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BACHELOR THESIS

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

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

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Abstrakt: Poškodenie biomolekúl pri procesoch oxidácie je sprevádzané javom ultra slabej emisie fotónov (UPE). V prírode dochádza k poškodeniu biomolekúl prirodzene pri metabolických oxidačných procesoch, ktoré sú spojené s reaktívnymi formami kyslíka (ROS). Pôsobeniu ROS sa organizmus bráni antioxidačnými mechanizmami, ktoré môžu za určitých podmienok pôsobiť pro-oxidatívne. Na detekciu javu UPE sa bežne používajú fotonásobiče (PMT).

V tejto bakalárskej práci bola skúmaná UPE z oxidatívne poškodeného hovädzieho sérového albumínu (BSA). Na základe dosiahnutej úrovne UPE sme skúmali efekt ROS a antioxidantov. Najrozsiahlejšie poškodenie vznikalo pôsobením pri UPE indukovanej hydroxylovým radikálom ($HO^{\bullet}$). Naopak najnižšie hodnoty UPE boli dosahované u vzoriek so singletným kyslíkom ($^1O_2$). Kyselina askorbová (Asc) vykazovala lepšie antioxidačné účinky, kdežto alfa-tokoferol (Toc) v niektorých typoch meraní pôsobil pro-oxidatívne, čo sa prejavilo zvýšením UPE.

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

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Abstract: Damage of biomolecules in oxidation processes is accompanied by phenomenon of ultra-weak photon emission (UPE). In nature, biomolecules are damaged naturally by metabolic oxidation processes, which are associated with reactive oxygen species (ROS). The effect of ROS is hindered by antioxidant mechanisms, which may act as pro-oxidants under certain conditions. Photomultipliers (PMTs) are commonly used equipments to detect the phenomena of UPE.

In this bachelor thesis, UPE from oxidatively damaged bovine serum albumin (BSA) was investigated. Based on the achieved level of UPE, we investigated the effect of ROS and antioxidants. The most extensive damage was caused by hydroxyl radical ($HO^{\bullet}$)-induced UPE. In contrast, the lowest levels of UPE were achieved in measurements with singlet oxygen ($^1O_2$). Ascorbic acid (Asc) showed better antioxidant effects, while alpha-tocopherol (Toc) showed pro-oxidative behaviour few types of measurements, which was reflected as an increase in UPE.

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

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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
$FeSO_4 \cdot 7H_2O$	iron (II) sulfate heptahydrate
H_2O_2	hydrogen peroxide
$HO^\bullet$	hydroxyl radical
MoO_3	molybdenum (VI) oxide
O_2	molecular oxygen
$O_2^{\bullet -}$	superoxide anion radical
PMT	photomultiplier tube
CCD	couple-charged divice
C^*	excited pigment
$R^\bullet$	carbonyl radical
$RO^\bullet$	alkoxyl radical
$ROO^\bullet$	peroxyl radical
ROOH	hydroperoxide
ROOR	dioxetane
ROOOOR	tetroxide
ROS	reactive oxygen species
RNS	reactive nitrogen species
UPE	ultra-weak photon emission
EPR	electron aparamagnetic resonance
Asc	ascorbic acid
DHA	dehydroascorbate
Toc	alpha-tocopherol

Toc-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 oxidized biomolecules by ROS can be detected from near UVA, through VIS and near IR regions of spectra using filters or in combination with other techniques.

An effective defense mechanism against ROS in organisms are 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 it´s one-electron reduction or donation of atom H where less reactive species are formed. Enzymatic antioxidants convert oxidative products to H_2O through multi-step process (*Nimse and Pal 2015*).

Under few condition antioxidants may exhibit pro-oxidant behaviour (*Kondakçi et al. 2013*). One of the best observed factors is presence of transition metal ions (Fe^{2+}, Mn^{2+}, Zn^{2+} or Cu^{2+}). Another trigger is imabalance between number of antioxidants and oxidants in system. Few studies expose pro-oxidant behaviour of separate alpha-tocopherol (Toc), which is not in composition with other antioxidants such as coenzyme Q_{10} or ascorbic acid (Asc). Antioxidants reduce metal ions, which generate free radicals instead of reduction of oxidizing substances.

In this bachelor thesis, PMT in range of VIS part of spectra was used. Oxidative damage of bovine serum albumin (BSA), frequently investigated model protein was studied after addition of endogenous ROS. Study was focused on effect of namely three ROS, hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$) and singlet oxygen ($^1O_2$). 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 take place on physical, chemical and biological basis. 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 occur 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 Figure 1 beneath. Type I reactions can be both enzymatic and non-enzymatic and occurre mainly in mitochondria and chloroplasts but also in plasma membranes, microbodies and endoplasmic reticulum.

Type II reactions explain $^1O_2$ formation. Pathway that leads to $^1O_2$ formation is connected with absorbtion of excitation energy by skin photosensitizers and subsequent $^1O_2$ formation through intersystem crossing. Except mentioned pathway, other feasible way of $^1O_2$ formation occurs. One is decomposition of dioxetane (ROOR) through triplet excited carbonyl ($^3(R{=}O)^*$) and carbonyls with O_2 and subsequent triplet energy transfer. Other unstable intermediate, tetroxide (ROOOOR) can decay and form $^1O_2$ through same mechanism, but also a direct decomposition occurs. Tetroxide decomposes to $^1O_2$, carbonyls/ketones and alcohols.

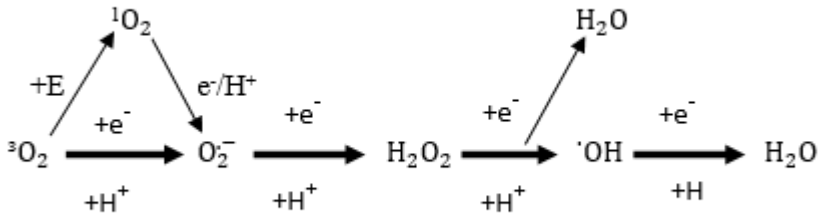

Figure 1: Subsequent one-electron reduction of O_2.

Formation of ROS plays a crucial role in cell signalling, ageing and products of ROS 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^{2+}, Mn^{2+}, Zn^{2+} or Cu^{2+} are natively in cell environment and are often bounded in metalloproteins and transport proteins called ferritins. These proteins scavenge metal ions to prevent running of Fenton´s reaction (*Shi et al. 2013*). Basically, the course of Fenton´s reaction can be described as reactions of divalent iron with H_2O_2, which lead to formation of trivalent iron, $HO^{\bullet}$ and hydroxyl anion (HO^-). Reaction is noticed in scheme 1.

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

Scheme 1: 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,Cifra et al. 2017).* 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 from chemical reactions; biophoton emission signify the biological origin 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 2014A*).

Ultra-weak photon emission can be divided in the same way as ROS according to different phases of metabolic processes or its 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 2014B*).

Forming 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 (Figure 2) is accompanied with photon emission and thus those species are considered being responsible for UPE. Maximum of UPE from $^3(R{=}O)^*$ is 350-550 nm and in case of dimolar UPE from $^1O_2$ is at 634 nm and 703 nm. Another electronically excited species responsible for UPE are singlet excited pigment ($^1C^*$) and triplet excited pigment ($^3C^*$). Maximum of UPE from $^1C^*$ is at 360-560 nm for melanin and 680-740 nm for

chlorophyll. Triplet excited pigment has maximum of photon emission at 870-100nm (chlorophyll).

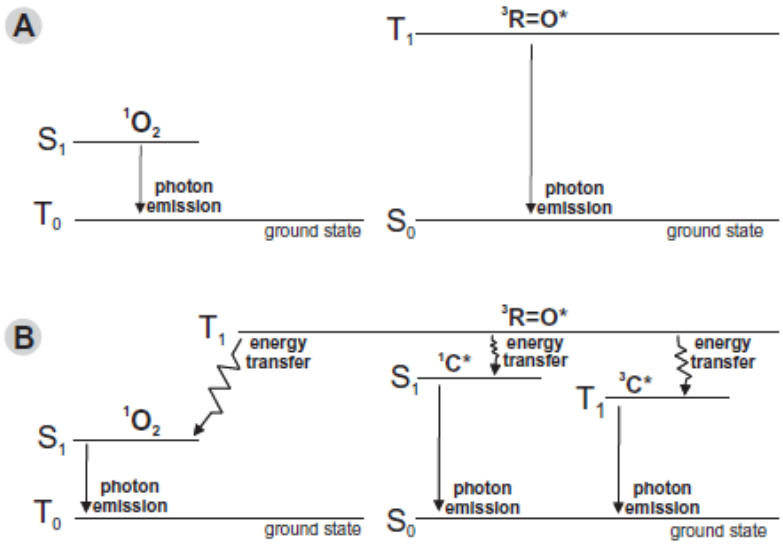

Figure 2: Transition from excited energy states of electronically excited species. A) Energy level of ^{3}(R=O)* and $^1O_2$. B) Energy level of $^1C^*$, $^3C^*$ and it´s transition from ^{3}(R=O)* (taken from Cifra and Pospíšil 2014)).

Applications of UPE are associated with wide-spread research on various oxidative damage by various reactive species (reactive oxygen and nitrogen species (RNS), 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. Below are a few studies as an example, where the technique of UPE was used.

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 made in divers studies. It is considered that lipid peroxidation is responsible for those damages. 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 PMT 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 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,Cifra et al. 2017,B*). Oxidation products after attack with ROS on biomolecule may lead to oxidation of other biomolecules and trigger 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 free radicals which oxidized DNA was made by *Davies et al. 1999*. Other study display how one electron reduction of lipid ROOH leads to damages in DNA structures (*Crean et al. 2009*).

Oxidation of lipids are considered as first step of cell damage as basic biomolecules present in every phospholipid bilayer. Polyunsaturated fatty acids are often affected parts of lipids due to multiple methylene groups position 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 hydroperoxides (*Shimizu et al. 2018*). The oxidation process itself begins when hydrogen atom is abstracted from polyunsaturated fatty acid by ROS. This part of chain reaction, iniciation, leads to the formation of fatty acid radical. Next step, propagation, is reaction of unstable fatty acid radical with O_2 which creates peroxyl radical ($ROO^{\bullet}$). It also lead to reaction where $ROO^{\bullet}$ reacts with another free fatty acid (non-radical molecule) and produce ROOH by abstraction of hydrogen atom. Termination lead to reaction of two radicals formed during previous reaction and the non-radical species are formed. Specific 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 and reduce ROOH to alkoxyl radicals ($RO^{\bullet}$). 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 ketons *(Prasad et al. 2016).*

Modification caused by oxidation of DNA structure occurs in the form of base oxidation and rarely sugar-phosphate backbone oxidation. More oxidized bases are purine rather than pyrimidine. Guanine has the lowest redox potential, which makes it the main target from nitrogenous bases. Ability to directly oxidize DNA manifest $H_2O_2, HO^\bullet$ and $^1O_2$. Publication of *Ma et al. (1998)* provided experimental evidence of UPE from oxidized (e.g. by H_2O_2) guanine, 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*. Mechanism of oxidation starts when ROS attack carbons from the base ring. This attack leads to formation of carbonyl radicals also called alkyl radicals ($R^\bullet$). Various measurements by HPLC technique provide information, which carbon is attacked. In connection with guanine it is C5. Presence of O_2 shapes in $ROO^\bullet$ formation but more often is observed reaction, where C5 radical predominantly react with $O_2^{\bullet-}$ to form $ROO^\bullet$ and later ROOH. Formation of $ROO^\bullet$ on thymine was studied by *Lozinova and Lander in 2015*. 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. Various reactions with $ROO^\bullet$ was however described through different mechanisms and is still under research. 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).* Guanine adduct formed and used as biomarker of cancer 8-hydroxy-2′-deoxyguanosine (8-OHdG) is often described. Formation of 8-OHdG was described in study of *Ma et al. 2016* on retinal mDNA. Oxidation processes within DNA can also produce 8-OHdG. *Goto et al. 2008* provided experiment where peroxized thymidine formed this biomarker.

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 reaction 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 H abstraction from R-H bonds. Major reactions occur on Cys and Met residues, but in few cases a further distance from amide group lead to damage also in other amino acids (e.g. Trp or Gly).

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 as 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 formation of $R^{\bullet}$,which in presence of O_2 can give rise to $ROO^{\bullet}$. Subsequent hydrogen transfer from protein structure forms ROOH (Figure 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, $ROO^{\cdot}$ 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 ketons/alcohols.

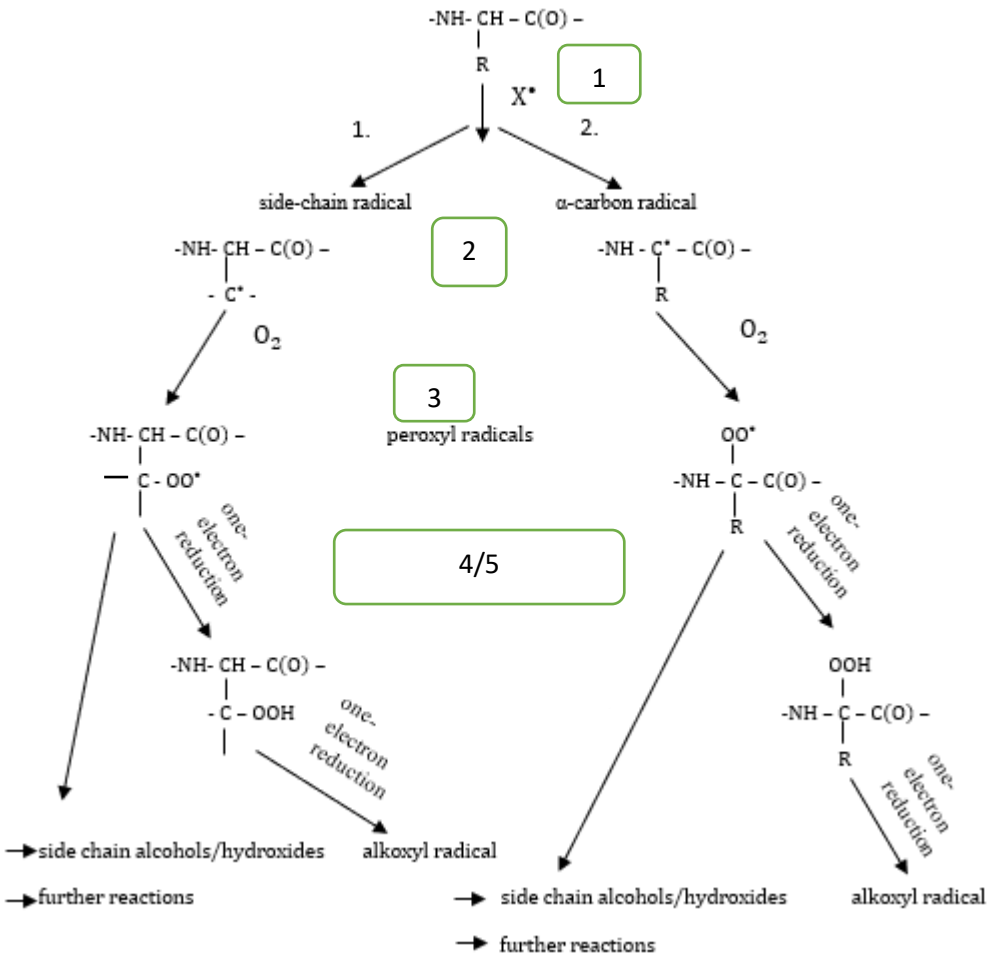

Figure 3: Major reactions on 1. side chain during protein oxidation and 2. backbone protein oxidation in presence of O_2. First step is attack of exogenous/endogenous radical $X^{\bullet}$. Second step is formation of $R^{\bullet}$, which continously lead to third step, which is $ROO^{\bullet}$ formation in presne of O_2. Fourth and fifth step is formation of protein ROOH through unstable intermediates like ROOOOR. Further reactions may give rise to $RO^{\bullet}$, which trigger another oxidation processes.

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 $ROO^{\cdot}$ 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 Figure 4.

Secondary reactions of protein ROOH often occur and can give rise to another radicals. One-electron reduction of hydroperoxides 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).*

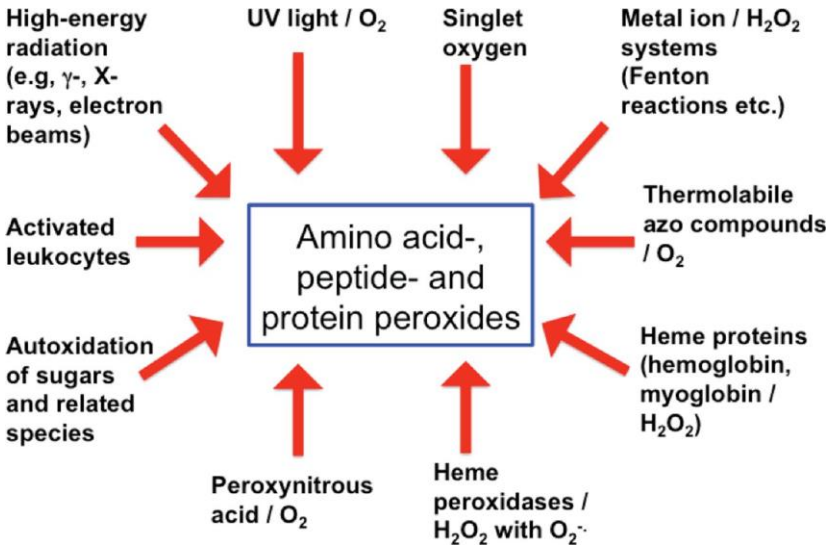

Figure 4: Known pathways for protein oxidation systems, few of them mentioned above. Viewed pathways give rise to amino acid-, peptide- and protein-hydroperoxides in the presence of O_2 (taken from Davis et al. (2016)).

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 defense 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 reason of their reactivity is replaced by unpaired electron taken antioxidant. Described mechanism is enlight protective feature of antioxidants.

There are two types of antioxidants. Enzymatic and non-enzymatic, Non-enzymatic defence components (low molecular weight components) as Asc and Toc are often connected with their beneficial abilities. Chemical structure of Asc and Toc predicts their ability to transfer atoms of hydrogen rather than donate electrons. Ability to donate atoms of hydrogen making them efficient antioxidants. Important role of Asc as a major non-enzymatic scavenger of $O_2^{\bullet -}$ in mangroves exposed to oxidation was described in study of *Jitesh et al. 2006.* Reduced glutathione has strong reductant ability and hence is considered as one of the most active scavengers of ROS. Second group of antioxidants, enzymatic antioxidants are also essential in scavenging of ROS after their accumulation in cells. Well-known enzymatic antioxidants are (SOD) and chloramphenicol acetyltransferase (CAT). Activity of SOD is described as convertion of $O_2^{\bullet -}$ to H_2O_2 and O_2 and principle of CAT reaction is convertion of H_2O_2 to O_2 and H_2O_2 *(Awoniyi et al. 2012).* Study explaining effects of both enzymatic and non-enzymatic antioxidants has been made on different types of teas by *Awoniyi et al. (2012).*

Different types of antioxidants affect in different parts of cell. According to this criteria antioxidant can be divided into extracellular, intracellular and membrane´s antioxidants.

One of the most important membrane antioxidants is considered Toc. α-tocopherol or some other kinds of lipid-soluble antioxidants are able to terminate lipid oxidation by changing its position in lipid membrane and change the structure. *Gulec et al. (2006)* studied protective effects of Toc against oxidative damage caused by formaldehyde in the liver and plasma of rats. Addition of Toc before formaldehyde showed observable improvement of antioxidant status. They suggested this might be the

result of free radical scavenging effect of this vitamin. Ability of Toc to reduce level of NO oxidants in the liver tissues of FA-injected rats has been perceptible.

The group of extracellular antioxidants contains e.g. uric acid, glutathione or Asc. Ascorbic acid is considered as a good reducing agent and thus could be oxidized by most of the ROS. The reaction of Asc with various types of ROS forms semidehydroAscorbate, which is weak reactive radical. Another significant property of Asc is its ability to recover Toc radical to origin state. Plenty of studies have been made to show the protective effects of Asc in agriculture, pharmacology, dermatology etc. Thus, Asc has potential in transdermal activity in skin care. *Shibamaya et al. (2014)* showed that Asc in connection with its derivate disodium isostearyl 2-O-L-ascorbyl phosphate is an effective protective intercellular agent against UVA-radiation.

2.6 Pro-oxidant behaviour

Studies aimed to detect and compare antioxidant activity showed few abnormalities and brought unlikely results. Antioxidants act as pro-oxidants under special circumstances. Switch to pro-oxidant behaviour could be caused by several factors.

First factor is presence of transition metal ions. Asc, which reduce ROS such as H_2O_2 can also reduce transition metal ions and through Fenton reaction forms free radicals. 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)*. Study of *Shi et al. (2017)* aimed to prove direct production of $HO^{\bullet}$ using electron paramagnetic resonance (EPR) and TEMPO (molecule containing stable nitroxide). Nitroxides used as antioxidants in this study, are considered as potential therapeutic antioxidants. However, in connection with iron (II) indicate pro-oxidant behaviour even though nitroxides reducing potential suggest a favour to take an electron than to donate one.

Capacity to scavenge specific ROS or other forming radicals occurs as feature of Toc. α-tocopherol, lipophilic antioxidant shows scavenge activity to $ROO^{\bullet}$ and partially $^1O_2$ and $O_2^{\bullet-}$. Lack of scavenging capacity of $HO^{\bullet}$, $RO^{\bullet}$ or thyil radical is typical for Toc *(E. Niki 2014)*. Process of Toc oxidation forms tocopheroxyl radical (Toc-$Q^{\bullet}$). 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. In their absence, Toc appear to act as pro-oxidant, especially in laboratories experiments. Accumulation of Toc-$Q^{\bullet}$ cause formation of other reactive species even though Toc-$Q^{\bullet}$ is moderately reactive *(Rietjens et al. 2002)*.

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.

Specific pro-oxidant toxicity is used as anticancer agents, stimulants for anti-inflammatory enzymes and many other applications. Excessive consumption of food supplements may cause pro-oxidant activity and thus it can be dangerous for health.

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 methods

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´s 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).

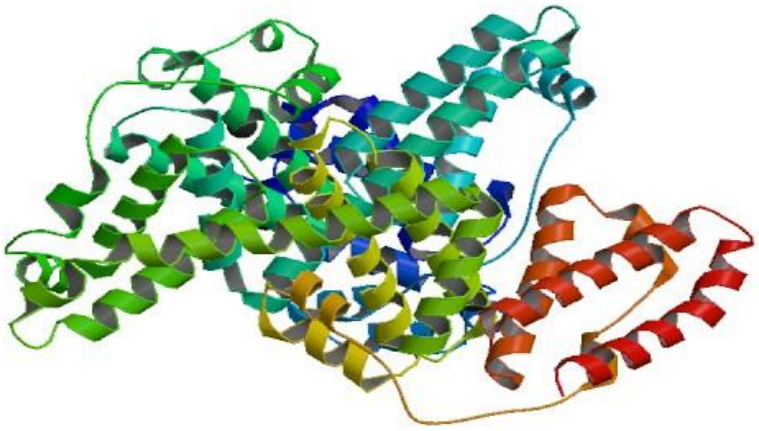

Figure 5: Structure of bovine serum albumin imaged in MD model.

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.

After first 100s of observing the UPE from BSA we added a chemical (around 120s) and measured its effect until the other chemical was added. 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. Then we made averages of the UPE in concrete parts of measurement as we gradually added chemicals. After that, we made difference between averages of UPE from BSA with added chemicals and emission from pure BSA, last chemical added minus penultimate chemical added. This showed as the effect of added chemicals on UPE and oxidation of BSA. 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, where effects of ROS and antioxidants on BSA are compared. Due to noise from PMT, we decided to set UPE from BSA on 1

counts·s^{-1}. This value allowed us better comparison of effects after addition of chemicals. During some measurements the cooling system wasn´t stabilized at ideal temperature -30°C, which caused higher level of signal-to-noise ratio.

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

Increase in UPE emission from BSA was observed after exposure to H_2O_2. 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 (around 120s, see Fig.5). Immediately after addition of H_2O_2 UPE increased rapidly (Fig.5) and then it fell to steady-state level (around 700s).

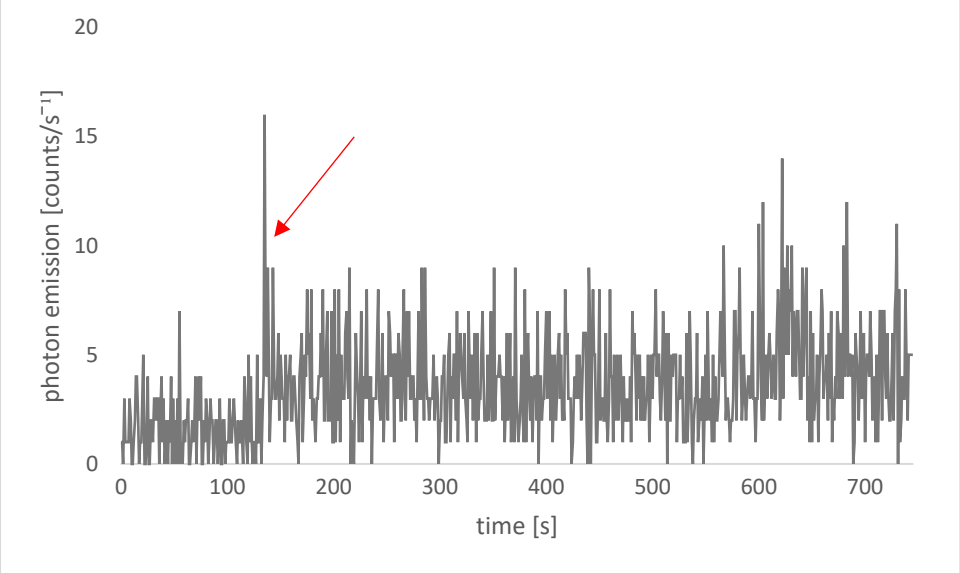

Figure 6: Effect of 5mM H_2O_2 on UPE from 0,15mM BSA. Moment of H_2O_2 addition is indexed with red arrow, from where sharp increase and later (around 140s) decline of UPE is observable. Stady-state level can be seen from 350s.

5.2 Effect of antioxidants on UPE induced by H_2O_2

Effect of Asc and Toc on BSA oxidation induced by H_2O_2 was studied using UPE measurements (Figure 7a and b). Addition of Asc decreased UPE from sample treated with 5mM H_2O_2. Alpha-tocopherol, on the contrary increased UPE from the BSA treated with H_2O_2 but achieved level UPE was still lower than from sample without antioxidant treatment. We also measured effect of antioxidants on UPE before addition of H_2O_2 (Figure 7c 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. 7-a,b).

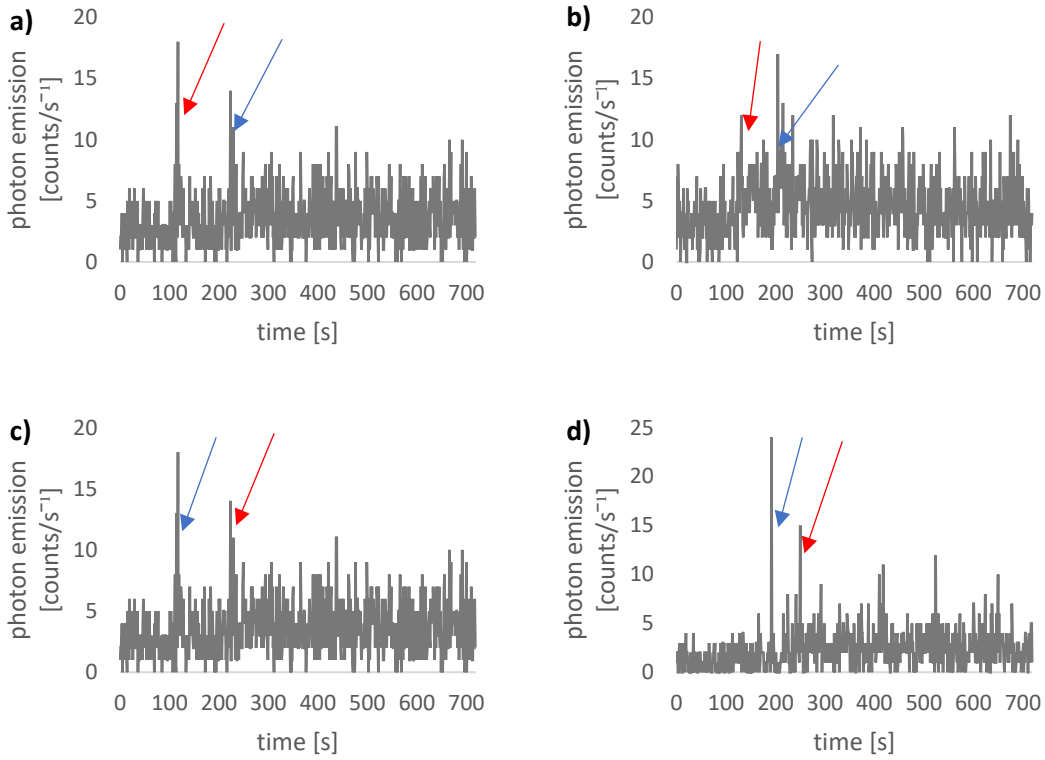

Figure 7: Effect of antixidants on UPE induced by 5mM H_2O_2. Part a) shows effect of 5mM Asc added after H_2O_2 and part c) its addition before. Parts b) and d) represents samples with 5mM Toc. Red arrows represents addition of H_2O_2 and blue arrow addition of antioxidant. Graphs show current UPE and effect of added chemicals on BSA oxidation during measurement. Termination of measurement was after UPE achieved steady-state level (700s).

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 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 and also 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	BSA	Δ((BSA+H_2O_2)-BSA) [counts/s^{-1}]	Δ[A-(H_2O_2+BSA)]
BSA+H_2O_2	1,00	2,72	-
BSA+H_2O_2+Asc	1,00	2,59	-0,61
BSA+H_2O_2+Toc	1,00	4,41	2,44
Sample	BSA	Δ((BSA+A)-BSA) [counts/s^{-1}]	Δ[H_2O_2-(A+BSA)]
BSA+Asc+H_2O_2	1,00	0,57	1,15
BSA+Toc+H_2O_2	1,00	0,39	1,73

Comparison of reached levels of UPE from different types of samples is shown on Figure 8. 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.

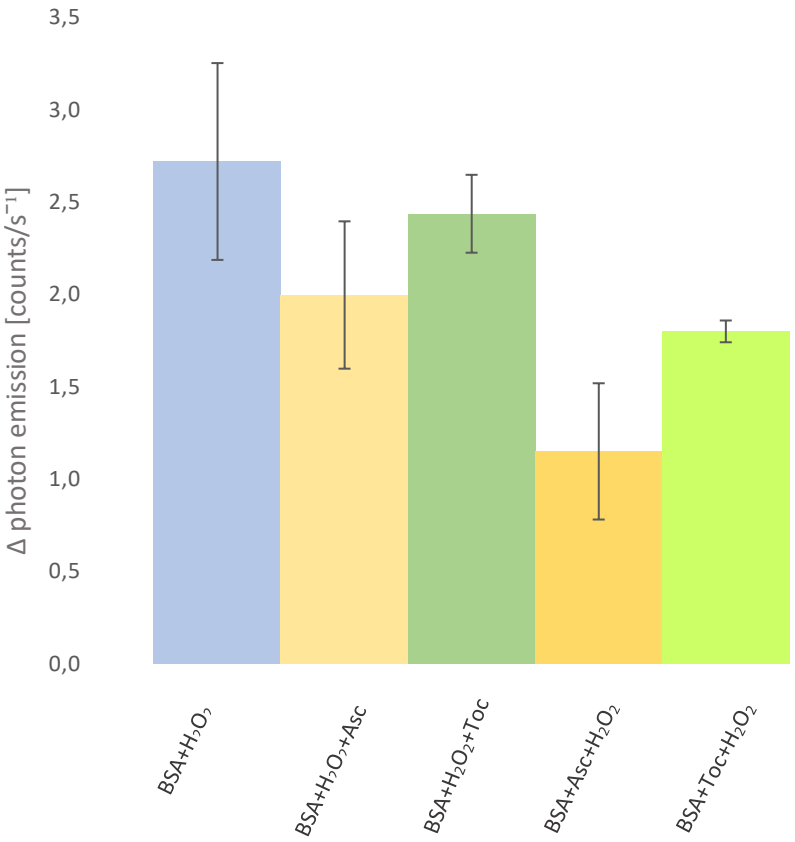

***Figure 8*:** Comparison between UPE (averages of the count rates) from samples treated with H_2O_2 and antioxidants and UPE from BSA. The deviations represent the course and the different reached values of UPE through measurement.

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 Figure 8.

5.3 Effect of HO· on UPE from BSA

UPE from BSA was measured after exposure to HO•. We observed the increase in UPE from 0,15mM BSA after addition of 1mM $FeSO_4$ and 5mM H_2O_2, which produce HO• through Fenton reaction. 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 (Figure 9). Between 400s and 500s, steady-state level was achieved and we stopped measurement around 700s.

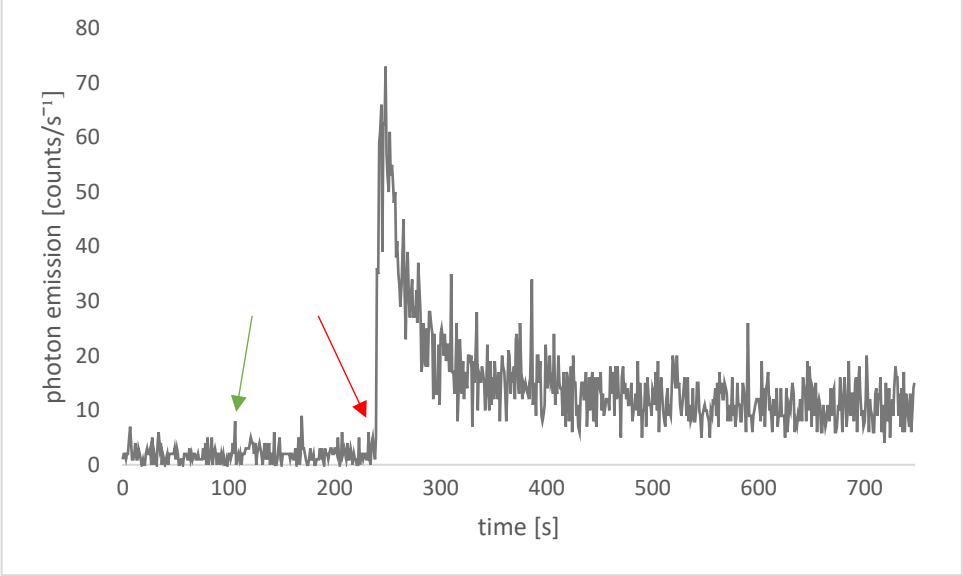

Figure 9: Effect of HO• on UPE from 0,15mM BSA. Moment of addition of1mM $FeSO_4$ is indexed with green arrow and addition of 5mM H_2O_2 by red. Formation of HO• and it´s effect on BSA is accompanied with sharp increase of UPE. Stady-state level can be set from 400s.

5.4 Effect of antioxidants on UPE induced by HO•

Effect of antioxidants (Asc and Toc) on oxidation of BSA stimulated by HO• was measured. Addition of Asc was accompanied with strong increase of UPE and later decrease (Figure 10a). Its addition suppressed UPE induced by HO•. Same course occurs in samples with Toc (Figure 10b). 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 $HO^{\bullet}$ and its previous addition before H_2O_2 led to small increasement of UPE in comparison with afterwards treatment (Figure 10c). Increase of UPE in treatment with H_2O_2 after Toc occurred, where UPE remained higher (Figure 10d).

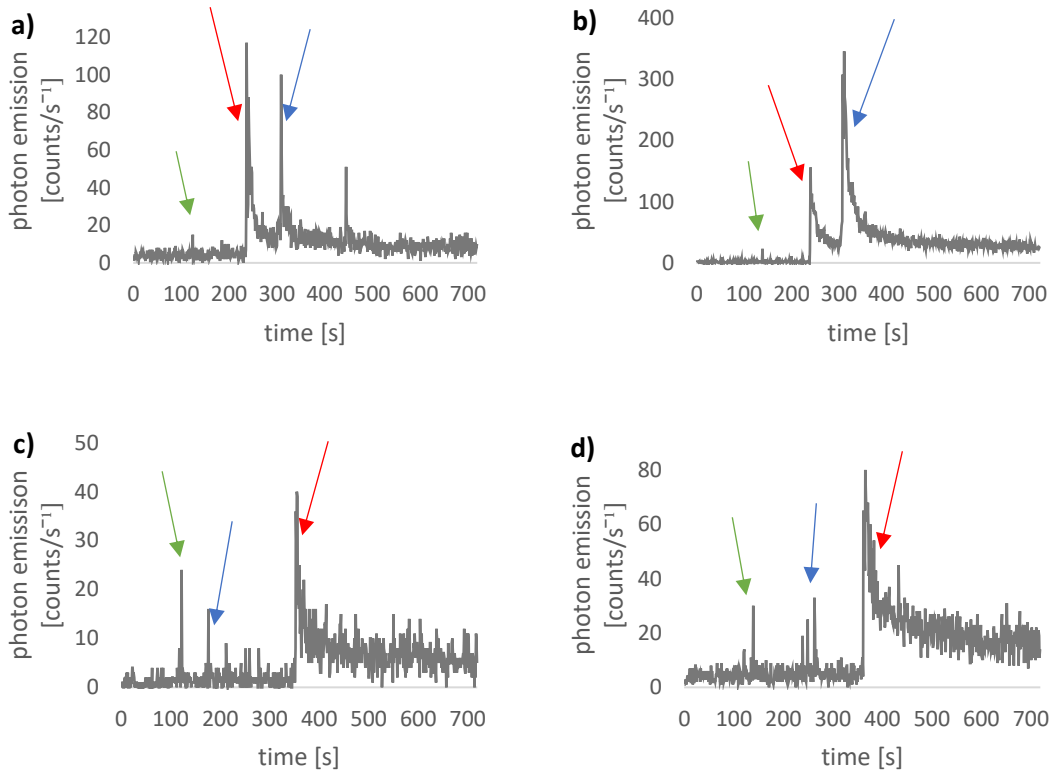

Figure 10: Effects of antixidants added after (a,b) and before (c,d) 5mM H_2O_2 and $HO^{\bullet}$ formation. Samples with 5mM Asc are shown on parts a) and c) and with 5mM Toc are b) and d). Graphs show current UPE and effect of added chemical to BSA oxidation during measurement. Termination of measurement 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 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 $HO^{\bullet}$ increase UPE from all the samples in similar values. Antioxidants added before $HO^{\bullet}$ 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	BSA	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	1,00	0,52	36,24	-
BSA+$FeSO_4$+H_2O_2+asc	1,00	0,68	26,23	-11,17
BSA+ $FeSO_4$+H_2O_2+toc	1,00	0,68	39,15	42,57
Sample	BSA	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	1,00	0,85	-0,39	8,61
BSA+ $FeSO_4$+toc+H_2O_2	1,00	0,31	0,78	24,29

Column graph (Figure 11) display comparison of UPE in samples, where induction was reached by $HO^{\bullet}$ formation. Samples with Asc reach lower UPE, while Toc act as pro-oxidant. In comparison with Figure 9, we observe higher levels of UPE.

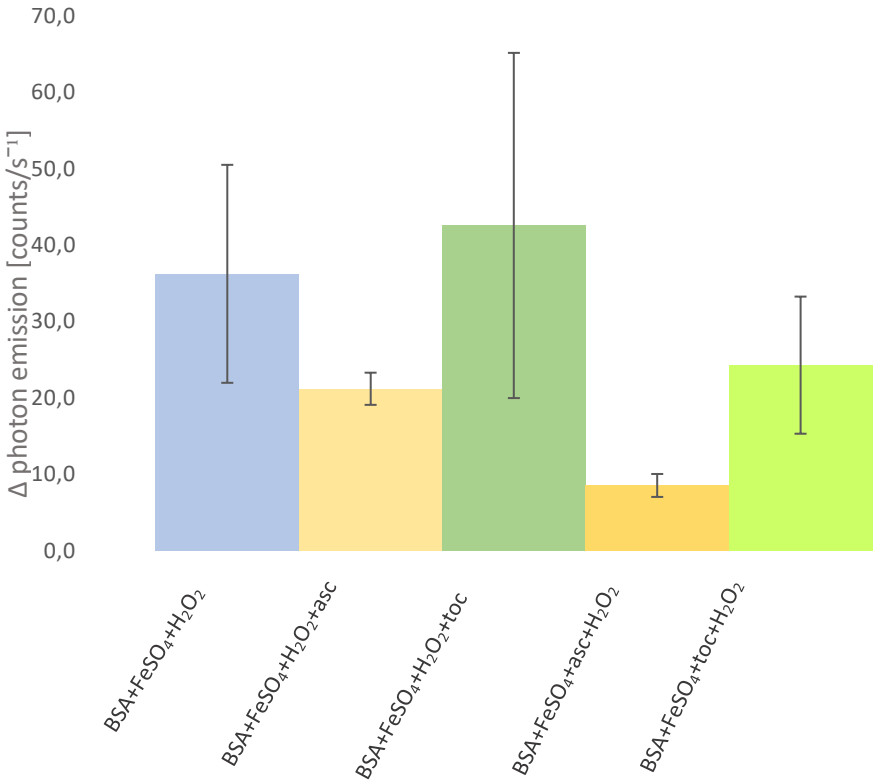

Figure 11: Comparison between UPE (averages of the count rates) from samples treated with $HO^{\bullet}$ and antioxidants and UPE from BSA. The deviations represent the course and the different reached values of UPE through measurement.

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 (around 120s, see Fig.12). 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 (Figure 12).

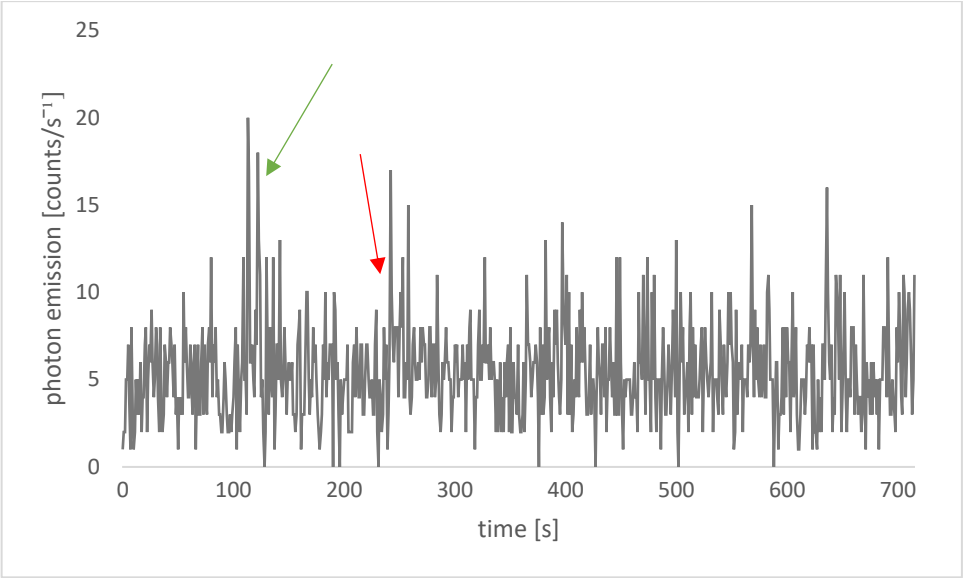

Figure 12: Effect of $^1O_2$ on UPE from 0,15mM BSA. Moment of 2,5mM MoO_3 addition is indexed with green arrow and addition of 5mM H_2O_2 by red. Formation of $^1O_2$ and it´s effect on BSA is accompanied with hardly noticeable increase in UPE. Stady-state level can be set from 400s.

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

Last examined effect 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 (Figure 13a). Sharp increase of UPE occurred after addition of Toc (Figure 13b) 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.

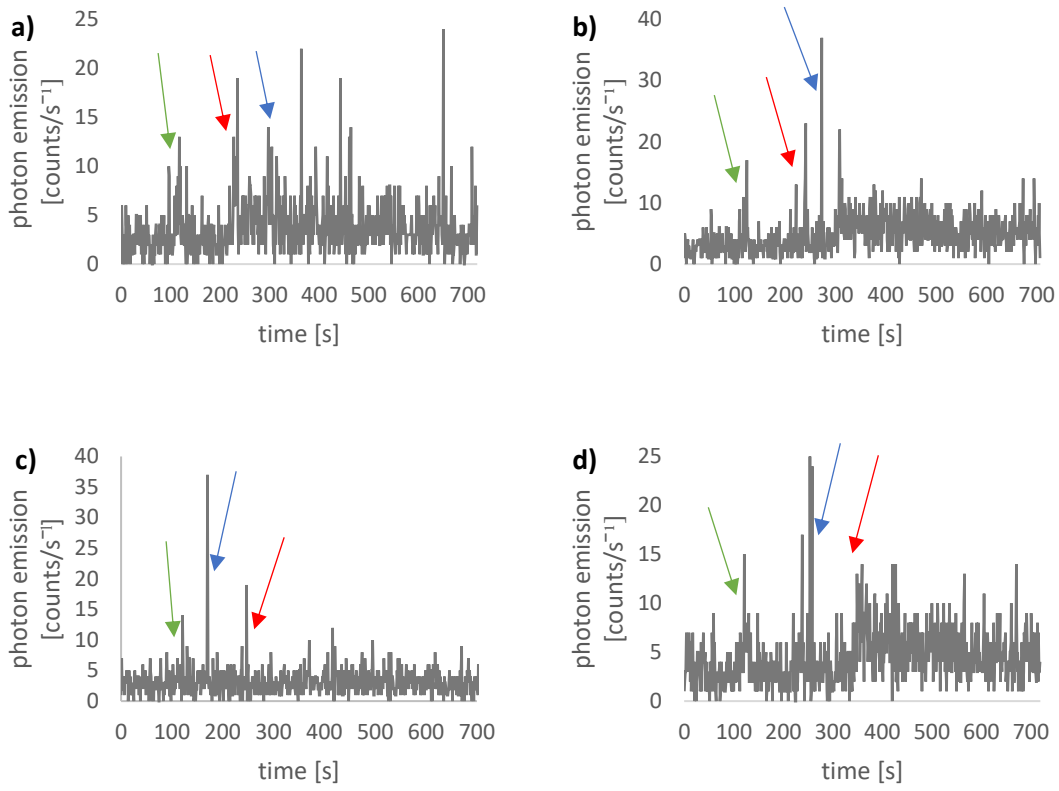

Figure 13: Comparison of samples with antixidants added after (a,b) and before (c,d) 5mM H_2O_2 and $^1O_2$ formation. Samples with 5mM Asc are shown on parts a) and c) and with 5mM Toc are b) and d). Graphs show current UPE and effect of added chemical to BSA oxidation during measurement. Termination of measurement was after UPE remained unchanged (700s).

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 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	BSA	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	1,00	0,37	1,11	-
BSA+ MoO_3+H_2O_2+asc	1,00	0,40	1,77	-0,41
BSA+ MoO3+H_2O_2+toc	1,00	0,29	1,79	4,32
Sample	BSA	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	1,00	0,87	-0,37	0,80
BSA+ MoO_3+toc+H_2O_2	1,00	0,51	0,34	1,66

Column graph (Figure 14) 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.

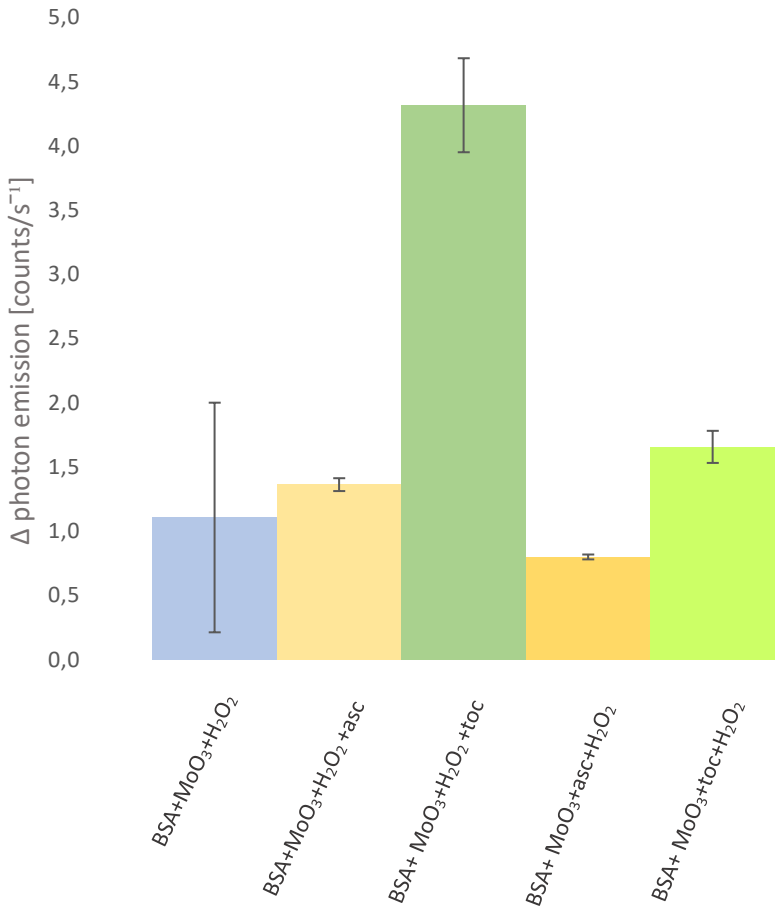

Figure 14: Comparison between UPE (averages of the count rates) from samples treated with $^1O_2$ and antioxidants and UPE from BSA. The deviations represent the course and the different reached values of UPE through measurement.

6 Discussion

Comparison of effects of exogenous ROS and particularly various 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. Ultra-weak photon emission from samples treated with Asc was lower in comparision with Toc as well as from sample treated only with H_2O_2 as can be seen in Table 1 and Figure 8. Ascorbic acid added after H_2O_2 slightly decreased UPE by stabilizing formed radicals ($R^{\bullet}, ROO^{\bullet}$). In comparison with sample BSA+H_2O_2 was UPE 1,35x smaller. Ascorbic acid reduced oxidizing H_2O_2 and formed ascorbic 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 again. Final UPE was 1,4 lower than from sample without antioxidant treatment. α-tocopherol added to sample treated with H_2O_2 increased UPE, specifically 1,13x in comparison with BSA+H_2O_2 sample. Effect of Toc added after H_2O_2 include stabilization of $ROO^{\bullet}$ by its reduction to ROOH by Toc supply with atom of hydrogen. Equation describing ongoing reaction is $\alpha - toc - OH + ROO^{\bullet} \rightarrow \alpha - Toc - Q^{\bullet} + ROOH$. 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. α-tocopherol supplied atom of hydrogen and quenched effect of H_2O_2 *(Niki et al. 2014)*, final UPE was 1,5x smaller than from sample without antioxidant treatment. 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 $HO^{\cdot}$ started. 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 $ROO^{\bullet}$ were formed. Alkyl radicals formed on amino acids reacted with Asc and decreased UPE by its reduction ($R^{\bullet} + Asc \rightarrow Asc^{\bullet} + RH$). 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 (Figure 10). Addition of Toc to sample with BSA oxidized by $HO^{\bullet}$ caused increase of UPE by 1,16x in comparison with treatment only with $HO^{\cdot}$. Pro-oxidant behavior of Toc was expressed in this measurement. Strong instant increase of UPE occurred (Figure 10b). 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 (Figure 12). 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)* and thus UPE is higher due to triggering of cascade reaction. Even Toc-$Q^{\bullet}$ is weak radical with small reaction constant, in abundance may cause further oxidation processes.

Singlet oxygen formation and its oxidative effect on BSA is showed on Figure 12. 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

$^1O_2$**.** 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 ($^1O_2 + \mathrm{Asc} \rightarrow H_2O_2 + \mathrm{DHA}$) show that reaction of $^1O_2$ with Asc leads to H_2O_2 formation *(Kramarenko et al. 2006)* and thereby other oxidation of BSA starts, which is projected to increased UPE. We consider that due to high concentration of Asc is increasement only moderate. In samples with Asc addition before H_2O_2 and later $^1O_2$ formation, the lowest level of UPE was achieved. We attribute this to the reaction between MoO_3 and Asc, where further reaction between MoO_3 and H_2O_2 and thus the formation of $^1O_2$ was not entirely possible. 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. Reaction scheme known for reaction between Toc and $^1O_2$ ($\mathrm{Toc} + {}^1O_2 \rightarrow \mathrm{Toc\text{-}Q}^{\bullet} + HO_2^{\bullet}$) shows that product of this reaction is $\mathrm{Toc\text{-}Q}^{\bullet}$ and $HO_2^{\bullet}$, which might cause secondary rection in BSA solution, which might seen as increased UPE. On the other hand, Toc display pro-oxidant activity in laboratory conditions, where $\mathrm{Toc\text{-}Q}^{\bullet}$ 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·s^{-1}. In presence of transition metal ions ($FeSO_4$) , $HO^{\cdot}$ is formed. Average of UPE induced by $HO^{\bullet}$ is 36,24 counts·s^{-1} and strong oxidant capability of this species occurred. Addition of H_2O_2 to BSA treated with MoO_3 before leaded to $^1O_2$ formation. Enhanced UPE report to 1,11 counts ·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.

Another studied aspect was effect of non-enzymatic antioxidants (Asc and Toc) and its effect on UPE from BSA. Ascorbic acid showed antioxidant capacity and chelating effects in measurements with UPE induced by H_2O_2 and $HO^{\bullet}$. 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 showed its capacity to quench ROS only with H_2O_2. Evidence of pro-oxidant switch of Toc was provided in both types of treatment in samples with $^1O_2$. Ultra-weak photon emission from the samples wasn´t significant. Since we also measured whether increasement of UPE isn´t caused by Toc solvent (ethanol) and results didn´t make change we consider our results relevant. Addition of Toc to samples treated with $HO^{\bullet}$ led to sharp increase of UPE. The inability of Toc to stabilize $HO^{\bullet}$ occurs and pro-oxidant activity is present as a result of presence of transition metal ions, which is one of the interpreted preconditions to pro-oxidant switch.

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