Stopped-flow-optical and -ESR Study on Oxidative Reaction of Quercetin by Nitrosodisulfonate Radical as a Model of Reactive Oxygen Species

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By means of the stopped-flow-ESR and -optical methods, oxidation of quercetin by nitrsodisulfonate (NDS) radical anion was studied. The results of optical measurements demonstrated that NDS readily oxidize quercetin to the quinone form. The rapid field-sweep ESR spectrum revealed the formation of short-lived quercetin-derived *o*-semiquinone anion radical. The radical was confirmed to be a transient intermediate species for generation of the quinone of form.

Flavonoids are naturally occurring polyphenolic metabolites found in vegetables, herbs, and teas.[1] Many flavonoids possess anti-viral, anti-inflammatory activities, which may be consequence of their superior antioxidant activity to reactive oxygen species (ROS).[2,3] One of the most intensively investigated flavonoids, quercetin (3,3′,4′,5,7-pentahydroxyflavone), exhibits superior antioxidant activity to ROS. The fast pulse radiolysis experiments demonstrated the transient UV–vis spectra due to the quercetin radical, and kinetic parameters of the radical were also evaluated.[4,5] So far, however, little is known about the ESR spectroscopic property of the quercetin radical. Since the both formation and decay rates of the radical are too fast and quickly decomposed to diamagnetic products, thus keeping its steady-state concentration below the ESR detection limit.

Recently, nitrosodisulfonate radical anion (NDS, $K_2(SO_3)_2$-NO•), which is a product of dissociation of Fremy's salt, has been used as a model for hydroperoxyl radical in studies designed to investigate the antioxidant activity of natural products.[6] In addition, Fremy's salt is known to oxidize phenols, and catechols to the corresponding quinones.[7] By means of newly developed stopped-flow-ESR and -optical methods, spectroscopic investigations are performed for the oxidative reaction between quercetin and NDS, with focusing on detection of the quercetin radical.

Stopped-flow apparatus composed of a micro-4-jet-mixer and nitrogen gas drive syringe pump (JEOL), were applied for both optical absorption and ESR measurements. By using quartz cell (optical path-length 2.0 mm), optical spectra were recorded by photodiode array spectrometer (Photal, MCPD 3000), which has time resolution 16 ms. The dead time of this system was evaluated to be about 20 ms. ESR was record by field- and time-sweep modes using modified TE-100 and data analyzing system (JEOL), which has 1.0 ms time resolution. A quartz capillary (i.d. 0.86 mm, Wilmad) was used as ESR cell to reduce the dead time (below 5 ms). The concentrations of radicals were calibrated using Fremy's salt as a standard. Quercetin mono-hydrate (Wako Pure Chemical) and Fremy's salt (Sigma-Aldrich) were used without further purification. The concentration of NDS was determined using extinction coefficient 1690 M^{-1} ($M = mol/dm^3$) cm^{-1} (248 nm) and 20.8 $M^{-1}\,cm^{-1}$ (545 nm).[8] Sample solutions were prepared in Na_2CO_3–$NaHCO_3$ (0.1 M) buffer solution (pH 10.0) of Q-II grade pure water in order to stabilize the semiquinone anion radical. All the reaction solutions were prepared under nitrogen atmosphere and just before each measurement to prevent the autoxidation.[9]

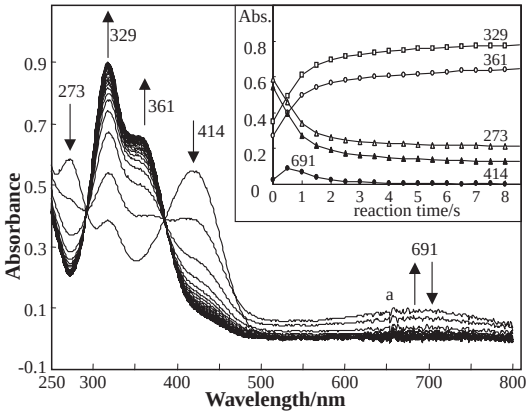

Figure 1. Optical absorption spectra recorded 500 ms interval after mixing aqueous solutions (pH 10.0) of NDS and quercetin. Initial concentrations after mixing are 0.5 mM NDS and 0.25 mM quercetin. Arrows indicate the direction of the spectral changes during the reaction. [a], Noise of spectrometer.

Figure 1 shows spectral changes observed 500 ms interval after mixing NDS (0.5 mM) and quercetin (0.25 mM). The absorption maxima at 273, 330, and 414 nm were of characteristic to the dianion form of quercetin.[10] Within about 5 s from mixing, these absorption bands were shifted to 329 and 361 nm.[11] Remarkably, a weak and rapidly decaying absorption band was observed at 691 nm during about 1.5 s after mixing. The kinetic traces of absorbance (Figure 1, Inset) indicated that this is an intermediate involved in the present system. Similar stopped-flow measurements made by 100 ms interval provided that the absorbance at 691 nm reached to the maximum at about 600 ms after mixing.

The observed spectral changes are analogous to those observed for oxidation of quercetin by $NaIO_4$ in aqueous solution.[12] Based on the spectral parameters, the intermediate species is attributed to the mixture of quercetin-derived *o*-quinone of B-ring, and its charge-transfer complex.[13] On the other hand, the oxidation product is postulated to be a benzofuranone derivative, which was formed by well known chalcan-trione ring-chain

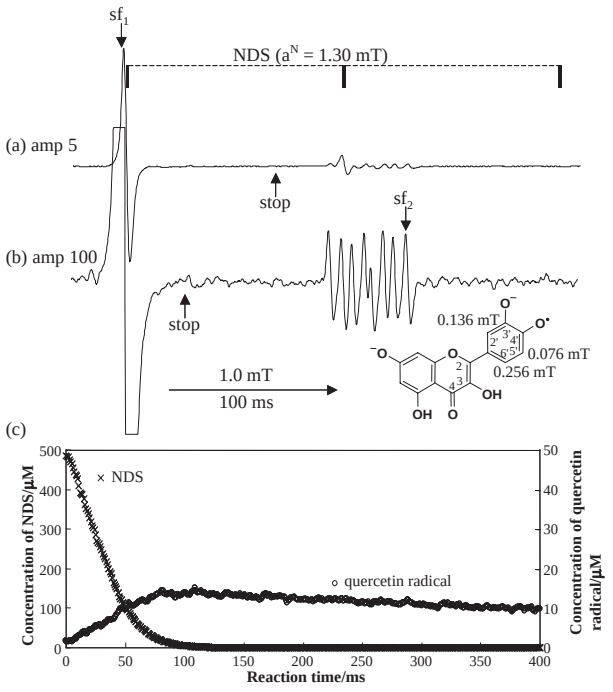

Figure 2. Stopped-flow ESR recorded by field-sweep (a), (b), and time-sweep (c) modes for aqueous (pH 10.0) solutions of NDS and quercetin at room temperature. The flow of solutions and the rapid field-sweep were started simultaneously, and stopped the flow at (a) after 70 ms, and (b) 130 ms, as indicated by upward arrows. The time-sweep ESR measurements were performed at static fields, indicated by down arrows (sf_1) and (sf_2), for NDS and the quercetin radical, respectively. Initial concentrations after mixing are NDS (0.5 mM) and quercetin (0.25 mM).

tautomeric rearrangement occurring at C-ring, followed by the water addition at 2-position.[14] These observations provide experimental evidence that NDS readily oxidize quercetin to the quinone form.[12]

In order to detect the paramagnetic intermediate, the stopped-flow-ESR measurements coupled with the field-sweep method were performed for the same reaction solutions of NDS and quercetin. As shown Figure 2a, a strong ESR signal recorded before the stop of flow was assigned to the lower field signal of NDS. After about 45 ms from the stop of flow, the intensity of the central line of NDS was remarkably diminished, and unknown weak ESR signals were also recorded. The higher filed signal of NDS was completely disappeared. As depicted in Figure 2b, the unknown species ($g = 2.0044$) was exclusively recorded at after about 90 ms from the stop of flow. The observed eight lines hyperfine structure was explained to be three sets of doublet splitting. The proton hyperfine coupling constants (hfcc) were evaluated to be 0.076, 0.136, and 0.256 mT, respectively. Based on the *g*- and hfcc values, the unknown species is assigned to be the *o*-semiquinone anion radical of quercetin B-ring (Figure 2).[14]

The time-sweep stopped-flow ESR measurements were made by the same reaction condition, for the both NDS and the quercetin radical. As illustrated in Figure 2c, the concentration of NDS quickly diminished and completely disappeared within about 100 ms. After the trial and error curve fitting, the apparent second order rate constants defined for the decay of NDS was evaluated to be $\approx 2 \times 10^3\ M^{-1}\ s^{-1}$. While the concentration of the quercetin radical reached to the maximum (15 μM) during about 110 ms, and gradually decreased with time course. Such a low concentration of the radical is indicative that the rate of formation must be much slower than the rate of decay.[5] In addition, the concentration of the radical was too low to be detected by UV–vis method, if its absorption coefficient were comparable to those of quercetin.[4] These findings provide that the combination of stopped-flow-optical and -ESR methods is a useful tool to detect short-lived intermediates involved in the antioxidant action of naturally occurring substances.

With combining the results of the stopped-flow-optical measurements, the quercetin-derived quinone is thought to be the most probable reaction product derived from the radical.[3] The quercetin radical may be changed to its quinone form not only by re-oxidation with NDS,[11] but also through the disproportionate[5] reaction of the radical. Consequently, the quercetin radical is concluded to be a transient intermediate species for formation of quinone derivatives of quercetin. Further kinetic and mechanistic investigations on the quercetin radical are now in progress.

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References and Notes

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