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OLIGOMERIC OXIDATION PRODUCTS OF THE FLAVONOID QUERCETIN

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Some of the main oxidation products of quercetin were shown to be compounds formed by oligomerization of the starting flavonoid. Conditions for the preparative synthesis of these compounds were developed. Their structures were established using HPLC—MS and NMR methods. Quercetin oligomers in the natural sample, outer leaves of modified runners of Allium cepa *L., were found using chromatographic procedures. The use of quercetin oligomers as indicators of its oxidation was proposed.*

Key words: quercetin, oxidation products, *Allium cepa*, redox reactions, NMR.

We have previously shown that oxidation of quercetin *in vitro* produces primarily oligomeric products without any significant accumulation of hydroxybenzoic acid derivatives [1]. The question of the existence of oligomeric quercetin oxidation products *in vivo* remains open. Herein we describe possible formation pathways and structural properties of the two main oligomeric oxidation products of the flavonoid. The possibility of using data on the physical chemical properties of these compounds to search for them in biological samples is discussed.

It was shown previously that quercetin was chemically modified upon reaction with certain hemoproteins of the *b* and *c* types [1]. As a result, several oxidation products, the principal ones of which were product B, a dimer of the starting compound, and product C, the structure of which could not be established, were formed. Use of hemoproteins as oxidants did not produce product C in quantities required for structural studies. The main obstacle was the limited solubility of quercetin in the aqueous solutions that were used to carry out the oxidation. Addition of an organic solvent to the reaction mixture could have increased the solubility of quercetin. However, this would have made it impossible to use proteinaceous oxidants because of their possible denaturation. We used $K_3[Fe(CN)_6]$ as an alternate oxidant that is not destroyed in organic media. The redox potential of $K_3[Fe(CN)_6]$ is high and positive, like that of cytochrome *c* [2, 3].

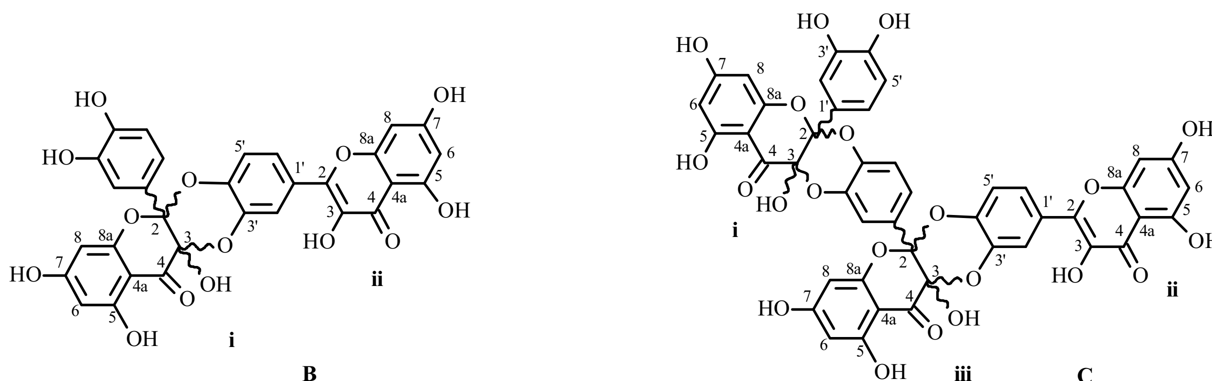

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TABLE 1. PMR Spectra for Dimer B and Trimer C (δ, J/Hz)

H atom	Subunits				
	i		ii		iii
	C	B	C	B	C
H-6	6.00 (1H, 8d, J = 2.1)	6.03 (d, J = 2.1)	6.27 (1H, 8d, J = 2.1)	6.28 (d, J = 2.1) and 6.29 (d, J = 2.1)	6.04 (0.5H, 3d, J = 2.1) 6.06 (0.5H, 2d, J = 2.1)
H-8	6.03 (0.2H, 2d, J = 2.1) 6.05 (0.8H, 6d, J = 2.1)	6.08 (d, J = 2.1)	6.59 (1H, m)	6.61 (d, J = 2.1) and 6.63 (d, J = 2.1)	6.13 (0.3H, 2d, J = 2.1) 6.15 (0.2H, 2d, J = 2.1) 6.155 (0.25H, d, J = 2.1) 6.165 (0.25H, d, J = 2.1)
H-5′	6.76 (0.5H, 4d, J = 8.5) 6.77 (0.5H, 2d, J = 8.5)	6.82 (d, J = 8.4)	7.17 (0.2H, 2d, J = 0.3; 8.7) 7.18 (0.3H, 2d, J = 0.3; 8.7) 7.31 (0.5H, m)	7.17 (d, J = 8.7) and 7.29 (d, J = 8.7)	7.02 (0.5H, 4dd, J = 0.3; 8.6) 7.15 (0.5H, 2d, J = 8.6)
H-6′	7.09 (1H, 4dd, J = 2.4; 8.5)	7.17 (dd, J = 2.4; 8.4) and 7.19 (dd, J = 2.4; 8.4)	7.98 (0.7H, 3dd, J = 2.1; 8.7) 8.00 (0.3H, m)	7.98 (dd, J = 2.2; 8.7) 8.01 (dd, J = 2.2; 8.7)	7.46 (1H, m)
H-2′	7.29 (1H, 2d, J = 2.4)	7.35 (d, J = 2.4) and 7.36 (d, J = 2.4)	7.915 (0.3H, 2dd, J = 0.3; 2.1) 7.925 (0.3H, 2d, J = 2.1) 8.04 (0.2H, 2dd, J = 0.3; 2.1) 8.06 (0.2H, 2d, J = 2.1)	7.91 (d, J = 2.2) and 8.05 (d, J = 2.2)	7.43 (0.5H, m) 7.56 (0.1H, 2d, J = 0.3; 2.4) 7.58 (0.3H, 2dd, J = 0.3; 2.4) 7.59 (0.1H, 2d, J = 0.3; 2.4)

HPLC studies of the reaction products showed that the qualitative compositions of the reaction mixtures obtained using cytochrome *c* and $K_3[Fe(CN)_6]$ were identical. However, the quantitative compositions were slightly different. The quercetin content varied in the range of 30%; product B, 35%; product C, 17%. Use of acetone as the solvent for preparative purposes could increase the quercetin content in the reaction mixture and; therefore, the overall yield of the oxidation products.

Chromatographic separation produced highly pure products B and C. Product B was the conjugate of two quercetin molecules joined through a dioxane ring [1, 4-10]. Product C was most probably a trimer of quercetin, as was demonstrated earlier using HPLC—MS [1]. An attempt was made to analyze spectra of the trimeric adduct of this flavonoid [4]. However, some ambiguity remained in the assignments of certain resonances. Use of acetone-d_6 as solvent enabled all resonances in the spectrum to be assigned. Chemical shifts and spin—spin coupling constants (SSCC) in the PMR spectrum of fragments (i) and (ii) of the trimeric adduct (Table 1) turned out to be close to those for compound B [1, 4, 7]. This suggests that the principal fragments of B and C had similar chemical structures and allowed the trimeric structure to be proposed for C. Use of acetone-d_6 as solvent made it possible to observe resonances for hydroxyls of compound C, for which a detailed spectral analysis was also carried out using 2D NMR spectroscopy methods (HSQC, HMBC, COSY, NOESY) and all resonances in the PMR and ^{13}C NMR spectra were assigned.

Table 2 shows clearly that the described correlations for resonances of H atoms in the PMR spectra between chemical shifts of pyrone and phenol parts of subunits (i), (ii), and (iii) of product C and pyrone and phenol parts of subunits (i) and (ii) of product B remained valid. Differences in chemical shifts of resonances in the ^{13}C NMR spectrum of subunits (i) and (ii) of the dimeric and trimeric adducts were less than 0.2 ppm for most C atoms; for subunit (iii), 0.3 ppm.

The presence of resonances for C atoms that did not correlate with the overall pattern could be explained by the effect of the through-space (more than four bonds) chemical environment. For example, the differences of chemical shifts for C1′ and C2′ of the phenol part of subunit (iii) of the trimer relative to the phenol part of subunit (ii) of the dimer were 2.95 and 1.00 ppm, respectively. This was due to the different substitutions on C3′ and C4′. For the trimer, C3′-C4′ are fused to subunit (i); for the dimer, C3′ and C4′ have hydroxyls. Furthermore, an Overhauser effect between protons on C-8 of the corresponding pyrone part of the molecule and protons on C-2′ and C-6′ of the phenol part was observed using the 2D NMR NOESY method. According to high-resolution ^{13}C NMR and PMR spectra for the trimer, it was concluded that product C is a chromatographically inseparable mixture of eight diastereomers.

All resonances corresponding to the stereomers have not yet been assigned because of the large number of them.

TABLE 2. ^{13}C NMR Spectra for Dimer B and Trimer C

C atom	Subunits				
	i		ii		iii
	C	B	C	B	C
2	101.38	101.05	145.33	145.4	101.02
3	91.49	91.5	137.65	137.6	91.29
4	188.95	189.05	176.78	176.7	188.71
4a	100.80	100.85	104.18	104.2	100.71
5	165.04	165.1	162.2	162.3	165.18
6	97.95	98.0	99.23	99.3	98.28
7	169.25	169.2	165.33	165.2	169.49
8	97.28	97.3	94.64	94.6	97.38
8a	160.68	160.82	157.84	157.8	160.4
1′	126.15	126.25	127.05	126.8	129.75
2′	116.49	116.5	117.62	117.6	118.6
3′	145.43	145.4	141.35	141.6	141.1
4′	147.66	147.6	142.98	143.2	143.15
5′	115.39	115.4	118.35	118.3	117.92
6′	121.02	121.1	123.6	123.5	123.65

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Average values are given for mixtures of diastereomers of B and C.

Because quercetin is readily oxidized, it can be assumed that oligomeric oxidation products will be encountered in living organisms in addition to the starting flavonoid. *Allium cepa* L. is a natural sample that contains significant quantities of quercetin in various chemical forms. The flavonoid is localized in the leaves of a modified runner (bulbs). It is observed as the aglycon in outer dry leaves; as glycosides, in inner moist ones. In view of this information, we selected outer bulb leaves (skin) to search for quercetin derivatives. Phenolic compounds were extracted by EtOAc. HPLC—MS analysis of the extract showed the presence of compounds similar to products B and C (retention times 9.6 and 11.7 min) that were formed upon oxidation of quercetin by cytochrome *c* and $K_3[Fe(CN)_6]$. Mass spectra of the first compound gave two ions at 601 (43, $[M - 1]^-$) and 299 (100) *m/z*; of the second, 901 (49, $[M - 1]^-$) and 299 (100) *m/z*. Addition of highly pure products B and C to the onion skin extract as an internal chromatographic standard did not produce new peaks. These facts indirectly indicate that onion skin contains oligomeric quercetin derivatives, the structures of which agree fully with those published for products B and C [4]. However, there are reports in which other oligomeric forms of quercetin are described [11, 12].

We tend to believe that the natural oligomers of the flavonoid contains a dioxane fusion as indicated by the detailed NMR spectral analyses obtained in our and previous work [4]. Enzymes of the oxidoreductase class, namely peroxidase, are most probably involved in the conversion of quercetin to oligomeric adducts in onion skin. Onion skin has been shown to have peroxidase activity [13, 14]. Herein we used a system containing peroxidase from horseradish root, H_2O_2, and quercetin in phosphate buffer as a model of peroxidase oxidation. Peroxidase oxidation of quercetin formed a rather large amount of oxidation products compared with systems containing cytochrome *c* or $K_3[Fe(CN)_6]$. Using the aforementioned methods, compounds with properties identical to those of products B and C could be observed. This suggested that the formation of oligomeric oxidation products of quercetin in onion skin involves enzymatic systems. The reaction mechanism presumably included oxidation to form a C-3′–C-4′ semiquinone of quercetin with subsequent Diels—Alder heterocyclization with an activated C-2–C-3 double bond of the pyrone part of the other quercetin molecule. Apparently the cyclization process occurred randomly because mixtures of all possible isomers were formed for the dimer and trimer. Only steric factors and hindered rotation of large fragments of quercetin determined the predominance of one product or another. Proteins that could influence the selectivity of the process were not involved in the reaction at this stage. It has been demonstrated here and previously that oligomeric quercetin adducts are formed in reactions with a variety of oxidants [1, 5, 7, 8]. They have been analyzed by chromatographic and spectral methods. These facts suggest that these compounds can be used as indicators of the biological oxidation of the flavonoid regardless of the pathway taken. The extent of the oxidative processes occurring in living systems,

in particular plants, can be estimated using them. The relationship of these processes to changes in environmental factors of the plant habitat can be determined.

EXPERIMENTAL

Analytical Methods. HPLC, mass spectrometry, and NMR conditions have been published [1].

Oxidation of Quercetin in Reactions with Various Electron Acceptors. Aqueous phosphate buffer (100 mL, 0.05 M, pH 7.4) containing quercetin (3.02 mg, 0.01 mmol) was stirred vigorously at 25°C and treated over 3 min with oxidants a) ferricytochrome *c* (125 mg, 0.01 mmol); b) $K_3[Fe(CN)_6]$ (3.29 mg, 0.01 mmol); or c) horseradish peroxidase solution (0.01 mL, 0.01 μmol) and H_2O_2 solution (0.01 mL, 0.1 mmol). The mixture was stirred for 30 min. The course of the reaction was monitored by TLC. The reaction mixture was evaporated to one half the volume and treated with EtOAc (30 mL). The organic fraction was separated. The aqueous layer was extracted with EtOAc (3 × 20 mL). The combined organic fractions were dried over Na_2SO_4. The precipitate was filtered off. The filtrate was evaporated. The solid was analyzed by HPLC—MS.

Preparative Synthesis and Production of Pure Oligomeric Quercetin Oxidation Products. Aqueous acetone phosphate buffer (100 mL, 0.05 M, 40/60 by vol., pH 7.4) containing quercetin (604 mg, 0.2 mmol) was stirred vigorously at 25°C and treated over 3 min with $K_3[Fe(CN)_6]$ (658 mg, 0.2 mmol). The mixture was stirred for 30 min. Completion of the reaction was monitored by TLC. The reaction mixture was evaporated to one half the volume, extracted with EtOAc, and dried over Na_2SO_4. The solid was chromatographed over a column of Sephadex LH-20 [1] to produce (in order of elution) unreacted quercetin (181 mg, 30%) and oligomeric products B (52.1 mg, 9%) and C (43.3 mg, 7%).

1,3,11a-Trihydroxy-9-(3,5,7-trihydroxy-4*H*-1-benzopyran-4-on-2-yl)-5a-(3,4-dihydroxyphenyl)-5,6,11-hexahydro-5,6,11-trioxanaphthacen-12-one (B). UV spectrum (EtOH, λ_{max}, nm): 270, 303, 365 (log ε 4.31, 4.36, 4.33). Mass spectrum (EI, 4.5 kV, *m/z*, I_{rel}, %): 601 (43) $[M - 1]^-$, 299 (100).

1,3,11a-Trihydroxy-9-(3,5,7-trihydroxy-4*H*-1-benzopyran-4-on-2-yl)-5a-[1,3,11a-trihydroxy-5a-(3,4-dihydroxyphenyl)-5,6,11-hexahydro-5,6,11-trioxanaphthacen-12-on-9-yl]-5,6,11-hexahydro-5,6,11-trioxanaphthacen-12-one (C). UV spectrum (EtOH, λ_{max}, nm): 302, 363 (log ε 4.61, 4.39). Mass spectrum (EI, 4.5 kV, *m/z*, I_{rel}, %): 901 (49) $[M - 1]^-$, 299 (100).

Extraction of Phenolic Compounds from Outer Skin of *Allium cepa*. Outer light-brown onion skin was separated manually from the bulb and ground in a Waring 1L Blender. The ground skin (4 g) was placed in a flask and treated with EtOAc (50 mL). The resulting mixture was incubated at 25°C for 12 h with constant stirring. The extract was clarified by filtration and evaporated to dryness. The solid was dissolved in EtOAc or EtOH.

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