Quercetin oxidation by horseradish peroxidase: the effect of UV-B irradiation

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Abstract

Horseradish peroxidase (HRP), a highly investigated member of the peroxidase family, has been known – among many other biological activities – to catalyze the oxidation of flavonoids and phenolic substrates overall, quercetin among them. On the other hand, quercetin is very well known for its expressed antioxidant activities, which in the case of UV external radiation can be expressed partly in a preventive manner: quercetin is an excellent UV-absorber. Therefore, the aim of this investigation was to study quercetin oxidation by HRP in phosphate buffer, under conditions of UV-stress, i.e. continuous, prolonged UV-B irradiation. The results show that while UV-B irradiation had definitely affected the activity of HRP, and the overal rate of quercetin oxidation by HRP, it had probably very little effect on it for longer UV-B-irradiation periods (>30 min).

Keywords: quercetin, peroxidase, UV-B radiation, kinetics, oxidation.

Available online at the Journal website: http://www.ache.org.rs/HI/

SCIENTIFIC PAPER

UDC 577.972:577.152.1:54:66

Hem. Ind. **67** (3) 411–418 (2013)

doi: 10.2298/HEMIND120607093S

Peroxidases are enzymes that are widely distributed in eukaryotes and prokaryotes, and have a pivotal role in biology [1]. Most of peroxidases are hemoproteins, which contain ferriprotoporphyrin IX as the prosthetic group. The family of peroxidases includes plant ascorbate peroxidases, yeast cytochrome c peroxidase, the mammalian haloperoxidases (myeloperoxidase and lactoperoxidase), fungal peroxidases and other plant peroxidases [2,3].

Horseradish peroxidase (HRP, EC 1.11.1.7.) is a well-known and highly investigated member of the peroxidase family that catalyzes the oxidation of flavornoids and phenolic substrates to the free phenoxyl or semiquinone radicals [1–2,4–7]. Previous studies have shown that the oxidation process of phenolic compounds can be considered as a modified type of ping-pong kinetics [8,9]. HRP exhibits wide substrate specificity to donor hydrogens, and recently attention has been focused on polyphenols with complex structures [1,2,8], such as flavonoids.

Flavonoids are a large group of polyphenols which are widely distributed in plants, vegetables, fruit juices, and a variety of beverages (tea, coffee, wines, and fruit drinks) [10–15]. Many experimental studies have demonstrated that they possess numerous biological and pharmacological effects including antioxidant, antimutagenic, anticarcinogenic, antiulcer, probiotic, antimicrobial, antiallergic, antiviral and antiinflammatory properties [10–12,14,16]. Because of its ubiquitous nature, quercetin (3,3',4',5,7-pentahydroxyflavone) is the most studied of the dietary flavonoids and one of the most abundant plant-derived polyphenols widely consumed in a human diet [17–26]. Quercetin (Figure 1), containing the 3',4'-dihydroxy structure of the B ring (catechol B ring), is known to possess a high antioxidant ability expressed through scavenging of free radicals (a "chain-breaking role"). It has a strong antioxidant effect against lipid peroxidation in phospholipid bilayers and in human low-density lipoprotein [13,21,27–28]. However, when lipid peroxidation chain reaction is initiated by UV-radiation then another quercetin antioxidant ability takes place. Since it is a strong UV-absorber, it can react as a preventive antioxidant by absorbing incident UV-radiation, therefore reducing initiation step of the LP chain mechanism, *i.e*., formation of reactive oxygen species (ROS) [29]. The UV energy absorbed by quercetin may be dissipated as heat [30] or converted into decomposition products [31]. The UV-irradiation of flavonoids in solutions and in liposome systems *in vitro*, as well as *in vivo*, results in their irreversible breakdown, accompanied by the appearance of a number of decomposition products [32,33]. In a recently published paper, by using HPLC techniques to analyse UV-B (300 nm) and UV-C (254 nm) irradiated quercetin in methanol solution, we have detected at least four decomposition products, some of them belonging to quinones family, and established the formation kinetics [34]. Based on this, we have explored the relationship between quercetin stability to UV-irradiation and it has antioxidant ability (under the same UV-irradiation regime) by comparing it to the one of its flavonoid counterpart, rutin, with glycoside residue at the 3-position [35].

The 3-position (at the ring C) makes the crucial difference between quercetin and rutin [31]. Because

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Paper received: 7 June, 2012
Paper accepted: 22 August, 2012

quercetin has a free hydroxyl group at the 3-position, this compound can also act as an inhibitor of the tyrosinase [36,37]. Thus, this molecule is simultaneously a substrate and an inhibitor, depending on the way in which it binds to the copper atoms of the active site. Since UV-irradiation evidently affects quercetin structure, it would be interesting to see how it would affect quercetin-involving enzymatic oxidation reactions.

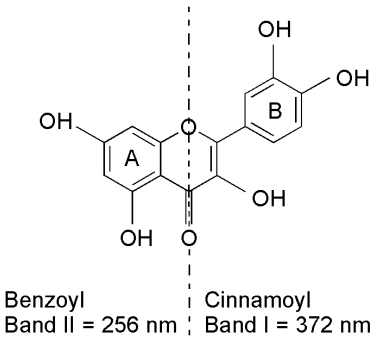

Figure 1. Quercetin structure with the indicated (benzoyl and cinnamoyl) parts contributing to spectral band I and II [20].

Previous studies have shown that the enzymatic oxidation of quercetin primarily produces quinones, which can react with various nucleophilic agents building adducts [36]. We have already investigated the oxidation of quercetin by HRP in the presence of L-cysteine and possible related mechanisms (unpublished). In the extension, the objective of this work is to study possible effects that continuous, prolonged UV-B-irradiation might have on oxidation of quercetin by HRP.

EXPERIMENTAL

Chemicals and preparation of sample solutions

Quercetin (Sigma-Aldrich) of the highest quality available (98%) was used without purification. A 10 mM stock solution of quercetin was prepared in methanol (HPLC grade, J.T.Baker) immediately before the experiments. For all experiments, freshly prepared solutions of quercetin were made by dilution of the appropriate amount of the stock solution with phosphate buffer at pH 7.4.

Horseradish peroxidase (298 U/mg; using pyrogallol) was purchased from Sigma-Aldrich (Germany). A 2 μM stock solution of HRP was prepared by dissolving the 0.34 mg of the solid HRP in 10 ml of cold 50 mM phosphate buffer, pH 6.0. The enzyme concentration was calculated using ε_{403} = 102.0 mM^{-1} cm^{-1}.

Spectrophotometric assays

Quercetin degradation

Kinetic assays were carried out by tracing the disappearance of quercetin (at 380 nm) in the reaction medium on Varian Cary-100 spectrophotometer equipped with UV-Winlab software in quartz cuvettes dimensions, 1 cm×1 cm×4.5 cm. The measured samples consisted of 1.6 mM quercetin only, quercertin with 0.1 mM H_2O_2, quercetin with 8 nM HRP, and finally the reaction mixture (quercetin, H_2O_2 and HRP), all in 100 mM phosphate buffer (pH 7.4), and all irradiated in 2.5 ml volumes with UV-B at increasing time periods (10–60 min).

Determination of reaction rate

To determine the overall reaction rate, aliquots from HRP solution in 50 mM phosphate buffer (pH 6.0) were synchronously irradiated with the above samples under the same irradiation conditions, and then subjected to a series of consecutive absorbance measurements at λ_{max} = 380 nm (corresponding to increasing irradiation periods) every 10 s, for a period of 120 s. All measurements were performed at temperature of 20 °C. The blank was represented by 100 mM phosphate buffer pH 7.4.

Enzymatic reaction was performed by mixing irradiated HRP solution with non-irradiated quercetin phosphate buffer (pH 7.4) solution, followed by subsequent addition of 50 μl 10 mM hydrogen peroxide solution; the reaction was carried out by vigorous vortexing.

Determination of the proportion of enzymatic reaction in the overall reaction was done by comparing the reaction rate in the presence and absence of the enzyme.

Activity measurements

Peroxidase activity was determined by Soysal and Söylemez [38], with slight modification. A 2.1 ml of 100 mM acetate buffer (pH 6) was measured followed by addition of 0.5 ml 8.8 mM H_2O_2, 0.2 ml of 0.125% solution of *o*-dianisidine in methanol; the mixture was vigorously vortexed, and finally 0.2 ml solution of the irradiated HRP was added. Change of absorbance was recorded as a function of time at 460 nm and activity of HRP was calculated by using following equation:

$$A[\mathrm{U/ml}] = (R/\varepsilon)\mathrm{tg}\alpha \quad (1)$$

where tgα is a slope, R – ratio of the total volume of the reaction mixture and the enzyme volume, ε – molar extinction coefficient (ε_{460} = 11.3 mM^{-1} cm^{-1}).

The reference blank contained all reagents except the hydrogen peroxide, which was replaced by aqua destillata.

One unit of peroxidase activity was defined as the amount of enzyme that transformed 1 μmol of *o*-dianisidine per min.

UV-irradiation

Continuous irradiation of the samples was performed in a cylindrical photochemical reactor “Rayonnet”,

with 7 symmetricaly placed lamps with emission maxima at 300 nm (UV-B). The samples were irradiated in quartz cuvettes (1 cm×1 cm×4.5 cm) placed on a rotating circular holder. The total measured energy flux is about 10.5 W/m^2 at 10 cm distance from the lamps.

RESULTS AND DISCUSSION

UV-B radiation effects on plants have been well documented. Reduced photosynthesis, decreased protein synthesis and production of ROS species are among them [39–44]. However, there are other types of abiotic and biotic stresses that can also produce ROS, such as singlet oxygen, hydrogen peroxide, and hydroxyl radicals, in plants [41–44]. Plants have developed several defense systems against ROS-induced harmful effects. Peroxidase, as a member of a group of antioxidant enzymes, plays an important role by converting hydrogen peroxide to water molecules. It is known that HRP usually occurs in multiple molecular forms (isoenzymes), but their function and regulation remain largely unknown. On the other hand, as a part of an entire answer against UV-radiation inducing effects, plants react preventively through a synthesis of UV-absorbing, protective pigments, such as flavonoids [45–47].

The absorption spectrum of quercetin features two major peaks, one belonging to the band I as a result of absorption of the catechol B-ring in UV-A range, between 360 and 370 nm (λ_{max} = 365 nm, ε = 28400 M^{-1} cm^{-1}), and the other one, belonging to band II as a result of benzoyl A-C system in UV-C range, around 260 nm (λ_{max} = 256 nm, ε = 28.300 M^{-1} cm^{-1}). When quercetin is irradiated with continuous prolonged UV-radiation, its stability is affected. In order to estimate it, quercetin stability toward UV-irradiation from the three belonging sub-ranges (UV-A, UV-B and UV-C) was compared to the one of its flavonoid counterpart, rutin, under the same irradiation conditions [35]. It was found that the stability of both flavonoids towards UV-irradiation is highly affected by the incident photons energy input (declining from UV-A *via* UV-B to UV-C), but inside the same UV-sub-range quercetin was found much more sensitive compared to rutin (*i.e.*, its degradation was faster), which was attributed to the presence of the very reactive 3-OH position in the C-ring of quercetin [31,35]. The same result has been confirmed in the subsequent paper by using HPLC to analyse irradiated quercetin and rutin solutions in methanol [34]; at least four different quercetin-originating UV-B&C induced products have been clearly recorded on the corresponding chromatograms (with A_{max} values in 295–300 nm range) and the anticipated structures have been proposed, at least two of them implying the opening of the C-ring [29,34].

A question emerges on the relevancy of these facts (UV-induced structural changes in quercetin structure) when quercetin is subjected to oxidation by HRP under condition of UV-sttress. To answer this, quercetin was irradiated with the increasing UV-B irradiation time periods in the same phosphate buffer mediums, in the absence and presence of HRP. The spectral changes are shown in Figure 2 (A,B – left), while the related quercetin degradation plots expressing first order type of behavior (including some points scattering) is shown in Figure 2 (A,B – right). The changes in the quercetin spectra, expressed through decrease of A_{max} values of both major bands (A_{380nm} and A_{267nm}, both in the absence, as well as in the presence of HRP) clearly prove that quercetin undergoes degradation with the increase of UV-B irradiation periods, while a synchronous rise of absorbance with A_{max} at 329 nm was detected; the peak position (λ_{max} = 329 nm) is significantly red-shifted compared to the one found in our previous paper when quercetin itself was irradiated in methanol (λ_{max} = 295 nm) [34]. This shift can certainly be attributed to the change of medium (phosphate buffer, pH 7.4, versus MeOH), since it is already known that quercetin spectrum itself is very much affected by change of pH in the range of 2–12 [48]. However, when quercetin is oxidized by myeloperoxidase in 50 mM phosphate buffer (pH 6.5) in the presence of H_2O_2 and in the absence of any radiation, the detected product had A_{max} at 336 nm [49], suggesting some possible correlation between the two (UV-induced and enzymatic) mechanisms. Awad *et al.* [13] have listed structures of compunds obtained as a result of various flavonoids oxidation by different types of peroxidases with H_2O_2. Most of them have been created through C-ring opening preceding final structures formation (benzoic acid derivatives – like the ones obtained by UV-oxidation of quercetin in methanol [29]). On the other hand, the calculated quercetin degradation rate constants (from the slopes of the respected plots, Figure 2 (A,B – right)) are 0.020, for quercetin itself, and 0.022 for quercetin in the presence of HRP. Evidently, quercetin degrades at very similar rate in the absence and the presence of HRP (the difference is roughly about 10%). Based on this fact, a question emerges if quercetin oxidation by HRP is affected at all by a prolonged, continuous UV-irradiation.

Part of the answer might come from investigation of possible change of HRP activity as a result of UV-B irradiation. Unlike several previous *in vivo* studies [50], this report represents *in vitro* study of UV-B irradiation effects on HRP activity.

Temporal absorbance changes of the added substrate during measurements of activity of irradiated HRP are shown in Figure 3. The results indicate that the activity of HRP decreases with increasing periods of UV-B

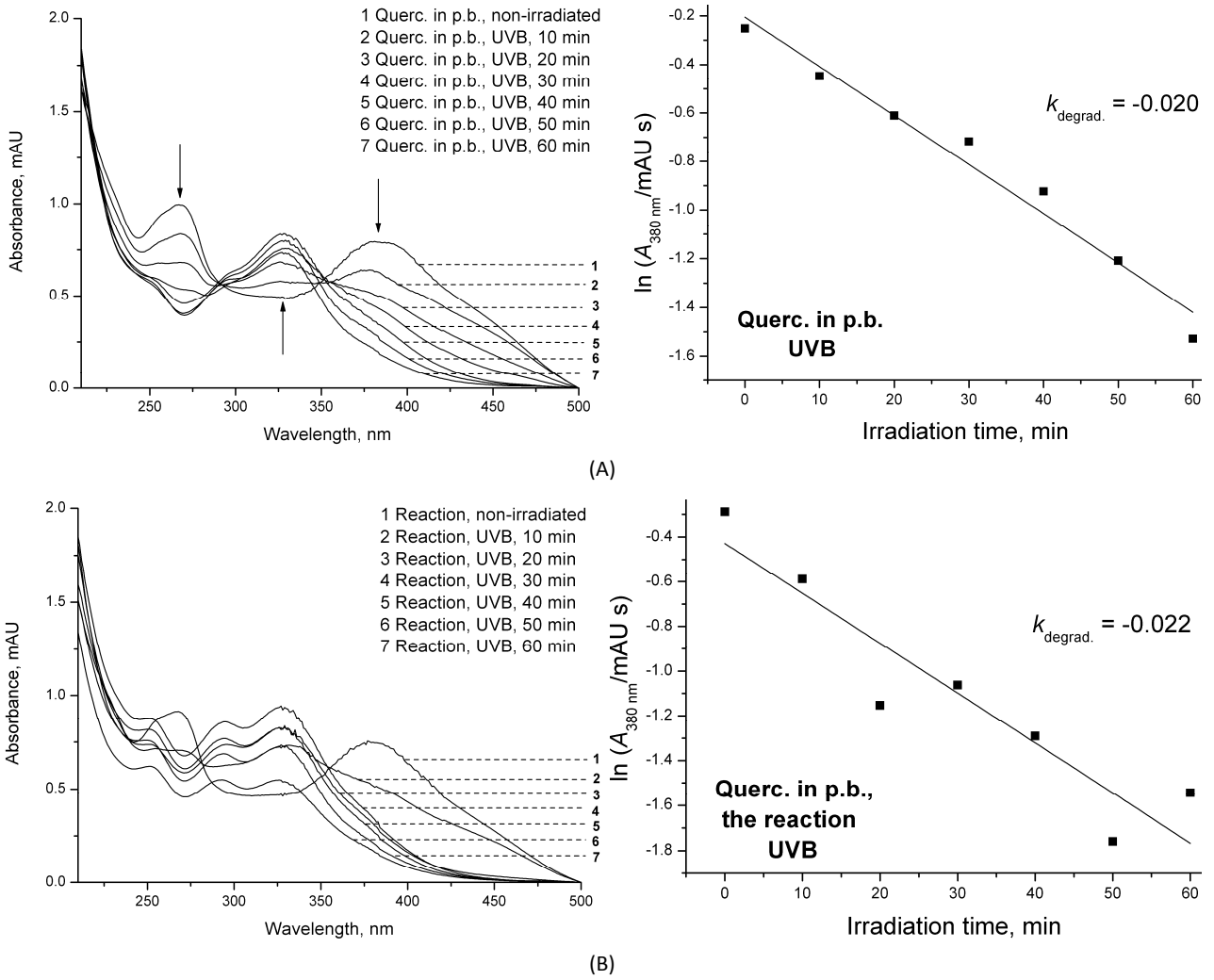

Figure 2. Changes in the absorption spectra of quercetin in phosphate buffer only and in the reaction mixture, irradiated with continuous UV-B irradiation (A,B, left, respectively). The exposure time periods were displayed on the graphs, and the arrows show the changes direction: down (↓), in the case of quercetin, and up (↑) in the case of the possible degradation products formation. Kinetic ln plots of UV-B induced quercetin only (A, right), as well as in the reaction mixture (B, right), detected as decrease of the band I absorbance (recorded at 380 nm), as a function of UV-B irradiation: ln A_{380nm} as a function t_{irr}. The plots show linear fitting, with $R^2 \approx 0.85$ (B) and $R^2 \approx 0.97$ (A). Corresponding rate constants calculated from the slopes of the linear plots are displayed on the graphs.

irradiation (of HRP), which is in accordance with already published results [51]. HRP activity falls to 50% of the initial activity after 20 min UV-B irradiation (Table 1). Mechanism of HRP inactivation takes place most probably through change of some amino acid residues; the increasing irradiation doses leads to disorganization of enzyme structure [1,51].

Figure 4 shows the change of the rate of quercetin oxidation by increasingly irradiated HRP. The non-irradiation case is shown in the increment**,** indicating that this reaction is mostly enzymatic; the minor, non-enzymatic reaction makes 10% of the total reaction. Table 2 (presenting the rate reaction values) suggests that the increased irradiation periods of HRP lead to reducement of the overall rate reaction, which looks compatibile with irradiated-HRP activity results.

However, the compatibility is not complete: it does exist until 30 min of irradiation (Figure 4), whereas for UV-B irradiation periods longer than 30 min the values of quercetin oxidation rates (for 30, 40, 50 and 60 min irradiated HRP) remain the same as in the case of HRP absence, *i.e.*, in the non-enzymatic case (Figure 4 – increment, the upper plot). Since HRP expressed some "survived" activity after 60 min of UV-B irradiation, this non-compatibility can be explained by an additional quercetin role: it might also act as an HRP inhibitor depending on a binding mode [36]. In fact, previous studies have shown that quercetin could bind to HRP by occupying the enzyme active positions by its hyd roxyl groups leading to a block of the HRP heme group [1].

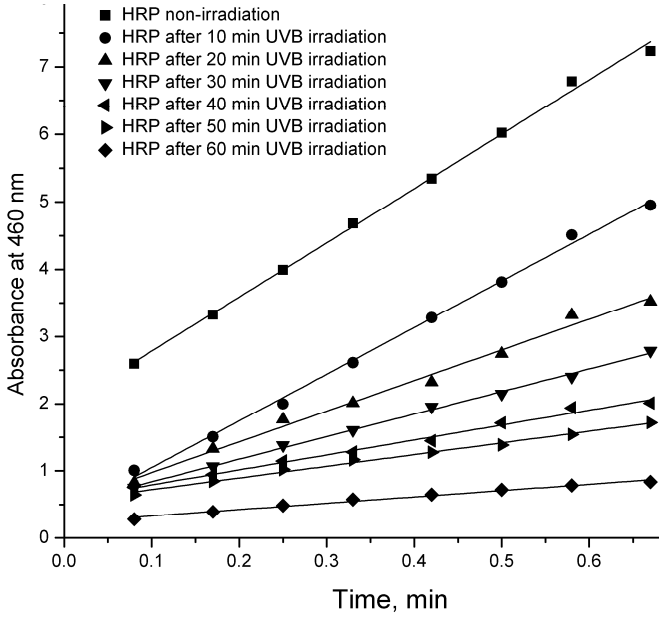

Figure 3. Change of activity of HRP as a result of UV-B irradiation.

Table 1. Change of activity of HRP as a result of UV-B irradiation

Time, min	*U* / ml	%
0	17.803	100.00
10	15.365	86.31
20	10.095	56.74
30	7.429	41.71
40	4.915	27.60
50	3.838	21.56
60	2.081	11.73

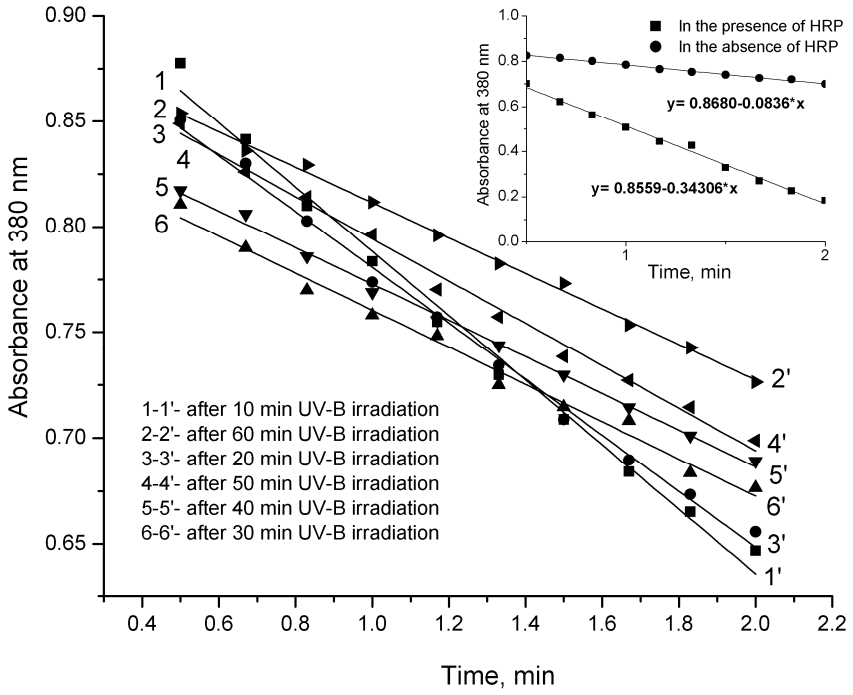

Figure 4. Change of overall reaction rate (oxidation of quercetin by HRP) as a result of UV-B irradiation. The increment shows the non-irradiation case in the presence and in the absence of HRP, recorded at 380 nm.

Table 2. Change of reaction rates: overall, and the enzymatic parts, separately, as a result of increasing UVB irradiation periods

Time of UV-B irradiation, min	Rate of overall reaction, ΔA_{380} / min	Change of the rate of the enzymatic reaction	
		ΔA_{380} / min	%
0	0.343	0.260	100.00
10	0.152	0.069	26.54
20	0.132	0.049	18.85
30	0.087	0.004	1.54
40	0.086	0.003	1.15
50	0.100	0.017	6.54
60	0.084	0.001	0.38

CONCLUSION

In conclusion, it seems that while UV-B irradiation has definitely affected the activity of HRP and the overal rate of quercetin oxidation by HRP, it probably had very little effect on it for UV-B irradiation periods longer than 30 min. In other words, after 30 min of continuous irradiation it seems that the reaction followed the non-enzymatic pattern.

Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia under Project No.TR-34012 and OI-172044.

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IZVOD

OKSIDACIJA KVERCETINA POMOĆU PEROKSIDAZE IZ RENA: EFEKAT UV-B ZRAČENJA

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Peroksidaza iz rena (HRP) jedan je od najviše istraživanih enzima iz grupe peroksidaza, koji poseduje pored drugih bioloških aktivnosti i sposobnost da katalizuje oksidaciju mnogih flavonoida i fenolnih supstrata, između ostalog i kvercetina. Sa druge strane, poznato je da kvercetin poseduje izraženu antioksidativnu aktivnost, koja u slučaju UV zračenja može biti izražena delom i na preventivni način: kvercetin je odličan UV-absorber. Stoga je cilj ovog istraživanja bio da se ispita oksidacija kvercetina pomoću peroksidaze iz rena u fosfatnom puferu pod uslovima UV-stresa, tj. pod dejstvom kontinualnog produženog UV-B ozračivanja. Rezultati pokazuju da definitivno, UV-B ozračivanje utiče na aktivnost HRP, kao i na ukupnu brzinu oksidacije kvercetina pomoću HRP, s tim da je uticaj na brzinu oksidacije (kvercetina) veoma mali nakon dužeg perioda UV-B ozračivanja (>30 min).

Ključne reči: Kvercetin • Peroksidaza • UV-B zračenje • Kinetika • Oksidacija