

SPIN TRAPPING STUDY OF THE INFLUENCE OF TAXIFOLIN ON FENTON REACTION IN ETHANOL AND METHANOL

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To examine the effect of solvents on the nature and amount of the spin adducts, spin trapping experiments with 4-POBN, the Fenton reagents and taxifolin were performed in solutions of ethanol or methanol and water. The addition of taxifolin resulted in a decrease of the spectrum intensity of the spectrum that was dependent on taxifolin concentration. Computer simulation revealed that 4-POBN/•CH(CH₃)OH adduct dominated in ethanol whereas 4-POBN/•OH and 4-POBN/•CH₂OH adducts are present in methanol.

INTRODUCTION

Flavonoids are a group of compounds abundant in plants and also present in human diet. Many studies confirm the role of flavonoids in preventing diseases like coronary heart disease, cancer and age-related neuropathologies. Flavonoids are able to scavenge free radicals, chelate transition metal ions and interact with other antioxidants (Teixeira, Siquet, Alves, Boal, Marques, Borges, Lima & Reis, 2005). Taxifolin, which is present in the plants from pinus genus (in pinus maritima), in the milk thistle seeds (Kim, Graf, Sparacino, Wani & Wall, 2003), and in citrus fruits, has a flavanone structure and can act as an antioxidant due to reducing properties of its hydroxyl groups. The radical-scavenging activity of fruit extracts, juices and herbal teas was estimated by various methods including ESR technique (Oszmiański, Wolniak, Wojdyło & Wawer, 2007; Wasek, Nartowska, Wawer & Tudruj, 2001; Oszmiański, Wolniak, Wojdyło & Wawer, 2008).

The aim of this research was to study the radicals produced in Fenton reaction with methanol or ethanol and the mechanism of their reaction with taxifolin. The ESR technique, coupled with spin trapping methods and computer simulation has been extensively used for the detection and identification of short-lived free radicals. The method of spin trapping is based on the scavenging of the radicals by a spin trap, leading to the formation of a spin adduct with higher stability. In this work a 4-POBN spin trap was chosen due to its stability and its selectivity toward trapping of carbon-centered radical species. Normally, it is easy to establish the presence of the radical and more of a challenge to identify it.

Computer simulation is the most powerful technique in the analysis of multi component ESR spectra.

EXPERIMENTAL DETAILS

ESR spectroscopy

ESR spectra were measured on a MiniScope MS 200 spectrometer from Magnettech at room temperature (23-24°C) in 50 µl capillary tubes. Typical instrument settings were: microwave attenuation 10 dB, modulation amplitude of 0.5G, sweep time 20s. Measurements of kinetics were performed every 3 minutes unless indicated otherwise. The simulations of fast motion ESR spectra were performed with routines implemented in EasySpin toolbox (Stoll & Schweiger, 2006) for Matlab. The ESR spectra of 4-POBN spin adducts exhibit hyperfine splittings from one ¹⁴N and one ¹H – nuclei. The hyperfine data provided a good initial guess for the fitting (Janzen, Wang & Shetty, 2006; Finkelstein, Rosen & Rauckman, 1982; Sridhar, Beaumont & Powers, 1986).

Generation of free radicals for ESR

α-(4-Pyridyl N-oxide)-N-tert-butyl nitron (4-POBN) was purchased from Sigma Aldrich. Solutions were prepared using distilled and degassed water. Taxifolin solutions (0.010 M) in methanol and (0.012 M) in 96% ethanol were prepared, kept in a refrigerator and protected from light. Samples were prepared using 20 µl of 20mM 4-POBN dissolved in water. POBN was mixed with 20 µl of 5mM FeSO₄ prepared freshly from 0.1M stock solution and 20 µl of 25mM H₂O₂ solution,

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prepared from 30% solution. Finally, 20 μl of taxifolin sample (or solvent only) was added. The taxifolin concentration varied from 0.1mM to 2mM.

RESULTS AND DISCUSSIONS

The Fenton reaction was the method for generating free radicals. To examine the effect of solvents on the nature and amount of the spin adducts, spin trapping experiments in the Fenton reagents in ethanol, methanol and water were performed first. The second set of experiments was performed in the presence of taxifolin solutions.

Spin Trapping in solvents

The 4-POBN/ $\bullet\text{OH}$ radical adduct was the only species obtained in the Fenton reactions based on Fe(II) in water. The 4-POBN/ $\bullet\text{OH}$ adducts were unstable and decayed fast to ESR silent products. The hyperfine splittings obtained by computer simulations are $a_{\text{N}} =$

14.9G and $a_{\text{H}} = 1.63$ G. These values are in agreement with previous data (Janzen *et al.*, 2006) and were used for further simulations.

A 6-line ESR spectrum due to 4-POBN radical adducts was recorded when mixing Fe^{2+} (as FeSO_4), H_2O_2 , 4-POBN and methanol. The spectra were registered every 3 minutes, starting from the 4th minute after the addition of H_2O_2 to the sample. Computer simulation of the spectrum (Fig. 1) revealed a species having hyperfine coupling constants of $a_{\text{N}} = 15.5$ G and $a_{\text{H}} = 2.8$ G and $a_{\text{N}} = 15.0$ G and $a_{\text{H}} = 1.6$ G (Table 1) values reasonably close to 4-POBN/ $\bullet\text{CH}_2\text{OH}$ and 4-POBN/ $\bullet\text{OH}$ adducts in aqueous solution (Sridhar *et al.*, 1986). The ESR spectrum measured 4 minutes after the addition of H_2O_2 to the sample and the spectrum measured after 140 minutes of the reaction had a similar (30%) ratio of 4-POBN/ $\bullet\text{OH}$ (Fig. 1). The ESR signal measured 20-80 minutes after H_2O_2 addition to the sample was dominated by 4-POBN/ $\bullet\text{CH}_2\text{OH}$ radical adduct (Fig. 1).

Table 1. Isotropic hyperfine splitting of 4-POBN adducts in solutions

System	Adduct	hyperfine splitting, G	
		a_{N}	a_{H}
Fe(II)/H ₂ O/4-POBN	4-POBN/ OH	14.9	1.6
Fe(II)/MeOH/4-POBN	4-POBN/ OH	15.0	1.6
	4-POBN/ CH ₂ OH	15.4	2.8
Fe(II)/EtOH/4-POBN	4-POBN/ CH(CH ₃)OH	15.5	2.5

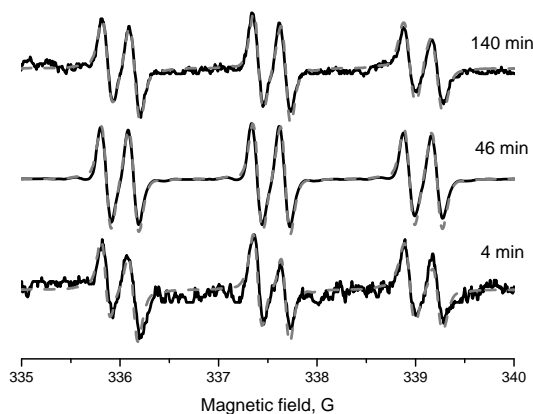


Fig. 1. X-band ESR spectra of 4-POBN/ $\bullet\text{OH}$ and 4-POBN/ $\bullet\text{CH}_2\text{OH}$ spin adduct in Fenton reaction with methanol recorded after 4, 46 and 140 minutes of reaction (solid line). The dotted lines are calculated spectra using hyperfine splitting constants from Table 1.

The Fenton reaction in ethanol can generate a number of radicals (Bosnjakovic & Schlick, 2006). Computer simulation of radicals that were trapped by 4-POBN revealed a mixture of C-centered radical adducts. The 4-POBN/ $\bullet\text{CH}(\text{CH}_3)\text{OH}$ adduct, the most commonly

detected, dominates the spectrum with hyperfine splittings: of $a_{\text{N}} = 15.5$ G and $a_{\text{H}} = 2.5$ G (Table 1). However, the 4-POBN/ $\bullet\text{OH}$ radical adduct was not detected in Fenton reaction with ethanol. This result suggested that $\bullet\text{OH}$ radicals rather react with ethanol

producing $\bullet\text{CH}(\text{CH}_3)\text{OH}$ radicals, and then they are trapped by 4-POBN. It is important to note that the reaction of ferryl radical, usually produced in Fenton reaction, with ethanol (Yamazaki & Piette, 1990) is another mechanism of $\bullet\text{CH}(\text{CH}_3)\text{OH}$ radicals production.

Spin trapping in Fenton reagents in the presence of taxifolin solutions

Taxifolin concentration had a significant effect on the intensity of the ESR signal and kinetics of its decay. The taxifolin concentration of 0.1mM decreased the intensity by about 50% in comparison with the reference. The

concentration of 2mM caused signal decay during 15 minutes. The spin adducts which were detected in the presence of taxifolin in methanol were similar to the situation in the absence of taxifolin, i.e. 4-POBN/ $\bullet\text{OH}$ and 4-POBN/ $\bullet\text{CH}_2\text{OH}$ appeared. The first stage of the reaction, where $\bullet\text{OH}$ radicals were formed and trapped by 4-POBN remained unchanged, however higher concentrations of taxifolin decreased the intensity of carbon-centered radical adduct signal and shortened the period of carbon-centered radical domination in the spectrum (Fig. 2).

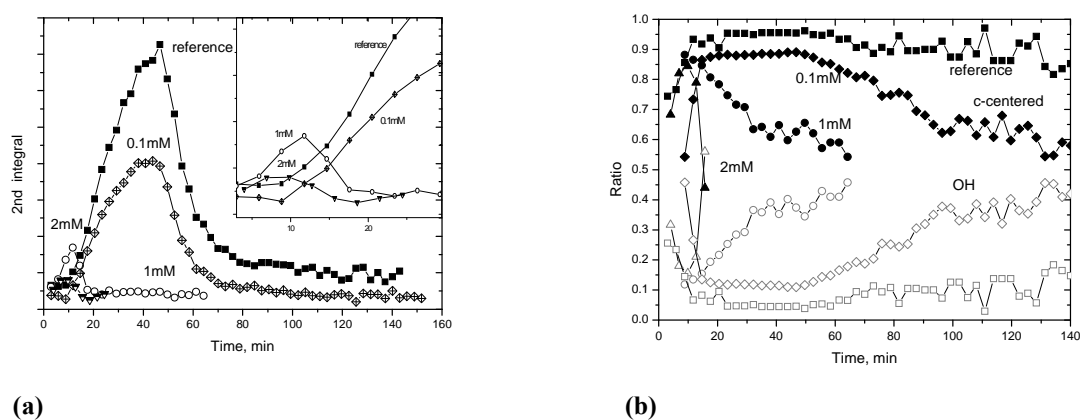


Fig. 2. (a) 2nd integral of ESR signal intensity of 4-POBN radical adducts from Fenton reaction with methanol (■) in the presence of 0.1mM (◇), 1mM (○) and 2mM (▼) of taxifolin. (b) ratio between 4-POBN/OH (grey symbols) and 4-POBN/CH₂OH (black symbols) components in reference (■) system and in the presence of 0.1mM (◇), 1mM (○) and 2mM (▼) of taxifolin.

The same dominating adduct (4-POBN/ $\bullet\text{CH}(\text{CH}_3)\text{OH}$) was identified in the presence of taxifolin in ethanol. The effect of taxifolin concentration on the ESR signal intensity was similar to the system with methanol. The taxifolin concentration of 0.1mM decreased the intensity by about 50 % in comparison with the reference, higher concentrations (1mM and 11.7mM) caused a very fast decay of the ESR signal (Fig.3).

The general conclusion is that the presence of taxifolin affects the intensity of carbon-centered 4-POBN adduct component. There are several possible explanations for this effect: (i) taxifolin could react with 4-POBN radical adducts resulting in ESR silent products; (ii) taxifolin could react with radicals formed in the experimental systems (scavenging), and (iii) the reaction of taxifolin with Fe(II) or intermediate radicals that are involved in 1-hydroxyethyl or hydroxymethyl radical production.

CONCLUSIONS

The radicals formed in the Fenton reaction with methanol and ethanol were studied with ESR spin trapping technique and analyzed with the aid of computer simulations. The fitting of experimental spectra made it possible to identify radical adducts that were formed in these reactions and to follow the kinetics of each component. It was shown that the presence of taxifolin decreased the ESR signal intensity, affecting mainly the c-centered radical adduct component. Taxifolin mechanism of the reaction with free radicals requires further investigation. Further experiments are in progress.

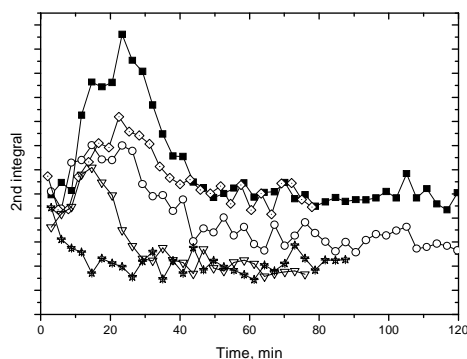


Fig. 3. 2nd integral of ESR signal intensity of 4-POBN radical adduct from Fenton reaction with ethanol (■) in the presence of 0.05mM (◇), 0.1mM (○), 1mM (▼) and 11.7mM (★) of taxifolin.

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