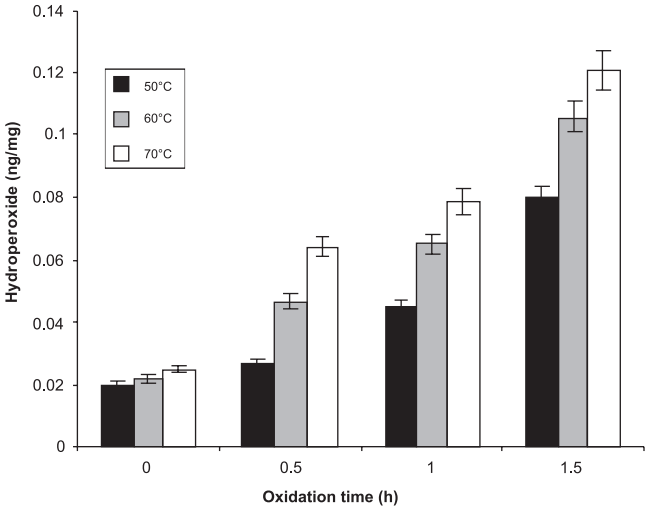

FIGURE 3. Hydroperoxides formation during thermal oxidation of PC membrane at 50°C, 60°C and 70°C measured after 0, 30, 60 and 90 min of oxidation.

Figure 4 presents the temporal results obtained for α-tocopherol, a membrane-soluble antioxidant, present in the sample at different concentrations during PC oxidation. It shows that the inhibitory effect of the antioxidant is observed up to 10 μmol/L. Similar results were reported by [Hirayama *et al.*, 1997] who determined antioxidant activity for natural antioxidant with the LCL method using an isoluminol/microperoxidase system. However, as shown in Figure 4, at a higher tocopherol concentration (*i.e.* above

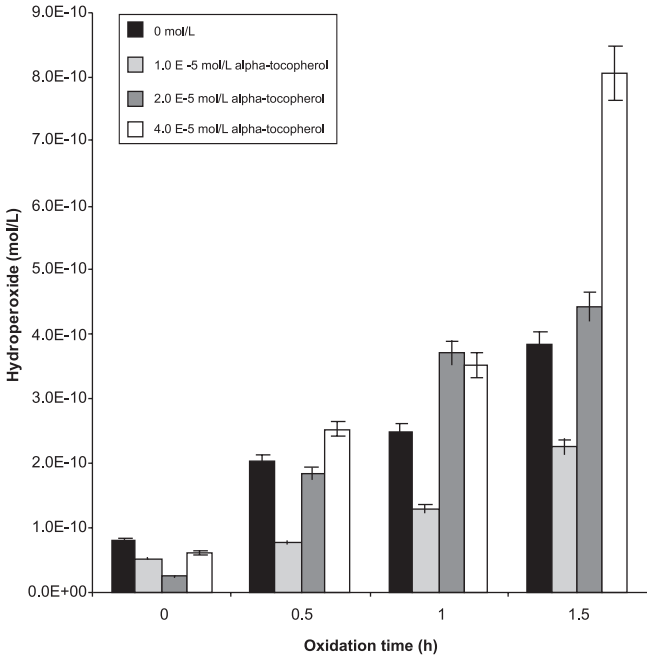

FIGURE 4. The amount of hydroperoxides measured in the presence of 0, 1×10^{-5}, 2×10^{-5} and 4×10^{-5} mol/L of alpha-tocopherol during oxidation of 2 mg/mL PC at 50°C.

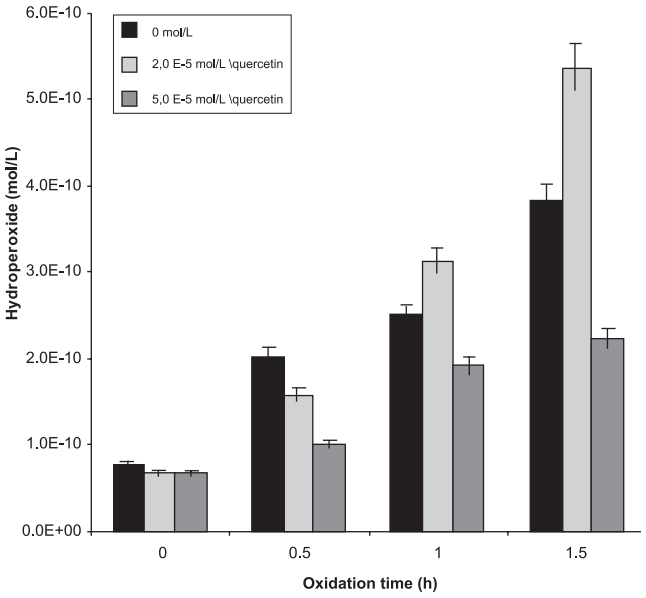

FIGURE 5. Hydroperoxides formation in the presence of 0, 2×10^{-6} and 5×10^{-6} mol/L of quercetin during 2 mg/mL PC oxidation at 50°C.

20 μmol/L), LCL concentration was observed to increase. This appeared to be contradictory to HPLC and conjugated dienes results giving lower hydroperoxide values at 20 and 40 μmol/L tocopherol concentration. At given conditions of the experiment, the 40 μmol/L concentration refers to 15 mol% of tocopherol in the PC membrane. Our previous studies [Dwiecki *et al.*, 2007] and these of other authors [Massey, 2001; Naumowicz & Figaszewski, 2005; Quinn, 1995; Wang *et al.*, 2000] have shown that 10 to 15 mol% is solubility limit of tocopherol in the membrane. In such a case, even higher tocopherol concentrations in the sample but not embedded into membrane do not protect oxidation of PC. The elevated LCL intensity reflects increasing amount of the hydroperoxides produced and very likely also the other types of the peroxides formed.

Figure 5 presents hydroperoxide formation under different concentrations of quercetin, a water-soluble antioxidant. In concentrations of up to 2 μmol/L quercetin exhibits prooxidative properties. Above that concentration LCL intensity decreases and above 10 μmol/L LCL emission is quenched in 90%. Such a behaviour is similar to that obtained by conjugated dienes and HPLC methods (data not shown). The peroxidation of unsaturated fatty acids in phospholipid liposomes may be inhibited by free radical traps as quercetin, which prevents further formation of lipid hydroperoxides. The decrease of LCL amplitude accompanying Fe^{+2}-induced peroxidation in phospholipid liposomes under higher concentrations of α-tocopherol and quercetin has been reported previously [Vasiljeva *et al.*, 2000].

Antioxidant activity is defined according to the formula (1):

$$AA = (I_0 - I_A)/ (I_0 xc) \quad (1)$$

where: (I_0) and (I) – LCL intensity (area) without and with antioxidant, respectively; (c) – antioxidant concentration.

The antioxidant activity calculated according to formula (1) at 10 μmol/L concentration equals 0.11 (1/μmol/L) for quercetin and 0.045 (1/μmol/L) for α-tocopherol. This better antioxidant activity of quercetin is due to the fact that water-soluble molecule resides in the membrane-water interphase, thus effectively preventing the formation of hydroperoxides inside the membrane.

CONCLUSIONS

The described chemiluminescent method provided very sensitive, fast and simple assay for antioxidant activity determination of natural antioxidants. According to the calibration curve, this method may detect hydroperoxides ranging from 50 pmol/L to 40 μmol/L. However, care should be taken regarding the range of antioxidant concentration. The LCL method is not specific for lipid hydroperoxides but may be used to follow enzymatic or non-enzymatic oxidation processes. The addition of an antioxidant, being a scavenger of an active free radical, results in LCL quenching providing a tool for antioxidant activity estimation.

ACKNOWLEDGEMENTS

This work was partially supported by a research grant from Poznan University of Life Sciences and grant N312 1410 33 from the Polish Ministry of Science and Higher Education.

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Received September 2008. Revision received November and accepted December 2008.