

MECHANISMS RESPONSIBLE FOR THE ANTIOXIDANT PROPERTIES OF CHLOROPHYLLIN: A PULSE RADIOLYSIS STUDY

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Abstract

Chlorophyllin, the water-soluble analogue of the ubiquitous green pigment chlorophyll has been shown to be a potent antioxidant and radioprotector under both in vitro and in vivo conditions. It is a potent protector of membrane damage and prevents protein oxidation, lipid peroxidation besides depletion of thiols induced by radiation, photosensitization and other oxidants. To analyze the possible mechanisms involved, we have studied the reaction of chlorophyllin with biologically relevant radical species in the form of linoleic acid peroxy radical (LOO^{\bullet}), chloroperoxy radical ($CCl_3O_2^{\bullet}$), azide radical (N_3^{\bullet}) and thiyl radical (RS^{\bullet}). The summary of results obtained are given below: Pulse radiolysis studies show that these radicals ($CCl_3O_2^{\bullet}$, N_3^{\bullet} , RS^{\bullet}) react with chlorophyllin in a similar way producing transient absorption bands at 370 nm and in the region of 430-450 nm with bleaching at 410 nm. The one electron oxidized species was long lived without any decay upto 1 millisecond. The high rate constant values reveal that chlorophyllin is able to scavenge these radicals effectively. However, the reaction of LOO^{\bullet} radical with chlorophyllin was slow and the yield of one-electron oxidized species of chlorophyllin was also small. This indicates that inhibition of lipid peroxidation observed with chlorophyllin may involve other mechanisms.

Introduction

In recent years there has been an increased interest in areas related to newer developments in the prevention of disease especially those involving natural compounds with antioxidant activities. These antioxidants neutralize free radicals or their activities. Oxidative stress involving the enhanced generation of reactive oxygen species (ROS) including free radicals and other related reactive species has been implicated in a variety of toxicities and ailments [Ames, 1993; Sies, 1996;

Yoshikawa et al. 2000]. ROS have been implicated as possible causative factors for more than 100 diseases. These include cardiovascular ailments, neural disorders, diabetes, various forms of cancer, hepatitis, urolithiasis, tissue injury and immune disorders [Sies, 1996; Thomas & Kalyanaraman, 1997; Yoshikawa et al. 2000; Klaunig & Kamendulis, 2004]. Hence compounds with potent antioxidant activity can help in the prevention/cure of diseases as well as in toxicities. Such compounds, especially derived from natural sources and capable of minimizing oxidative damage *in situ*, have

potential uses to control human diseases. These compounds, capable of interacting with DNA, cellular membranes and other biomolecules have potential protective properties against deleterious free radicals [Sies, 1996; Packer & Ong, 1998; Surh, 2003].

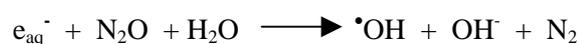
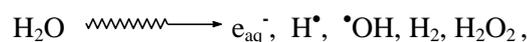
Chlorophyllin is a water-soluble analogue of chlorophyll, the ubiquitous photosynthetic green pigment present in food materials of plant origin as well as in nutritional supplements such as extracts from *Spirulina* and *Chlorella vulgaris*. Chlorophyll has been credited with several beneficial properties. Chlorophyllin (CHL), has proved to be better than the parent compound as evident from studies employing model systems [Negishi, 1997; Dashwood et al, 1998]. It possesses highly potent antioxidant activity [Sato et al, 1984; Sato et al, 1985; Kamat et al, 2000; Kumar et al, 2001]. It has been used, without apparent toxic side effects, to treat a number of human ailments. As a food pigment it can be widely used in cakes, beverages, sweets, ice creams etc. On a daily basis, it is used in green toothpaste and cosmetics. It has been found to exhibit anticarcinogenic and antimutagenic properties. Our earlier studies have shown chlorophyllin to be a potent antioxidant against oxidative stress induced by radiation, photosensitization and peroxyxynitrite as measured by inhibition of lipid peroxidation, protein oxidation, DNA damage and restoration of endogenous antioxidants [Kamat et al, 2000; Kumar et al, 2001].

Though its scavenging ability of primary radicals such as hydroxyl radical, superoxide and singlet oxygen has been established, its ability to react with biologically important secondary radicals such as peroxy radicals and thiyl radicals has not been examined. The present investigation is towards this objective. The secondary radicals studied in the present investigation are lipid peroxy radicals (LOO[•]), chloroperoxy radical (CCl₃O[•]) and thiyl radicals (RS[•]). Peroxy radicals are able to abstract hydrogen atom from another lipid molecule

(adjacent fatty acid), especially in the presence of metals such as copper and iron, thus causing an autolytic chain reaction. LOO[•] can combine with H to give LOOH and this reaction characterizes the propagation stage of lipid peroxidation. Chloroperoxy radical has been used as a representative peroxy radical and also for its inherent simplicity in performing experiments. The action of cytochrome P450 system on toxicants like CCl₄ generates trichloromethyl radical which is able to react with oxygen to give chloroperoxy radical. Thiols are present in living systems which in addition to participating in cellular redox processes also take part in scavenging free radicals. This reaction is known as an antioxidant reaction. The thiyl radicals thus generated in living systems are considered to be reactive oxidants as they are able to initiate lipid peroxidation by abstracting bis-allylic hydrogen from poly-unsaturated fatty acids. The thiyl radicals can also add to the double bond leading to efficient cis/trans isomerization of mono and polyunsaturated fatty acids.

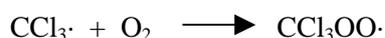
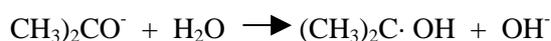
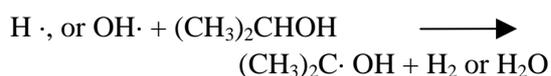
Materials and Methods

The pulse radiolysis system using 7 MeV electrons has been described earlier [Mukharjee, 1997; Scotl et al, 1993]. The dosimetry was carried out using an air-saturated aqueous solution containing 5 x 10⁻² mol dm⁻³ KSCN (G_e = 23,889 dm³ mol⁻¹ cm⁻¹ per 100 eV at 500 nm) [Buxton and Stuart, 1995]. The kinetic spectrophotometric detection system covered the wavelength range from 250 to 800 nm. The optical path length of the cell was 1.0 cm. The width of the electron pulse was 50 ns. Time-dependent absorbance differences were recorded on a digital oscilloscope. High purity (> 99.9 %) N₂O, from BOC India Pvt. Ltd. was used.

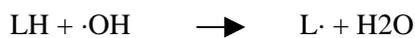


•Chloroperoxy radical (CCl₃OO[•]) was generated by the reaction of carbon tetrachloride

(CCl₄) with oxygen. ·CCl₄ generates trichloromethyl radical (·CCl₃) which is able to react with oxygen to give CCl₃OO·. This radical species was generated in aerated water-isopropanol-acetone (50:40:10 v/v) mixtures containing carbon tetrachloride by the following reactions:



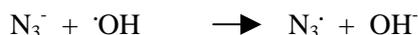
Lipid peroxy radical was generated by pulse radiolysis carried out in aerated aqueous solution containing 2 x 10⁻² mol dm⁻³ linoleic acid.



Thiyl radicals were generated on pulse radiolysis of cysteine (10⁻³ M) in N₂O saturated solution containing chlorophyllin (6.5 x 10⁻⁵ M).



• Azide radicals were generated on pulse radiolysis of N₂O-saturated aqueous solution of NaN₃ (0.1 M).



The bimolecular rate constants were calculated by plotting pseudo-first order rate of formation of the transient against the concerned solute concentration. The uncertainty in the measurement in bimolecular rate constant is <10%. The transients obtained in the pulse radiolysis study were used to characterize the product radical. The rate constants determined and presented in the text are not mere radiation chemical parameters, but they reflect on the efficiency of scavenging free radicals and the ease with which competing reactions occur.

Results and Discussion

Figure 1 shows the transient optical absorption spectrum obtained on reaction of N₃· with chlorophyllin. One-electron oxidized species is observed to have transient absorption bands at 320, 370 and in the region of 430-450 nm with bleaching at 410 nm. The one-electron oxidized species is observed to be stable at least upto 1ms and recovery of bleaching is also not observed in this time scale.

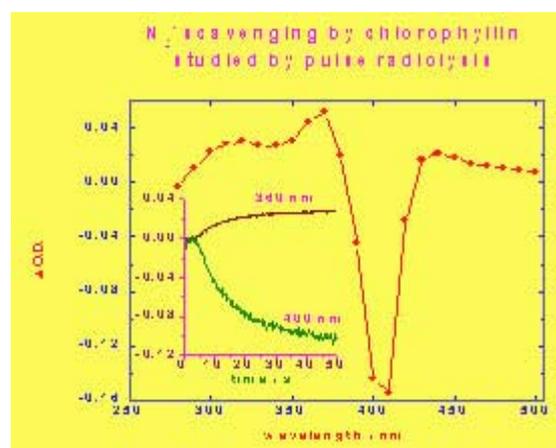


Fig. 1

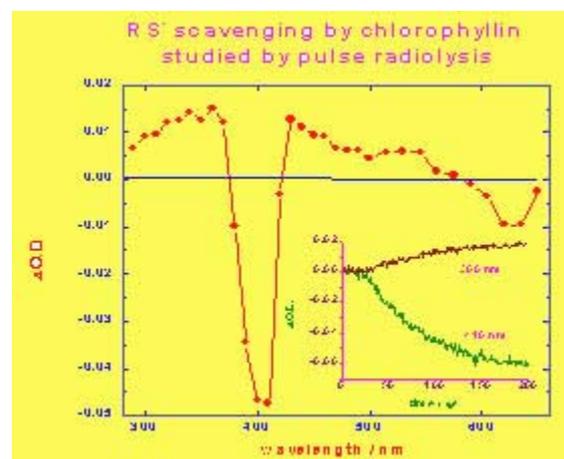


Fig. 2

Chloroperoxy radicals are also observed to react with chlorophyllin with a bimolecular rate constant of ~7x10⁸ M⁻¹S⁻¹ and the transient absorption spectra was similar to that obtained on reaction with azide and thiyl radicals. Azide radicals are observed to react with chlorophyllin

with a bimolecular rate constant of $1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ as determined from the growth of the transient band at 360 nm and bleaching of ground state absorption at 400 nm.

Thiyl radicals generated on pulse radiolysis of cysteine (10^{-3} M) in N_2O saturated solution of chlorophyllin ($6.5 \times 10^{-5} \text{ M}$) showed the formation of a transient band $\sim 370 \text{ nm}$ with bleaching at 410 nm with a bimolecular rate constant of $\sim 2 \times 10^8 \text{ m}^{-1} \text{ s}^{-1}$. The spectra is similar to that obtained on reaction with azide radicals, but only the rate constant value was lower.

Table 1: Reaction rate constants of various radicals studied with chlorophyllin determined using pulse radiolysis

Reaction of CHL with	Rate constant ($\text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$)
$\text{N}_3\cdot$	1×10^9
$\text{CCl}_3\text{O}_2\cdot$	7×10^8
$\text{RS}\cdot$	2×10^8

Pulse radiolysis studies reveal that chloroperoxyl, azide and thiyl radicals react with chlorophyllin in similar way. The high rate constant values reveal that chlorophyllin is able to scavenge chloroperoxyl, azide and thiyl radicals effectively.

Values are mean \pm S.E. of 4 experiments. LOOH – lipid hydroperoxide.

[Kamat JP, Bloor KK & Devasagayam TPA Chlorophyllin as an effective antioxidant against membrane damage *in vitro* and *in vivo*. *Biochimica et Biophysica Acta* 1487, 113-127, 2000]

However the reaction of $\text{LOO}\cdot$ radical with chlorophyllin was slow and the yield of one-electron oxidized species of chlorophyllin was also small. Our earlier studies have indicated chlorophyllin to be a potent protector of lipids and proteins in rat liver mitochondria (Table 2).

Table 2 : Protective effect of chlorophyllin against radiation-induced lipid peroxidation and inactivation of superoxide dismutase as compared to established antioxidants at equimolar concentration of $50 \mu\text{M}$

Treatment	Lipid peroxidation (nmoles LOOH/mg protein)	Superoxide dismutase (units/mg)
Control	7.38 ± 0.17	6.37 ± 0.10
Rad	43.55 ± 1.54	1.50 ± 0.17
Rad+CHL	20.15 ± 3.71	5.25 ± 0.14
Rad+Asc	40.21 ± 1.47	3.00 ± 0.28
Rad+GSH	25.50 ± 1.83	4.80 ± 0.10
Rad+Mann	35.66 ± 2.01	2.62 ± 0.21
Rad+t-BuOH	34.39 ± 1.25	2.25 ± 0.43

This indicates that inhibition of lipid peroxidation observed with chlorophyllin may involve other mechanism apart from radical scavenging.

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This paper won the Best Poster award at the International Conference on "Role of Free Radicals and Anti-oxidants in Health and Disease" held at Lucknow during February 10-12, 2003

About the authors ...

Dr K.K. Boloor received his Ph.D. degree carried out under the guidance of Dr T.P.A. Devasagayam from Radiation Biology and Health Sciences Division, BARC, on 'Mechanisms and modulation of membrane damage induced by ionizing radiation and reactive oxygen species'. Presently, he is working as a senior research scientist in Vimta Laboratories, Hyderabad.



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Dr Hari Mohan joined BARC in 1967. Since then, he has been actively involved in the study of fast reaction kinetics using accelerators and lasers. His current research interests include free radical reactions of halogenated and sulphur compounds and biomolecules of natural origin. He has co-authored more than 150 research papers in international journals. Presently, he is the Head, Radiation Chemistry Section of Radiation Chemistry and Chemical Dynamics Division, BARC.