

Antioxidant Activity of Phytic Acid in Lipid Model System

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Received: March 16, 2009

Accepted: July 12, 2010

Summary

Free radicals formed during thermal (60 °C, 24 h) and catalytic (Fe²⁺ ions, room temperature (23±1) °C, 3 h) oxidative degradation of hydroperoxyde-enriched soybean oil (HESO) were stabilized in the presence of spin trap *N-tert-butyl- α -phenylnitron* (PBN) and detected by electron spin resonance (ESR) spectrometry. In both thermal and catalytic oxidation of HESO, the same hyperfine coupling parameters ($a_N=14.75$ G and $a_H^{\beta}=2.80$ G) confirmed the generation of PBN-OOL/-OL spin adducts. The antiradical activity (AA) of phytic acid, in the 0.076–0.30 mM concentration range, was tested by measuring its ability to inhibit the generation of PBN-OOL/-OL spin adducts during thermal and catalytic oxidation of HESO. Phytic acid did not inhibit the thermal oxidation of HESO and showed no effect in the β -carotene bleaching test (AOA). Contrary to this, phytic acid exhibited antioxidant effect on the catalytic oxidation of HESO by chelating Fe²⁺ ions. The mechanism of antioxidant activity was confirmed by the results of chelating activity on Fe²⁺ in Fe²⁺-ferrozine test where a dose-dependent chelating activity of phytic acid was obtained.

Key words: phytic acid, lipid oxyradicals, antioxidant activity, ESR, chelating activity

Introduction

Lipid peroxidation is one of the undesirable reactions in food production and storage, which can be inhibited or retarded by antioxidants. Wide range of artificial antioxidants have been suspected of acting as promoters of carcinogenesis (1). Presently, there is an increasing interest both in the industry and the scientific research to substitute synthetic antioxidants with natural compounds and/or to produce foods by preserving antioxidant properties of raw materials.

Phytic acid (myo-inositol hexaphosphate) can be found in many cereals, legumes, oil seeds and nuts (1–5 %) and represents the major phosphorus storage compound in plant seeds (2,3). Additionally, it has been considered as an antinutrient, due to its ability to bind minerals and proteins and thus, to change their solubility, function-

ality, absorption and digestibility (4). Phytic acid-mineral complexes are insoluble at physiological pH and, consequently, they exhibit low bioavailability (5).

Regarding its relatively high binding affinity towards minerals, especially to iron, phytic acid has been recognized as a potent antioxidant (6). It is a potent inhibitor of iron-catalyzed hydroxyl radical formation by chelating the iron required for generation of hydroxyl radical *via* the Fenton-type reaction (7). Obata (8) reported that phytic acid exhibited antioxidant effect by suppressing iron(II)-enhanced hydroxyl radical formation induced by 1-methyl-4-phenylpyridinium ion (MPP⁺) in rat striatum. Furthermore, Dexter *et al.* (9) confirmed that iron-mediated oxidative damage involved in the progression of Parkinson's disease could be inhibited by phytic acid. Some experiments demonstrated anticancer effects of

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phytic acid in preventive and therapeutic actions (10,11). The possibility of utilizing phytic acid as an antioxidant in food processing and storage was documented by Stodolak *et al.* (12) in beneficially improving oxidative stability of raw and cooked meat.

Although the estimation of the antioxidant activity of phytic acid has been documented, as stated above, there is a lack of data on the effect of phytic acid on the formation of lipid radicals using electron spin resonance (ESR) spectrometry spin trapping technique. The information about the trapped radical is contained in the hyperfine splitting of the spin adducts, whereas the amplitude of the signal enables the amount of generated free radicals to be quantified (13).

The study presented in this paper is focused on the measurement of inhibition of lipid peroxidation by phytic acid during thermal and catalytic oxidation of hydroperoxide-enriched soybean oil (HESO) by ESR spectrometry using spin trapping. The objective of this research is to investigate antioxidant activity of phytic acid on lipid radicals which are formed in the chain reaction of lipid peroxidation.

Materials and Methods

Materials

Soybean oil was purchased from the local market. Oil was exposed to air for 72 h at room temperature ((23±1) °C) to allow its peroxidation and the obtained HESO was used to prepare the systems for thermal and catalytic oxidation.

Phytic acid dodecasodium salt (97 %), linoleic acid (99 %), β-carotene, Tween 40, 3-(2-pyridyl)-5-6-bis(4-phenylsulphonic acid)-1,2,4-triazine (ferrozine), ferrous sulphate heptahydrate, butylated hydroxytoluene (BHT), ethylene-diaminetetraacetic acid disodium salt dihydrate (EDTA) and α-tocopherol were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents (ethanol and chloroform) were of analytical grade.

Thermal oxidation of HESO

The system for investigation of thermal oxidation of HESO (model system I) was prepared by mixing 6.0 mL of HESO with 21.3 mg (0.12 mmol) of spin trap *N-tert-butyl-α-phenylnitrone* (PBN) (blank probe I). Reaction mixture was heated at 60 °C. Thermal oxidative degradation of HESO was detected by measuring the formation of PBN lipid radical spin adducts after 24 h.

The influence of phytic acid on the thermal oxidation of HESO was investigated by adding phytic acid at 0.076–0.30 mM concentrations to blank probe I.

Antiradical activity (AA in %) of phytic acid was calculated according to the following equation:

$$AA = 100 \cdot (h_o - h_x) / h_o \quad /1/$$

where h_o and h_x are the heights of the first peak in the ESR spectrum of PBN lipid radical spin adducts of blank probe I and probe I, respectively.

Catalytic oxidation of HESO

The system for investigation of catalytic oxidation of HESO (model system II) was prepared by mixing 6.0 mL of HESO, 3.0 mL of distilled water, 21.3 mg (0.12 mmol) of spin trap *N-tert-butyl-α-phenylnitrone* (PBN) and 3.9 mg (0.015 mmol) of ferrous sulphate heptahydrate (blank probe II). Reaction mixture was allowed to stay at room temperature ((23±1) °C). Catalytic oxidative degradation of HESO was detected by measuring the formation of PBN radical spin adducts after 3 h.

The influence of phytic acid on catalytic oxidation of HESO was investigated by adding phytic acid at 0.076–0.30 mM concentrations to blank probe II.

Antiradical activity (AA in %) of phytic acid was calculated according to Eq. 1, where h_o and h_x are the heights of the first peak in the ESR spectrum of PBN radical spin adducts of blank probe II and probe II, respectively.

Detection of lipid radicals

ESR spectra were recorded with spin trapping technique using Bruker 300E ESR spectrometer (Bruker, Rheinstetten, Germany) under the following conditions: modulation field 100 kHz, modulation amplitude 0.204 G, receiver gain 10^3 , time constant 327.68 ms, conversion time 1310.72 ms, centre field 3440.00 G, sweep width 100.00 G, microwave frequency 9.64 GHz, microwave power 20.0 mW, and temperature (23±1) °C.

Magnetic field scanning was calibrated using Fremy's salt (peroxylamine disulphonate). A quartz flat cell Bruker ER-160FC was used for detection. Splitting constants were calculated from computer-generated second derivatives of the spectra after optimizing signal-to-noise ratios and were verified by computer simulations. ESR spectral files were imported into the WinSim program (WinSim, Sugar Land, TX, USA) for the analysis of the hyperfine splitting constants (14).

Chelating activity on Fe^{2+} ions

Chelating activity of phytic acid on Fe^{2+} ions was measured by following the method of Decker and Welch (15). In brief, 1 mL of phytic acid solution (3.7–117 μM) was mixed with 3.7 mL of deionized water. After the addition of ferrous sulphate heptahydrate (2 mM, 0.1 mL) and ferrozine (5 mM, 0.2 mL), the mixture was left to react for 10 min at room temperature ((23±1) °C). Absorbance of the reaction mixture was measured at 562 nm by Jenway 6405 UV/VIS spectrophotometer (Bibby Scientific Ltd, Stone, UK). Blank probe contained deionized water instead of phytic acid solution. Lower absorbance indicates a higher chelating power. Chelating activity of phytic acid on Fe^{2+} ions was compared with that of EDTA in the 8.0–403 μM concentration range.

Chelating activity (in %) was calculated according to the following equation:

$$\text{Chelating activity} = 100 - (A \cdot 100) / A_o \quad /2/$$

where A_o and A are absorbances of the control and the sample, respectively at 562 nm. IC_{50} value (μM) is the effective concentration at which the chelating activity is 50 %.

Antioxidant activity (AOA) measured by β -carotene bleaching method

The oxidative loss of β -carotene in a β -carotene/linoleic acid emulsion was used to assess the antioxidant activity of phytic acid (16). β -carotene (2 mg) was dissolved in 10 mL of chloroform and 1 mL of β -carotene solution was mixed with 20 mg of purified linoleic acid and 200 mg of Tween 40 in a round-bottomed flask. Chloroform was removed by purging with nitrogen. Distilled water (50 mL) was added into β -carotene/linoleic acid emulsion and thoroughly mixed by using a vortex mixer (V1 plus BOECO, Hamburg, Germany). Phytic acid water solution at various concentrations (3.7, 7.3, 14.7, 29.3, and 58.6 μ M) (0.2 mL) and aliquots (5 mL) of the β -carotene/linoleic acid emulsion were placed in capped culture tubes and mixed thoroughly. The tubes were immediately placed in a water bath and incubated at 50 °C. Oxidation of β -carotene/linoleic acid emulsion was monitored spectrophotometrically at 470 nm (6405 UV/VIS, Jenway spectrophotometer). Absorbance was measured at 0, 15, 30, 45, 60, 75, 90, 105 and 120 min. A control was prepared using 0.2 mL of distilled water instead of phytic acid solution.

Degradation rate of phytic acid solution was calculated according to the first order kinetics using the equation (17):

$$\ln(a/b) \times 1/t = \text{sample degradation rate} \quad /3/$$

where \ln is natural log, a is initial absorbance (at 470 nm) at time zero, b is absorbance (at 470 nm), and t is time (min).

The antioxidant activity (AOA) was expressed as inhibition (in %) relative to the control using the equation:

$$\text{AOA} = (dr_0 - dr) \cdot 100 / dr_0 \quad /4/$$

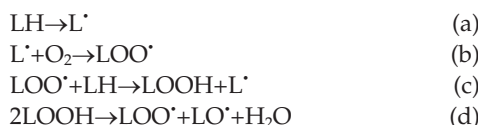
where dr_0 and dr are the degradation rates of the control and the sample, respectively.

BHT at the concentration of 9.08 mM and α -tocopherol at 4.64 mM were used as positive controls.

Results and Discussion

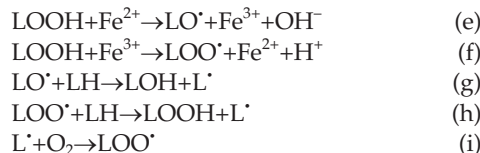
Traces of hydroperoxides, which are often present in oils, may have been formed by lipooxygenase action in the plant prior to and during the extraction of oil. Secondary initiation by homolytic cleavage of hydroperoxides is usually the main initiation reaction in edible oils. This reaction is commonly catalysed by metal ions. After initiation, lipid radical is converted into a different lipid radical.

Among the reactions occurring during lipid peroxidation the following reactions (a–d) indicate the most important processes during thermal oxidation of HESO (18):



According to Kadiiska and Mason (19) transition metal ions are involved in lipid peroxidation by decomposing lipid peroxides into their peroxy and alkoxy radicals

(reactions e and f). These radicals then abstract hydrogen from LH (reactions g and h) and perpetuate a chain reaction of lipid peroxidation:



Thermal treatment of soybean oil in model system I and catalytic treatment in model system II induced a decomposition of hydroperoxides formed during exposure to air, resulting in the formation of free radicals trapped by PBN. PBN spin adducts of formed radicals are registered in the ESR spectrum of blank probe I (Fig. 1) and blank probe II (Fig. 2a).

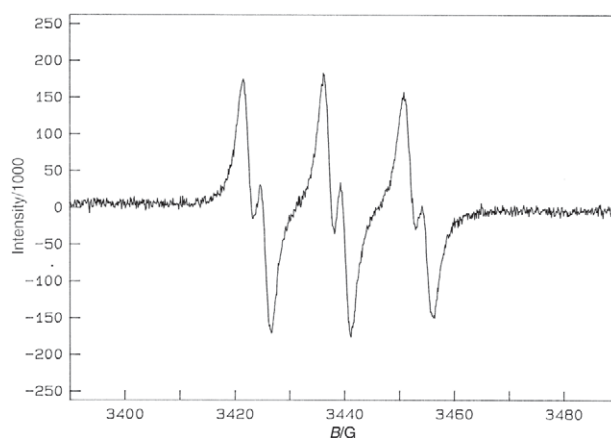


Fig. 1. ESR spectrum of PBN spin adducts formed during thermal oxidation of HESO in model system I (blank probe I)

The ESR spectra presented in Figs. 1 and 2a consist of six lines of the same relative intensities, characteristic for the interaction of unpaired electron and ^{14}N -atom ($I=1$) and ^1H -atom ($I=1/2$), with hyperfine coupling parameters $a_{\text{N}}=14.75$ G and $a_{\text{H}}^{\beta}=2.80$ G. After computer simulation of the ESR spectrum with these hyperfine coupling parameters, the obtained simulated spectrum had the same appearance as the experimental spectrum.

Buettner (13) reported that PBN spin adduct parameters in oxidizing methyl linoleate in the presence of Fe^{2+} ions are $a_{\text{N}}=14.8$ G and $a_{\text{H}}=1.8$ –2.0 G, assigned to alkoxy ($\text{LO} \cdot$) radicals, and in the presence of Fe^{3+} ions are $a_{\text{N}}=14.4$ G and $a_{\text{H}}=2.2$ G, assigned to peroxy ($\text{LOO} \cdot$) radicals. Kinetics of LOOH -induced lipid peroxidation depends on $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio during the time course of lipid peroxidation (20).

Since iron is present at both oxidation states in the investigated catalytic system (reactions e and f), and the hyperfine coupling constants are nearly the same, it is impossible to distinguish these two lipid oxyradicals. According to this, radicals trapped in the investigated catalytic system are recognized as lipid oxyradicals. Spin adducts obtained in this system are marked as PBN-OOL/-OL spin adducts. The same parameters were obtained for the ESR spectra obtained in model system I and in a previous paper for PBN-OOL spin adducts detected in

catalytic oxidation of hydroperoxide-enriched methyl esters of sunflower oil (21).

The formation of PBN-OOL/-OL spin adducts in model system I was not affected by the addition of phytic acid in the investigated concentration range. This finding is in accordance with those of Ahn *et al.* (22) in comparing irradiated phytic acid and other antioxidants for antioxidant activity where DPPH radical scavenging of phytic acid was not detected. On the basis of our results and those of Ahn *et al.* (22), it can be concluded that phytic acid does not act as the chainbreaking antioxidant, capable of scavenging free radicals and thus preventing lipid oxidation. This is predictable since the structural feature of phytic acid is the lack of a hydrogen atom needed for transfer to peroxy radicals.

This conclusion was further confirmed by the results of AOA test in β -carotene/linoleic acid model system. Phytic acid did not exhibit any antioxidant activity in the tested concentration range (3.7–58.6 μ M).

Contrary to this, BHT and α -tocopherol, used as positive controls, were highly effective in suppressing β -carotene bleaching in the experimental model system (AOA of these antioxidants was (56.2 \pm 1.06) and (55.3 \pm 0.80) %, respectively).

Regarding its chelating activity on Fe^{2+} ions (2,23), the effect of phytic acid addition at 0.076–0.30 mM concentration on the catalytic oxidation of HESO in model system II (Figs. 2b and c) was investigated.

Intensities of ESR signal for PBN-OOL/-OL spin adducts registered in the model system II in the presence of phytic acid were lower than that of blank probe II (Figs. 2b and 2c). AA of phytic acid in the model system II is presented in Fig. 3.

Linear regression ($R^2=0.9880$) was observed between AA and phytic acid concentration of 0.076–0.30 mM (Fig. 3).

The obtained results indicate that phytic acid is an antioxidant acting as a potent inhibitor of iron-catalyzed radical formation by chelating the free iron and then blocking its coordination site. Graf and Eaton (6) found the inhibition of hydroxyl radical generation over a wide range of phytate/iron ratios from 1:4 to 21:1.

In our experiment, the range of phytate/iron ratios from 1:22 (0.076 mM phytic acid in model system II) to 1:5.5 (0.30 mM phytic acid in model system II) was tested. It was found that the addition of phytic acid to model system II showed significant effect on the formation of PBN-OOL/-OL spin adducts. Our results are in accordance with the finding that one phytate molecule can bind up to 6 divalent cations (6). The addition of 0.30 mM phytic acid to model system II, which corresponds to phytate/iron ratio of 1:5.5, caused almost complete inhibition of the formation of PBN-OOL/-OL spin adducts (Fig. 3).

Moreover, Graf *et al.* (3) demonstrated that phytate prevented the peroxidation of arachidonic acid driven by ascorbic acid and iron by shifting the redox potential of iron, *i.e.* it accelerated $\text{Fe}^{2+} \rightarrow \text{Fe}^{3+}$ by molecular oxygen, which could explain the inhibition of lipid peroxidation. This occurs especially at high phytate/iron ratios.

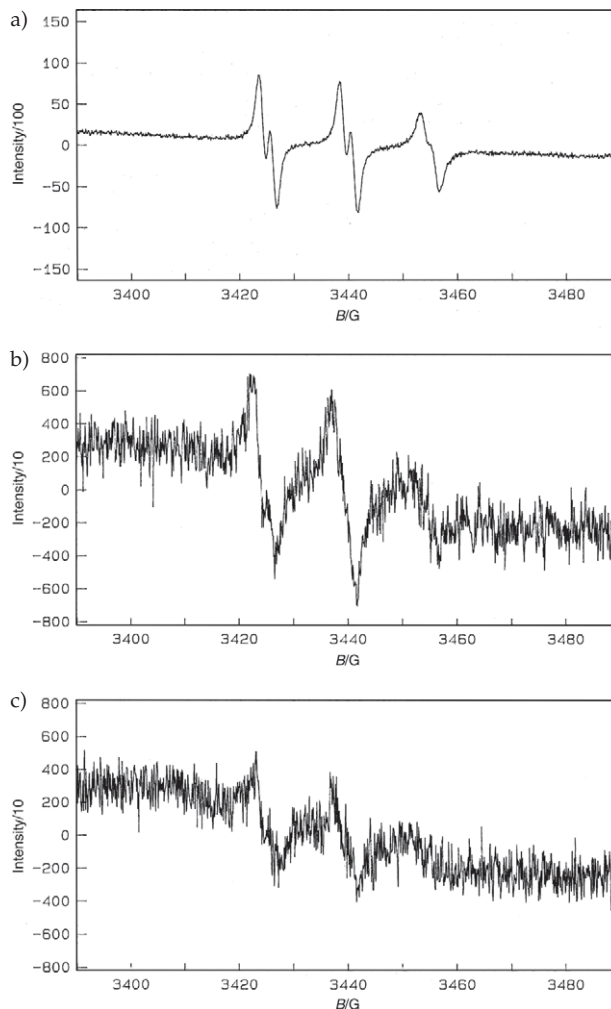


Fig. 2. ESR spectrum of PBN spin adducts formed during catalytic oxidation of HESO in model system II: (a) in the absence of phytic acid (blank probe II), (b) in the presence of 0.15 mM phytic acid and (c) in the presence of 0.30 mM phytic acid

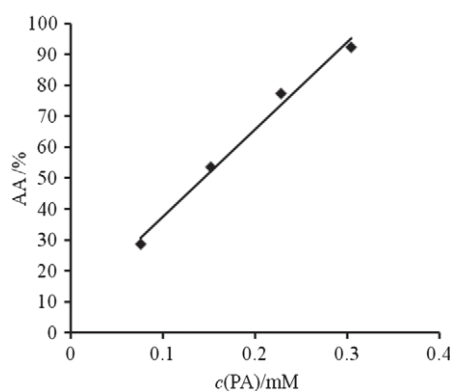


Fig. 3. Antiradical activity (AA) of different concentrations of phytic acid (PA) on PBN-OOL/-OL spin adducts formed during catalytic oxidation of HESO in model system II

An important mechanism of antioxidant activity is the ability of an antioxidant to chelate/deactivate transition metals that can catalyze hydroperoxide decomposition and Fenton-type reactions. One of the most com-

monly used systems for testing the chelating activity on Fe^{2+} is ferrozine/ FeCl_2 . In the light of our experience, the usage of FeCl_2 in the test is connected with poor reproducibility of the results due to the instability of FeCl_2 . Also, Fe^{2+} ions in the FeCl_2 solution are readily oxidized to Fe^{3+} .

Much better repeatability and reproducibility are obtained by using FeSO_4 instead of FeCl_2 because of better oxidative stability of Fe^{2+} ions in the FeSO_4 solution. The obtained results of chelating activity on Fe^{2+} in ferrozine/ FeSO_4 system are presented in Fig. 4.

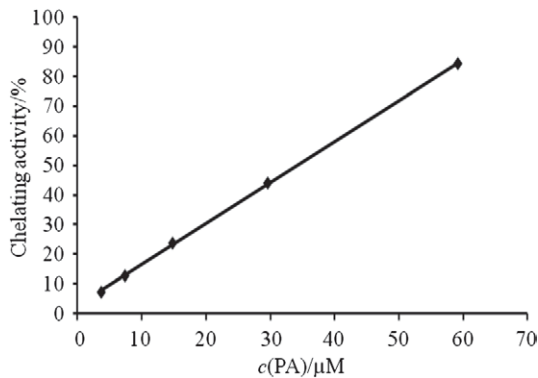


Fig. 4. Chelating activity on Fe^{2+} of different concentrations of phytic acid

As expected, linear regression ($y=1398.7x+2.759$, $R^2=0.9990$) was observed between chelating activity on Fe^{2+} and phytic acid concentration from 3.7 to 59 μM . At the concentration of 117 μM phytic acid exhibited chelating activity of 96 %. Similar results were obtained for EDTA, linear regression ($y=355.52x-1.1563$, $R^2=0.9996$) between chelating activity on Fe^{2+} and EDTA concentration from 8.0 to 201 μM . Chelating activity was 98 % at the concentration of EDTA of 403 μM . Regarding the calculated IC_{50} values for phytic acid (33.8 μM) and EDTA (144 μM), it can be concluded that phytic acid is more efficient chelator than EDTA. Graf *et al.* (24) demonstrated that in free iron and iron chelates the availability of at least one coordination site is required for catalysis of hydroxyl radical generation. According to the same authors, occupation of all iron coordination sites by a chelator with displacement of water in the first coordination sphere precludes the binding of H_2O_2 to Fe^{2+} chelates and the subsequent Fenton reaction. Contrary to iron-EDTA chelates with free coordination site, the iron-phytate chelate is totally inert in the Fenton reaction (24).

Despite the fact that it is widely used, ferrozine/ Fe^{2+} test does not provide any information about the availability of aquo sites, and thus the efficiency of a chelator as an antioxidant. This fact can explain poor correlation between the results of chelating activity and the results of antioxidant activity estimated by other tests, which is often found in the literature (25,26). The proposed system for investigation of catalytic oxidation of HESO provides an opportunity for the direct measurement and characterization of free radicals and thus presents a better choice for estimating the efficiency of a chelator as an antioxidant.

Conclusions

Based on our results, we can conclude that phytic acid does not inhibit the thermal oxidation of HESO, but influences catalytic oxidation of HESO by chelating Fe^{2+} ions, and thus inhibits generation of lipid oxyradicals that were detected in the form of PBN-OOL/-OL spin adducts. By the same mechanism, dietary phytic acid could lower the incidence of cancer (11) or inhibit oxidation during processing, preservation and storage of foods (27).

Acknowledgements

This work was supported by the Ministry of Science and Technological Development, the Republic of Serbia (Project: TR-20068).

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