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Bleaching of chlorophylls by UV irradiation *in vitro*: the effects on chlorophyll organization in acetone and *n*-hexane

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Abstract: The stability of chlorophylls toward UV irradiation was studied by Vis spectrophotometry in extracts containing mixtures of photosynthetic pigments in acetone and *n*-hexane. The chlorophylls underwent destruction (bleaching) obeying first-order kinetics. The bleaching was governed by three major factors: the energy input of the UV photons, the concentration of the chlorophylls and the polarity of the solvent, implying different molecular organizations of the chlorophylls in the two solvents.

Keywords: chlorophyll; UV-irradiation; kinetics; acetone; hexane.

INTRODUCTION

Beyond being the ultimate driving force of photosynthesis and its important regulatory factor, solar light is also a major source of stress to photosynthetic organisms. Depletion of stratospheric ozone has led to an increase of biologically damaging UV light at ambient levels (mainly UV-B light, 280–320 nm). Though photosynthetic pigments, such as chlorophylls and carotenoids, primarily absorb in the Vis region, their composure is significantly altered when exposed to UV light *in vivo* and *in vitro*.¹ This may cause an impairment of their photosynthetic function.² Plants generally respond to UV-B irradiation through very different mechanisms, including the synthesis of protective pigments and degradation of chlorophyll.³

Chlorophyll (Chl) is a major photosynthesis pigment. Its major function in photosynthesis is related to light collection and light conversion processes,⁴ since chlorophylls perform a light-harvesting function in the antennas of the photosynthetic apparatus or act as exciton traps and electron-carriers in reaction centers (RC).⁵ Chlorophyll is a chlorin, porphyrin derivative, a cyclic tetrapyrrole with an isocyclic cyclopentanone ring fused at the edge of the right-bottom pyrrole ring; the central Mg-atom plays a coordinating role⁶ (Fig. 1). Significant progress has been made in the understanding of the *in vitro* properties of Chl and

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this has contributed to a better understanding of the role of Chl on the molecular level in photosynthesis.⁷





Chlorophylls are not efficient UV-absorbers but are still able to absorb UV radiation, especially around 350 nm. The irradiation of chlorophyll solutions with UV and visible light results in the irreversible breakdown of chlorophyll, accompanied by the appearance of a number of intermediate and final products.⁸ The chemical structure of these products is largely unknown because of the diversity of the pathways involved and the lability of the primary photoproducts.⁹ Photobleaching of chlorophyll in solution may proceed without pheophytinization and could involve the opening of the porphyrin ring,¹⁰ although small amounts of allomers have also been detected.¹¹

The stability of chlorophylls in extracts of photosynthetic pigments (extracted chlorophylls), extracted from spinach leaves, against UV irradiation of three different ranges (UV-A, UV-B and UV-C) and for three different concentrations of Chl (c(Chla + Chlb) $\approx 10^{-6}$, 10^{-5} and 10^{-4} mol dm⁻³) was studied in this work. The irradiation was performed in acetone and *n*-hexane solutions for different irradiation periods, providing for the possibilities of kinetics analysis.

EXPERIMENTAL

All experiments were performed under dim light as much as possible and inside vessels and equipment covered with aluminum foil or black cloth to prevent possible chlorophyll photo-oxidation with visible light.

Extraction of plant pigments

The plant pigments were extracted from spinach leaves (*Spinacia oleracea* L.) using a previously reported method.^{12,13} The extraction and re-extraction mixtures were methanol and 40–75 °C petroleum ether in a 2:1 ratio, and 40–75 °C petroleum ether and diethyl ether in a 1:1 ratio, respectively. The methanol removes water from the plant material and the petroleum ether picks up the pigments before they undergo secondary reactions. The diethyl ether increases pigments solubility in the organic phase. The final extract was a mixture of pigments containing large amounts of various Chl-forms (with a predominant contribution of chlorophyll *a* – Chl*a*), as well as accessory pigments, carotenoids (carotenes and xanthophylls). For Chl*a* identification, HPLC chromatography was used with a Chl*a* standard (C₅₅H₇₂MgN₄O₅, $M_r = 893.5$ g mol⁻¹, Sigma-Aldrich) in acetone. The pigment extracts were evaporated and diluted in acetone or *n*-hexane and then subjected to HPLC, which showed a large content of Chl*a* in each of them with a significant contribution of Chl*b*.

The Chl*a* + Chl*b* content in the extracts was calculated as reported^{14,15} in acetone and adjusted to give three ranges of concentration: $c(Chla + Chlb) \approx 1.2 \times 10^{-6}$, 1.2×10^{-5} and 1.4×10^{-4} mol dm⁻³ ($c(Chla) \approx 8.5 \times 10^{-7}$, 8.5×10^{-6} and 1.0×10^{-4} mol dm⁻³ and $c(Chlb) \approx 3.4 \times 10^{-7}$, 3.4×10^{-6} and 3.5×10^{-5} mol dm⁻³, respectively). The content of carotenoids (xanthophylls + carotenes – (x + c)) in the extracts was calculated as reported¹⁴ and the c(Chla + Chlb)/c(x + c) ratio was found to be about 3:1. The molar extinction coefficient (ε) for Chl*a* in 100 % acetone at 661.6 nm is 8.260×10^4 dm³ mol⁻¹ cm⁻¹ and for Chl*b* at 644.8 nm is 4.686×10^4 dm³ mol⁻¹ cm⁻¹.¹⁶ The molar extinction coefficient (ε) for Chl*a* in s 9.01×10^4 dm³ mol⁻¹ cm⁻¹.¹⁷ Chlorophyll bleaching values (%) were calculated using Eq. (1), in which the chlorophyll concentration after one of the UV-irradiation treatments (c_1) represents X% of chlorophyll and (100 - X) % represents the percentage of UV-induced chlorophyll bleaching.¹⁸

Bleaching of chlorophyll (%) =
$$100(c_0 - c_1)/c_0$$
 (1)

Chla standard in acetone

To compare the kinetics of the Chl*a* bleaching processes in the pigment extract and in pure solution (in the same concentration range), a Chl*a* standard dissolved in acetone and irradiated at all three UV-irradiation ranges was employed. The concentration of the standard was $\approx 8.26 \times 10^{-6}$ mol dm⁻³.

UV treatment

Continuous irradiation of the chlorophylls in acetone and in *n*-hexane was performed in a cylindrical photochemical reactor "Rayonnet", with 8 symmetrically placed lamps having emission maxima at: 350 nm (UV-A), 300 nm (UV-B) and 254 nm (UV-C). The samples were irradiated in quartz cells $(1\times1\times4.5 \text{ cm}^3)$ placed on the rotating circular holder. The total measured energy flux (hitting the samples) was about 10.3 W m⁻² for 350 nm, 12.0 W m⁻² for 300 nm and 14.3 W m⁻² for 254 nm radiation.

Vis spectroscopy

The spectrophotometric measurements were made on a Varian Cary-100 spectrophotometer equipped with 1.0 cm quartz cells. All spectra before and after irradiation were recorded from 300 to 800 nm with 1.0 bandwidth.

HPLC analysis

HPLC analysis of the extracted chlorophylls and Chl*a* standard solutions was performed under isocratic conditions; apparatus: Agilent 1100 Series, Waldborn, Germany; column: Zorbax Eclipse XDB-C18; mobile phase: acetonitrile/methanol/ethyl acetate, 60:20:20; flow rate: 0.5 cm³ min⁻¹ at 25 °C. The monitoring wavelength was 430 nm.

RESULTS AND DISCUSSION

Chlorophylls have two major absorption regions in the visible range, due to their extended π -delocalization at the edge of cyclic tetrapyrrole (porphyrin) skeleton: a "red" (Q) band and a "blue" (Soret or B) band.¹⁹ The Q-band absorption maxima (A_{max}) for Chla and Chlb in acetone are located at 662.1 nm and 645.5 nm, respectively.²⁰ The UV-induced changes of the chlorophylls were detected in the mixtures of pigments using the Q-bands as sensitive indicators, since they are exhibited by only the chlorophylls and not by carotenoids.²¹

The absorption spectra of the Chla-standard sample measured after its bleaching in acetone by UV-C radiation are shown in Fig. 2A. The irradiation induces a gradual decrease in the intensity of the Q-band, i.e., a hypochromic effect was clearly observed for irradiated Chla relative to irradiation time, $t_{\rm irr.}$ Apparently, bleaching of Chla resulted in a progressive flattening of the absorption spectra in the "red" region and a slower absorption decrease in the "blue" region. An increase in the absorbance was observed between 450 and 560 nm for all three UV ranges. The absorbance ratio A_{430}/A_{411} (where A_{430} and A_{411} correspond to the absorbance intensity measured at 430 and 411 nm, respectively) decreased during UV-C irradiation and was proportional to the extent of bleaching of the chlorophylls - Fig. 2B (plot 1). The ratio behavior indirectly suggests the formation of UV-induced bleaching products which absorb above 410 nm.¹⁸ Also, the ratios of the O-band maximum at 661 nm to the intersection points of the absorption maxima at 450 and 560 nm $(A_0/A_1, i.e., A_{661}/A_{450})$ and A_{661}/A_{560} , respectively) markedly decreased during irradiation, and is linearly proportional to the extent of chlorophylls bleaching (Fig. 2B, plots 2 and 3, respectively, with an average correlation coefficient, $R \approx 0.99$). A similar behavior was observed for UV-B and UV-A induced bleaching of the Chla standard in acetone (not shown).

The absorption spectra of the chlorophylls from the extracted chlorophylls in acetone and *n*-hexane showed very similar behavior during the corresponding regime of irradiation with UV-A, UV-B and UV-C light, for all three concentration ranges. The absorption spectra of the pigments extract during its bleaching in *n*-hexane by UV-B light, with a calculated concentration of chlorophylls of 1.2×10^{-5} mol dm⁻³, are shown in Fig. 3A.

In this case, the absorption spectra have intersection points at 490 and 570 nm. The first intersection point is "red shifted" compared to the Chla standard solution (Fig. 2). The probable reason for this is the absorption of accessory pigments in the spectral region about 450 nm.²¹ The absorbance ratios A_{665}/A_{488} and A_{665}/A_{570} , as well as A_{430}/A_{410} plotted *versus* the bleaching of the chlorophylls are shown in Fig. 3B (plots 2 and 3 and plot 1, respectively, the average correlation coefficient is $R \approx 0.99$). A clear linear decrease was found. These results indicate the possible formation of UV-induced bleaching products of the chlorophylls in spectral regions above 410 and 480–570 nm.¹⁸ Moreover, Merzlyak

found that irradiation of solutions containing purified carotenoids resulted in a uniform bleaching of these pigments without any appreciable formation of products absorbing between 350 and 500 nm.¹⁸ Similar Vis spectral behavior (to that shown in Figs. 2 and 3) was observed in flash photolysis experiments of chlorophylls. The bleaching of chlorophylls was claimed to be related to the absorption by the triplet state of Chl (³Chl) in organic solvents.²²



Fig. 2. (A) Changes in the Chl*a* standard absorption spectra following its exposure to UV-C radiation in acetone. The exposure time periods were: (0) 0, (1) 2, (2) 3, (3) 4, (4) 5 and (5) 7 min. (B) Dependence of the absorbance ratios of the red (Q) peak to the pseudo--isosbestic points, A_Q/A_i (plots 2 and 3 refer to the left ordinate) and of the ratio of the Soret bands, A_{430}/A_{410} (plot 1 refers to the right ordinate) on the extent of chlorophyll bleaching. (1) Soret band maximums are at 430 and 410 nm; (2) $\lambda_{Qmax} = 661 \text{ nm}$, $\lambda_{imax} = 450 \text{ nm}$; (3) $\lambda_{Qmax} = 661 \text{ nm}$, $\lambda_{imax} = 560 \text{ nm}$. The % chlorophyll bleaching was calculated according to Eq. (1). The absorption spectra showed very similar responses during irradiation with UV-A and UV-B light.

In chlorophyll, the central magnesium coordinates four nitrogen atoms of the pyrrole rings, providing for one or both Mg axial positions to be occupied by a molecule possessing a lone electron pair capable of acting as an electron donor. When Chl is dissolved in a nucleophilic polar solvent, such as acetone, the solvent acts as an electron donor to Mg and the chlorophyll then appears as "monomer", with five- and/or six-coordinated Mg.²³ On the other hand, Chls may also act as electron donors, due to presence of a few keto groups, mostly belonging to the peripheric ester groups (C=O group in position C–13¹ in the Chl*a* molecule, an additional C=O group in the Chl*b* molecule in position C–7 and two ester groups in positions C–13³ and C–17³, Fig. 1).

Hence, one chlorophyll may act as an electron donor (*via* its ring E, keto group, $C-13^1$ position, Fig. 1) and the other Chl molecule can act as an electron

acceptor *via* its central Mg atom.²⁴ Thus, in nonpolar solvents, such as *n*-hexane, chlorophyll predominantly appears in aggregated forms ("dimers" and "oligomers") at higher concentrations (over 10^{-5} mol dm⁻³)^{23,25} and as a "monomer" at low concentrations. The Q-band A_{max} of "monomeric" Chl in *n*-hexane lies at ≈ 660 nm at $c(\text{Chl}a + \text{Chl}b) = 1.2 \times 10^{-6}$ mol dm⁻³. In the concentration range about 10^{-5} to 10^{-4} mol dm⁻³, the Q-band A_{max} is "red" shifted due to Chl–Chl interaction in the "dimeric" chlorophyll form (≈ 665 nm).²³



Fig. 3. UV-B induced bleaching of the extracted chlorophylls (Chla + Chlb) in *n*-hexane. (A) Changes in the pigments absorption spectra following their exposure to UV-B radiation. The exposure time periods were: (0) 0, (1) 5, (2) 7, (3) 10, (4) 15, (5) 20 and (6) 30 min. (B) Dependence of the absorbance ratios of the red (Q) peak to the pseudo-isosbestic points A_Q/A_i (plots 2 and 3 refer to the left ordinate) and of the maximum of the Soret bands, A_{430}/A_{410} (plot 1 refers to the right ordinate) on the extent of chlorophyll bleaching. (1) Soret band maximums are 430 and 410 nm; (2) $\lambda_{Qmax} = 665 \text{ nm}, \lambda_{imax} = 488 \text{ nm};$ (3) $\lambda_{Qmax} = 665 \text{ nm}, \lambda_{imax} = 570 \text{ nm}.$ The % chlorophyll bleaching was calculated according to Eq. (1). The absorption spectra showed very similar responses during irradiation with UV-A and UV-C light.

Bearing this in mind, chlorophylls *in vitro* should show a different behavior toward UV-irradiation in solvents with different polarities and in different concentration ranges.

Ultraviolet radiation can induce the generation of free radicals in organic compounds, especially those containing C=C bonds. In chlorophylls these bonds appear in great numbers (see Fig. 1).²⁶ Once created, free radicals can initiate chain reactions, which, in some cases, may be linked particularly to the presence of C=C and C=O bonds.²⁷ Absorption of electromagnetic radiation resulting in the generation of free radicals may occur through two competing mechanisms:

(*i*) Excitation by energy absorption and subsequent decomposition into radicals;

(ii) photosensitized energy transfer.²⁸

To assess the possibility of direct UV absorption by photosynthetic pigments, Johnson and Day measured the absorbance of extracted chlorophylls and noticed that Chl*a* had absorbance peaks at 340 and 389 nm, which were 49 and 72 % of the Soret peak absorbance, and Chl*b* had absorbance peaks at 315 and 346 nm, which were both 35 % of the Soret peak value.²⁹ The excited chlorophylls can be oxidized photochemically to their π -cation radicals (*i*).^{30,31} On the other hand, both the proposed mechanisms (*i*) and (*ii*) may lead to Chl bleaching in more complicated ways, which include the relatively stable excited triplet state (³Chl, lifetime of 10⁻⁵–10⁻³ s) and the presence of reactive oxygen species (ROS), by three most probable pathways:³²

(1) formation of an active complex between ³Chl and the ground state of oxygen (${}^{3}O_{2}$), which can then oxidize HO⁻ to the extremely powerful oxidizing agent HO[•];

(2) energy transfer from ³Chl to ³O₂, leading to very reactive singlet oxygen $(^{1}O_{2})$;

(3) electron transfer from ³Chl to ³O₂ resulting in formation of superoxide radical anion (O_2^-) which can then generate HOO[•], H₂O₂, HO[•] and ¹O₂. This type of reactions deals with a mechanism in which the initial attack of oxygen is directed toward position C-5 (Fig. 1).³³ These mechanisms may be called Chl self-destruction mechanisms due to their pronounced photosensitizing capability.

Exposure of Chls to UV-A and UV-B radiation *in vivo* is believed to enhance the amount of ROS.^{34–36} Singlet oxygen also attacks the double bonds of the phytyl chain leading to the formation of numerous isoprenoid photoproducts.³⁷

A report about ROS production *in vitro* under photosynthetically active radiation (PAR) in hexane solutions of chlorophylls³⁸ is relevant for this work, because chlorophyll absorbs UV-A and UV-B²⁹ and oxygen was certainly present, due to oxygen diffusion in acetone and hexane.^{22,39} In addition, acetone and *n*-hexane have similar viscosities (the viscosity of acetone is $\eta = 0.303 \times 10^{-3}$ Pa s and of *n*-hexane 0.29×10^{-3} Pa s),^{22,40} which should imply that the concentrations of oxygen in these solvents is similar. The authors³⁸ concluded that in disorganized organic solutions (such as those studied in the present work), UV-induced bleaching of chlorophyll seems to be related to the photochemical generation of ¹O₂ by mechanism (*ii*); no role of O₂⁻ or H₂O₂ was proposed.

The kinetics of the bleaching of chlorophylls seems to obey a first-order law, as already reported.⁴¹⁻⁴³ The corresponding kinetic logarithmic plots as a result of increasing time of irradiation for the extracted chlorophylls in *n*-hexane, for all three UV-ranges, are shown in Fig. 4. The corresponding kinetic ln plots for all three concentrations of chlorophylls in both solvents are of very similar shape to the presented ones (not shown).



Fig. 4. The kinetic plots of the bleaching of the extracted chlorophylls (Chla + + Chlb) in *n*-hexane with increasing irradiation time of UV-A, UV-B and UV-C radiation. The absorbance of Chla was followed at A_{max} of the Q-band (≈ 665 nm). The chlorophyll concentration was 1.2×10^{-5} mol dm⁻³. The corresponding slopes (in min⁻¹) are displayed for all three UV irradiation ranges (UV-A, UV-B and UV-C).

The bleaching rate constants, k, for the Chla standard and the extracted chlorophylls, for all three UV-ranges and the same Chla concentration, calculated from the slopes given in Fig. 4,

$$y = -kx + n \tag{2}$$

where y is log of the extract absorbance in acetone or *n*-hexane at 662 nm and 660—665 nm, respectively, x is the UV-irradiation time and k is the rate constant for the bleaching of chlorophyll, are shown in Table I.

TABLE I. Kinetics of chlorophylls bleaching in acetone (Chla-standard and the pigments extract) with increasing UV irradiation time for the three different UV-ranges: UV-A, UV-B and UV-C. Chlorophylls absorbencies were followed at A_{max} of the Q-band (661 nm for the Chla standard and 662 nm for the Chls in the pigments extract)

$\lambda_{\rm UV}$ / nm	Chla-standard solution	Chls in pig	Chls in pigment extract	
	c_{Chla} and $c / 10^{-6}$ mol dm ⁻³			
	8.26	8.02 (~c _{Chla})	8.54 (~ <i>c</i> _{Chla})	
-	k / \min^{-1}			
350 (UV-A)	0.10616	0.12797	0.08587	
300 (UV-B)	0.32170	0.29548	0.23985	
254 (UV-C)	0.26800	0.26788	0.22602	

The bleaching rate constants of the chlorophylls in the extract were very similar to those of the standard for the same concentration range (Table I, 0.26800, 0.26788 and 0.22602 min⁻¹ for UV-C irradiated Chl*a*-standard and the extracted chlorophylls of the concentration of 8.02×10^{-6} and 8.54×10^{-6} mol dm⁻³, respectively). This proves that bleaching rate of Chls in solution does not depend significantly on the presence of the accessory pigments in the environment. The

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very similar k values for the Chla standard and the extracted Chls also indicates that Chlb is possibly also very sensitive to UV irradiation; consequently, Chla is the major contributor to the bleaching of the extracted Chls.⁴¹

The calculated bleaching rate constants for the extracted chlorophylls, for all three UV-ranges in the two solvents and for all three concentrations $(1.2 \times 10^{-6}, 1.2 \times 10^{-5} \text{ and } 1.4 \times 10^{-4} \text{ mol dm}^{-3})$ are shown in Table II.

TABLE II. Bleaching kinetics of the extracted chlorophylls in acetone and *n*-hexane with increasing UV-irradiation time for the three different UV-ranges: UV-A, UV-B and UV-C at three different concentration ranges ($\approx 10^{-6}$, 10^{-5} and 10^{-4} mol dm⁻³). Chlorophylls absorbances were followed at A_{max} of the Q-band (662 nm in acetone, 660 nm in *n*-hexane at 1.2×10^{-6} mol dm⁻³ and at 665 nm in *n*-hexane in the 10^{-5} – 10^{-4} mol dm⁻³ concentration range).

$c(\text{Chl}a+\text{Chl}b) / 10^{-5} \text{ mol dm}^{-3}$		UV-A (350 nm)	UV-B (300 nm)	UV-C (254 nm)
			<i>k</i> / min ⁻¹	
0.12	In acetone	0.41480	0.70000	0.42526
	In <i>n</i> -hexane	0.03265	0.21101	1.43295
1.2	In acetone	0.08587	0.23985	0.22602
	In <i>n</i> -hexane	0.00625	0.03877	0.37076
14	In acetone	0.01246	0.03961	0.10130
	In <i>n</i> -hexane	0.00180	0.00364	0.01716

The presented data (Tables I and II) suggest that the bleaching rate of chlorophylls *in vitro* depends on the UV irradiation range (*i.e.*, the energy of the photons), the chlorophylls concentration and the solvent polarity.

n-Hexane is transparent in the UV range down to 201 nm and the UV radiations used in this work should have no influence on the solvent molecules in terms of their excitation, ionization and formation of *n*-hexyl cation-radicals, which could then participate in the bleaching of the extracted chlorophylls. The influence of energy on photobleaching rate constants in non-polar *n*-hexane progressively increased from UV-A to UV-C for all three studied concentrations (Table II). The ratios of bleaching rate constants obtained for the concentration when Chl is "monomeric" and the two concentrations when Chl is "aggregated" $(10^{-6}, 10^{-5} \text{ and } 10^{-4} \text{ mol } \text{dm}^{-3}$, respectively) are: for UV-A, $k_{10^{-6}}/k_{10^{-5}}$ $(k_{\text{mon}}/k_{\text{agg}}) = 5, k_{10}-5/k_{10}-4 (k_{\text{agg}}) = 3$; for UV-B, $k_{10}-6/k_{10}-5 (k_{\text{mon}}/k_{\text{agg}}) = 5$, $k_{10}-5/k_{10}-4$ (k_{agg}) = 11; for UV-C, $k_{10}-6/k_{10}-5$ (k_{mon}/k_{agg}) = 4, $k_{10}-5/k_{10}-4$ (k_{agg}) = 22. Increasing the chlorophyll concentration by about one order of magnitude distinctly decreases the ratios of the bleaching rates of the extracted chlorophylls (Table II). It would be expected that aggregated extracted chlorophylls should be much more stable against UV-A radiation (as was found for UV-B and UV-C induced bleaching). However, the data from Table II suggest that chlorophylls aggregation probable does not play a major stability role against UV-A bleaching of the extracted chlorophylls in *n*-hexane (decreases of the bleaching rates ratios of 5 and 3, respectively, Table II). On the other hand, aggregation of Chl mole-

cules plays a significant stability role against UV-B and, especially, UV-C irradiation (Table II). Chlorophylls aggregates are reportedly excellent quenchers, showing an extreme shortening of the lifetime of the chlorophyll triplet excited state.^{23,44} The possibility of the existence of a free radical "dimeric" form⁴⁵ (*i.e.*, *in vitro* UV-induced formation of "dimeric" chlorophyll cation radicals (Chl₂)⁺) was not considered because no available information was provided.

Acetone absorbs in the UV range below 350 nm (two absorption maximums at 280 and 190 nm, related to the forbidden $n-\pi^*$ transition and the highly intensive $\pi - \pi^*$ transition, respectively).⁴⁶ Considering the influence of the energy of the photon input on the stability of the extracted chlorophylls in acetone, it is evident that the bleaching rates decline by a factor of 1.7-3.2 when going from UV-A to UV-C radiation for all three studied concentrations: for $\approx 10^{-6}$ mol dm⁻³, $k_{\text{UV-B}}/k_{\text{UV-A}} = 1.7$; for $\approx 10^{-5}$ mol dm⁻³, $k_{\text{UV-B}}/k_{\text{UV-A}} = 2.8$; for $\approx 10^{-4}$ mol dm⁻³, $k_{\rm UV-B}/k_{\rm UV-A} = 3.2$. The only exception was noted in the case of UV-C radiation, for irradiated extracted chlorophylls in the concentrations ranges of $\approx 10^{-6}$ and 10^{-5} mol dm⁻³ (Table II). In the former case, the observed bleaching rate constant for UV-C irradiation was slightly higher than the one obtained for UV-A irradiation ($k_{UV-C}/k_{UV-A} = 1.02$), while in the latter case the observed bleaching rate constant for UV-C irradiation was slightly lower than the one for UV-B irradiation ($k_{\rm UV-C}/k_{\rm UV-B} = 0.94$). However, the $k_{\rm UV-C}/k_{\rm UV-B}$ ratios progressively increased with increasing concentration of chlorophylls (for 1.2×10^{-6} mol dm⁻³, $k_{\text{UV-C}}/k_{\text{UV-B}} = 0.61$, while for 1.4×10^{-4} mol dm⁻³, $k_{\text{UV-C}}/k_{\text{UV-B}} = 2.56$).

When acetone molecules interact with "monomeric" extracted chlorophylls via acetone carbonyl "bridges", the electron density of the chlorin ring is not arranged between the two chlorophyll molecules (as in the Chl "dimer" formed at higher Chl concentrations in *n*-hexane) but is distributed between one chlorophyll molecule and one or two acetone molecules.²³ Since acetone absorbs over the whole UV range (which overlaps with the UV-ranges used in this study),⁴⁶ this may lead to a decreased stability of the extracted chlorophylls against UV radiation, compared to the extracted chlorophylls in *n*-hexane. If one compares the photobleaching rate constants for the three chlorophyll concentration ranges obtained in the two solvents, then it is obvious that the photobleaching rate constants obtained with UV-A radiation in acetone were about 12, 13 and 7 times higher than the corresponding values obtained in *n*-hexane, while the corresponding relations obtained for UV-B radiation were about 3, 6 and 11 higher. In fact, going from UV-A to UV-C, and for increasing Chls concentrations ($10^{-6} \rightarrow$ $\rightarrow 10^{-5} \rightarrow 10^{-4}$ mol dm⁻³), the extracted "aggregated" chlorophylls appear more stable in *n*-hexane than in acetone, probably due to "dimer" formation. On the other hand, the "monomeric" extracted chlorophylls (1.2×10⁻⁶ mol dm⁻³) expressed lower stability against UV-C irradiation in *n*-hexane than in acetone (by a factor of 3).

CONCLUSIONS

1. The UV-induced bleaching of chlorophylls in the pigment extract fits the first-order kinetic model.

2. The bleaching rate of the extracted Chls *in vitro* did not significantly depend on the presence of accessory pigments (carotenoids) in the pigment extract.

3. UV-induced bleaching of the extracted chlorophylls is probably followed by the formation of chlorophyll bleaching products which absorb in spectral regions above 410 nm and 480–570 nm.

4. The bleaching rate of Chls *in vitro* depended on the energy input of the UV-photons, the concentration of chlorophylls and the molecular organization of the chlorophylls in n-hexane and acetone. The Chls aggregates in *n*-hexane protect best against UV-C and UV-B radiation and to a less extent against UV-A irradiation.

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ИЗВОД

ОБЕЗБОЈАВАЊЕ ХЛОРОФИЛА UV ЗРАЧЕЊЕМ *IN VITRO*: ЕФЕКТИ НА ОРГАНИЗАЦИЈУ ХЛОРОФИЛА У АЦЕТОНУ И ХЕКСАНУ

ЈЕЛЕНА ЗВЕЗДАНОВИЋ и ДЕЈАН МАРКОВИЋ

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Стабилност хлорофила према UV зрачењу проучавана је Vis спектрофотометријом у екстрактима који садрже смеше фотосинтетских пигмената у ацетону и хексану. Хлорофили подлежу деструкцији (обезбојавању) покоравајући се кинетици првог реда. На кинетику обезбојавања утичу три главна фактора: енергија UV фотона, концентрација хлорофила и поларност растварача, која подразумева различите молекуларне организације хлорофила у њима.

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