PALACKY UNIVERSITY IN OLOMOUC

Faculty of Science

Department of Biophysics

BACHELOR THESIS

Effect of antioxidants on oxidative damage of albumin detected by ultra-weak photon emission

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Olomouc 2021

Bibliografická identifikácia

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Názov práce: Vplyv antioxidantov na oxidatívne poškodenie albumínu študovaný pomocou ultra-slabej fotónovej emisie

Typ práce: experimentálna

Pracovisko: Katedra Biofyziky

Vedúci práce: Mgr. Marek Rác, Ph.D.

Rok obhajoby práce: 2021

Abstrakt: Poškodenie biomolekúl a teda aj ich oxidácia je sprevádzaná slabou biofotónovou emisiou alebo inak nazývanou ultra slabá emisia fotónov (UPE). V tejto bakalárskej práci bola skúmaná UPE z oxidatívne poškodeného hovädzieho sérového albumínu (BSA). Merania sa uskutočnili za použitia vysoko citlivého fotonásobiča (PMT). Cieľom práce bolo zmerať UPE z BSA poškodeného rôznymi reaktívnymi formami kyslíka (ROS). Efekt antioxidantov na oxidatívne poškodenie bolo taktiež sledované technikou UPE. Na základe dosiahnutej úrovne UPE sme diskutovali rozpätie daného poškodenia, prípadne stupeň dosiahnutej ochrany vďaka pridaným antioxidantom. Najrozsiahlejšie poškodenie BSA podľa nameraných dát vzniklo po pridaní hydroxylového radikálu ($HO^{\bullet}$) naopak najnižšia UPE bola pozorovaná u vzoriek so singletným kyslíkom ($^1O_2$). Rozsah poškodenia peroxidom vodíka (H_2O_2) bolo niekde na pomedzí. Kyselina askorbová (Asc) vykazovala antioxidačnú kapacitu u všetkých typov indukovanej UPE. Mierne zvýšenie UPE po pridaní Asc sa prejavil u vzorku so singletným kyslíkom ($^1O_2$). Rozdielne efekty vykazoval alfa-tokoferol (Toc), ktorý prejavoval ako antioxidačné účinky tak aj pro-oxidačné. Mierne pro-oxidačné účinky u vzoriek so $^1O_2$, kde Toc zvýšil UPE pred aj po formovaní ROS a silnejší pro-oxidačný účinok Toc pri UPE indukovanej $HO^{\bullet}$. Dáta uvedené v tejto práci môžu byť použité pre ďalší výskum napríklad pre iné dôležité proteíny v organizmoch za použitia UPE, prípadne pre BSA za použitia iných antioxidantov.

Kľúčové slová: reaktívne formy kyslíka, ultra-slabá fotónová emisia, oxidácia proteínov, albumín, antioxidanty

Počet strán: 47

Počet príloh: 0

Jazyk: anglický

Bibliographical identification

Author´s first name and surname: Alžbeta Mydlárová

Title of thesis: Effect of antioxidants on oxidative damage of albumin detected by ultra-weak photon emission

Type of thesis: experimental

Department: Biophysics

Supervisor: Mgr. Marek Rác, Ph.D.

The year of defence: 2021

Abstract: The damage to biomolecules and thus their oxidation is accompanied by a weak biophoton emission or otherwise called ultra-weak photon emission (UPE). In this bachelor thesis, UPE from oxidatively damaged bovine serum albumin (BSA) was investigated. Measurements were performed using a high sensitivity photomultiplier (PMT). The aim of the work was to measure UPE from BSA damaged by various reactive oxygen species (ROS). The effect of antioxidants on oxidative damage was also monitored by the UPE technique. Based on the achieved level of UPE, we discussed the extent of the damage, or the degree of protection achieved due to the added antioxidants. According to the measured data, the most extensive BSA damage occurred after the addition of the hydroxyl radical ($HO^{\bullet}$), on the contrary, the lowest UPE was observed in samples with singlet oxygen ($^1O_2$). The extent of hydrogen peroxide (H_2O_2) damage was somewhere in frontier. Ascorbic acid (Asc) showed antioxidant capacity in all types of induced UPE. A slight increase in UPE after the addition of Asc was observed in the sample with $^1O_2$. Alpha-tocopherol (Toc) showed different effects, showing both antioxidant and pro-oxidant effects. Slight pro-oxidant effects in samples with $^1O_2$, where Toc increased UPE before and after ROS formation and stronger pro-oxidatant effect of Toc in UPE induced by $HO^{\bullet}$. The data presented in this work can be used for further research, for example for other important proteins in organisms using UPE, or for BSA using other antioxidants.

Key words: reactive oxygen species, ultra-weak photon emission, protein oxidation, albumin, antioxidants

Number of pages: 47

Number of attachments: 0

Language: english

Acknowledgments

I would like to thank to my supervisor Mgr. Marek Rác, Ph.D. for his leading during the process of creation on this bachelor thesis, providing of literature, advices and endless patience. I would also like to thank my consultant Doc., RNDr. Pavel Pospíšil, Ph.D.

Statement

I declare that I have written this bachelor thesis myself under the direction of Mgr. Marek Rác, Ph.D. and with the help of the literature mentioned in the end of the thesis.

In Olomouc dated …….. ……

List of Abbreviations

$^1O_2$	singlet oxygen
$^3(R{=}O)^*$	triplet excited carbonyl
$FeSO_4 \cdot 7\ H_2O$	iron (II) sulfate heptahydrate
H_2O_2	hydrogen peroxide
$HO^\bullet$	hydroxyl radical
HO^-	hydroxyl anion
MoO_3	molybdenum (VI) oxide
O_2	molecular oxygen
$O_2^{\bullet -}$	superoxide anion radical
8-oxoG	8-oxo-7,8-guanosine
8-OHdG	8-hydroxy-2′-deoxyguanosine
PMT	photomultiplier tube
CCD	couple-charged device
EPR	electron paramagnetic resonance
$R^\bullet$	carbonyl radical
$RO^\bullet$	alkoxyl radical
$ROO^\bullet$	peroxyl radical
ROOH	hydroperoxide
ROOR	dioxetane
ROOOOR	tetroxide
$^1C^*$	singlet excited pigment
$^3C^*$	triplet excited pigment
ROS	reactive oxygen species
RNS	reactive nitrogen species

UPE	ultra-weak photon emission (equivalents: biophoton emission, low-level chemiluminescence)
SOD	superoxide dismutase
GSH	glutathione
GPx	glutathione peroxidase
CAT	catalase
Asc	ascorbic acid
$Asc^{\bullet}$	ascorbyl radical
MDA	monodehydroascorbic acid
DHA	dehydroascorbic acid
Toc	alpha-tocopherol
$Toc\text{-}Q^{\bullet}$	tocopheroxyl radical
BSA	bovine serum albumin

Content

1 Introduction

Oxidative damage of different biological systems is widely studied by phenomenon of ultra-weak photon emission (UPE). Origin of UPE comes from electronically excited species formed during oxidative processes in organisms. Pioneering discoveries in recent decades in technical and theoretical fields of UPE enable to rigorously study oxidative damage of biomacromolecules (lipids, nucleic acids and proteins). Standard devices for UPE detection are low-noise multiplier tubes (PMT) and charge-coupled devices (CCD) cameras. Technique of UPE can be used as non-invasive diagnostic tool in wide range of fields from medicine, agriculture to food industry (*Cifra and Pospíšil, 2014*).

Reactive oxygen species (ROS) are one of the considered oxidation agents. The most common classifications of ROS are radical and non-radical or by formation to excited or reduced forms of molecular oxygen (O_2). These species are formed during oxidative metabolic processes and oxidative stress processes. The products of these processes as well as their course can be detected by UPE (*Pospíšil et al. 2019*). The photon emission from biomolecules oxidized by ROS can be detected from near UV, through VIS and near IR regions of spectra using filters or in combination with other techniques.

An effective defense mechanism against ROS in organisms includes antioxidants. Antioxidants are substances able to inhibit or restrain oxidazing substances mentioned above. If we divide antioxidants by activity, we can talk about enzymatic and non-enzymatic substances. In this thesis effect of non-enzymatic antioxidants was studied. Non enzymatic scavenging stabilize ROS by its one-electron reduction or donation of atom of hydrogen, where less reactive species are formed. Enzymatic antioxidants convert oxidative products to H_2O through multi-step process (*Nimse and Pal 2015*).

Under specific conditions antioxidants may exhibit pro-oxidant behaviour (*Kondakçi et al. 2013*). One of the best observed factor is presence of transition metal ions (Fe^{3+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{+}, Cu^{2+} or Cr^{3+}), which are important elements in chemistry of living systems. They provide transport of electrons, ions and affect the actions of enzymes, vitamins etc. Many proteins obtain transition metal ions, e.g. proteins of respiratory chain. Antioxidants reduce metal ions instead of oxidizing substances, which leads to generation of more ROS. Another trigger of pro-oxidant behaviour is

imabalance between number of antioxidants and oxidants in system. Few studies expose pro-oxidant behaviour of separate alpha-tocopherol (Toc), where it is not in complex with other antioxidants such as coenzyme Q_{10} or ascorbic acid (Asc).

In this bachelor thesis, PMT sensitive in VIS part of spectra was used. Oxidative damage of bovine serum albumin (BSA), frequently investigated model protein was studied after exogenous addition of ROS. Study was focused on effect of namely three ROS, hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$) and singlet oxygen ($^1O_2$). Based on the literature, we assumed that the level of detected UPE matched the extension of oxidative damage and processes associated with formation of other reactive intermediates. Antioxidant activity and pro-oxidant behaviour of Asc and Toc was detected and defined by measured level of UPE as well.

2 Theoretical part

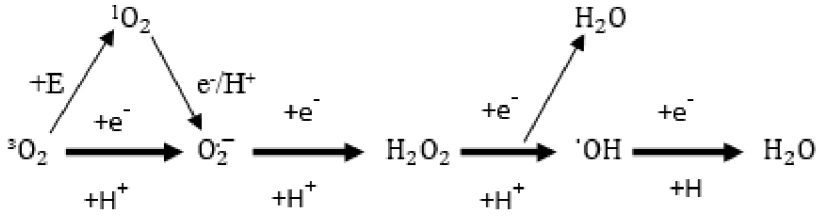
2.1 Reactive oxygen species

Formation of ROS is a common part of metabolic processes where these species are frequent by-products of reactions. The ongoing processes at the cellular level can give rise to different types of ROS in different cellular compartments. Those species can be divided into two groups, radical and non-radical ROS. Radical ROS have one or two unpaired electrons on the oxygen atom and into this group belongs superoxide anion radical ($O_2^{\bullet -}$) and $HO^{\bullet}$. Non-radical ROS have no unpaired electron and H_2O_2 and $^1O_2$ belongs into this group. Two separate pathways lead to the formation of mentioned ROS. Sequential one-electron reduction of O_2, also called Type I reaction, or triplet-singlet energy transfer from triplet excited molecules (chlorophylls, melanin etc.) to $^1O_2$, called Type II reaction (*Pospíšil et al. 2014*).

Type I reaction is connected with leakage of electrons. This reaction has chain character and takes place in biological systems. During partial reduction of O_2 (e.g. in mitochondria) by-products of electron transport chain such as $O_2^{\bullet -}$ (*Chernyak et al. 2006*) are formed. Subsequent one-electron reduction of $O_2^{\bullet -}$ forms H_2O_2 but also two-electron reduction of O_2 can lead to formation of H_2O_2 supported by enzyme water oxidase, which occurs in cell systems. One-electron reduction of H_2O_2 in presence of transition metal ions forms $HO^{\bullet}$ through Fenton´s reaction, see 2.1.1. One-electron reduction reactions are shown in Fig. 1 beneath. Type I reactions can be both enzymatic and non-enzymatic and occurrs mainly in mitochondria and chloroplasts but also in plasma membranes, microbodies and endoplasmic reticulum.

Type II reactions explains $^1O_2$ formation. One of the pathways, which leads to $^1O_2$ formation relates to absorption of excitation energy by skin photosenzitizers (either in plant or animal skin). Subsequent reactions are going through intersystem crossing and end by formation of $^1O_2$ (*Schmidt 2007*). Except mentioned pathway, other feasible way of $^1O_2$ formation occurs. These reactions are decompositions of unstable intermediates such as dioxetane (ROOR) or tetroxide (ROOOOR), (*Miyamoto et al. 2014*). Decomposition of these intermediates is through triplet excited carbonyl (^{3}(R=O)*) and carbonyls. In the presence of O_2, (^{3}(R=O)*) form $^1O_2$ through triplet energy transfer. In

case of ROOOOR direct decomposition to $^1O_2$, carbonyls and alcohols is described. Another pathway leading to $^1O_2$ formation is through Russell mechanism, where recombination of two peroxyl radicals (ROO$^\bullet$) forms ROOOOR, which rapidly decompose to carbonyl, alcohol and $^1O_2$. Various reactions with ROO$^\bullet$ was however described through different mechanisms and is still under research.



Reaction 1: One-electron reduction of molecular oxygen.

Formation of ROS plays a crucial role in cell signalling, ageing and products of oxidation reactions are often used as markers for oxidation of biomolecules and hence their presence can be used as biomarkers for various diseases (*Kristiansen et al. 2009*).

2.1.1 Fenton´s reaction

Transition metals such as Fe^{3+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{+}, Cu^{2+} or Cr^{3+} are natively in cell environment providing the transport of electrons, ions and affecting the actions of various elements. They are often bounded in metalloproteins, transport proteins or storage proteins. These proteins also scavenge metal ions to prevent running of Fenton´s reaction (*Mandéz-Garridol et al. 2014*). Basically, the course of Fenton´s reaction can be described as reactions of Fe^{2+} with H_2O_2, which lead to formation of trivalent iron, HO$^\bullet$ and hydroxyl anion (HO^-). Reaction is noticed in scheme 1.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^\bullet + HO^-$$

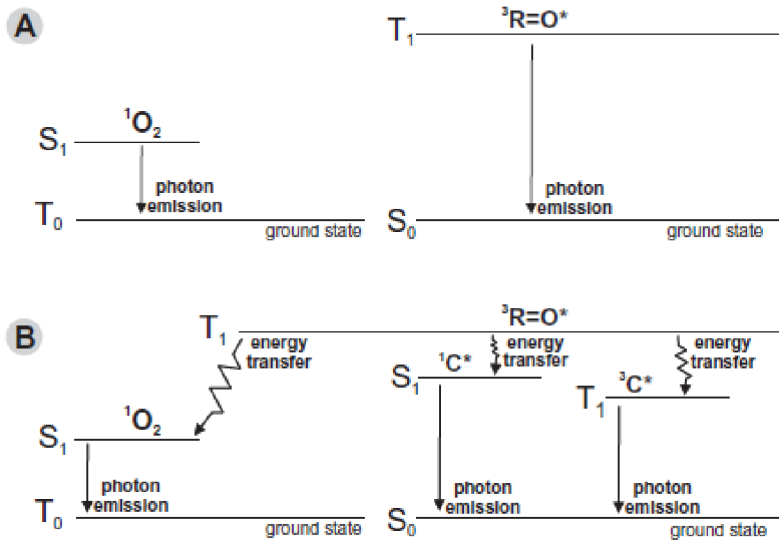
Reaction 2: Course of Fenton´s reaction. In presence of transition metal ions, H_2O_2 is reduced to highly reactive HO$^\bullet$.

2.2 Ultra-weak photon emission

Ultra-weak photon emission is characterised as non-thermal radiation from near-ultraviolet to visible region (100–800 nm) of the electromagnetic spectrum, possibly reaching the near-infrared region (801–1300 nm) (*Burgos et al. 2017*). Non-thermal radiation is observed due to the presence of excited molecules formed during chemical reactions. Based on attitude of concrete author, we can find other terms in literature such as chemiluminescence, biophoton emission or autoluminescence. Chemiluminescence is connected with fact, that observation of UPE is directly from chemical reactions; biophoton emission signify the biological origin within chemical reactions inside cells and autoluminescence is linked to the fact that it originates without any stimuli from environs. Phenomena of UPE is observable from all living biological systems from bacteria, fungi, plants, organs to whole organisms (*Cifra and Pospíšil 2014*).

Ultra-weak photon emission can be divided into groups according to different phases of metabolic processes or their different character into spontaneous and stress-induced. Spontaneous UPE is formed during common oxidative metabolic processes (e.g. photosynthesis, cellular respiration) without any stimuli from outside. On the other hand, the stressed-induced UPE is stimulated by biotic or abiotic stress factors and processes. Biotic stress factors are virus, fungi or bacteria. Abiotic stress factors can be divided into physical and chemical. Factors as pH, surrounding gases along with others are considered as chemical and UV or ionizing radiation, temperature are referred as physical stress factors. The difference between spontaneous and induced UPE can be identified from its intensity. The spontaneous UPE is in the range from several units to several hundred photons $s^{-1}cm^{-2}$, the stress induced UPE is in the range from several hundreds to several thousands of photons s^{-1} cm^{-2} (*Cifra and Pospíšil 2014*).

By-products of mentioned metabolic processes include $^3(R=O)^*$ and $^1O_2$. Transition from singlet or triplet excited state to the ground state of these species (Fig. 2) is accompanied with photon emission and thus those species are considered being responsible for UPE. Maximum of UPE from $^3(R=O)^*$ is 350-550nm and in case of dimolar UPE from $^1O_2$ is at 634nm and 703nm. Another electronically excited species responsible for UPE are singlet excited pigment ($^1C^*$) and triplet excited pigment ($^3C^*$). Maximum of UPE from $^1C^*$ is at 360-560nm for melanin and 680-740nm for chlorophyll. Triplet excited pigment has maximum of photon emission at 870-100nm (chlorophyll).



Scheme 1: Transition from excited energy states of electronically excited species. A) Energy level of ^{3}(R=O)* and $^1O_2$. B) Energy level of ^{1}C*, ^{3}C* and it´s transition from ^{3}(R=O)* (taken from Cifra and Pospíšil 2014)).

Applications of UPE are associated with wide-spread research on various oxidative damage by various reactive species (reactive oxygen and nitrogen species, hydroperoxides, electronically excited species etc.). Main targets of oxidative damage are biomolecules (proteins, lipids, nucleic acids). In a field of UPE, many studies were aimed to detect changes in biomolecules as it is non-invasive tool for its detection.

(*Van Wijk et al. 2010*) made a study based on detection of oxidative stress from human skin using UPE. They considered higher level of protein carbonyls, which are protein oxidation product as source of signal in detection of oxidative stress-induced UPE from the skin. Based on this, they studied protective effects of oligomeric proanthocyanidins creams on skin and compared UPE before and after its application. Ultra-weak photon emission from oxidatively damaged polyunsaturated fatty acids has been examined in divers studies. Evidence of involvement of lipid peroxidation in UPE measured in *Chlamydomonas reinhardtii* cells was provided in study of (*Prasad and Pospíšil 2011*). Oxidative damage of nucleic acids and its detection by UPE is limited. In study made by (*Ma et al. 1998*) was shown that from adenine, guanine, cytosine and thymine, addition of copper/Asc/H_2O_2 system, results in enhanced UPE only from guanine.

Detection of UPE is still under development but PMTs are conventional equipment with long scale of advantages and future development possibilities (*Kobayashi and Inaba*

2000). They are constructed to measure different parts of spectra (NIR region of spectrum, UV/VIS part of spectrum). These photodetection technologies are based on photoelectric phenomenon.

2.3 Oxidation of biomolecules

Oxidation of biomolecules is closely connected with formation of ROS. Formation of ROS in living systems triggers the cascade reaction on biomolecules, which leads to stress-induced metabolism. Oxidative stress metabolism has been reported as a critical dimension of pathogenicity in many diseases in connection with oxidation of biomolecules (proteins, lipids, nucleic acids) in cells (*Burgos et al. 2017*). After ROS attack on biomolecules, the resulted oxidation products may trigger the oxidation of other biomolecules in cascade reactions. Common attribute is formation of hydroperoxides (ROOH), which might be reduced and cause subsequent oxidation. Study where histone H_1 protein ROOH gave rise to ROS which oxidized DNA was made by (*Davies et al. 1999*). Other study displays how one electron reduction of lipid ROOH leads to damages in DNA structures (*Crean et al. 2009*).

Oxidation of lipids/lipid peroxidation is a process in which lipids and their structure is changed through chain reactions propagated by ROS and other types of free-radicals. Polyunsaturated fatty acids are often affected parts of lipids due to multiple methylene groups positioned between double bonds (*Takashi et al. 2015*). Since oxidation of lipids is considered as key chemical event, which might be implicated in alternation of other biomolecules various studies have been made to study effect of concrete ROS. Hydroxyl radical is undispusible oxidation agent (*Poyton et al. 2016*) and various studies also show that presence of $^1O_2$ lead to the formation of lipid ROOH (*Shimizu et al. 2018*). The oxidation process itself begins when atom of hydrogen is abstracted from polyunsaturated fatty acid by ROS. This part of chain reaction, iniciation, leads to the formation of fatty acid alkyl radical ($R^{\bullet}$). Next step is propagation. In this phase, $R^{\bullet}$ rapidly reacts with O_2 which creates $ROO^{\bullet}$. Peroxyl radical abstracts a hydrogen from another lipid molecule, which creates second $R^{\bullet}$. This process is responsible for chain reaction character. Another product of propagation reaction is ROOH. Termination is the reaction of radicals formed during previous reaction and the reagents like vitamines, which products have non-radical character. Sufficient concentration of radicals in system is required condition for the course of termination. Secondary reaction in process of oxidation of polyunsaturated fatty acid occurs under reducing conditions such as presented free and bound metals. Presence of these molecules leads to reduction of ROOH to alkoxyl radicals ($RO^{\bullet}$) or $ROO^{\bullet}$. These radicals may trigger another cascade of

reactions. High concentration of forming ROO$^{\bullet}$ can lead to its recombination to ROOOOR, which might decompose to triplet excited carbonyls ^{3}(R=O).* Transfer of triplet energy can lead to decomposition on excited pigment C* or O_2, which lead to formation of $^1O_2$. Direct decomposition of ROOOOR give rise to $^1O_2$ through Russell mechanism together with by products alcohols and carbonyls (*Prasad et al. 2016*). Alkoxyl radical is highly capable of hydrogen atom abstraction. These reactions lead to a formation of R$^{\bullet}$ and these reactions further trigger lipid autoxidation and promote the propagation of lipid peroxidation (*Xie et al. 2019*).

Modification caused by oxidation of DNA leads to cleavage of DNA strands, modifications in genome or various mutations. Oxidation of DNA is located on heterocyclic bases or sugar-phosphate backbone. Ability to directly oxidize DNA manifest H_2O_2, HO$^{\bullet}$ and $^1O_2$ but also ROO$^{\bullet}$. More oxidized bases are purine rather than pyrimidine. Guanine has the lowest redox potential, which makes it the main target from nitrogenous bases. Publication of (*Ma et al. 1998*) provided experimental evidence of UPE from oxidized guanine (e.g. by H_2O_2), which was exposed to antioxidants. Ultra-weak photon emission detected during these measurements was assigned to the region of spectra characteristic for ^{3}(R=O)*. Review on topic of DNA oxidizied by $^1O_2$ was made by (*Agnez-Lima et al. 2012*). Main product of reaction between ROS and guanine is 8-oxo-7,8-guanosine (8-oxoG). Intermediate product and the first product after attack of ROS is guanine radical G$^{\bullet+}$ which undergoes hydrogenation and forms 8-oxoG (*Cadet et al. 2009*). Reaction of three ROS with guanine along DNA and its products was studies by (*Matter et al. 2018*). Reaction where recombination of ROO$^{\bullet}$ and subsequent formation of ROOOOR later led to $^1O_2$ formation through Russell mechanism was described in DNA oxidation processes. Damage caused by ROO$^{\bullet}$ produced by peroxized thymidine led to the formation of 8-hydroxy-2´-deoxyguanosine (8-OHdG), adduct used as biomarker of cancer (*Goto et al. 2008*). Formation and amount of formed 8-OHdG was described in study of (*Ma et al. 2016*) on retinal mDNA. Oxidation of sugar-phosphate backbone is still under subject of studies, but few aspects of this process is partially evaluated. The C5 atoms are accessible and carbon-hydrogn bonds are weaker than other bonds in DNA. As the most effective oxidant in this part is considered HO• and (*Roginskaya et al. 2015*) also studied effect carbonate radicals. Described consequence of DNA oxidation is mispair and misincorporation of bases. Formation of DNA cross-links increased in study, where samples were treated with metal-ions. Subsequent

oxidatants and misincorporation of adenine and guanin caused further lesions formation (*Hailer et al. 2005*).

2.4 Protein oxidation

Oxidation of proteins has many different pathways and complex description is difficult due to various structures composed of various residues. Proteins are composed from amino acids connected by peptide bond. In this assumption one considers various residues as different composition of amino acids (20 common amino acids). Review articles, which covered the essence of processes leading to protein oxidation and it´s distribution used in this thesis are written by (*Dean et al. 1997 and Davies 2016*).

Oxidation of proteins occurs on backbone or side-chain. In proteins, consistent with length of chain (in order free amino acids<peptides<proteins) and apparent increased α-carbonyl radical formation on backbone (*Morgan et al. 2008*). Stability of α-carbonyl radical depends on secondary protein structure. Extent of backbone damage occurs predominantly in antiparallel β-sheets, where is abundance of Gly residues (*Stringfellow et al. 2014*). Limiting access to attacking radicals can traverse structural factors of proteins and so in globular and sheet proteins side-chain reactions occur more frequently. In connection with extent of backbone damage, it is also established that plenty of enzymes obtain endogenous radicals in active form. These radicals can be found on Tyr, Try, Gly and thiols. Reaction of these protein radicals with O_2 can conduct inactivation of enzymes on backbone (rather than side chains) (*Pedersen and Finazzi 1993*). In general, main pathway is hydrogen abstraction from R-H bonds. Major reactions occur on Tyr, Try and Gly but also Cys and Met residues because of their features connected with presence of sulfur. In few cases a further distance from amide group lead to damage also in other amino acids (e.g. Trp and Lys).

Proteins are susceptible to damage by various ROS. It is well established that less reactive species are more selective in damage. Level of reactivity depends on character of attacking radicals. Radicals with electrophilic nature ($HO^{\bullet}$, $RO^{\bullet}$, $ROO^{\bullet}$) often attack electron rich sites of proteins. Hydroxyl radical is ROS with highest redox potential and is able to oxidize all amino acid residues. Nucleophilic radicals such as $R^{\bullet}$ attack electron deficient sites of proteins preferentially. Addition of exogenous H_2O_2 to proteins relates to increased susceptibility to hydrolysis and proteolysis (*Fligiel et al. 1984*). In study made by (*Ghesquiere at al. 2011*), H_2O_2-treated T-lymphocytes were used to identify and quantify Met oxidation of cellular proteome. Since Cys residues are connected with cell signaling and sulfide bonding between proteins structures in connection with naturally

forming ROS in cells various studies are aimed to understand the mechanism behind it. (*Reest at al. 2018*) made proteome analyses to prove Cys oxidation and chemistry behind it is a key amino acid behind redox stress induced by H_2O_2. Various studies were aimed to detected oxidation of Cys and Met residues after UV radiation or by $HO^{\bullet}$ and other ROS. Modification on Trp residues by ROS or derived species together with its detection was explained by (*Erhenshaft et al. 2015*) to enlighten specific protein oxidation.

First step of oxidation is attack of protein chain by radical (general designation - $X^{\bullet}$) and the second step is subsequent formation of $R^{\bullet}$,which in presence of O_2 can give rise to $ROO^{\bullet}$. Subsequent hydrogen transfer from protein structure forms ROOH (Fig. 3). Hydrogen abstraction is easier in the presence of -OH group, e.g. of Ser and Thr (*Thomas et al. 2014*). However, in lack or absence of C-H (S-H in Cys) bonds, $ROO^{\bullet}$ are likely to undergo recombination. Product of which is unstable ROOOOR and subsequent Russell mechanism leads to formation of $^1O_2$ directly or through $^3(R=O)^*$. By products of these reactions are carbonyls/alcohols.

Formation of proteins ROOH depends not only on the presence of certain bond but also on concrete amino acid and location. Addition of O_2 to C3 radicals on the indole ring of Trp resulting in formation of $ROO^{\bullet}$ and subsequent ROOH formation was observed in study of (*DeGray et al. 1997*). This selectivity for specific residue also occurs in Tyr radical of myoglobin reacting with $O_2^{\bullet -}$ to give rise ROOH (*Das et al. 2010*). Different pathways, which lead to ROOH formation are shown in Fig. 4.

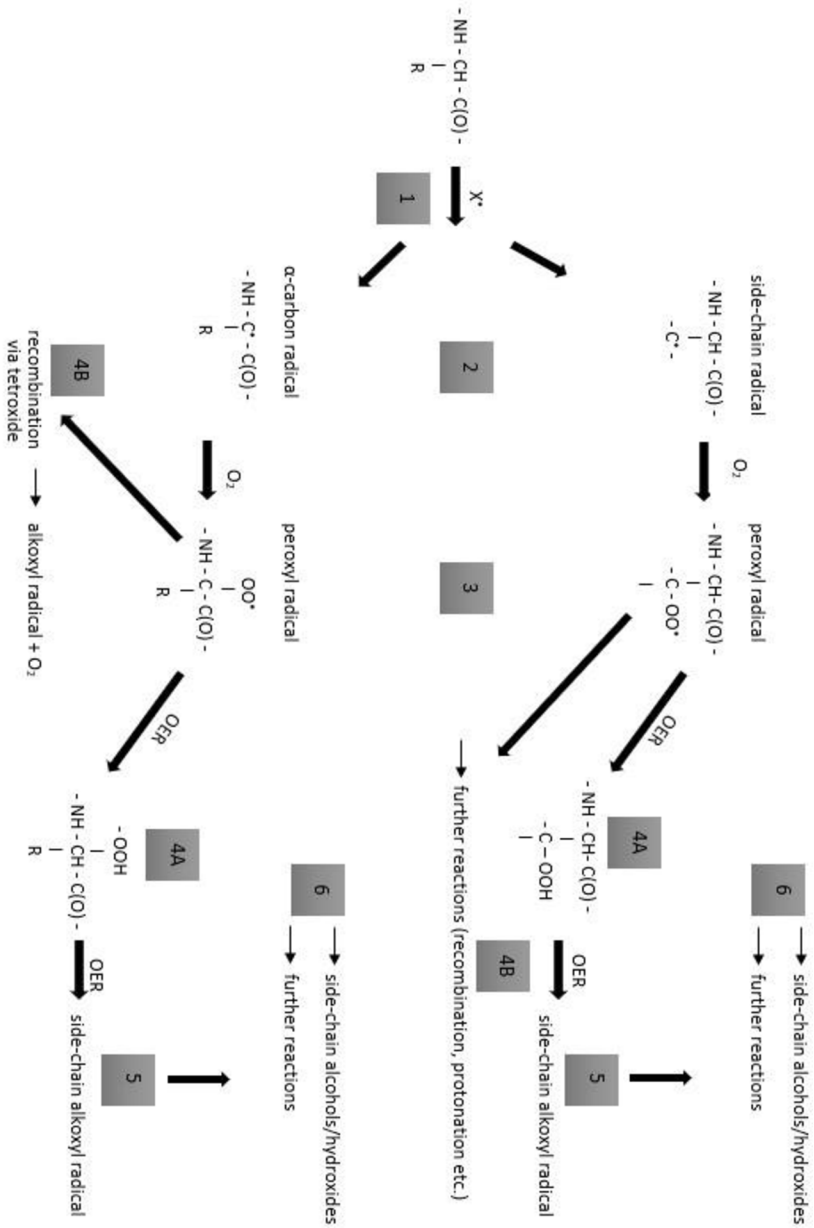


Figure 1: The upper part of scheme represents the reactions on side-chain and the lower part represents the reactions on backbone during protein oxidation in presence of O_2. First step (1) is same for both and it is attack of exogenous/endogenous X•. Second step (2) is immediate formation of R•, which continuously lead to third step (3), which is ROO• formation in presence of O_2. Peroxyl radical in case of protein oxidation on side chain undergo two possible reactions. Mainly, it gives rise to protein ROOH through one-elctron reduction (4A) but ROO• may undergo other multiple reactions (4B). Formed ROOH often undergo one-electron reduction (5) and give rise to side-chain RO•, which triggers other reactions (6). Once ROO• is formed on protein backbone the course of reactions are alike. However, ROO• is only subject to recombination (4B) because of lack of required molecules to go further.

Secondary reactions of protein ROOH often occur and can give rise to another radicals. One-electron reduction of ROOH can lead to formation of $RO^{\bullet}$. Alkoxyl radical is capable of hydrogen abstraction, which leads to formation of alcohols, aldehydes and carbonyls. These carbonyls are often used as biomarkers of protein oxidation (*Chandra et al. 2016*).

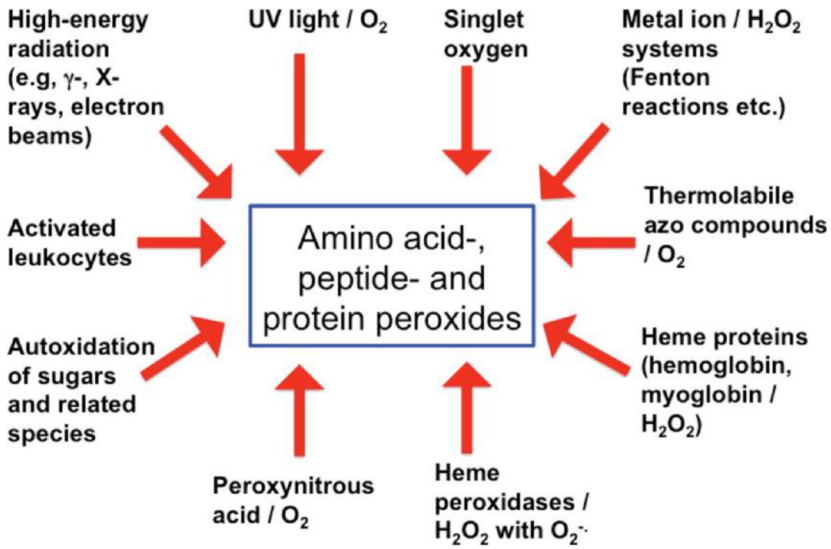


Figure 2: Known pathways for protein oxidation systems, few of them mentioned above. Viewed pathways give rise to amino acid-, peptide- and protein-hydroperoxides in the presence of O_2 (taken from (*Davis et al. 2016*)).

All the reactions mentioned above cause often irreversible damage to proteins. Most of them like unfolding, loss of structural and functional activity leads to an accumulation of damaged proteins and relates to aging and pathological disorders.

2.5 Antioxidants

As response on oxidative damage caused by ROS, organisms have developed few defence mechanisms. One of the most important is antioxidants substances. In general, the term antioxidant is very frequent although not always in a proper meaning. Antioxidants are substances able to restrict an activity of ROS (or various radicals such as RNS). Few antioxidants have one unpaired electron, which can be donated to ROS. Thus, effort of ROS to fill their valence state, which is one of the reasons of their reactivity is replaced by unpaired electron taken antioxidant. Described mechanism is enlight protective feature of antioxidants.

There are two types of antioxidants based on their structure, enzymatic and non-enzymatic. Enzymatic antioxidants work on principle of decomposition and removal of free radicals formed after attack of ROS. Oxidation products are converted to H_2O_2 and later H_2O through multiple steps reactions. This group obtain enzymes such as superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase (GPx). Activity of SOD is described as conversion of $O_2^{\bullet -}$ to H_2O_2 and O_2 and principle of CAT reaction is conversion of H_2O_2 to O_2 and H_2O (*Awoniyi et al. 2012*). Different types of teas were studied in work made by (*Awoniyi et al. 2012*) that explains effects of both types of antioxidants. Examples of non-enzymatic antioxidants are Asc, mentioned Toc, carotenoids, flavonoids, glutathione (GSH) etc. Action of non-enzymatic antioxidants (low molecular weight components) is described as discontinuation of oxidation chain reactions. (*Gulec et al. 2006*) studied protective effects of Toc against oxidative damage caused by formaldehyde in the liver and plasma of rats by termination of lipid peroxidation.

Ascorbic acid can reduce oxidants such as $HO^{\bullet}$, $RO^{\bullet}$, $O_2^{\bullet -}$ or $^1O_2$. It can transform to ascorbic radical $Asc^{\bullet}$ by donating electron to free radicals. It is enediol group on C2 and C3, which enables Asc to donate one or two electrons to form monodehydroascorbic acid (MDA). By losing second electron, dehydroascorbic acid is formed (DHA), the course of this reaction is shown down (Reaction 3). This form of Asc has no antioxidant capability. Formation of DHA is described with or without presence of other enzymatic antioxidants. Important role of Asc as a scavenger of $O_2^{\bullet -}$ in mangroves exposed to oxidation was described in study of (*Jitesh et al. 2006*). Rapid reaction of Asc with $^1O_2$

was studied by (*Kramarenko et al. 2006*). Course of reaction (Reaction 4) show that reaction of $^1O_2$ with Asc leads to H_2O_2 formation and thereby other oxidation is triggered.

$$2Asc_2+H_2O_2 \rightarrow DHA+H_2O$$

Reaction 3: Course of formation of DHA by oxidation of Asc by H_2O_2.

$$^1O_2 + Asc \rightarrow H_2O_2 + DHA$$

Reaction 4: Course of formation of DHA by oxidation of Asc by $^1O_2$.

Capacity to scavenge specific ROS or other forming radicals occurs as feature of Toc. Chain-breaking activity of Toc is considered as one of the best features in lipid peroxidation processes. Alpha-tocopherol shows scavenge activity to $ROO^{\bullet}$ and partially $^1O_2$ and $O_2^{\bullet -}$. Ongoing reaction between $ROO^{\bullet}$ and Toc, where $ROO^{\bullet}$ is stabilized to ROOH and tocopheroxyl radical (Toc-$Q^{\bullet}$) is formed shows Reaction 5. Peroxyl radical abstracts hydrogen atom from 6-hydroxy group on chromanol ring of Toc. This reaction produces alkyl hydroperoxide and Toc-$Q^{\bullet}$. Lack of scavenging capacity of $HO^{\bullet}$, $RO^{\bullet}$ or thyil radical is typical for Toc (*E. Niki 2014*). Reaction between $^1O_2$ and Toc is two-electron oxidation. Product of this reaction is 8-hydroxy-α-tocopheron. This mechanism is widely studied in photosystem II (*Kruk et al. 2005*). Oxidation products of Toc and its homologues was studied by (*Gruzska et al. 2008*).

$$Toc\text{-}OH + ROO^{\bullet} \rightarrow Toc\text{-}Q^{\bullet} + ROOH$$

Reaction 5: Course of formation of Toc-$Q^{\bullet}$ and ROOH by stabilizing $ROO^{\bullet}$.

In living systems, the synergic cooperation of antioxidants is described as mentioned above. This mechanism provide stabilisation in forming less reactive antioxidant radicals or overall stabilization. (*Shibamaya et al. 2014*) showed that Asc in connection with his own derivate disodium isostearyl 2-O-L-ascorbyl phosphate are an effective protective agent against UVA-radiation. Reaction between Toc and methylene groups (e.g. of polyunsaturated fatty acids) during oxidation of biomolecules can lead to increased Toc-$Q^{\bullet}$ formation. This radical in presence of other antioxidants (Asc, coenzyme Q_{10}) may be transform to less reactive forms (*Bursać-Mitrovič et al. 2016*). In plant systems, the complex known as Asada-Halliwel (Asc+ glutathione (GSH)) represents the beneficial cooperation of antioxidants. Among these reagents other are

present. DHA is reduced by GSH and GSSH is produced. Another example of synergic cooperation in plants antioxidant system is called ascorbate-glutathione-tocopherol triad (*Szarka et al. 2012*).

2.6 Pro-oxidant behaviour of antioxidants

Pro-oxidants are defined as chemicals that pronounce oxidative stress during the process of ROS formation or by inhibiting system of antioxidants (*Sotler et al. 2019*). Antioxidants capacity studies and antioxidants activity studies showed few abnormalities and brought unlikely results. Antioxidants act as pro-oxidants under special circumstances. Switch to pro-oxidant behaviour is caused by several factors. There are various known, described factors from which are presence of metal ions, high concentration of antioxidants in system and redox potential of antioxidants. Structural properties of antioxidants are also considered in mechanisms and aspects in which they act as pro-oxidants.

Factor of presence of metal ions in pro-oxidant switch is described in connection with various antioxidants. Ascorbic acid, which reduce ROS such as H_2O_2 can also reduce metal ions and through Fenton reaction forms other ROS. Redox cycling, where repeated reduction of metal ions occur can cause abundant ROS formation and increase pro-oxidant activities. Switch of Asc to pro-oxidant behaviour occurs in low concentrations of Asc as treatment. Pro-oxidant behaviour may be related to weak chelating actions toward ferrous ions (*Yen et al. 2002*).

Toc appear to act as pro-oxidant, especially in laboratories experiments. This phenomena is connected with high concentration of Toc. Accumulation of formed Toc-Q$^{\bullet}$ cause formation of other reactive species even though Toc-Q$^{\bullet}$ is moderately reactive (*Rietjens et al. 2002*).

Lack of oxidative stress could be assign to the aspect of high concentration of antioxidants. In this aspect various studies aimed on effect of antioxidants in high dose to deal with oxidative stress was made. Pro-oxidant factors such as smoking, long-term stress or environmental contamination lead people to employ high doses of antioxidants. Study of (*Retana-Ugalde et al. 2008*) proved that high dosage of antioxidants didn´t lead to diminish oxidative stress. On the contraty high dosage of Toc and Asc caused increase in oxidative stress and pro-oxidant biomarkers were observed.

Few studies showed pro-oxidant activity of antioxidants treatment in the absence of exogenous oxidative stress. Effect of Toc, D-erythroAscorbic acid and coenzyme Q_{10} on aging and their pro-oxidant activity has been studied by (*Lam et al. 2010*). Result of exogenously added Toc showed increased cellular oxidative stress and shortened lifespan of studied organism without exogenous oxidative stress. D-erythroAscorbic acid had only

a little effect on cell lifespan. Surprisingly treatment with coenzym Q_{10} showed same results as decreased lifespan by Toc.

3 Aim of work

Aim of this work was to measure and compare effect of various oxidants and antioxidants on bovine serum albumin using method of ultra-weak photon emission in visible part of spectra. We also discuss background of effects of various reactive oxygen species as well as effects of antioxidants.

4 Material and methonds

4.1 Albumin

Albumin is serum protein set in plasma and participate on maintaining of oncotic pressure in plasma. Transport function of this protein is connected with transportation of hem, bilirubin, steroid substances, metals, drugs etc. Contributing to protecting cell mechanism is considered as antioxidant protein. Structurally it is small and stable protein consisting from 607 amino acids in length. Bovine serum albumin used in this thesis is standard protein used in laboratory experiments. Used albumin is from Sigma Aldrich company, and it´s purity is ≥ 98%. Albumin has been prepared in needed concentration 1 hour before every measurement. Volume of albumin solution in Petri dish (average 5,5cm) was 5ml. Concentration of albumin was set on 10mg/ml (0,15mM).

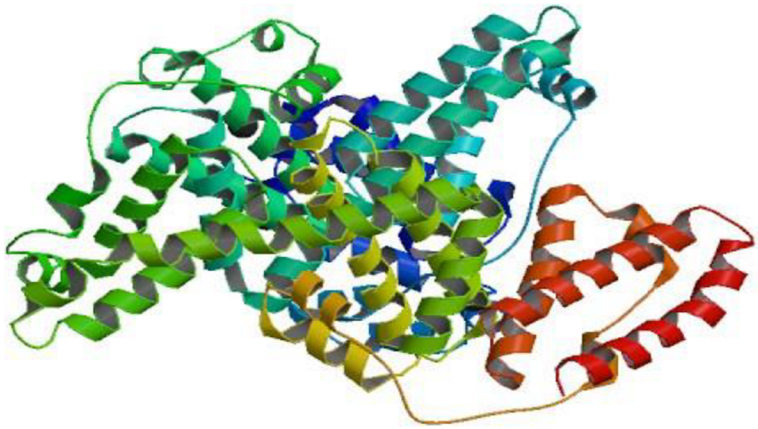


Figure 3: Molecular structure of BSA in the form of secondary structure with color-coded domains (*Majorek et al. 2012*).

4.2 PMT measurements

Measurements were done by photomultiplier tube Hamamatsu R7518P (Hamamatsu Photonics KK, Iwata City, Japan) with spectral range from 185nm to

730nm. Photon counting was mediated by counting unit C9744 (Hamamatsu Photonics KK, Iwata City, Japan). Cooling of PMT to -30°C was provided with thermoelectric cooler C9143 allowed measurements with ideal ratio of the signal from the sample to noise from the detector. Voltage have been set to -960V. All measurements were done at room temperature. PMT is placed in dark room painted with black colour for proper circumstances for measurements. Inside of PMT housing were placed stand and clamp, where Petri dish was placed. Computer with control program is placed in next room also with dark conditions.

Each chemical used and added to Petri dish during measurement in PMT had volume 50µl. First 100s UPE from BSA was measured, then we added individual chemicals. Addition of chemicals was divided in around 100s periods (50s if ROS was added before antioxidants).

4.3 Evaluation of measured data

Output data from program are in *.txt format. Data obtained from measurements were processed and evaluated in MS Office – Excel. Each sample was plotted in graph. Time axis was set up on maximum 700s, where the samples were stable, and no reactions and fluctuations were observed (stady-state level). Noise counts have been deleted by filter in MS Excel. Deviation were counted as standard deviation function (STDEV.P) uses formula $\sqrt{\frac{\sum(x-\bar{x})^2}{n}}$, where n is the sample size, x is the value of sample.

Then we made averages of the UPE in concrete parts of measurement as we gradually added chemicals. After first 100s of measurement we added a chemical and measured their effect until the other chemical was added. After all chemicals were added we run the measurements until the steady-state level of UPE was achieved. From these parts of spectra, the average of UPE was made. Averages were made from 100s parts (or 50s parts if antioxidant was added after ROS) of measurements. After that, we made difference between averages of UPE from BSA with added chemicals and UPE from pure BSA, last chemical added minus penultimate chemical added. From each type of sample (viz section 4.4), we selected three measurements with similar course. These numbers are presented in Table 2,3 and 4 and column graphs.

4.4 Types of samples

Table 1: Types of samples measured in experiments set in thesis.

Types of samples		
$BSA+H_2O_2$	$BSA+FeSO_4+H_2O_2$	$BSA+MoO_3+H_2O_2$
$BSA+ H_2O_2$+Asc	$BSA+ FeSO_4+ H_2O_2$+Asc	$BSA+ MoO_3+ H_2O_2$+Asc
BSA+Asc+ H_2O_2	$BSA+ FeSO_4$+Asc+ H_2O_2	$BSA+ MoO_3$+Asc+ H_2O_2
$BSA+ H_2O_2$+Toc	$BSA+ FeSO_4+ H_2O_2$+Toc	$BSA+ MoO_3+ H_2O_2$+Toc
BSA+Toc+ H_2O_2	$BSA+ FeSO_4$+Toc+ H_2O_2	$BSA+ MoO_3$+Toc+ H_2O_2

5 Results

5.1 Effect of H_2O_2 on UPE from BSA

First we measured UPE from BSA after exposure to H_2O_2. Hydrogen peroxide is widely used in technique of UPE as initiative stressor. We measured UPE from 0,15mM BSA in VIS part of spectra. After first 100s of observing spontaneous UPE, 50μl of 5mM H_2O_2 was added to Petri dish. Immediately after addition of H_2O_2, UPE increased significantly (Fig.4) and then it fell to steady-state level (around 700s). The average value of induced UPE was 2,72 counts· s^{-1}.

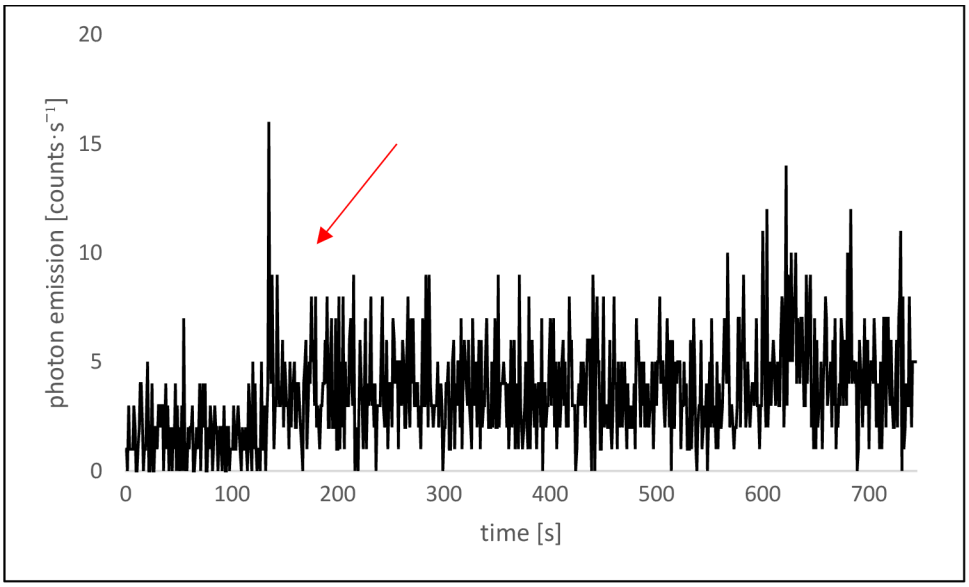


Figure 4: Effect of 5mM H_2O_2 on UPE from 0,15mM BSA. Moment of H_2O_2 addition is indexed with red arrow, from where sharp increase and later (around 140s) decline of UPE is observable. Stady-state level can be observed from 350s.

5.2 Effect of antioxidants on UPE induced by H_2O_2

After we observed effect of H_2O_2 on UPE, we investigated effect of Asc and Toc (Fig. 5a and b) on this type of samples. Addition of Asc suppressed UPE from sample treated with 5mM H_2O_2. Alpha-tocopherol, on the contrary increased UPE from the BSA treated with H_2O_2. We also measured effect of antioxidants on UPE before addition of

H_2O_2 (Fig. 5c and d). Antioxidants added before oxidation of BSA by H_2O_2 suppressed increase of UPE to lower values. Samples with treatment with Asc reached lower levels of UPE after addition of H_2O_2. In case of Toc reached level of UPE was higher than in case of Asc but still lower than level reached without any antioxidant treatment.

Because H_2O_2 has short lifetime in reaction with biomolecules, we added antioxidants to sample 50s after addition of H_2O_2, around 170s (Fig. 5a and b).

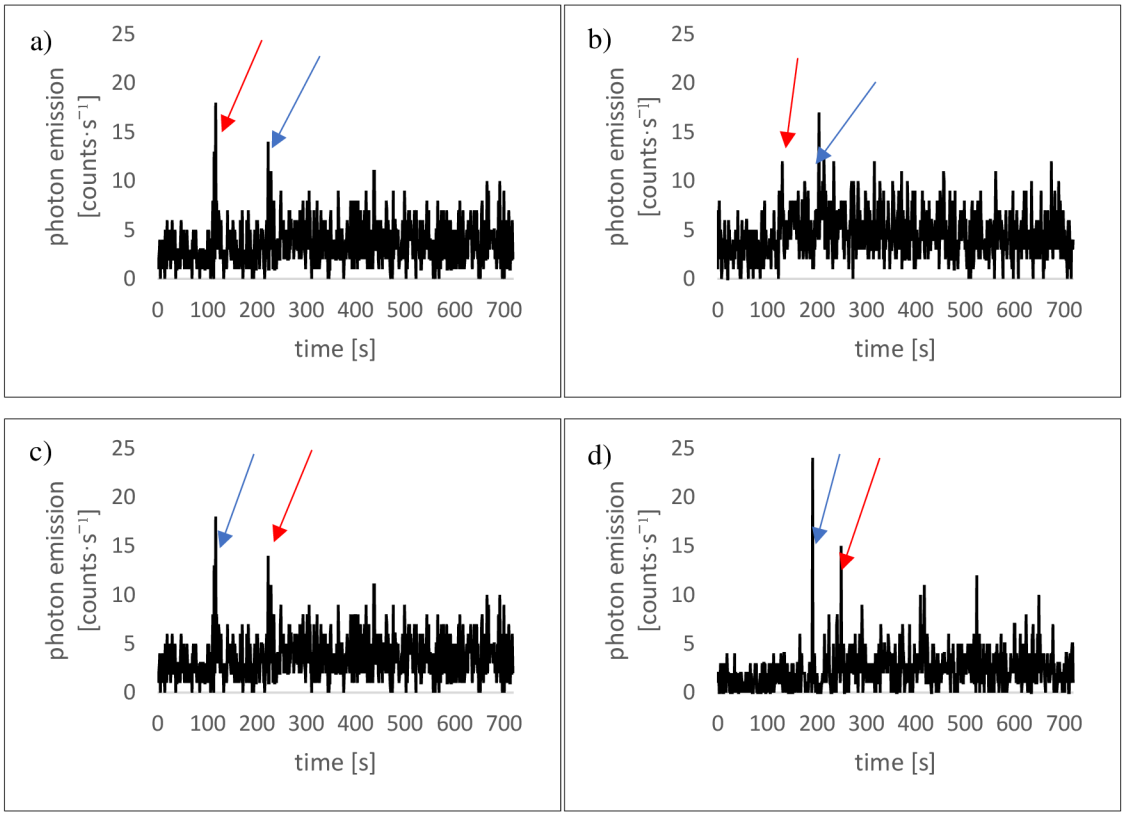


Figure 5: Effect of antixidants on UPE induced by 5mM H_2O_2. Part a) shows effect of 5mM Asc added after H_2O_2 and part c) its addition before. Parts b) and d) represents samples with 5mM Toc. Red arrows represents addition of H_2O_2 and blue arrow addition of antioxidant. Graphs show current UPE and effect of added chemicals on BSA oxidation during measurement.

In Table 2, comparison of UPE from different types of samples treated with H_2O_2, Asc and Toc are shown. Values represented in Table 2 are averages from 3 chosen measurements in the count-rate. As we can observe from the Table 2, samples with Asc reached low level of UPE. Addition of Toc added after treatment with H_2O_2 was accompanied with increase in UPE, however reverse types of samples show only small incresement in UPE.

Table 2: Differences between UPE from the samples after addition of individual chemicals. The achieved emission values represent the effect of individual chemicals. Each value is the average of 3 selected measurements. Symbol A represents antioxidant (Asc or Toc).

Sample	$\Delta((BSA+H_2O_2)-BSA)$	$\Delta[A-(H_2O_2+BSA)]$
	[counts· s^{-1}]	
$BSA+H_2O_2$	2,72	-
$BSA+H_2O_2+Asc$	2,59	-0,61
$BSA+H_2O_2+Toc$	4,41	2,44
Sample	$\Delta((BSA+A)-BSA)$	$\Delta[H_2O_2-(A+BSA)]$
	[counts· s^{-1}]	
$BSA+Asc+H_2O_2$	0,57	1,15
$BSA+Toc+H_2O_2$	0,39	1,73

Comparison of reached levels of UPE from different types of samples is shown on Fig. 6. Achieved level of UPE is difference between the averages of the count rates of UPE. Induced UPE from BSA by H_2O_2 were the highest achieved. Antioxidant activity of Asc is above activity of Toc. For comparison, we also added a column with control, basic measurement.

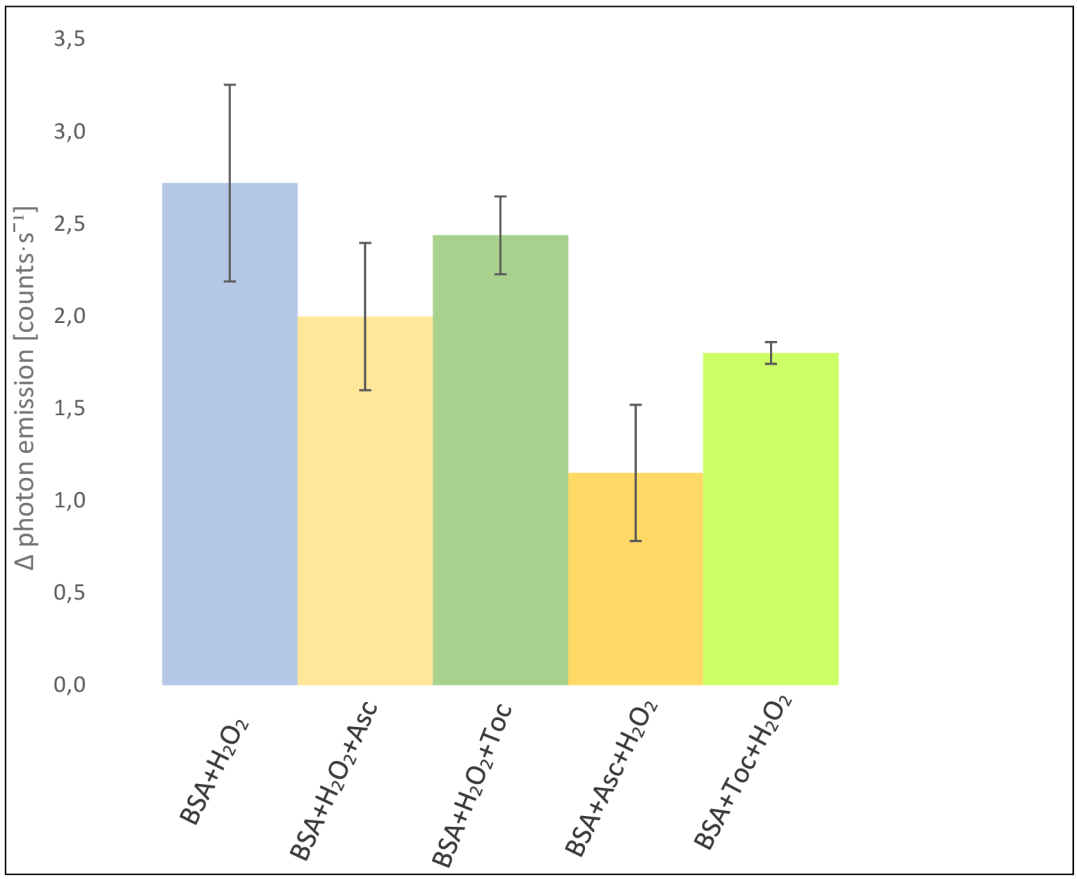


***Figure 6*:** Comparison between UPE (averages of the count rates) from samples treated with H_2O_2 and antioxidants and UPE from BSA. The deviations represent the course and the different reached values of UPE through measurement.

Variation between measurements in same type samples occurs due to high noise in background of PMT. Deviation from concrete measurements vary due to the different course of UPE induction as seen on Fig. 6.

5.3 Effect of HO˙ on UPE from BSA

Involvement of HO˙ in UPE was studied as a second type of measurements. Hydroxyl radical was produced through Fenton reaction using $FeSO_4$. Increase in UPE from 0,15mM BSA after addition of 1mM $FeSO_4$ and 5mM H_2O_2 was observed. Addition of 50µl $FeSO_4$ caused a negligible enhancement of UPE. The formation of HO˙ after addition of 50µl H_2O_2 was accompanied by sharp increase of UPE followed by decline (Fig. 7), however UPE remained elevated. The average value of induced UPE was 36,24 counts· s^{-1}.

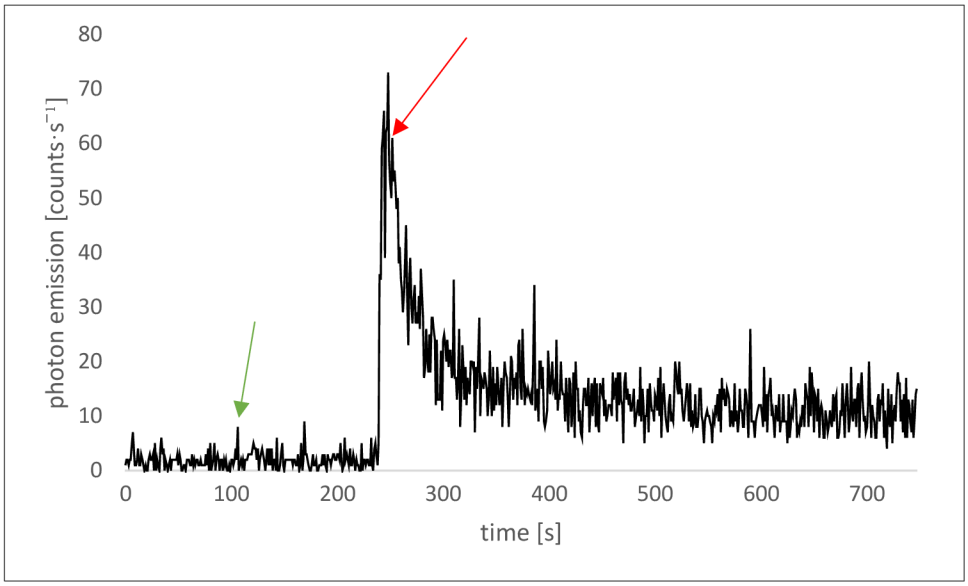


Figure 7: Effect of HO˙ on UPE from 0,15mM BSA. Moment of addition of1mM $FeSO_4$ is indexed with green arrow and addition of 5mM H_2O_2 by red. Formation of HO˙ and it´s effect on BSA is accompanied with sharp increase of UPE.

5.4 Effect of antioxidants on UPE induced by HO˙

Second measurement with antioxidants was aimed on their effect on UPE stimulated by HO˙. Addition of Asc was accompanied with strong increase of UPE and later decrease (Fig. 8a). Its addition suppressed UPE induced by HO˙. Same course occurs in samples with Toc (Fig. 8b). However, treatment with Toc increased UPE from the sample, behaviour ascribed to pro-oxidant effect. Reverse measurements, where we tested antioxidant capacity of Asc and Toc by its addition before HO˙ formation resulted in

following. Ascorbic acid suppressed oxidation of BSA by HO• and its previous addition before H_2O_2 led to small increasement of UPE in comparison with afterwards treatment (Fig. 8c). Increase of UPE in treatment with H_2O_2 after Toc occurred, where UPE remained higher (Fig. 8d).

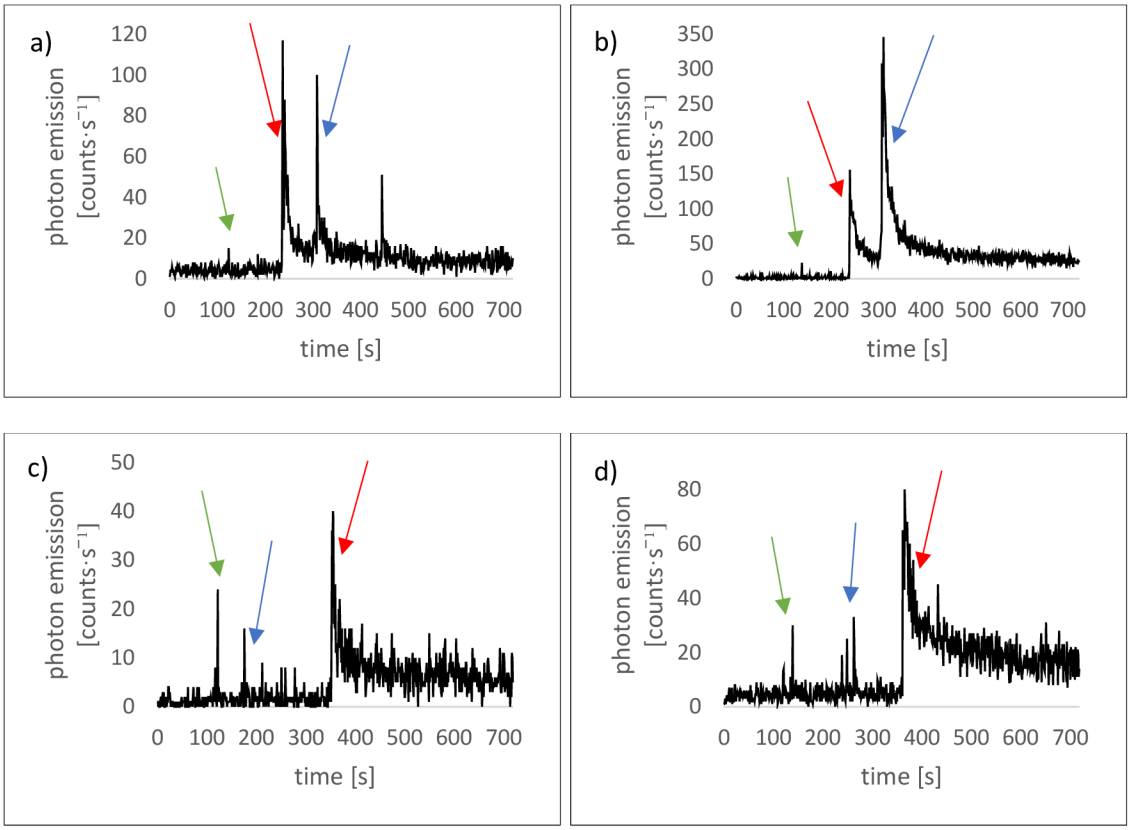


Figure 8: Effects of antixidants added after (a,b) and before (c,d) 5mM H_2O_2 and HO• formation. Samples with 5mM Asc are shown on parts a) and c) and with 5mM Toc are b) and d). Graphs show current UPE and effect of added chemical to BSA oxidation during measurement. Termination of measurement was after UPE remained unchanged. Addition of $FeSO_4$ is indexed with green arrow, addition of antioxidants by blue and red arrow represents addition of H_2O_2.

Comparison of UPE after addition of individual chemicals in the count-rate is shown in Table 3. Values represented in Table 3 are averages from 3 chosen measurements in the count-rate. We can observe minimum enhancement of UPE after addition of $FeSO_4$. Addition of H_2O_2 and subsequent formation of HO• increase UPE from all the samples in similar values. Antioxidants added before HO• formation either decrease UPE in case of Asc or rather slightly increased it in case of Toc.

Table 3: Differences between UPE from the samples after addition of individual chemicals. The achieved emission values represent the effect of individual chemicals. Each value is the average of 3 selected measurements. Symbol A represents antioxidant (Asc or Toc).

Sample	Δ((BSA+$FeSO_4$)-BSA)	Δ[H_2O_2-($FeSO_4$+BSA] [counts·s^{-1}]	Δ[A-(H_2O_2+($FeSO_4$+BSA)]
BSA+$FeSO_4$+H_2O_2	0,52	36,24	-
BSA+$FeSO_4$+H_2O_2+asc	0,68	26,23	-11,17
BSA+ $FeSO_4$+H_2O_2+toc	0,68	39,15	42,57
Sample	Δ((BSA+$FeSO_4$)-BSA)	Δ[H_2O_2-($FeSO_4$+BSA)] [counts·s^{-1}]	Δ[A-(H_2O_2+($FeSO_4$+BSA)]
BSA+ $FeSO_4$+asc+H_2O_2	0,85	-0,39	8,61
BSA+ $FeSO_4$+toc+H_2O_2	0,31	0,78	24,29

Column graph (Fig. 9) display comparison of UPE in samples, where induction was reached by $HO^{\bullet}$ formation. Samples with Asc reach lower UPE on the contraty with Toc.

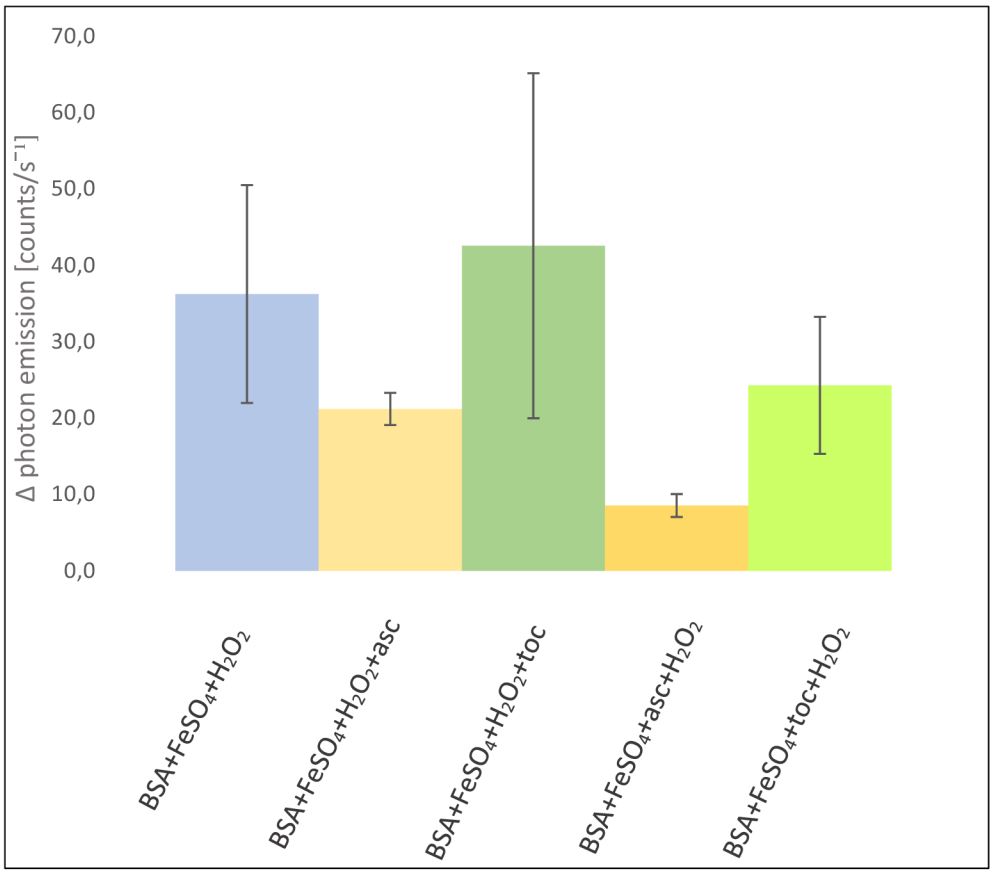


Figure 9: Comparison between UPE (averages of the count rates) from samples treated with HO˙ and antioxidants and UPE from BSA. The deviations represent the course and the different reached values of UPE through measurement.

5.5 Effect of $^1O_2$ on UPE from BSA

Effect of the last tested ROS, $^1O_2$ was measured on 0,15mM BSA. We observed low increase in UPE after addition of 2,5mM MoO_3. Addition of 50µl 5mM H_2O_2 to the Petri dish led to the formation of $^1O_2$ and insificantly increased UPE. After 400s no further changes were observed from the sample and we stopped the measurements (Fig. 10). The average value of induced UPE was 1,11 counts· s^{-1}.

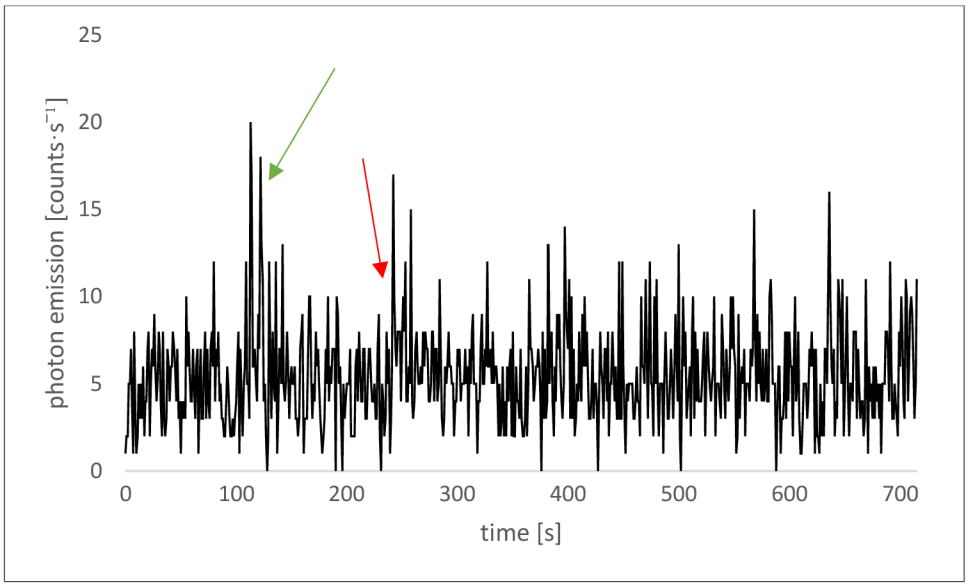


Figure 10: Effect of $^1O_2$ on UPE from 0,15mM BSA. Moment of 2,5mM MoO_3 addition is indexed with green arrow and addition of 5mM H_2O_2 by red. Formation of $^1O_2$ and it´s effect on BSA is accompanied with hardly noticeable increase in UPE.

5.6 Effect of antioxidants on UPE induced by $^1O_2$

Last examined investigation was effect of Asc and Toc on BSA oxidation induced by $^1O_2$. Treatment of oxidized BSA by $^1O_2$ with Asc led to slight decrease of UPE (Fig. 11a). Sharp increase of UPE occurred after addition of Toc (Fig. 11b) indicating pro-oxidative behaviour. In second type of samples we studied oxidation of BSA by $^1O_2$ previously treated with antioxidantds. While Asc suppressed UPE, Toc increased UPE (Fig. 11c and d).

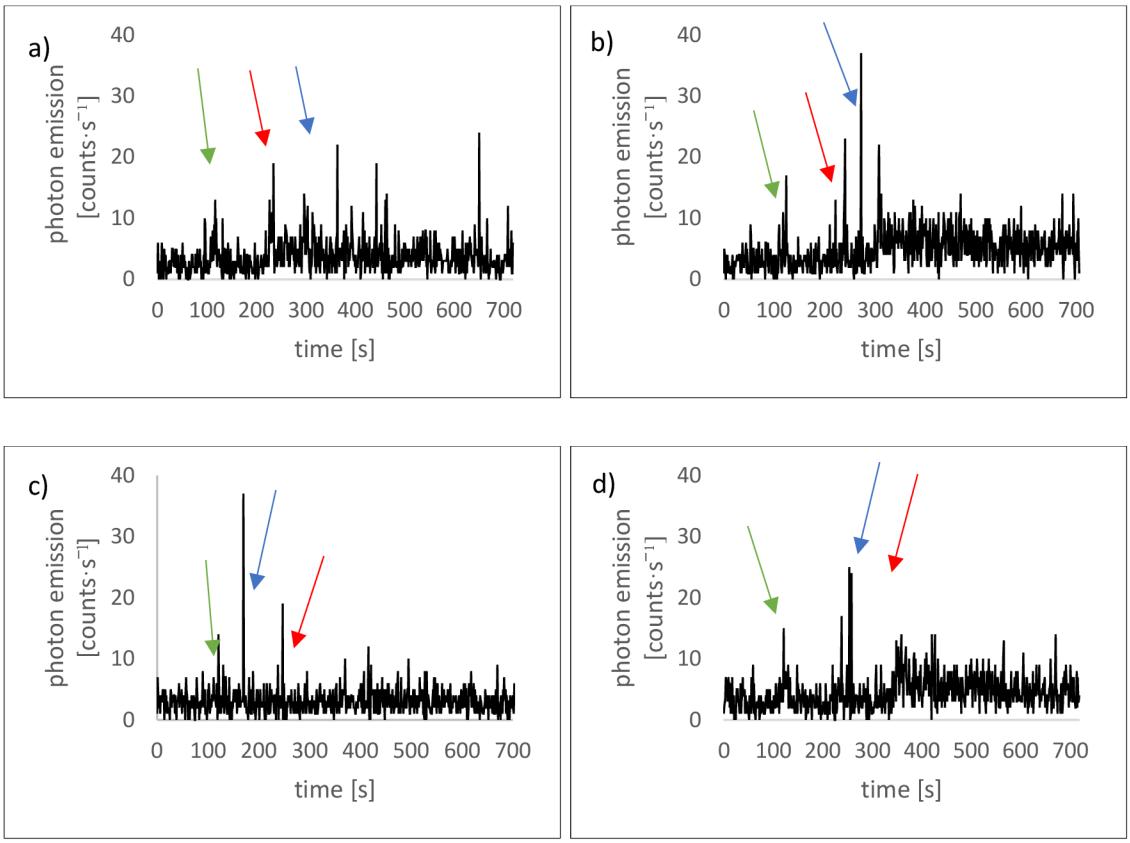


Figure 11: Comparison of samples with antixidants added after (a,b) and before (c,d) 5mM H_2O_2 and $^1O_2$ formation. Samples with 5mM Asc are shown on parts a) and c) and with 5mM Toc are b) and d). Graphs show current UPE and effect of added chemical to BSA oxidation during measurement.

Comparison of UPE after addition of individual chemicals in the count-rate is shown in Table 4. Values represented in Table 4 are averages from 3 chosen measurements in the count-rate. Molybdenum oxide increased UPE moderately. Addition of Asc decreased UPE after MoO_3, while Toc slightly increased UPE. Addition of H_2O_2 and subsequent formation of $^1O_2$ subtly increased UPE from all the samples in similar values.

Table 4: Differences between UPE from the samples after addition of individual chemicals. The achieved emission values represent the effect of individual chemicals. Each value is the average of 3 selected measurements. Symbol A represents antioxidant (Asc or Toc).

Sample	Δ((BSA+MoO_3)-BSA)	Δ[H_2O_2-(MoO_3+BSA)] [counts· s^{-1}]	Δ[A-(H_2O_2+(MoO_3+BSA)]
BSA+ MoO_3+H_2O_2	0,37	1,11	-
BSA+ MoO_3+H_2O_2+asc	0,40	1,77	-0,41
BSA+ MoO3+H_2O_2+toc	0,29	1,79	4,32
Sample	Δ((BSA+MoO_3)-BSA)	Δ[H_2O_2-(MoO_3+BSA)] [counts·s^{-1}]	Δ[A-(H_2O_2+(MoO_3+BSA)]
BSA+ MoO_3+asc+H_2O_2	0,87	-0,37	0,80
BSA+ MoO_3+toc+H_2O_2	0,51	0,34	1,66

Column graph (Fig. 12) display comparison of UPE in samples, where induction was reached by $^1O_2$ formation. In comparison with treatment by H_2O_2 and $HO^{\bullet}$ in these types of measurements are the lowest levels of UPE.

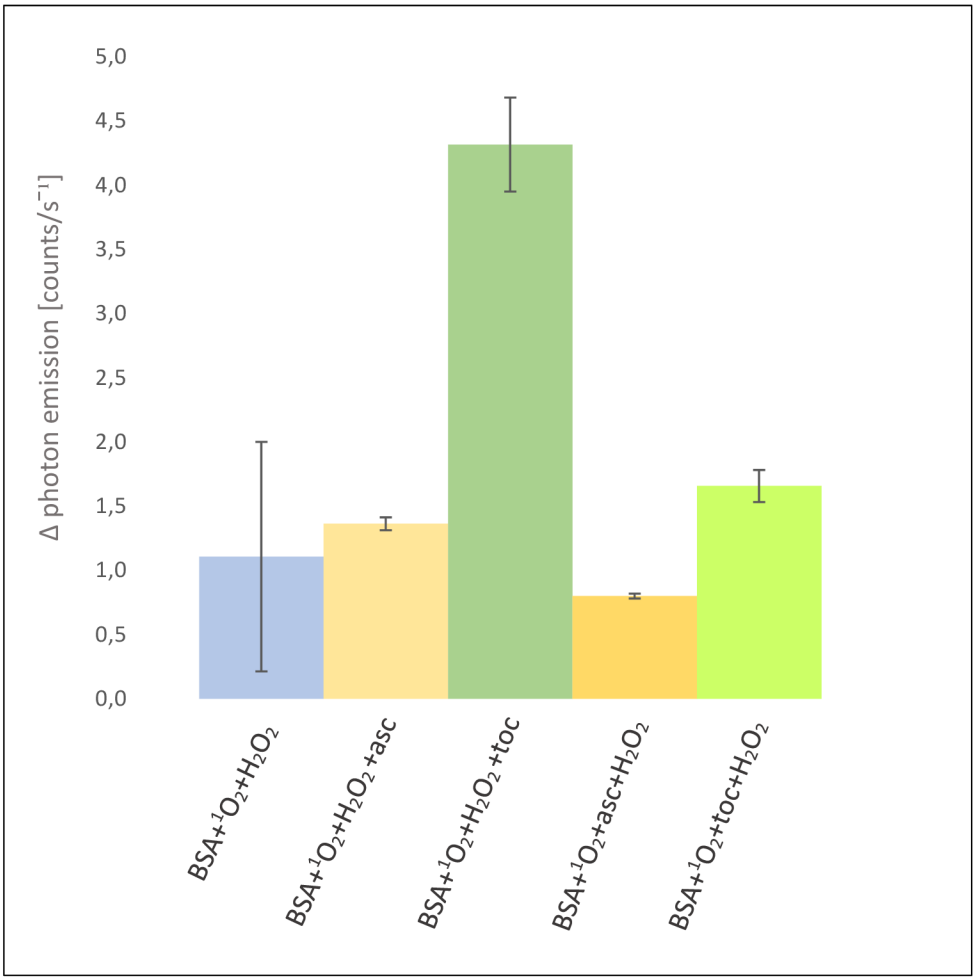


Figure 12: Comparison between UPE (averages of the count rates) from samples treated with $^1O_2$ and antioxidants and UPE from BSA. The deviations represent the course and the different reached values of UPE through measurement.

6 Discussion

Comparison of effects of exogenous ROS and particularly two non-enzymatic antioxidants on UPE from BSA was main aim of this bachelor thesis.

First we measured UPE from BSA induced by H_2O_2. Difference between the count rate of UPE from BSA and BSA with added H_2O_2 is on average of the count rate 2,72 counts·s^{-1}. Enhancement of UPE caused by oxidation of BSA by H_2O_2 is related to oxidation of amino acid residues with slow rate constante. Treatment of lens proteins with H_2O_2 was observed in study of *McNamara and Augusteyn (1984)*, where Cys and Met residues where rapidly oxidized. Other residues were only marginally oxidized. Measurements, where samples were treated with antioxidants were made to compare its effect on oxidation by H_2O_2. We were able to compare effect of Asc and Toc added after oxidation was triggered as well as its antioxidant capacity before its addition. Antioxidant capacity of both antioxidants on BSA oxidized by H_2O_2 was transparent (Tabble 1 and Fig. 8). Ascorbic acid added after H_2O_2 slightly decrease UPE by stabilizing formed radicals (ROOH, R$^{\bullet}$, ROO$^{\bullet}$). In comparison with sample BSA+ H_2O_2 was UPE 1,35x smaller. Ascorbic acid stabilized oxidizing H_2O_2 and formed ascorbate radicals (Asc$^{\bullet}$). Product of this reaction is dehydroascorbate (DHA) and H_2O_2 is reduced to H_2O ($2Asc_2+H_2O_2 \rightarrow DHA+H_2O$). Similar effect of Asc was shown in study of (*Shibamaya et al. 2014*). In measurements, where Asc was added before oxidation final UPE reached only low levels, which proves its antioxidant capacity. Final UPE was 1,4x lower than from sample without antioxidant treatment. Alpha-tocopherol added to sample treated with H_2O_2 increased UPE, specifically 1,13x. Effect of Toc added after H_2O_2 include stabilization of ROO$^{\bullet}$ by its reduction to ROOH. Equation describing ongoing reaction is α-Toc-OH + ROO$^{\bullet} \rightarrow$ α-Toc-Q$^{\bullet}$ + ROOH. Created Toc-Q$^{\bullet}$ can cause further oxidation but it has smaller oxidative effect than H_2O_2. Reverse type of samples provide information about antioxidant activity of Toc, where UPE reached lower levels. Added Toc supply atom of H and quenched effect of H_2O_2 *(Niki et al. 2014)*, final UPE was 1,5x smaller. Antioxidants successfully suppressed oxidation of BSA in this type of samples, which is confirmed by the fact that UPE from samples treated with them is lower.

Second studied oxidant was HO$^{\bullet}$, where we observed the highest achieved level of UPE. By addition of $FeSO_4$ and later H_2O_2, formation of HO$^{\bullet}$ started through Fenton

reaction. Final UPE from BSA+$FeSO_4$+H_2O_2 was in average of the count rate 36,24 counts·s^{-1}. Since $HO^{\bullet}$ is capable of oxidizing all amino acids and biomolecules in general, we suppose that this is the reason of highest achieved UPE from all the measurements. As products of protein oxidation by $HO^{\bullet}$ are considered mainly $R^{\bullet}$, we discussed effects of antioxidants on this species. Since we measured in presence of O_2, it is propable that before antioxidants were added also $ROO^{\bullet}$s were formed. Alkyl radicals formed on amino acids reacted with Asc and decreased UPE by its reduction ($R^{\bullet}$ + Asc → $Asc^{\bullet}$ + RH). Final UPE from this sample was 1,7x smaller than from sample treated only with $HO^{\bullet}$. Ascorbic acid showed antioxidant activity also before H_2O_2 addition *(Nauser and Gebicki 2017).* High concentration (in comparison of measurements *in vivo*) of Asc contributed to protection against oxidation *(Yen et al. 2002)*. Final UPE was 5x smaller in comparison with basic sample (Fig. 8). Addition of Toc to sample with BSA oxidized by $HO^{\bullet}$ caused increase of UPE by 1,17x in comparison with treatment only with $HO^{\bullet}$. Pro-oxidant behavior of Toc was expressed in this measurement. Strong instant increase of UPE occurred (Fig. 8b). Hence, we investigated the possibility of ethanol (as solvent of Toc) to be responsible for enhancement. Article of *Dubbs and Gupta (1998)* provided information on concentration of ethanol as solvent. We made measurements, where solvent was added to samples instead of Toc. Results from these measurements showed only small difference in UPE (in average of the count rate around 15 counts·s^{-1}). Weak ability of Toc to scavenge $HO^{\bullet}$ did not prevented oxidation of BSA *(Kontush et al. 1996).* Ultra-weak photon emission from samples, where Toc was added before H_2O_2 shows smaller enhancement. Ultra-weak photon emission from this sample is 1,49x smaller than from standard sample (Fig. 9). Donation of atom H from phenolic group of Toc protected BSA from oxidation. Higher concentration of Toc in protein solution can results in Toc-$Q^{\bullet}$ formation *(Mayo et al. 2003)*, which accumulates without present of other antioxidants and thus UPE is higher.

Singlet oxygen formation and its oxidative effect on BSA is showed on Fig. 11. Difference between average of the count rate from UPE before and after $^1O_2$ formation is 1,48 counts·s^{-1}. Samples with $^1O_2$ achieved the lowest level of UPE from all samples and was difficult to observe. Molybdenum oxide added to sample together with H_2O_2 creates $^1O_2$. Singlet oxygen is formed through Russell mechanism. Treatment of BSA oxidized by $^1O_2$ with antioxidants showed both, decrease and increase of UPE during measurements. Ascorbic acid moderately increased UPE, 1,25x. Singlet oxygen is

electrophilic species and rapidly reacts with electron rich moieties like Asc. Course of reaction ($^1O_2$ + Asc → H_2O_2 + DHA) show that reaction of $^1O_2$ with Asc leads to H_2O_2 formation and thereby other oxidation of BSA starts, which is projected to increased UPE *(Kramarenko et al. 2006)*. We consider that due to high concentration of Asc is increasement only moderate. Concequence of this treatment is low UPE signal. Alpha-tocopherol added to sample oxidized by $^1O_2$ enhanced UPE 3x higher than from basic sample BSA + MoO_3 + H_2O_2. Inverse addition of chemicals also brought 1,48x higher UPE from sample. Tocopherol is considered as one of the best scavenge agents for $^1O_2$ especially in plant PSII systems *(Rastogi et al. 2014, Trebst et al. 2002)*. We did not prove this effect in this thesis. Results of measurements in this thesis refer to pro-oxidant activity or weak antioxidant activity. 8-hydroperoxy-α-tocopheron, which is in systems hydrolyzed to stable tocopherolquinone. We can discuss high concentration of Toc in this experiment, which could lead to abundant formation of hydroperoxyl groups. If there was no decrease in ROS formation in our system, Toc didn´t act as antioxidant. This phenomena was described in review of (*Trebst 2003*), where high yieald of $^1O_2$ subsequently lead to oxidation of Toc and degradation of D1 protein in photosystem II. Another approach is that Toc display pro-oxidant activity in laboratory conditions, where various homologues of Toc is not stabilized by other antioxidants naturally present in organisms such as Asc or coenzyme Q_{10}. This phenomenon was widely described in food industry and dietary area and study of pro-oxidant activity of Toc and its analogue was made by (*Tafazoli et al. 2005*).

7 Conclusion

In this bachelor thesis, UPE was used to compare effect of different ROS on BSA using PMT in VIS part of spectra. The main aim of this work was to compare effects of non-enzymatic antioxidants (Asc, Toc) added before and after ROS caused oxidative damage to BSA.

Addition of endogenous H_2O_2 to BSA solution resulted in enhanced UPE to 2,72 counts$\cdot s^{-1}$. In presence of transition metal ions ($FeSO_4$) , $HO^{\bullet}$ is formed. Average of UPE induced by $HO^{\bullet}$ is 36,24 counts$\cdot s^{-1}$ and strong oxidant capability of this species occurred. Addition of H_2O_2 to BSA treated with MoO3 before leaded to $^1O_2$ formation. Enhanced UPE report to 1,11 counts $\cdot s^{-1}$. Confirmation that used ROS caused oxidative damage to model protein, BSA, is provided by enhancement of UPE. Hydroxyl radical taken as one of the most effective ROS caused highest increasement of UPE. Due to various noise during measurements, we made averages of UPE from separate parts (individual addition of chemicals) and difference between UPE is showed as average in the count rate of UPE. This approach allowed us to compare scale of BSA oxidation and compare effect of various ROS.

Another studied aspect was effect of Asc and Toc and its effect on UPE from BSA. In samples treated with H_2O_2, both Asc and Toc acted as antioxidants. Emissions from all the samples treated with them in comparison with samples in their absence reached lower levels of UPE. Oxidation of BSA by $HO^{\bullet}$ reached the highest level of UPE. Treatment with Asc and Toc brought various effects. Pro-oxidant behviour of Toc occuread after its addition to basic sample. Ascorbic acid showed antioxidant capacity and chelating effects in measurements with UPE induced by $HO^{\bullet}$ and H_2O_2. In measurements with $^1O_2$ Asc enhanced UPE slightly, which we attributed to electrophilic character of $^1O_2$, which reacts with electron rich moieties such as Asc. Effect of second antioxidant, Toc had pro-oxidant character. Since we also measured whether increasement of UPE is not caused by Toc solvent (ethanol) and results did not make change, we consider our results relevant.

8 References

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8.1 References – Images

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