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Oxidative modification of quercetin by hemeproteins

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Abstract

The ability of a number of hemeproteins to oxidize the flavonoid quercetin has been shown. It was found that quercetin undergoes chemical modification in the presence of cytochrome *c*, myoglobin, and hemoglobin but not cytochrome b_5. In the range of investigated proteins the most effective oxidant appears to be cytochrome *c*. Chromatographic analysis of the reaction mixture revealed a number of quercetin oxidation products. The main oxidation product was purified and characterized by means of LC-MS and NMR analyses. It has a dimeric structure similar to the product of quercetin oxidation by horseradish peroxidase and is formed during radical-driven reactions. Our results indicate that a number of hemeproteins can react and modify biologically active flavonoids. However, these reactions might also lead to the generation of active species with deleterious consequences for the cellular macromolecules.

Keywords: Quercetin; Flavonoid; Oxidation; Hemeprotein; Cytochrome *c*; Cytochrome b_5; Myoglobin; Hemoglobin; LC-MS; NMR

Quercetin is one of the most abundant plant-derived polyphenols widely consumed with a human diet [1]. Due to its structure quercetin possesses a prominent antioxidant and radical absorbing capacity [2,3]. On account of these properties it is widely used as beneficial food supplement that is recommended for prevention and suppression of many disease associated with an oxidative stress. It has become evident that antioxidant therapy may lead to adverse effects, as an ability of quercetin and other flavonoids to cause damage of cellular macromolecules and induce formation of reactive oxygen species has been discovered [4–6]. This finding provokes researchers to get deeper insight into the problem of biotransformation and utilization of foreign compounds such as flavonoids.

The most accepted explanation of the adverse effects is the formation of flavonoid oxidation products [7,8]. The main intermediates formed during oxidation are semiquinone and reactive electrophilic *o*-quinones. These compounds possess strong alkylation activity and their production results in damage of cellular proteins and DNA. From this point of view the investigation of plausible bioactivation sites takes a special significance. Some of the hemeproteins may act as flavonoid activating sites due to their relatively high redox potential and ability to participate in electron-transfer reactions with low molecular compounds.

The aim of the present study was to determine whether quercetin can be oxidized by a number of hemeproteins and investigate the formed reaction products to find clues to the mechanism of reaction with flavonoid as electron donor and an oxidant.

Materials and methods

Quercetin and acetone-d_6 were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate and acetonitrile were from Fluka (Buchs, Switzerland), Sephadex LH-20 was from Pharmacia (Uppsala, Sweden).

Proteins. Cytochrome *c* (horse heart, type IV) was purchased from Sigma. Its purity was assessed by means of SDS-electrophoresis and analytical gel-filtration chromatography. Preparation of cytochrome *c* was found to be pure and essentially free from dimeric forms. Thus,

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cytochrome *c* was used for further work without extra purification. Myoglobin (horse heart, type III) was purchased from Serva (Heidelberg, Germany). It was chromatographically purified on a Sephadex G-50 column (56 × 2 cm) using 50 mM Tris–HCl, pH 7.4, as eluting buffer. Hemoglobin (bovine) was purchased from Sigma. In this study, it was used without extra purification. Recombinant rat cytochrome b_5 with a histidine tag at the N-terminus was expressed and purified as described in [9]. The concentrations of hemeproteins were determined using $\varepsilon_{409} = 106.1\ mM^{-1}\ cm^{-1}$ for cytochrome *c*, $\varepsilon_{409} = 164\ mM^{-1}\ cm^{-1}$ for myoglobin, $\varepsilon_{407} = 136\ mM^{-1}\ cm^{-1}$ for hemoglobin, and $\varepsilon_{412} = 117\ mM^{-1}\ cm^{-1}$ for cytochrome b_5.

Spectrophotometric analysis. Different amounts of hemeproteins were diluted in 50 mM Tris–HCl or sodium phosphate buffer, pH 7.4. Quercetin in DMF (dimethyl formamide) was added to 1 ml of protein solutions to achieve a final concentration of 200 μM. The mixture was incubated for 5 min and then 3 ml of ethanol was added to precipitate the protein and stop the reaction. Protein was removed from solutions by centrifugation at 3000*g* for 10 min. Spectra of supernatants were recorded in the range 220–700 nm using a Shimadzu UV-3000 (Japan) spectrophotometer.

Quercetin caused conversion of the studied hemeproteins to their reduced forms with different rates. To study this process, the spectrophotometric titration of hemeproteins with quercetin was done. The 25 μM hemeprotein solution in 50 mM sodium phosphate buffer, pH 7.4, was placed in the cuvette and its temperature allowed to be equilibrated for 5 min to 25 °C. Then a 10 μl aliquot of quercetin solution in DMF was added to the cuvette to start the reaction. The flavonoid concentration for all experiments was 100 μM. The increases in absorbance at 550, 580, and 577 nm were recorded for the cytochrome *c*, myoglobin, and hemoglobin, respectively. The concentrations of reduced hemeproteins were determined using $\varepsilon_{550} = 29.5\ mM^{-1}\ cm^{-1}$ for cytochrome *c*, $\varepsilon_{580} = 14.4\ mM^{-1}\ cm^{-1}$ for myoglobin, and $\varepsilon_{577} = 14.6\ mM^{-1}\ cm^{-1}$ for hemoglobin. The apparent rate constants for the studied electron-transfer reactions were calculated using non-linear regression analysis.

Chromatographic separation of quercetin oxidation products. The quercetin solution in DMF was added to the hemeprotein solution in 50 mM sodium phosphate buffer, pH 7.4, to the final concentration of 200 μM. The mixture was incubated for 10 min and then an appropriate amount of 1 M HCl and an equal volume of ethyl acetate were added to stop the reaction and extract the phenolic compounds. The organic phase was collected and evaporated under a stream of argon. Dried samples were diluted with methanol and subjected to a chromatographic procedure. Analytical HPLC was conducted using Agilent 1100 (Agilent Technologies, USA) equipped with Diaspher C18 (250 × 4 mm, 5 μm) column. The column was eluted with a mixture of water containing 0.1% (v/v) acetic acid and acetonitrile. From 0 to 5 min a linear gradient from 20% to 50% of acetonitrile was applied, from 5 to 8 min acetonitrile in the mobile phase was maintained at 50%, followed by a gradient from 8 to 12 min to 70% of acetonitrile, form 12 to 14 min to 100% acetonitrile, and finally from 14 to 15 min the mobile phase was 100% acetonitrile. Detection was accomplished with a photodiode array detector at 290 and 375 nm. The spectra of eluted compounds were recorded between 220 and 500 nm. Separation was performed at the flow rate of 1 ml min^{-1} at room temperature. Preparative separation was carried out using the following scheme. The concentrated diethyl ether extract (about 0.2 ml) of reaction products from several preparations was applied on Sephadex LH-20 column (1 × 10 cm). Colored substances were fractionated with methanol as eluting solvent at the flow rate of 5 ml h^{-1} and were subjected to rechromatography under the same conditions.

Mass spectrometry. An LCMS-QP8000a (Shimadzu, Japan) chromatograph equipped with diode array and single quadrupole mass spectrometry detectors was used for mass spectrometric analysis. The mass spectrometric electrospray interface was set in the positive ion mode. The mass spectrometry parameters were as follows: nebulizer gas (nitrogen) flow rate, 4.5 L min^{-1}; electrospray voltage, 4.5 kV; curved desolvation line heater temperature, 250 °C.

NMR measurements. NMR spectra were recorded on a Bruker Avance 500 spectrometer at 500.13 MHz (^1H) and 125.75 MHz (^{13}C), respectively, using standard Bruker software. TMS was used as an internal standard. The chemical shifts are reported in δ-values and the coupling constants are in Hz.

Results and discussion

The direct interaction of quercetin with the studied hemeproteins was assessed by UV–visible spectroscopy. Fig. 1 shows the changes in the absorbance spectrum of quercetin incubated with the different amounts of cytochrome *c*. Addition of 50 μM hemeprotein to 200 μM quercetin solution in 50 mM sodium phosphate buffer, pH 7.4, resulted in a decrease in absorbance at 378 nm and the appearance of a shoulder around 330 nm. Consequent increase of cytochrome *c* content relative to flavonoid leads to further diminution of peak at 375 and appearance of a new absorption maximum at 335 nm. Three isobestic points at 279, 358, and 435 nm are observed in the spectra. Similar but less pronounced spectral changes were observed when 50 μM myoglobin and hemoglobin was used instead of cytochrome *c* (data not shown). The spectral changes are typical to quercetin oxidation products in peroxidase systems or in the presence of heavy metal ions [10–12] suggesting the same nature of end product(s) of flavonoid oxidation by either the hemeproteins studied here (cytochrome *c*, myoglobin, and hemoglobin) or the peroxidases studied previously. Unlike those proteins cytochrome b_5 failed to induce appreciable alteration of the flavonoid spectrum.

Incubation of 100 μM quercetin with 25 μM cytochrome *c*, myoglobin or hemoglobin in 50 mM sodium phosphate buffer, pH 7.4, leads to the reduction of these hemeproteins. The spectral changes in the Soret region are hard to interpret because of the super-imposition of two

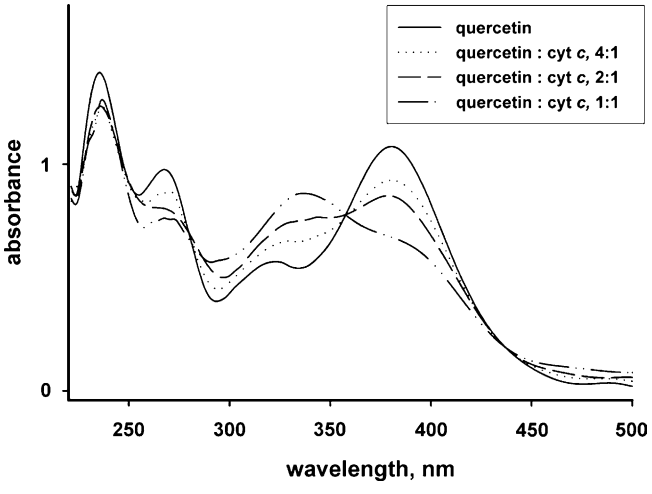

Fig. 1. Changes of UV–visible absorption of quercetin induced by different amounts of cytochrome *c*. Solid line—initial spectrum of quercetin; dotted line—spectrum of quercetin recorded after its incubation with cytochrome *c* in the ratio of 4:1; dashed line—spectrum of quercetin recorded after its incubation with cytochrome *c* in the ratio of 2:1; dash-dotted line—spectrum of quercetin recorded after its incubation with cytochrome *c* in the ratio of 1:1. Experimental conditions as described under Materials and methods.