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Photoinactivation of tyrosinase sensitized by folic acid photoproducts

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A B S T R A C T

Tyrosinase catalyzes in mammals the first and rate-limiting step in the biosynthesis of the melanin, the main pigment of the skin. Pterins, heterocyclic compounds able to photoinduce oxidation of biomolecules, accumulate in the skin of patients suffering from vitiligo, where there is a lack of melanin. Folic acid (PteGlu) is a conjugated pterin widespread in biological systems. Aqueous solutions of tyrosinase were exposed to UV-A irradiation (350 nm) in the presence of PteGlu and its photoproducts (6-formylpterin and 6-carboxypterin). The reactions were followed by UV–Vis spectrophotometry, enzyme activity measurement, fluorescence spectroscopy and HPLC. In this work, we present data that demonstrate unequivocally that solutions of tyrosinase exposed to UV-A irradiation in the presence of PteGlu, undergo enzyme inactivation. However, PteGlu itself causes a negligible effect on the activity of the enzyme. In contrast, PteGlu photoproducts are efficient photosensitizers. The tyrosinase inactivation involves two different pathways: (i) a photosensitization process and (ii) the oxidation of the enzyme by the hydrogen peroxide produced during the photooxidation of PteGlu and its photoproduct. The former pathway affects both the active site and the tryptophan residues, whereas the latter affects only the active site. The biological implications of the results are discussed.

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1. Introduction

Pterins, a family of heterocyclic compounds, are present in biological systems in multiple forms and play different roles ranging from pigments to enzymatic cofactors for numerous redox and one-carbon transfer reactions [1,2]. The most common pterin derivatives are 6-substituted compounds (Fig. 1). According to the molecular weight and the functional groups of these substituents, pterins can be divided into two groups: (i) *unconjugated pterins*, containing substituents with one carbon atom or a short hydrocarbon chain, and (ii) *conjugated pterins*, with larger substituents containing a *p*-aminobenzoic acid (PABA) moiety.

Folic acid, or pteroyl-L-glutamic acid (PteGlu), is a conjugated pterin widespread in biological systems, whose chemical structure is composed of three moieties: a 6-methylpterin (Mep) residue, a PABA residue, and a glutamic acid (Glu) residue (Fig. 1). In living systems, PteGlu is present in multiple forms including molecules attached to several glutamate residues and dihydro and tetrahydro pterin derivatives. Mammals do not synthesize folates and therefore they have to get them from food. The main form of folates in human plasma is 5-methyltetrahydrofolate (5CH$_3$-H$_4$PteGlu) [3,4]. This compound is oxidized to corresponding dihydropterin derivatives at pH close to 7 [5], such as skin pH. It is know that 7,8-dihydrofolic acid is oxidized by reactive oxygen species, like hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (^{1}O$_2$), yielding PteGlu as one of the products [6,7]. Taking into account that in some pathological conditions the concentration of reactive oxygen species increases significantly, the oxidation of reduced folic acid derivatives can be occurred, yielding a source of folic acid in the cell.

In particular, folates are involved in DNA biosynthesis, repair, DNA methylation and aminoacid metabolism [8]. Folate requirements increase in periods of rapid cell division and growth, and,
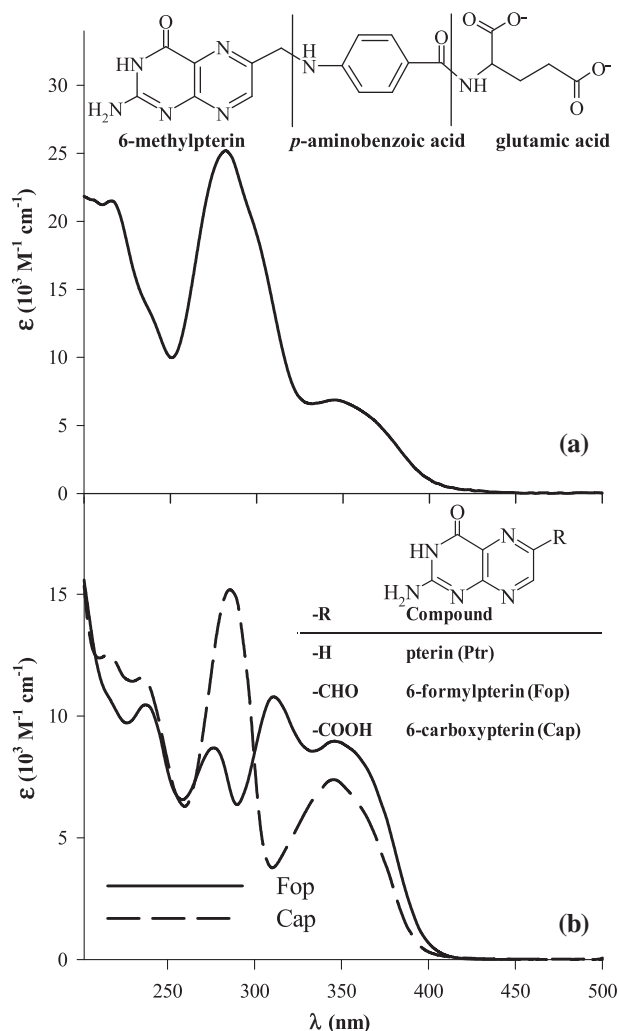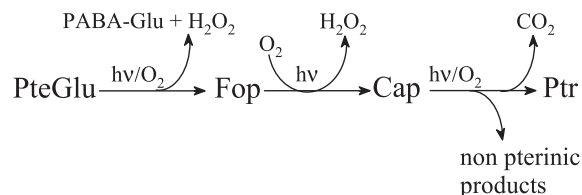
Fig. 1. Molecular structure of PteGlu and unconjugated oxidized pterins, and their corresponding absorption spectra in air-equilibrated aqueous solutions (pH 6.0). (a) PteGlu; (b) Fop and Cap. UV–Vis absorption spectra of Ptr is almost identical to that shown for Cap.

Scheme 1. Photooxidation of PteGlu in air-equilibrated aqueous solutions under UV-A irradiation.

Folates participate in photobiological processes, *e.g.* the derivative 5,10-methenyltetrahydrofolic acid acts as photoantenna in DNA photolyases and cryptochromes [19]. In addition, the photodegradation of PteGlu has been discussed as one of the reasons for the development of skin tanning in evolution because the PteGlu depletion by natural exposure to sunlight can become physiologically relevant [20–24]. PteGlu decomposes under UV-A radiation in the presence of oxygen, yielding Fop, and *p*-aminobenzoylglutamic acid (PABA–Glu) as photoproducts (Scheme 1) [25–30]. The rate of this process increases with irradiation time. The data point to an "auto-photo-catalytic" effect, wherein Fop acts as photosensitizer and photoinduces the oxidation of PteGlu [30]. In turn, Fop is transformed into Cap upon further photooxidation (Scheme 1). Reactive oxygen species, like H_2O_2 and superoxide anion (O_2^-) are formed during the photooxidation of PteGlu into Fop, and in the reaction where Fop is converted into Cap [30]. Therefore under irradiation of skin affected by vitiligo Fop and Cap are formed, very likely, due to the photolysis of both PteGlu and H_4Bip. In fact, Cap has been isolated from the skin of patients suffering from vitiligo [31].

Several studies have demonstrated that pterin (Ptr), the parent and unsubstituted compound of oxidized pterins (Fig. 1), is able to photosensitize free amino acids, such as tryptophan (Trp) [32] and Tyr [33], peptides [34] and proteins, such as bovine serum albumin [35,36]. A very recent study has demonstrated that tyrosinase is inactivated by Ptr through a photosensitized process and suggested that the process is initiated by an electron transfer reaction from the enzyme to the triplet excited state of Ptr [37]. In 2003 it was reported that PteGlu is able to photoinduce DNA damage *via* its photolysis products [38]. More recently it was informed that protein damage takes place when albumin is exposed to UV-A radiation in the presence of PteGlu [39,40].

Taking into account that in some skin disease, such as vitiligo, the protection against UV radiation fails due to the lack of melanin, the photodegradation of PteGlu *in vivo* may occur in the depigmented patches of patients with this disease. We chose this compound because: it is more stable than most member of folate's family and efficiently metabolized into biologically active derivatives, it is used in tablet form and in fortified foods for dietary supplementation [8,41] and its photooxidation products are the same oxidized pterins produced during the photooxidation of H_4Bip. Therefore, the main aim of this work was investigated the capability of PteGlu and its photoproducts to photoinduced the inactivation of tyrosinase, the key enzyme in melanin biosynthesis.

as such, it is imperative that pregnant women, for example, keep folate concentrations at an appropriate level [9]. Folate deficiency in pregnant women has clearly been shown to be related to neural tube defects, such as spina bifida and anencephaly [10,11].

Recent research has demonstrated that folates regulate melanin biosynthesis and, hence, is present in human skin, because it is required for the novo production of tetrahydrobiopterin (H_4Bip) in melanocytes and keratinocytes [12,13]. H_4Bip acts as a cofactor for hydroxylation of L-phenylalanine *via* phenylalanine hydroxilase and regulates tyrosinase activity in melanosome [14]. The synthesis of H_4Bip is highly activated in vitiligo, a skin disorder which is characterized by the acquired loss of constitutional pigmentation manifesting as white macules and patches. The photooxidation of H_4Bip yield biopterin (Bip) [15], which under UV-A (315–400 nm) irradiation leads to oxidation of the 6-substituent to yield 6-formylpterin (Fop, Fig. 1), which, in turn, is photooxidized to 6-carboxypterin (Cap, Fig. 1) [16,17].

Tyrosinase ((L-tyrosine, L-dopa: oxygen oxidoreductase, EC 1.14.18.1)) is a copper-containing glycoprotein that, in mammals, catalyzes the first and rate-limiting step in melanin biosynthesis, the hydroxylation of L-tyrosine (Tyr) to 3,4-dihydroxy-L-phenylalanine (DOPA) and the subsequent oxidation of DOPA to L-dopaquinone [18]. This compound, in turn, undergoes fast oxidation and rearrangement to yield L-dopachrome.

2. Materials and methods

2.1. General

Pterin derivatives (PteGlu, Fop, Cap and Ptr) were purchased from Schircks Laboratories and used without further purification. Tyrosinase from mushroom (lyophilized powder, $\geqslant$1000 unit/mg solid), catalase from bovine liver (lyophilized powder, 10,700 unit/mg solid) and other chemicals were provided by Sigma Chemical Co. H_2O_2 (30%) was from Merck.

The pH measurements were performed using a pH-meter PHM220 (Radiometer Copenhagen) combined with a pH electrode pHC2011-8 (Radiometer Analytical). The pH of the aqueous solutions was adjusted by adding drops of HCl and NaOH solutions from a micropipette. The concentration of the acid and the base used for this purpose ranged from 0.1 M to 2 M. When necessary the samples were incubated using a low temperature bath/circulator R1 (Grant Instruments).

2.2. Steady-state irradiation

2.2.1. Irradiation setup

Air equilibrated aqueous solutions containing a given pterin derivative and tyrosinase were irradiated in quartz cells (1.0 cm optical path length) at room temperature using a Rayonet RPR lamp (Southern N. E. Ultraviolet Co.) with emission centered at 350 nm (bandwidth ~20 nm). To regulate the intensity of the incident light, the photolyses were performed at two different distances between the lamp and the sample (4 and 20 mm).

2.2.2. Actinometry

Aberchrome 540 (Aberchromics Ltd.) was used as an actinometer for the measurement of the incident photon flux density ($q_{n,p}^{0,V}$) at the excitation wavelength, which is the amount of incident photons per time interval ($q_{n,p}^0$) and divided by the volume (V) of the sample [42]. Aberchrome 540 is the anhydride form of the (E)-α-(2,5-dimethyl-3-furylethylidene)(isopropylidene)succinic acid which, under irradiation in the spectral range 316–366 nm leads to a cyclized form. The method for the determination of $q_{n,p}^{0,V}$ has been described in detail elsewhere [43].

Taking into account the distance from the irradiation source to the sample, two different values of $q_{n,p}^{0,V}$ were measured ($q_{n,p}^{0,V,1}$ = 1.7 (±0.2) × 10^{-5} Einstein L^{-1} s^{-1} and $q_{n,p}^{0,V,2}$ = 3.4 (±0.4) × 10^{-6} Einstein L^{-1} s^{-1}, for 4 and 20 mm, respectively). Values of the absorbed photon flux density ($q_{n,p}^{a,V}$) were calculated from $q_{n,p}^{0,V}$ according to the Lambert–Beer law:

$$q_{n,p}^{a,V} = q_{n,p}^{0,V}(1 - 10^{-A}) \tag{1}$$

where A is the absorbance of the reactant at the excitation wavelength.

2.3. Analysis of irradiated solutions

2.3.1. UV–Vis analysis

Electronic absorption spectra were recorded on a Shimadzu UV-1800 spectrophotometer. Measurements were made using quartz cells of 1 cm optical pathlength. The absorption spectra of the solutions were recorded at regular intervals of irradiation time.

2.3.2. High-performance liquid chromatography (HPLC)

Chromatographic analysis was performed using a Prominence instrument from Shimadzu (solvent delivery module LC-20AT, on-line degasser DGU-20A5, auto sampler SIL-20A HT, column oven CTO-10AS VP, photodiode array detector SPD-M20A). A Synergi Polar-RP column (150 × 4.6 mm, 4 μm, Phenomenex) was used for product separation. Solutions containing 7% of methanol and 93% of 10 mM NH$_4$OAc aqueous solution (pH 7.0) were used as the mobile phase. HPLC runs were monitored by UV–visible spectroscopy at different wavelengths.

2.3.3. Enzyme activity

The tyrosinase activity was assayed according to the method of Pomerantz [44]. Briefly, the formation of L-dopachrome was measured spectrophotometrically at 475 nm (ε = 3600 M^{-1} cm^{-1}). The reaction was performed at 37 °C in KH$_2$PO$_4$ buffer 20 mM at pH 6.5 using L-tyrosine 0.15 mg/mL (0.83 mM) as the substrate. The conversion of an inactive form of the catalytic site of the enzyme into an active form gives rise to a lag period before the reaction reaches maximal rate, which is a characteristic of the overall activity of the tyrosinase (oxidation of L-tyrosine into L-dopachrome) [45]. Therefore the enzyme activity (rate of formation of L-dopachrome (v), μM min^{-1}) was determined in the linear phase by measuring the slope of the curve of absorbance at 475 nm *vs.* time after the addition of the substrate.

2.3.4. Fluorescence spectroscopy

Fluorescence measurements were performed on air-equilibrated aqueous solutions of pterin derivatives and tyrosinase using a Single-Photon-Counting equipment FL3 TCSPC-SP (Horiba Jobin Yvon). The equipment has been previously described in detail [46]. Briefly, in steady-state measurements the sample solution in a quartz cell was irradiated with a 450 W Xenon source through an excitation monochromator. The fluorescence, after passing through an emission monochromator, was registered at 90° with respect to the incident beam using a room-temperature R928P detector. The total fluorescence intensities (I_F) were calculated by integration of the fluorescence band centered at *ca.* 340 nm. The tyrosinase fluorescence intensity was corrected for inner filter effect using the following equation [47]

$$I_{Fcorr} = I_{Fobs} \, 10^{(A_{exc}+A_{emi})/2} \tag{2}$$

where I_{Fcorr} and I_{Fobs} are the corrected and observed fluorescence intensities, respectively, and A_{exc} and A_{emi} are the absorbance of the system at excitation and emission wavelengths, respectively.

3. Results and discussion

3.1. Evaluation of the photoinactivation of tyrosinase by PteGlu

To check the photochemical behavior of PteGlu, we carried out photolysis in the absence of substrates and the results observed were compatible with those reported in the literature [30]. Photooxidation of PteGlu under UV-A radiation may be divided into three stages (Fig. S1. Supporting material): (i) in the first phase, which follows a zero-order kinetics, PABA–Glu and Fop are photogenerated; (ii) in the second phase, Fop photoinduces the photooxidation of PteGlu and its degradation process is accelerated; (iii) in the third phase the degradation of Fop to Cap is the dominating process. This result indicated that the photodegradation of the sensitizer itself could not be avoided in the photolysis experiments performed in the presence of tyrosinase. Therefore, the irradiated samples were analyzed by HPLC to evaluate the PteGlu photooxidation.

To ascertain if PteGlu is able to photoinduce the inactivation of the enzyme, air-equilibrated aqueous solutions (1 mM KH$_2$PO$_4$, pH 6.0) containing PteGlu and tyrosinase were irradiated at 350 nm during different periods of time. After the irradiation, the samples were analyzed by UV–Vis spectrophotometry, enzyme activity measurement, fluorescence spectroscopy and HPLC. Under these experimental conditions only PteGlu was excited (Fig. 1), whereas tyrosinase did not absorb radiation. The analysis of the treated solution showed that the activity of tyrosinase decreased significantly when the enzyme was exposed to UV-A radiation in the presence of PteGlu.

In control experiments, tyrosinase solutions were exposed to UV-A radiation in the absence of PteGlu and no inactivation of the enzyme was detected, thus excluding the possibility that spurious effects of direct light absorption by the enzyme could affect its activity. In another set of control experiments, tyrosinase

inactivation was not observed in a solution of PteGlu with tyrosinase that was kept in the dark, thus excluding the possibility of thermal reactions. The same result was also observed in dark control experiments carried out with Fop, Cap and Ptr instead of PteGlu.

The comparison of the kinetic profiles of PteGlu photodegradation and tyrosinase photoinactivation indicated that in the first four minutes of irradiation, the concentration of PteGlu did not change significantly, whereas no lost of the enzyme activity was registered (Fig. 2). It can be assumed that, in this time range, no photoproduct of PteGlu was generated. Therefore, PteGlu itself presented a negligible capability to photoinactivate the enzyme.

After five min of irradiation, an important increase on the rate of photodegradation of PteGlu and on the rates of formation of Fop and Cap were observed (Fig. 2a). Therefore, a significant proportion of light was absorbed by Fop and Cap. Simultaneously, a considerable decrease of the enzyme activity was observed (Fig. 2b). Consequently, these results suggest that, in contrast to PteGlu, Fop and Cap photoinduce the inactivation of the enzyme.

3.2. Evaluation of the photoinactivation of tyrosinase by PteGlu photoproducts

To confirm that PteGlu photoproducts are able to photoinduced the inactivation of tyrosinase, the rate of enzyme inactivation was determined in experiments carried out under the same conditions as those used in the experiment of Fig. 2 (pH, absorbance at the irradiation wavelength (350 nm), irradiation source, optical geometry, etc), but changing the pterin derivatives used as a photosensitizer. After the irradiation, the samples were analyzed by UV–Vis spectrophotometry, enzyme activity measurement, and HPLC. The
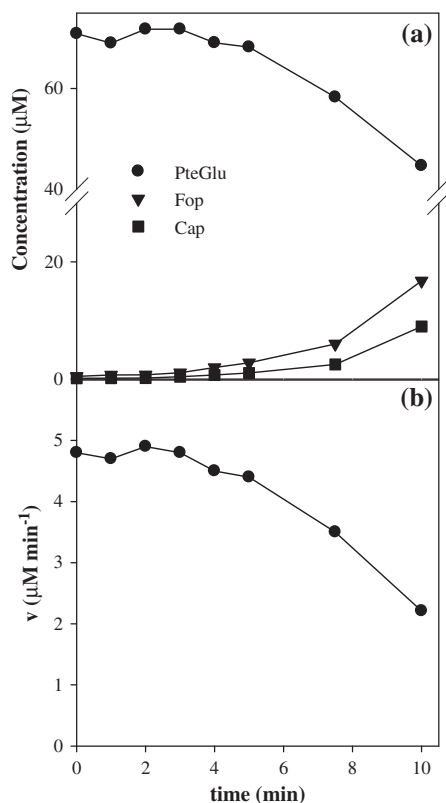

Fig. 2. Photolysis of air-equilibrated aqueous solution of PteGlu and tyrosinase exposed to UV-A radiation. Time evolution of: (a) PteGlu, Fop and Cap concentrations; (b) tyrosinase activity (v, rate of formation of L-dopachrome). $[PteGlu]_0 = 70\ \mu M$, $[tyrosinase]_0 = 28\ \mu g/mL$, 1 mM KH_2PO_4, pH = 6.0, $q_{n,p}^{0,V,1}$.

comparison of the rates of photoinactivation registered using the different pterin derivatives revealed that, in fact, Fop is a photosensitizer much more efficient than PteGlu (Fig. 3), which explains why the rate of tyrosinase photoinactivation increased with irradiation time when Fop is accumulated during the photooxidation of PteGlu. Cap and Ptr, in turn, have the same capability to generate inactivation of tyrosinase, and are less efficient than Fop (Fig. 3).

The comparison of the kinetic profiles of Fop degradation and enzyme activity in Fig. 3a, indicated that after 10 min of irradiation, almost 70% of the initial Fop was consumed to form Cap, with a linear decrease in the rate of enzyme inactivation. Taking into account that Cap is less efficient photosensitizer than Fop, it is expected that the rate of enzyme photoinactivation should be lower when the Fop concentration decreases as a function of irradiation time. In order to elucidate the reason of the kinetic behavior of the enzyme inactivation when Fop is used as photosensitizer, and taking into account that under UV-A radiation Fop generates one molecule of H_2O_2 for each molecule of Fop consumed (Scheme 1) [48], it was appropriate to explore the stability of tyrosinase in the presence of H_2O_2 (see next section).

3.3. Evaluation of the inactivation of tyrosinase by H_2O_2

The study of the stability of tyrosinase in the presence of H_2O_2 is very important from a biomedical point of view because patients with vitiligo accumulate high levels (mM) of H_2O_2 in their epidermis [49,50]. In addition, it has been suggested that photooxidation of pterins is one of the H_2O_2 sources in this depigmentation disorder [31]. Schallreuter et al. have studied the direct effect of H_2O_2 on the tyrosinase reaction. The results showed that the enzyme was activated by low concentrations of H_2O_2 (<300 μM), while at concentrations in the range 500–5000 μM the tyrosinase was inactivated [51]. Taking into account this report, since in the photooxidation of Fop, one molecule of H_2O_2 is produced per molecule of Fop consumed, the H_2O_2 formed in our experiments would not be able to produce the inactivation of tyrosinase. Therefore, to assess the participation of H_2O_2 in the photoinactivation of tyrosinase by Fop, air equilibrated aqueous solutions of tyrosinase and H_2O_2 (pH 6.0) were incubated at 20 °C in the dark for different times and then the activity of the enzyme was determined. Surprisingly, the data showed that tyrosinase was deactivated by low H_2O_2 concentrations (Fig. 4). These results, in disagreement with those reported by Wood et al. [51], suggest that the H_2O_2 produced during the photooxidation of PteGlu and its photoproducts is enough to cause, at least, a partial inactivation of the enzyme.

To further investigate the contribution of H_2O_2 in the deactivation of the enzyme during the photosensitized process, air equilibrated aqueous solutions of tyrosinase and Fop were exposed to UV-A radiation in the absence and in the presence of catalase, an enzyme that catalyzes specifically the decomposition of H_2O_2 into H_2O and O_2. Fop was chosen for this study because, according to the results shown so far, this derivative is the main sensitizer responsible for the photoinactivation of the enzyme. The data obtained clearly showed that the photoinactivation of tyrosinase was much more efficient in the absence than in the presence of catalase (Fig. 5), thus suggesting the participation of H_2O_2.

To discard potential interferences in the experiment shown in Fig. 5 and then to confirm the participation of H_2O_2 in the inactivation of tyrosinase upon irradiation in the presence of Fop, several controls were carried out. In first place, to determine if catalase is able to react with Fop under excitation, air equilibrated aqueous solution of the photosensitizer and catalase were irradiated with UV-A radiation. Concentration profiles showed that the rates of Fop photooxidation did not change in the presence of catalase (data not shown). In another control experiment, solutions of tyrosinase without photosensitizer were irradiated in the presence
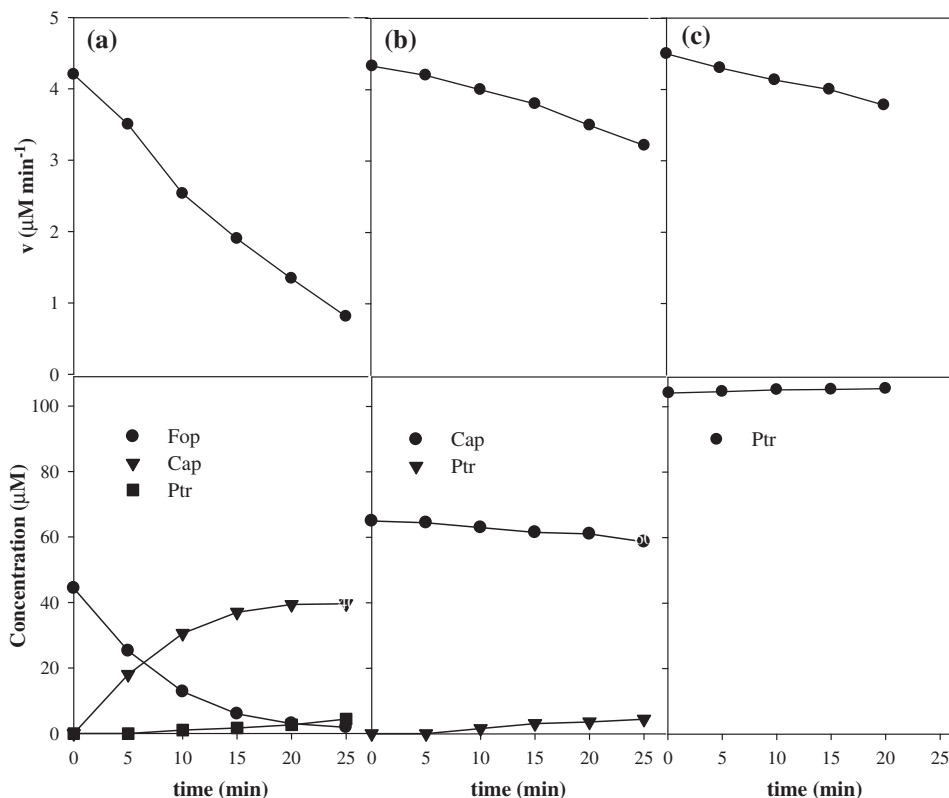
Fig. 3. Evolution of tyrosinase activity (v), and concentrations of pterins in air-equilibrated aqueous solution containing tyrosinase and a given pterin derivative exposed to UV-A radiation as a function of the elapsed irradiation time (λ_{exc} = 350 nm, [tyrosinase]$_0$ = 23 µg/mL, 1 mM KH$_2$PO$_4$, pH = 6.0, $q_{n,p}^{0,V,2}$). The photosensitizers used were: (a) Fop ($A_{0\,(350\,nm)}$ = 0.50, [Fop]$_0$ = 44 µM); (b) Cap ($A_{0\,(350\,nm)}$ = 0.50, [Cap]$_0$ = 65 µM); (c) Ptr ($A_{0\,(350\,nm)}$ = 0.50, [Ptr]$_0$ = 104 µM).
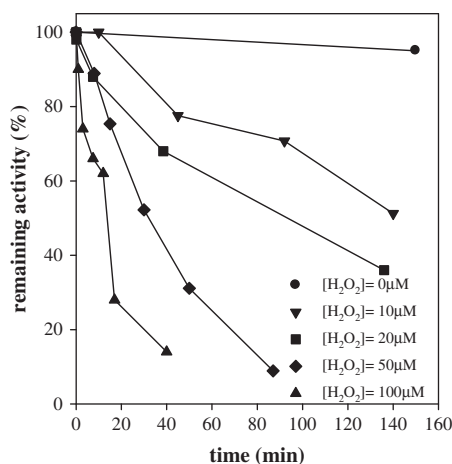

Fig. 4. Determination of the activity of tyrosinase as a function of incubation time at different concentrations of H$_2$O$_2$ ([tyrosinase]$_0$ = 23 µg/mL, T = 20 °C, 20 mM KH$_2$PO$_4$, pH = 6.0). For comparative purposes, the data are expressed as the percentage of the initial enzyme activity.
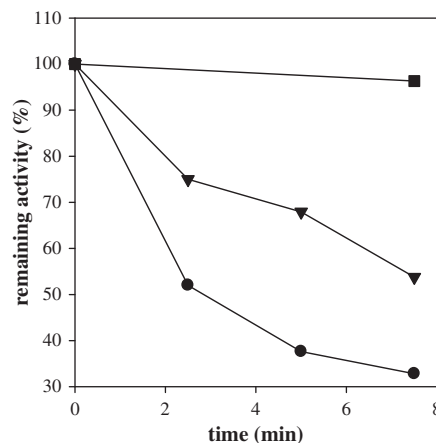

Fig. 5. Determination of the activity of tyrosinase as a function of irradiation time in the presence of catalase and absence of Fop (■), in the presence of Fop and absence of catalase (●) and in the presence of Fop and catalase (▼) ([tyrosinase]$_0$ = 50 µg/mL, 1 mM KH$_2$PO$_4$, λ_{exc} = 350 nm, pH = 6.0, catalase (5800 U/mL), [Fop]$_0$ = 44 µM, $q_{n,p}^{0,V,1}$). For comparative purposes, the data are expressed as the percentage of the initial enzyme activity.

and in the absence of catalase. No decrease of the activity of tyrosinase was registered in the presence of catalase, thus excluding the possibility that this enzyme directly affects the activity of tyrosinase (Fig. 5).

The results presented in this section clearly indicated that photoinactivation of tyrosinase in our reaction system takes place through two different types of pathways: (i) photosensitized reactions and (ii) the oxidation of the enzyme by H$_2$O$_2$. In the latter, H$_2$O$_2$ is produced in the photooxidation of PteGlu into Fop, and in the subsequent photooxidation of Fop into Cap [30] and then

this reactive oxygen species reacts with the enzyme causing its inactivation.

3.4. Effect on the tryptophan residues

Taking into account the photosensitization of Trp free [32] and in polypeptide chains [34] by Ptr previously reported, and that Trp is particularly susceptible to a variety of oxidizing agents [52], it is straightforward to expect that Trp residues of tyrosinase were
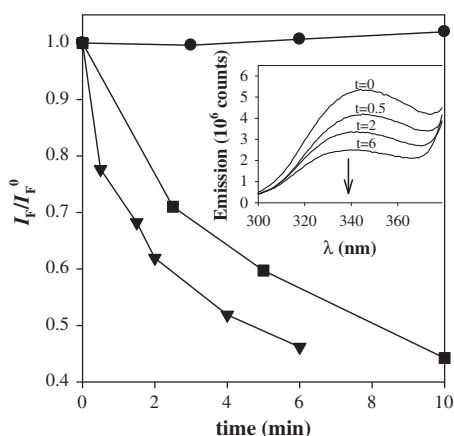
Fig. 6. Time evolution of the relative total fluorescence intensity (I_F/I_F^0) of an aqueous solution of tyrosinase irradiated at 350 nm in the absence of photosensitizer (●) and in the presence of Fop (($A_{0(350\ nm)}$ = 0.50, [Fop]$_0$ = 44 μM)) (▼) or Cap ($A_{0(350\ nm)}$ = 0.50, [Cap]$_0$ = 65 μM) (■). [Tyrosinase]$_0$ = 50 μg/mL, pH = 6.0, $q_{n,p}^{0,V,1}$. Corrected fluorescence spectra were obtained by excitation at 280 nm and recorded between 300 and 380 nm, T = 20 °C, optical path length 0.4 cm. Inset: Corrected fluorescence spectra registered using Fop as a photosensitizer. The irradiation time (min) appears above each spectrum.

affected by photosensitization with PteGlu and its photoproducts. Therefore, this point was investigated, taking advantage of the fluorescence properties of this amino acid [47], *i.e.* it is the only amino acid residue that absorbs at 295 nm and presents an intense emission band centered between 310 and 360 nm, depending on the protein local environment [47].

In tyrosinase the Trp residues are buried in hydrophobic environment when the protein is in the native state [53]. This enzyme contains also Tyr residues which act as intrinsic fluorescence probes, as well. In contrast to excitation at 295 nm, when excitation wavelength of 280 nm is used, both Tyr and Trp residues are excited. The comparison of fluorescence spectra of tyrosinase obtained by excitation at 280 and 295 nm showed that there are not changes in the shape and emission maximum of both spectra (data not shown), thus indicating that emission fluorescence comes mainly from Trp in tyrosinase, under this experimental conditions.

Therefore, to determine if the photosensitization process altered the Trp residues of the enzyme, emission spectra of air-equilibrated aqueous solutions containing tyrosinase and a given pterin derivative were recorded after different irradiation times. The typical emission band of Trp could be adequately registered when Fop and Cap were used as photosensitizers, since these pterin derivatives fluoresce with a broad band centered at approximately 450 nm [54]. In contrast, Trp emission could not be properly recorded when PteGlu was used as a photosensitizer because PABA–Glu, one of the products of the PteGlu photodegradation (Scheme 1), absorbs at the excitation wavelength and fluoresces with a broad and intense band centered at *ca* 350 nm.

As shown in Fig. 6, Trp emission decreased significantly as a function of time when tyrosinase was irradiated in the presence of Fop or Cap. This result suggests that, Trp residues are affected in the photosensitization process. The comparison of the Trp emission decrease registered using Fop or Cap as sensitizer revealed that, Fop is more efficient than Cap to produce an alteration in the Trp residues of tyrosinase.

As a control experiment, tyrosinase aqueous solutions were exposed to UV-A radiation during different periods of time in the absence of pterin derivatives. No changes in the total fluorescence intensity were detected, even after more than 10 min of irradiation, thus excluding the possibility that spurious effects of direct light absorption by the protein could affect its fluorescence (Fig. 6).
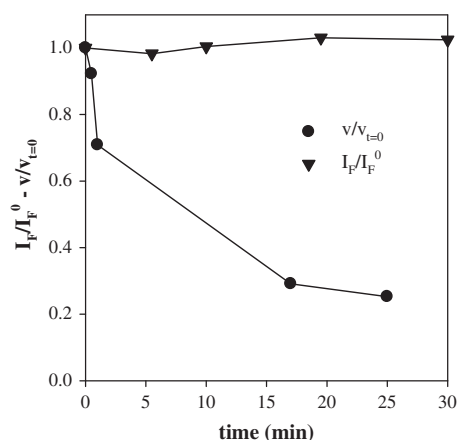

Fig. 7. Evolution of the relative total fluorescence intensity (I_F/I_F^0) and relative tyrosinase activity (v/v$_{t=0}$) of an aqueous solution of tyrosinase in the presence of H$_2$O$_2$ as a function of incubation time. [Tyrosinase]$_0$ = 33 μg/mL, [H$_2$O$_2$]$_0$ = 95.0 μM, 20 mM KH$_2$PO$_4$, pH = 6.0, T = 20 °C, λ_{exc} = 280 nm, optical path length 0.4 cm.
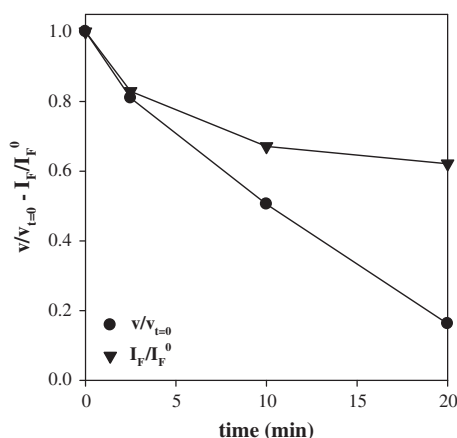

Fig. 8. Evolution of the relative total fluorescence intensity (I_F/I_F^0) (▼) and the relative tyrosinase activity (v/v$_{t=0}$) (●) of an aqueous solution of tyrosinase and Cap as a function of the irradiation time. [Tyrosinase]$_0$ = 50 μg/mL, [Cap]$_0$ = 60.0 μM, 1 mM KH$_2$PO$_4$, pH = 6.0, $q_{n,p}^{0,V,1}$.

To determine if the H$_2$O$_2$ is able to oxidize the Trp residues of tyrosinase, air equilibrated aqueous solutions of tyrosinase and H$_2$O$_2$ (pH 6.0) were incubated at 20 °C in the dark. After the incubation, the samples were analyzed by enzyme activity measurement and fluorescence spectroscopy. As shown in Fig. 7, Trp emission did not change in 30 min of incubation. For comparative purposes, the corresponding plot of activity as a function of time was included in Fig. 7. This result indicated that the alteration to Trp residues during the photosensitization process is only due to the reaction of excited state of pterin derivatives with the enzyme and, H$_2$O$_2$ does not contribute.

Taking into account the data shown so far, it is clear that the loss of enzyme activity is due to two different pathways: a photosensitized process and the reaction with H$_2$O$_2$. On the other hand, the Trp residues are affected only by the photosensitization. To compare rate of photoinactivation by photosensitization itself with the Trp alteration, additional experiments were carried out using Cap as a photosensitizer. This pterin derivative was chosen for this purpose because its photodegradation is negligible in the time window used in our experiments and, consequently, no H$_2$O$_2$ production is possible. Fig. 8 shows that the rate of photoinactivation is higher than the rate of Trp photoalteration, indicating that the active site of the enzyme is affected earlier than the amino acid residues.

4. Conclusions

The photosensitizing properties of folic acid (PteGlu) and its photoproducts, 6-formylpterin (Fop) and 6-carboxypterin (Cap), compounds that accumulate in the skin of patients suffering from vitiligo, were investigated using the tyrosinase as oxidizable target. When solutions of tyrosinase were exposed to UV-A irradiation in the presence of PteGlu, enzyme inactivation took place. However, PteGlu itself caused a negligible effect on the activity of the enzyme. In contrast, PteGlu photoproducts were shown to be efficient photosensitizers, being Fop the most efficient sensitizer of the series. The tyrosinase inactivation took place through two different pathways: (i) a photosensitization process, in which excited states of pterin derivatives reacted with the enzyme, and (ii) the oxidation of the enzyme by the H_2O_2 produced during the photooxidation of PteGlu and its photoproducts. Upon irradiation Trp residues were altered, but only through pathway i) and at a rate lower than that corresponding to the photoinactivation.

Taking into account that PteGlu is involved in the melanin biosynthesis and that its photodegradation may occur in skin regions were the protection against UV radiation fails, the study of inactivation of tyrosinase, the key enzyme in melanogenesis, photoinduced by PteGlu photoproducts is relevant from a biomedical point of view and can have important ramifications.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jphotobiol.2015.06.002.

References

[1] W. Pfleiderer, in: J.E. Ayling, M.G. Nair, C.M. Baugh (Eds.), Chemistry and Biology of Pteridines and Folates, Plenum Press, New York, 1993, pp. 1–16.

[2] T.J. Kappock, J.P. Caradonna, Pterin-dependent amino acid hydroxylases, Chem. Rev. 96 (1996) 2659–2756.

[3] V. Herbert, A.R. Larrabee, J.M. Buchanan, Studies on the identification of a folate compound of human serum, J. Clin. Invest. 41 (1962) 1134–1138.

[4] K.R. Thien, J.A. Blair, R.J. Leeming, W.T. Cooke, V. Melikian, Serum folates in man, J. Clin. Pathol. 30 (1977) 438–448.

[5] M.D. Lucock, M. Priestnall, I. Daskalakis, C.J. Schorah, J. Wild, M.I. Levene, Nonenzymatic degradation and salvage of dietary folate: physicochemical factors likely to influence bioavailability, Biomed. Mol. Med. 55 (1995) 43–53.

[6] M.L. Dántola, T.M. Schuler, M.P. Denofrio, M. Vignoni, A.L. Capparelli, C. Lorente, A.H. Thomas, Reaction between 7,8-dihydropterins and hydrogen peroxide under physiological conditions, Tetrahedron 64 (2008) 8692–8699.

[7] M.L. Dántola, A.H. Thomas, A.M. Braun, E. Oliveros, C. Lorente, Singlet oxygen ($O_2(^1\Delta_g)$) quenching by dihydropterins, J. Phys. Chem. A 111 (2007) 4280–4288.

[8] M. Lucock, Folic acid: nutritional biochemistry, molecular biology, and role in disease processes, Mol. Genet. Metab. 71 (2000) 121–138.

[9] L.B. Bailey, J.F. Gregory, Folate metabolism and requirements, J. Nutr. 129 (1999) 779–782.

[10] C. Bower, F.J. Stanley, Dietary folate as a risk factor for neural-tube defects: evidence from a case-control study in Western Australia, Med. J. Aust. 150 (1989) 613–619.

[11] L.E. Mitchell, Epidemiology of neural tube defects, Am. J. Med. Genet. C Semin Med. Genet. 135 (2005) 88–94.

[12] W. Shi, C.J. Meininger, T.E. Haynes, K. Hatakeyama, G. Wu, Regulation of tetrahydrobiopterin synthesis and bioavailability in endothelial cells, Cell Biochem. Biophys. 41 (2004) 415–434.

[13] K.U. Schallreuter, S. Kothari, B. Chavan, J.D. Spencer, Regulation of melanogenesis–controversies and new concepts, Exp. Dermatol. 17 (2008) 395–404.

[14] K.U. Schallreuter, Advances in melanocyte basic science research, Dermatol. Clin. 25 (2007) 283–291.

[15] A.A. Buglak, T.A. Telegina, T.A. Lyudnikova, Y.L. Vechtomova, M.S. Kritsky, Photooxidation of tetrahydrobiopterin under UV irradiation: possible pathways and mechanisms, Photochem. Photobiol. 90 (5) (2014) 1017–1026.

[16] M. Vignoni, F.M. Cabrerizo, C. Lorente, A.H. Thomas, New results on the photochemistry of biopterin and neopterin, Photochem. Photobiol. 85 (2009) 365–373.

[17] T.A. Lyudnikova, O. Dashina, T.A. Telegina, M.S. Kritsky, Investigation of the photochemical properties of biopterin and its reduced forms, Appl. Biochem. Microbiol. 45 (2009) 104–109.

[18] V.J. Hearing, K. Tsukamoto, Enzymatic control of pigmentation in mammals, FASEB J. 5 (1991) 2902–2909.

[19] A. Sancar, Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors, Chem. Rev. 103 (2003) 2203–2238.

[20] R.F. Branda, J. Eaton, Skin color and nutrient photolysis: an evolutionary hypothesis, Science 201 (1978) 625–626.

[21] A. Juzeniene, R. Setlow, A. Porojnicu, A.H. Steindal, J. Moan, Development of different human skin colors: a review highlighting photobiological and photobiophysical aspects, J. Photochem. Photobiol. B 96 (2009) 93–100.

[22] N.G. Jablonski, G. Chaplin, Human skin pigmentation as an adaptation to UV radiation, Proc. Natl. Acad. Sci. USA 107 (Suppl. 2) (2010) 8962–8968.

[23] D.C. Borradale, M.G. Kimlin, Folate degradation due to ultraviolet radiation: possible implications for human health and nutrition, Nutr. Rev. 70 (2012) 414–422.

[24] J. Moan, K.P. Nielsen, A. Juzeniene, Immediate pigment darkening: its evolutionary roles may include protection against folate photosensitization, FASEB J. 26 (2012) 971–975.

[25] M.J. Akhtar, M.A. Khan, I. Ahmad, Photodegradation of folic acid in aqueous solution, J. Pharm. Biomed. Anal. 25 (1999) 269–275.

[26] M.J. Akhtar, M.A. Khan, I. Ahmad, Identification of photoproducts of folic acid and its degradation pathways in aqueous solution, J. Pharm. Biomed. Anal. 31 (2003) 579–588.

[27] A.H. Thomas, G. Suárez, F.M. Cabrerizo, R. Martino, A.L. Capparelli, Study of the photolysis of folic acid and 6-formylpterin in acid aqueous solutions, J. Photochem. Photobiol. A 135 (2000) 147–154.

[28] A.H. Thomas, G. Suárez, F.M. Cabrerizo, F.S. García Einschlag, R. Martino, C. Baiocchi, E. Pramauro, A.L. Capparelli, Photochemical behavior of folic acid in alkaline aqueous solutions and evolution of its photoproducts, Helv. Chim. Acta 85 (2002) 2300–2315.

[29] M.K. Off, A.E. Steindal, A.C. Porojnicu, A. Juzeniene, A. Vorobey, A. Johnsson, J. Moan, Ultraviolet photodegradation of folic acid, J. Photochem. Photobiol. B 80 (2005) 47–55.

[30] M.L. Dántola, M.P. Denofrio, B.N. Zurbano, C.S. Gimenez, P.R. Ogilby, C. Lorente, A.H. Thomas, Mechanism of photooxidation of folic acid sensitized by unconjugated pterins, Photochem. Photobiol. Sci. 9 (12) (2010) 1604–1612.

[31] H. Rokos, W.D. Beazley, K.U. Schallreuter, Oxidative stress in vitiligo: Photooxidation of pterins produces H_2O_2 and pterin-6-carboxylic acid, Biochem. Biophys. Res. Commun. 292 (2002) 805–811.

[32] A.H. Thomas, M.P. Serrano, V. Rahal, P. Vicendo, C. Claparols, E. Oliveros, C. Lorente, Tryptophan oxidation photosensitizided by pterin, Free Radic. Biol. Med. 63 (2013) 467–475.

[33] C. Castaño, M.L. Dántola, E. Oliveros, A.H. Thomas, C. Lorente, Oxidation of tyrosine photoinduced by pterin in aqueous solution, Photochem. Photobiol. 89 (2013) 1448–1455.

[34] C. Castaño, C. Lorente, N. Martins-Froment, E. Oliveros, A.H. Thomas, Degradation of α-melanocyte-stimulating hormone photosensitized by pterin, Org. Biomol. Chem. 12 (2014) 3877–3886.

[35] A.H. Thomas, C. Lorente, K. Roitman, M.M. Morales, M.L. Dántola, Photosensitization of bovine serum albumin by pterin: a mechanistic study, J. Photochem. Photobiol. B 120 (2013) 52–58.

[36] A.H. Thomas, B.N. Zurbano, C. Lorente, J. Santos, E.A. Roman, M.L. Dántola, Chemical changes in bovine serum albumin photoinduced by pterin, J. Photochem. Photobiol. B 141 (2014) 262–268.

[37] M.L. Dántola, A.D. Gojanovich, A.H. Thomas, Inactivation of tyrosinase photoinduced by pterin, Biochem. Biophys. Res. Commun. 424 (2012) 568–572.

[38] K. Hirakawa, H. Suzuki, S. Oikawa, S. Kawanishi, Sequence specific DNA damage induced by ultraviolet A-irradiated folic acid *via* its photolysis product, Arch. Biochem. Biophys. 410 (2003) 261–268.

[39] P. Vorobey, A.E. Steindal, M.K. Off, A. Vorobey, J. Moan, Influence of human serum albumin on photodegradation of folic acid in solution, Photochem. Photobiol. 82 (2006) 817–822.

[40] L. Liang, J. Zhang, P. Zhou, M. Subirade, Protective effect of ligand-binding proteins against folic acid loss due to photodecomposition, Food Chem. 141 (2) (2013) 754–761.

[41] G.J. Locksmith, P. Duff, Preventing neural tube defects: the importance of periconceptional folic acid supplements, Obstet. Gynecol. 91 (1998) 1027–1034.

[42] S.E. Braslavsky, Glossary of terms used in photochemistry, 3rd edition (IUPAC Recommendations 2006), Pure Appl. Chem. 79 (2007) 293–465.

[43] H.J. Kuhn, S.E. Braslavsky, R. Schmidt, Chemical actinometry (IUPAC technical report), Pure Appl. Chem. 76 (2004) 2105–2146.

[44] S.H. Pomerantz, J.P.C. Li, in: H. Tabor, C. W Tabor (Eds.), Tyrosinase in Method in Enzymology, vol. VII A, Academic Press, New York and London, 1970, pp. 620–626.

[45] F. García-Molina, J.L. Muñoz, R. Varón, J.N. Rodríguez-López, F. García-Cánovas, J. Tudela, A review on spectrophotometric methods for measuring the monophenolase and diphenolase activities of tyrosinase, J. Agric. Food Chem. 55 (2007) 9739–9749.

[46] M.P. Serrano, M. Vignoni, M.L. Dántola, E. Oliveros, C. Lorente, A.H. Thomas, Emission properties of dihydropterins in aqueous solutions, Phys. Chem. Chem. Phys. 13 (2011) 7419–7425.

[47] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, third ed., Springer, New York, 2006.

[48] G. Petroselli, J.M. Bartsch, A.H. Thomas, Photoinduced generation of H_2O_2 and O_2^- by 6-formylpterin in aqueous solutions, Pteridines 17 (2006) 82–89.

[49] K.U. Schallreuter, J. Moore, J.M. Wood, W.D. Beazley, D.C. Gaze, D.J. Tobin, H.S. Marshall, A. Panske, E. Panzig, N.A. Hibberts, In vivo and in vitro evidence for hydrogen peroxide (H_2O_2) accumulation in the epidermis of patients with vitiligo and its successful removal by UV B-activated pseudocatalase, J. Invest. Dermatol. Symp. Proc. 4 (1999) 91–96.

[50] K.U. Schallreuter, J. Moore, J.M. Wood, W.D. Beazley, E.M.J. Peters, L.K. Marles, S.C. Behrens-Williams, R. Dummer, N. Blau, B. Thöny, Epidermal H_2O_2 accumulation alters tetrahydrobiopterin ($6BH_4$) recycling in vitiligo: identification of a general mechanism in regulation of all $6BH_4$-dependent processes?, J Invest. Dermatol. 116 (2001) 167–174.

[51] J.M. Wood, B. Chavan, I. Hafeez, K.U. Schallreuter, Regulation of tyrosinase by tetrahydropteridines and H_2O_2, Biochem. Biophys. Res. Commun. 325 (2004) 1412–1417.

[52] D.I. Pattison, A.S. Rahmantoa, M.J. Davies, Photo-oxidation of proteins, Photochem. Photobiol. Sci. 11 (2012) 38–53.

[53] A. Powell, N. Siu, J.K. Inlow, W.H. Flurkey, Immobilized metal affinity chromatography (IMAC) of mushroom tyrosinase, J. Chem. 1 (2007) 1.

[54] C. Lorente, A.H. Thomas, Photophysics and photochemistry of pterins in aqueous solution, Acc. Chem. Res. 39 (2006) 395–402.