SPECTRAL PROPERTIES OF PROPYL GALLATE IN ORGANIC SOLVENTS AND IN HETEROGENEOUS ENVIRONMENT[*]

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Abstract. The spectral properties of propyl gallate (PG) in water, organic solvents, micelles and lipid vesicles were investigated. In homogenous solvents the positions of fluorescence and absorption maxima of PG depend on the solvent nature. In protic solvents the emission maximum is red shifted and Stokes shift is higher compared to aprotic solvents. In sodium dodecyl sulphate, SDS, micelles partition of PG between bulk water and micellar phase was observed. In lipid vesicles the observed spectral shift and fluorescence quenching experiments with iodide and acrylamide suggest that PG is located in the hydrophilic part of the phospholipid bilayer where it may act as an aqueous phase antioxidant.

Keywords: antioxidant, micelle, lipid vesicle, absorption, fluorescence, DPPC, lecithin

INTRODUCTION

Propyl gallate (PG) is a synthetic derivative of the naturally occurring antioxidant gallic acid. Gallate derivatives are commonly used as antioxidants in food [1,8] and exhibit antibacterial properties [5,12]. They are also used as antifading agents in fluorescence microscopy [7,22]. It has been proved that gallate esters are hydrophobic moieties and as such they may interact with aliphatic side chains of amino acids in proteins or with phospholipids and sugars through hydrophobic association. It has been shown that the gallotannin-protein and gallotannin-phospholipid interactions were the result of cooperative effects of hydrogen

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bonding and hydrophobic association, and hydrogen bonding was the predominant effect in the interactions between gallotannins and sugars [18]. Like other phenolic antioxidants, gallate esters are chain breaking antioxidants and inhibit lipid peroxidation by transferring a hydrogen atom from its phenolic hydroxyl group to lipid radicals or lipid peroxyl radicals. During this process gallate semiquinone free radicals [21] effectively couple with lipid radicals. The formed structures exhibit relatively high stability, which makes gallates an effective antioxidant. This has been shown by EPR spectroscopy and quantum chemistry calculation methods [3,4].

It is known that changing dielectric environment influences spectral and molecular properties of molecules. One may expect that PG molecules located in the aqueous phase but in proximity of protein or lipid membrane display different properties than those observed in bulk water. Estrification of carboxylic moiety in gallic acid changes the nature of the new molecule. One may expect that the presence of hydrophobic alkyl chain may change the interactions between PG and solvents compared to the parent compound. To explore the interaction mechanisms between PG molecules and organic solvents, heterogeneous structures like micelles and lipid bilayer, the absorption and fluorescence PG spectra were investigated.

MATERIALS AND METHODS

The membranes were formed using <99% purity egg yolk L-α-phosphatydylcholine (PC) with fatty acid contents of approximately 33% palmitic (16:0), 13% stearic (18:0), 31% oleic (18:1) and 15% linoleic (18:2), [20], and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine 99% (DPPC) from Sigma Aldrich (Germany). Propyl gallate (PG) >98% (HPLC) was purchased from Fluka Chemie (Germany). Sodium dodecyl sulfate (SDS) and tetradecyltrimethylammonium bromide (TTABr) were from Sigma Chemical Co. (USA). Acrylamide and potassium iodide were from POCh (Poland). Organic solvents: acetonitryle, 1,4-dioxane, dimethylsulfoxide (DMSO), ethanol, methanol, ethyl acetate, propanol-2 were purchased from Merck.

For micelle studies, propyl gallate was dissolved in methanol and added to a detergent solution. For the membrane studies, PC or DPPC were dissolved in chloroform and mixed with methanol solution of PG. Chloroform-methanol solutions containing from 0 to 1000 μM of propyl gallate in 0.2 mg ml^{-1} of PC or DPPC were dried in order to obtain a thin film on the bottom of the flask. Then the sample was kept under vacuum to remove traces of the solvent. Multilamelar vesicles were formed by incubating the dried lipid in distilled water under vigorous vortexing at 324 K, temperature above the gel-liquid crystalline transition. Then the sample was cooled down to 295 K.

Fluorescence spectra were measured with Shimadzu 1501 PC fluorimeter in 1 cm cuvette at room temperature, 295 K, at excitation wavelength of 260 nm and emission from 300 to 550 nm. Absorption spectra were taken with S1000 spectrometer from Ocean Optics, from 200 to 700 nm in 1 cm cell.

RESULTS AND DISCUSSION

Organic solvents

Organic solvents that differ in dielectric constant and protic properties influence the spectral properties of the solute. In order to determine the nature of the interactions between PG and various organic solvents the absorption and fluorescence spectra of PG in those solvents were investigated. The measured absorption and fluorescence maxima together with dielectric constant of the solvents are given in Table 1.

Table 1. Absorption, emission maxima and Stokes shifts of 100 µM propyl gallate in solvents with different dielectric constant ε, SDS and TTABR micelles and PC and DPPC membranes

Solvent	Dielectric constant, ε	Peak maximum (nm)		Stokes shift (cm⁻¹)
		Absorbance	Fluorescence	
Acetonitrile	37.5	253	340	10114
1,4-dioxan	2.2	257	334	8970
DMSO	46.7	270 (327)	355	8868
Ethanol	24.5	262	373	11286
Methanol	32.4	261	375	11647
Ethyl acetate	6.1	252	332	9562
Propanol-2	19.9	263	373	10775
SDS	na*	258	364	11287
TTABr	na*	258	370	11732
PC	na*	258	355	10590
DPPC	na*	258	367	11511
Water	78.4	257	378	12455

*na – not applied.

In water, having the highest dielectric constant, the position of fluorescence maximum is most shifted to the red part of the spectrum. In ethyl acetate, the solvent with a low dielectric constant, the emission maximum is blue shifted compared to that recorded in water. The observed 46 nm shift between the

fluorescence maxima in water and ethyl acetate indicates that changing dielectric environment influences fluorescence and absorption spectra of PG. From Table 1 we may notice that with regard to the position of the fluorescence maximum we may group solvents into two classes. Those where the emission maximum is above 367 nm and posses protic character, and aprotic solvents where the maximum is located bellow 355 nm. The high values of the Stokes shift listed for all the solvents indicate that during excitation PG molecule undergoes serious electron rearrangement. In order to elucidate the nature of the interactions between PG and the used solvents the solvation model for dipole-dipole interaction between solute and solvent was applied. In this approach the fluorescence maximum, given in wavenumber, measured in different solvents plotted versus Onsager function, given by equation 1, should give a straight line

$$f(D) = 2(D\text{-}1) / (2D\text{+}1) \qquad (1)$$

where D is the dielectric constant of the solvent.

The obtained graph is presented in Figure 1; it consists of two lines with different slopes. The upper line represents aprotic solvents. The line in the right lower corner of the graph represents protic solvents. Those results indicate that the observed solvatochromism is connected with the changing microenvironment of the PG molecule, which is also evident from Table 1. In this mechanism the relaxation process between dipole moments of solute and solvent molecules leads to the observed spectral shift. This knowledge will be used to determine the microenvironment of the PG located in micelles or in lipid bilayers.
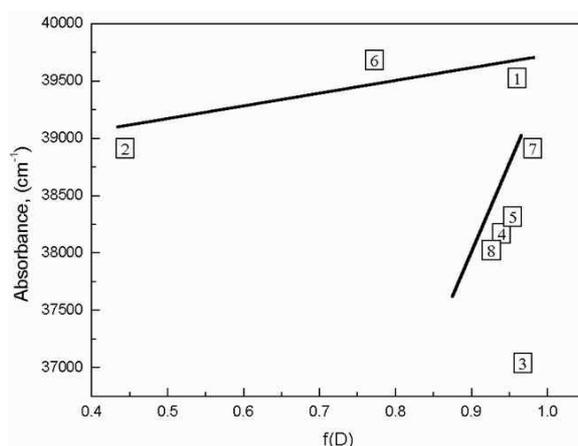

Fig. 1. Absorption maxima of 50 μM PG in different solvents versus Onsager function f(D). 1 – acetonitryle, 2 – 1,4-dioxane, 3 – DMSO, 4 – ethanol, 5 – methanol, 6 – ethyl acetate, 7 – water, 8 – propanol-2

Micelles

A micelle is a cluster of amphiphilic molecules in water. The non-polar part of the molecule forms a hydrophobic interior. The external, neutral or polar part of the micelle forms the shell where the interactions with water molecules occur. One may expect that PG, having a hydrophobic propyl tail, should easily incorporate into the micelle. To prove this we carried out an experiment with solutions of ionic and neutral detergents with increasing concentrations PG molecules.

Titration of PG up to 900 μM concentration in micellar solutions of SDS, an anionic detergent, increases the fluorescence up to 100% compared with water. The maximum fluorescence intensity was reached at 160 μM PG, which refers to a mean micellar occupancy of 6 PG molecules per micelle. Further increase of solute concentration led to quenching of the fluorescence with maximum at 364 nm. The increasing fluorescence intensity and fluorescence maximum located at lower wavelength than in water, 378 nm, but higher than in 1,4-dioxan, 334 nm, indicates that PG molecule is embedded into micellar environment. The above data suggest its location rather in the hydrophilic surface than in the hydrophobic micellar core. At pH 6 the PG molecule is not ionized, then we may speculate that this inclusion into micellar core occurs through the propyl chain whereas the aromatic moiety with three hydroxyl groups remains in aqueous environment. Another possibility of the interaction is adsorption of the aromatic part of PG molecule on the hydrophilic surface of the micelle. However, in such a case no change in dielectric environment is expected because the molecule will remain in protic environment, therefore no change in fluorescence maximum position and intensity should be observed. The above consideration led to the conclusion that at least the hydrophobic part of the PG molecule is anchored in SDS micelle. In this picture the hindered flexibility of propyl chain in micellar structure on the observed increased intensity has to be also recognized.

Similar experiments conducted in the presence of a cationic detergent, TTABr gave only the fluorescence of PG at 370 nm, whereas the fluorescence intensity was comparable with that observed in water. Those results suggest that the observed shift may be connected with interaction between PG and detergent molecules rather than with the changing dielectric environment of PG in this micelle.

Membranes

Propyl gallate interacts with different components of cell including phosphatidylcholines, constituent of the membranes. The intrinsic fluorescence of PG molecule was used to follow the partitioning process of PG molecules into PC liposomes. At 250 μM PG concentration in the presence of 0.2 mg ml^{-1} PC

liposomes a fluorescence intensity increase with maximum located at 355 nm was observed. Similar experiments carried out with DPPC membranes did not show changes in fluorescence intensity with maximum at 367 nm despite the fact that the hydrophilic part of both phospholipids was the same. The increasing PG concentration in PC membrane increases its fluorescence, Figure 2. Such intensity increase indicates that PG molecule enters into a more rigid and lower dielectric environment of lipid bilayer compared to bulk water. The fluorescence quenching observed at higher PG concentrations reflects the concentrational quenching of PG molecules. The deactivation of the excited state by ground state fluorophore with simultaneous reabsorption of the emitted light due to high fluorophore concentration leads to the selfquenching process. The positions of absorption and fluorescence spectra maximum of PG in PC and DPPC are given in Table 1.
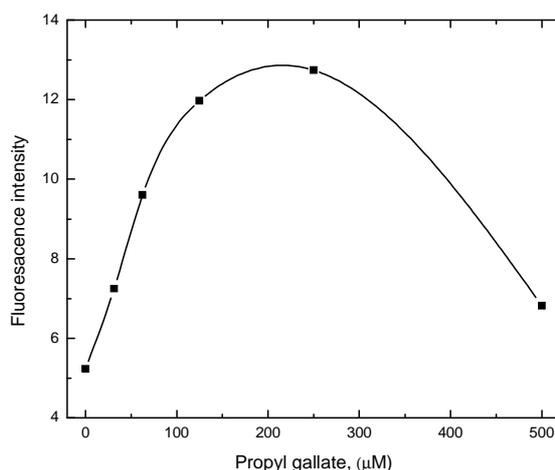

Fig. 2. The concentration influence on fluorescence intensity maximum of 50 μM PG in 0.2 mg ml^{-1} PC vesicle recorded at 355 nm

The observed differences in fluorescence intensity and emission maxima positions arise probably from different membrane morphology. DPPC membrane at room temperature exists in gel phase whereas PC is already in liquid crystalline phase. In DPPC the saturated fatty acids carbon chains are extended to a maximum. In this all-antiperiplanar conformation all torsion angles of -C-C- bonds of the hydrocarbon (polymethylene) chain are the same. In PC membrane in almost 50% of phospholipids, predominately in sn-2 position, unsaturated fatty acids are attached with one, two or three cis-double bonds [13]. In such a case the displacement

from extended trans form occurs, which causes that the surface area and volume per lipid molecule is higher than for DPPC [9]. PC membrane is prone to adopt another molecule into its structure, whereas the tight DPPC structure almost excludes any incorporation. A number of physical methods like DSC [19], X-ray diffraction [11], Raman and infrared spectroscopy [16], NMR [14] and neuron scattering [23] have confirmed that PC membrane exhibits higher degree of disorder and its ability to incorporate drugs, sensitizers and vitamins is much more efficient than that of DPPC membrane [2,6,10,15,17].

In order to find out where and how PG molecule is located relative to PC membrane the fluorescence quenchers were used. Potassium iodide quenches the fluorescence arising from aqueous phase because its permeability trough the membrane is low. Acrylamide, a water-soluble fluorescence quencher, penetrates the hydrophilic phase of the PC bilayer, thus it may quench fluorescence arising from the PG molecule located in this phase. We also used methanolic-water solution with dielectric constant $\varepsilon = 65$ for PG location in PC lipid bilayer and carried out the quenching studies of PG fluorescence by acrylamide in this solution. The ε value was estimated using the data from Table 1, 38750 cm^{-1}, and interpolated form Figure 1 the f(D) value of 0.975. During quenching studies in membranes no changes in the fluorescence spectra were observed, therefore the results were analyzed in terms of dynamic quenching mechanism with quenching constant described by Stern-Volmer equation (2).

$$F_0/F = 1 + K_{SV}(AC) \qquad (2)$$

where: F_0, F – fluorescence intensity without and with quencher, respectively; K_{SV} – dynamic quenching constant; (AC) – acrylamide concentration.

The results of the fluorescence quenching measurements are given in Figure 3. It shows that at 62 μM PG concentration in the PC membrane, iodide at concentrations bellow 100 mM does not show any fluorescence quenching. At higher quencher concentrations, above 100 mM, the quenching process with $K_{SV} = 8.6$ M^{-1} is observed. Acrylamide at low concentrations shows quenching with $K_{SV} = 40$ M^{-1}. Above 100 mM the quenching process is still efficient but, small upward curvature is observed. This indicates that at higher quencher concentration, static quenching, i.e. formation of ground state complex, also takes place. In methanolic-water solution the acrylamide quenching constant is one order higher than that in PC membrane. This indicates that although PG may have its aromatic moiety relatively close to the polar part of the bilayer, it is not sufficiently exposed to allow acrylamide to reach it as in homogenous methanolic-water solution.

The obtained results indicate that iodide quencher is much less efficient than acrylamide. Iodide is readily accessible only to fluorophores located in aqueous

phase, whereas acrylamide may quench in aqueous phase and hydrophilic membrane phase. When we recall molecular mechanism of dynamic quenching where the necessary condition for quenching is close encounter between fluorophore and quencher, then we may suggest a mechanism of the observed phenomenon. At low PG concentration most of the molecules are embedded into hydrophyllic part of PC bilayer, and quenchers, especially iodide due to its size and electric charge, do not have any possibility for close encounter. Increased quenchers concentration increases the probability of the encounter and quenching may occur, Figure 3.
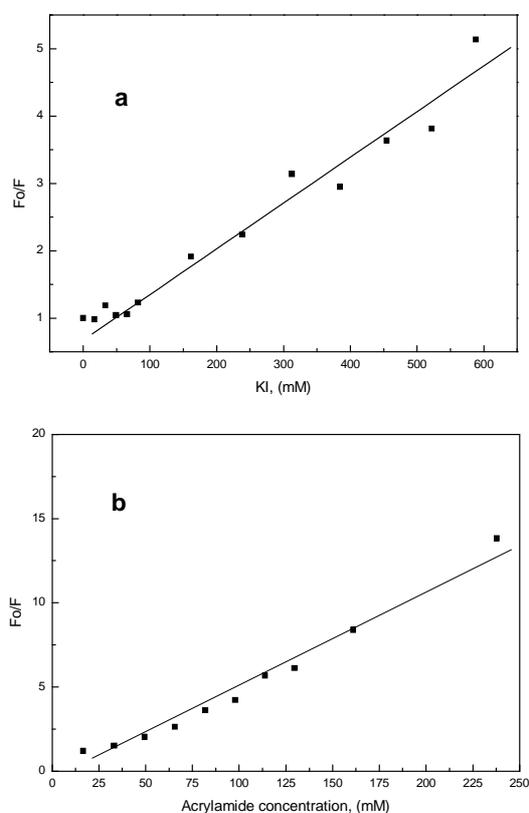

Fig. 3. The quenching of 62 μM PG fluorescence in 0.2 mg ml^{-1} PC membrane by: a) sodium iodide; b) acrylamide

CONCLUSION

The observed shift of fluorescence maximum, increased fluorescence intensity and quenching studies with acrylamide and iodide indicate that PG molecule may be embedded into the hydrophilic part of the PC membrane. We have to stress that the partitioning described above does not imply that the PG molecule is fully

embedded into hydrophobic core of the bilayer. This is rather an indication that the PG molecule interacts with the hydrophilic part of the membrane. The location of the PG molecule in the lipid/water interface could be of importance in explaining its mechanism of protection against oxidative agents present in lecithin bilayer.

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WŁASNOŚCI SPEKTRALNE GALUSANU PROPYLU W ROZPUSZCZALNIKACH ORGANICZNYCH I W ŚRODOWISKU HETEROGENICZNYM

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Streszczenie. Przeprowadzono pomiary własności spektroskopowych galusanu propylu (PG) w wodzie, rozpuszczalnikach organicznych, micelach i liposomach. Położenie maksimum absorpcji i fluorescencji zależy od natury rozpuszczalnika. W protycznych rozpuszczalnikach maksimum emisji jest przesunięte w stronę czerwonej części widma oraz przesunięcie Stokesa jest większe w porównaniu do rozpuszczalników aprotycznych. W miceli siarczanu dodecylu sodu (SDS) obserwuje się rozdział PG pomiędzy faza wodną a micelarną. W liposomach obserwowane przesunięcie maksimum fluorescencji oraz pomiary wygaszania fluorescencji z wygaszaczami jodkiem potasu oraz akrylamidem wskazują, że PG lokuje się w hydrofilowej części membrany lipidowej. PG w tym położeniu w membranie może funkcjonować jako antyutleniacz.

Słowa kluczowe: antyutleniacz, micela, liposom, fluorescencja, DPPC, lecytyna