Research Letter

Oxidation of Quercetin by Myeloperoxidase

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Study of effect of myeloperoxidase on quercetin at pH 6.0 indicated quercetin oxidation via the formation of the oxidation product. The stability of quercetin and oxidation product was investigated as a function of time by using spectrophotometric and HPLC techniques. The apparent pseudo first-order rate constants were calculated and discussed.

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1. Introduction

Flavonol quercetin (Q) (3,5,7,3',4'-pentahydroxyflavone) (Scheme 1) due to its phenolic structure is a strong antioxidant and free radical scavenger [1]. Oxidation of quercetin during its antioxidative functions is usually accompanied by the production of the quercetin radical anion, superoxide, and hydrogen peroxide [2]. Quercetin undergoes autoxidation—the nonenzymatic reaction with atmospheric oxygen. Also, it is known that one-electron oxidation of quercetin is catalyzed by different peroxidases like lactoperoxidase (LPO) [3] and horseradish peroxidase (HRP) [4–7] in the presence of H_2O_2 .

The heme enzyme myeloperoxidase (MPO) is oxidant enzyme in the process of inflammation and atherogenesis [8]. Myeloperoxidase is relatively nonspecific with respect to its reducing substrates. This enzyme is able to oxidize different substrates among which anilines and phenols [9–11]. This letter deals with quercetin oxidation by myeloperoxidase- H_2O_2 system.

2. Experimental

2.1. Chemicals. Quercetin dihydrate (Sigma-Aldrich) of the highest quality available (98%) was used without purification. 1×10^{-3} M stock solution of quercetin was prepared in methanol 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 6.0. Myeloperoxidase was purified from human neutrophils to a purity index

(A_{430}/A_{280}) greater than 0.70 as described previously [12]. Its concentration was calculated using $\epsilon_{430} = 91\,000\text{ M}^{-1}\text{cm}^{-1}$ per heme [13]. Hydrogen peroxide solutions were prepared daily by diluting a stock solution, and the concentration was determined using $\epsilon_{240} = 43.6\text{ M}^{-1}\text{cm}^{-1}$ [14]. Redistilled water was used in all experiments.

2.2. Oxidation of Quercetin by Myeloperoxidase. Quercetin (5×10^{-5} M) was incubated in 50 mM phosphate buffer, pH 6.0, with various concentrations of MPO. Reaction was started by the addition of $50\text{ }\mu\text{M}$ H_2O_2 , and UV absorbance changes were recorded. For the HPLC analysis, reaction was stopped after 30 minutes by adding catalase ($100\text{ }\mu\text{g/mL}$). The reaction mixture was centrifuged for 2 minutes at 10000 rpm. The clear supernatant was analyzed by HPLC.

2.3. HPLC Analysis. HPLC equipment consisted of an HP 1100 Series chromatograph coupled with a DAD. Chromatographic separations were run on a C18 Pinnacle ODS column (Restek, $250\text{ mm} \times 4.6\text{ mm}$, $5\text{ }\mu\text{m}$) using an 80 : 20 mixture of 2 vol% H_3PO_4 (A) and acetonitrile (J.T. Baker) (B) as the eluent for the first 2 minutes. The linear gradient was applied from 20% to 45% B from 2 to 7 minutes. An isocratic 55 : 45 mixture was applied between 7 and 13 minutes. The eluent flow rate was 1.0 mL min^{-1} , and the injection volume was $10\text{ }\mu\text{L}$. The elutions were monitored with DAD at different wavelengths between 200 and 450 nm.

2.4. UV-Vis Spectroscopic Studies. UV spectra were recorded on a Perkin Elmer Lambda 35 UV-Vis spectrophotometer

FIGURE 1: Absorption spectra and HPLC chromatograms of 5×10^{-5} M quercetin at pH 6.0 in absence (a), (b) and presence (c), (d) of 20 nM MPO. $50 \mu\text{M}$ H_2O_2 was present in all reaction solutions. Spectra were recorded over a period of 30 minutes; arrows indicate the direction of the change. Chromatograms were recorded after 30 minutes (b) at λ_{367} and (d) at λ_{336} .

TABLE 1: Rate constants of quercetin oxidation.

pH	Sample	Quercetin degradation	$k \text{ (min}^{-1}) \times 10^3$	
			Water adduct formation	Water adduct degradation
6.0	Q	3.61 ± 0.18	—	—
	Q + MPO (20 nM)	346.00 ± 21.15	524.60 ± 32.30	2.67 ± 0.09
10.0*	Non-irradiated Q	8.73 ± 0.34	25.12 ± 0.93	—
	Irradiated Q	110.00 ± 35.25	96.91 ± 0.12	10.13 ± 0.56

*Reference [17].

equipped with thermostatted quartz cell. The temperature in the cell was kept at $25 \pm 0.05^\circ\text{C}$ with a water-thermostatted bath.

3. Results and Discussion

We investigated the effect of MPO on 5×10^{-5} M quercetin in phosphate buffer, pH 6.0 in the presence of $50 \mu\text{M}$ H_2O_2 at 25°C , spectrophotometrically and by HPLC. The following concentrations of enzyme were used: 5, 11, 15, 20, 50, 100, 150, and 200 nM.

At the absorption spectra of samples in which concentration of MPO was ≤ 11 nM, two quercetin absorption bands (254 nm and 367 nm) were observed (Figure 1(a)). The same results were yielded when MPO or H_2O_2 was omitted from the mixture.

The absorption spectra of samples in which concentration of MPO was ≥ 15 nM showed the decay of quercetin absorption bands at 254 nm and 367 nm. At the same time simultaneous rise of the absorption band at 336–342 nm, depended on MPO concentration, was observed (Figure 1(c)), typical of oxidation product formation [16].

SCHEME 1: Oxidation of quercetin by MPO/ H_2O_2 system (modified from [15]). FIGURE 2: Change of absorbance of quercetin in the presence of various MPO concentrations in the function of time, at pH 6.0 (a) formation of quercetin oxidation product at λ_{336} ; Inset: 20 nM MPO and (b) degradation of quercetin oxidation product at λ_{367} .

Moreover, two well-defined isosbestic points at 290 and 364 nm indicate that there was no significant accumulation of intermediates in the reaction of oxidation product formation (Figure 1(c)). These spectral changes are analogous to those occurring upon autooxidation of quercetin in water [16] and UV irradiation [17] as well as oxidation with some oxidants in organic solvents [16].

The HPLC analysis of quercetin solution treated with MPO for 30 minutes (Figure 1(d)) revealed that concentration of quercetin (retention time 10 minutes) was negligible.

Also, formation of major oxidation product (retention time 5.26 minutes) which was more polar than quercetin was detected (Figure 1(d)). From literature data and the obtained spectrophotometric and HPLC results in this work, we assume that the oxidation product detectable at 336–342 nm results from H_2O addition on the *p*-quinonemethide formed by H-atom abstraction at 3-OH and 4'-OH of quercetin and subsequent rearrangement of the central ring [16]. The complete sequence of quercetin autooxidation induced by oxidants and catalyzed by metal ions is described earlier [15, 16] (Scheme 1).

According to spectrophotometric and HPLC experimental data, quercetin degradation (1) and oxidation product formation (2) as the function of time followed the relation

$$A_{Q,t} = A_0 e^{-kt}, \quad (1)$$

$$A_{\text{oxid},t} = A_{\infty} (1 - e^{-kt}), \quad (2)$$

where $A_{Q,t}$ and $A_{\text{oxid},t}$ are the absorbances proportional to the quercetin and its oxidation product concentrations, respectively, after an irradiation period of time t and k is the overall pseudo first-order rate constant. A_0 and A_{∞} are the absorbance proportional to the initial concentration of quercetin and the concentration of the oxidation product on the plateau of the kinetics curve, respectively.

The kinetic curves that describe the quercetin degradation and the formation of the oxidation product in the presence of different MPO concentrations are shown in Figure 2. In the presence of MPO concentration that was ≥ 15 nM oxidation product was formed at the first minute of reaction (Figure 2(a)). The overall apparent first-order rate constants for the oxidation product formation and quercetin transformation to its degradation products were determined from the kinetic curves and are presented in Table 1. For comparison, in Table 1 we also presented values for these constants obtained for quercetin oxidation by UV irradiation [17].

The rate of quercetin oxidation by MPO at pH 6.0 is 3-fold faster compared with the rate of quercetin oxidation by UV irradiation at pH 10.0. Moreover, product obtained for quercetin oxidation by MPO at pH 6.0 is more stable than product of quercetin oxidation by UV irradiation at pH 10.0.

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