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Hydroethidine as a probe for measuring superoxide formation rates during air oxidation of myricetin and quercetin

Yi Ling Quek, Dejian Huang *

Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore

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ABSTRACT

In the presence of the flavonols myricetin and quercetin, oxidation of hydroethidine (HE) by superoxide yielded ethidium (E^+) instead of 2-hydroxyethidium (2-OH-E^+). As a known pro-oxidant, myricetin alone was also found to be able to catalyze air oxidation of HE yielding exclusively E^+. The reaction is inhibited by added superoxide dismutase, suggesting that superoxide is involved in the rate limiting step of the oxidation.

Fluorescent probes capable of sensing reactive oxygen species (ROS), particularly hydrogen peroxide and superoxide, are needed because of the critical importance of these ROS in biological systems.[1,2] To delineate the mechanisms of reactive oxygen species, the availability of selective probes targeting specific ROS is important. For the past 40 years, 2,7-dichlorodihydrofluorescein has been commonly used as a redox sensitive probe; its usage however has been controversial due to its lack of selectivity.[3]

Although there are fluorescent probes available which are highly selective for H_2O_2 sensing,[4,5] detection and quantification of $O_2^{\cdot-}$ has been proven, time and again, a nontrivial task. Fe(III)–cytochrome c, lucigenin and luminol as well as hydroethidine are such examples. Being a mild reductant ($E^o = -0.137$ V),[6] $O_2^{\cdot-}$ reduces Fe(III)–cytochrome c to its ferrous counterpart; this was initially applied in the quantification of superoxide and is still used as a standard assay in quantifying the activity of superoxide dismutase.[7] However, reductants such as polyphenolic compounds can lead to false positive results by reducing Fe(III). Lucigenin and luminol, the once frequently used nitro-blue tetrazolium (NBT^{2+})[8] and chemiluminescent probes, have similar drawbacks and do not provide reliable results.[9]

On the other hand, $O_2^{\cdot-}$ oxidation of hydroethidine (HE) to ethidium (E^+) provided a seemingly attractive alternative due to there being no interference reported from reductants often present in the biological sample matrix. It was thus suggested to be suitable for measuring $O_2^{\cdot-}$ in cell lines.[9] Indeed, HE was used as the probe of choice for assaying $O_2^{\cdot-}$ over the last decade. However, the discovery that the oxidation product of HE was 2-OH-E^+ and not E^+ has put past studies that utilized HE into question.[10] 2-OH-E^+ is now regarded as the sole oxidation product due to $O_2^{\cdot-}$ and its concentration is suggested to be an indication of $O_2^{\cdot-}$ concentration in a wide range of sample types including soils and animal tissues.[11,12] It was later found that HE is an unsuitable probe for quantifying intracellular superoxide due to many interfering factors.[13] We report herein that in the presence of polyphenolic antioxidants, the oxidation product of HE by $O_2^{\cdot-}$ is solely E^+, instead of 2-OH-E^+. Our results show that E^+, instead of 2-OH-E^+, is a good indicator of the superoxide activity promoted by air oxidation of myricetin.

Polyphenolic compounds are known to exert pro-oxidant activity through the generation of free radicals.[14–16] Indeed, mixing equimolar amounts of myricetin (50 μM) and HE in pH 7.40 phosphate buffer for 2 h at 37 °C, led to clean formation of E^+ with no detectable amount of 2-OH-E^+, as revealed by HPLC analysis (Fig. 1). Addition of KO_2 to the HE solution under the same conditions yielded mainly 2-OH-E^+ with E^+ as the minor product (Fig. 1). Yet, when equal molar amounts of the flavonols, myricetin and quercetin were mixed with HE immediately before the addition of excess KO_2, E^+ was the only observed product (Scheme 1). The progress of the reaction can be conveniently monitored by studying the fluorescence signals of E^+. In the presence of a large excess of HE, the reaction was first order with respect to myricetin concentrations (Fig. 2). The plot of the rate of E^+ formation versus the concentration of myricetin gave a straight line and the slope was the apparent rate constant of E^+ formation. To test if

* Corresponding author. Tel.: +65 6516 8821; fax: +65 6775 7895.
E-mail address: chmhdj@nus.edu.sg (D. Huang).

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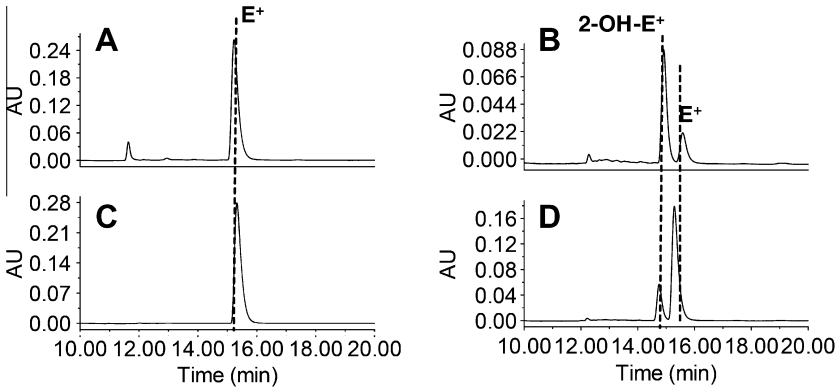
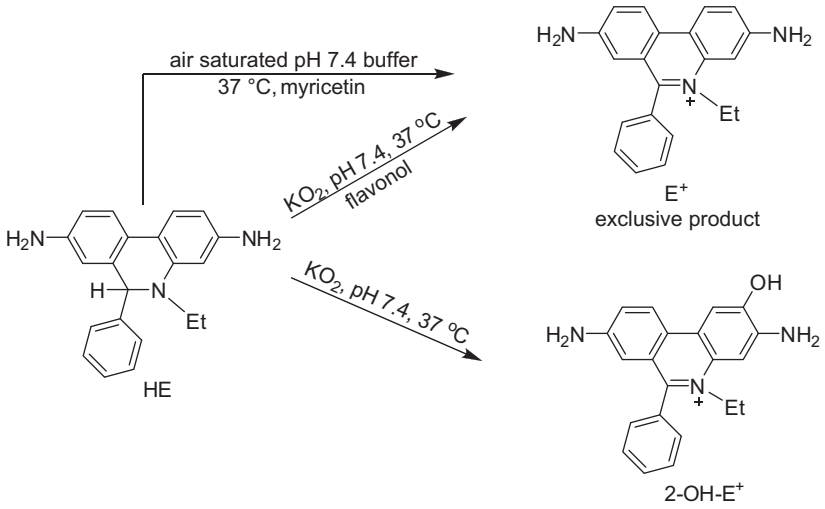


Figure 1. HPLC chromatograms of E^+, a potassium superoxide induced oxidation product of HE and the product formed from oxidation of HE by myricetin. (A) Mixtures of HE (50 μM) and myricetin (50 μM) in phosphate buffer (pH 7.4). (B) HPLC trace of authentic E^+ (50 μM) in phosphate buffer (pH 7.4). (C) Solution containing HE (50 μM) and KO_2 (excess) in phosphate buffer (pH 7.4). (D) Same as in (C) but spiked with authentic E^+ (50 μM). The detector was set at 290 nm.



Scheme 1. Reaction products of HE with superoxide anion in the presence or absence of myricetin or quercetin.

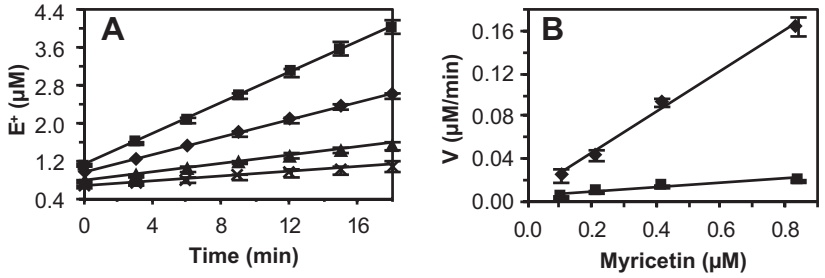


Figure 2. Kinetics of the HE/myricetin reaction in phosphate buffer (pH 7.4) monitored by fluorescence detection at λ_{ex} = 485 nm, λ_{em} = 645 nm. (A) Kinetic traces with [HE] = 25.6 μM and variable myricetin concentration (■: 0.833 μM; ♦: 0.417 μM; ▲: 0.208 μM; ×: 0.104 μM). (B) Oxidation rate of HE as a function of myricetin concentration in the absence (♦) and presence (■) of SOD (3 U/mL). n = 3.

$O_2^{\cdot-}$ was involved in the oxidation, superoxide dismutase (SOD) was first mixed with hydroethidine before myricetin was added; in the presence of SOD the oxidation rate decreased dramatically (Fig. 2B). This result clearly demonstrated involvement of $O_2^{\cdot-}$. It is also apparent from Figure 2 that the formation of E^+ was much greater with respect to the amount of myricetin added, indicating that myricetin acted as a catalyst for the reaction. This was verified independently by monitoring the decomposition kinetics of myricetin using UV–vis absorbance at 376 nm (Fig. 3A). Indeed, myricetin decomposition was slowed down in the presence of HE, indicating that myricetin was regenerated during HE oxidation. The best turn over number we measured was about 9 when 6.25% (mol) of myricetin was used to catalyze the oxidation. It is known that myricetin can generate free radicals under basic conditions due to deprotonation of myricetin to its phenolate, which is more sensitive to oxidation.[14] We also observed that the HE oxidation rate was highly pH dependent (Fig. 3B). The midpoint of this curve was found at pH 7.53, which is close to the reported pK_{a1} of myricetin (7.73).[17] Consistently, we observed similar trends of pH dependency on HE oxidation. The increasing pH value leads to increasing concentrations of the phenolates, and hence, increasing rates of radical formation through single electron reduction of

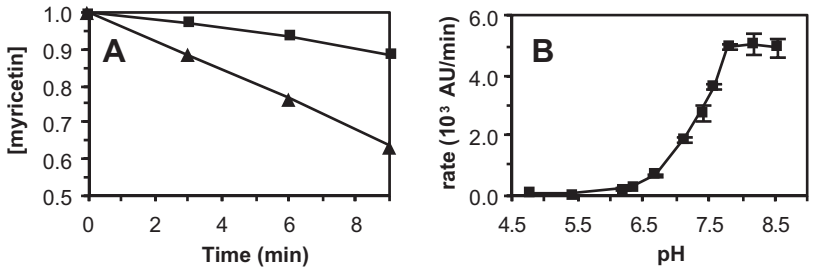


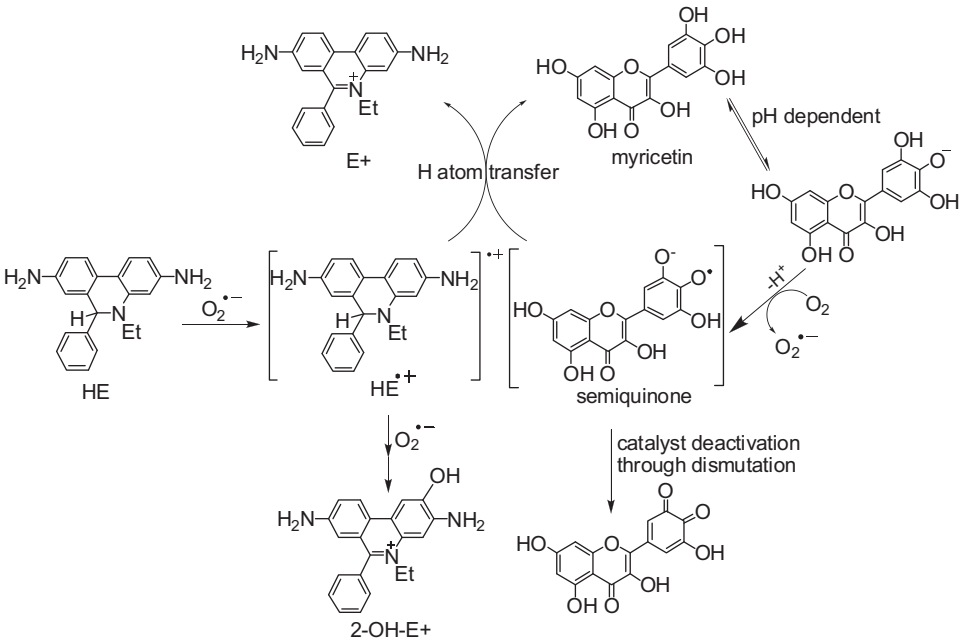
Figure 3. (A) Normalized myricetin concentration at 376 nm in the absence (▲) and presence (■) of HE (30 μM) in phosphate buffer (pH 7.4); initial [myricetin] = 7.5 μM. (B) pH dependency of HE oxidation by myricetin expressed as the increase in rate of the fluorescence signal at 37 °C, buffered solution.

dissolved molecular oxygen. It is reported that the rate of polyphenol autoxidation is highly sensitive to the pH of the medium studied.[14] We measured the oxidation potential (E_{pa}) of myricetin at pH 7.40 and 5.50 to be +0.030 and +0.179 V, respectively, using a three-electrode cyclic voltametry system made up of a glassy carbon working electrode, a platinum counter electrode and a silver/silver chloride reference electrode. Therefore, deprotonation of myricetin enables it to react with dissolved molecular oxygen.

Taken together, we propose a reaction mechanism for the myricetin-catalyzed oxidation of HE as follows (Scheme 2). The reaction of HE with the superoxide generated from the autoxidation of a myricetin anion leads to $HE^{\cdot+}$ as suggested by Zielonka et al.[18] $HE^{\cdot+}$ may react with another superoxide to form 2-OH-E^+.[19] Alternatively, a myricetin semiquinone radical may abstract a hydrogen atom from $HE^{\cdot+}$ and regenerate myricetin and form E^+. The reaction cycle stops when myricetin is irreversibly oxidized to myricetin quinone, which does not react with HE, as we found independently.

The formation of E^+ as the sole product of HE air oxidation promoted by flavonols makes HE ideal for quantifying radical generating pro-oxidation activity of flavonols. The dose response relationship between HE oxidation and the flavonol concentration provides a highly sensitive method to quantify their pro-oxidant activity. Under the assay conditions, the HE oxidation rate is pseudo first order with respect to the flavonol concentration; the apparent rate constant, k', of the reaction can be defined as the radical generating pro-oxidant activity. As shown in Table 1, the pro-oxidant activity increases with the increasing number of hydroxy groups on the B-ring, with myricetin being the most active. In contrast, galangin and kaempferol have no detectable pro-oxidant activity. This shows that the *ortho*-trihydroxy moiety on the B-ring is critical for the high radical forming pro-oxidant activity of myricetin. Our results are consistent with the earlier study by Canada et al. and this enhances the validity of HE as a probe for rapid and convenient quantification of radical forming pro-oxidant activity.[14] The pro-oxidant activity correlates with the pK_a values of myricetin and quercetin which fall in the range of 7.95–6.05 (Table 1). At pH 7.40, there are significant amounts of deprotonated flavonols for all four compounds, but their reactivity with oxygen is apparently different, as revealed by their oxidation potentials (E_{pa}), which increase from myricetin to galangin. The oxidation potentials at pH 5.50 (due to phenols) are all much higher than that at pH 7.40 (for phenolates), accounting for the lack of pro-oxidant activity under weak acidic conditions, which is typically the case in plant tissues where these polyphenolic compounds are normally found. The pH switch, after these compounds enter the small intestine of animals and in the bloodstream, will activate the radical generating pro-oxidant activity of these compounds. The toxicology effects of such reactivity switches are worthy of further study.

In summary, we have demonstrated that HE is oxidized to E^+ due to the pro-oxidative effect of flavonols under physiological conditions and is a sensitive fluorescent probe for rapid quantification of the radical-forming activity of polyphenolic compounds. Structurally, the number of hydroxy groups on the B-ring of flavonols has a great impact on their pro-oxidant activity. We are



Scheme 2. Proposed reaction mechanism for the oxidation of HE in the presence of myricetin.

Table 1
pK_{a1}, oxidation potential, and pseudo-first order rate constants of the oxidation of flavonols by oxygen at 37 °C, pH 7.40

Flavonol	k' (min^{-1})	pK_{a1}	Oxidation potential (V, vs Ag/AgCl)	
			pH 5.50	pH 7.40
Myricetin	0.194 ± 0.015	7.95	0.179	0.030
Quercetin	0.031 ± 0.001	6.05	0.256	0.089
Kaempferol	Not detectable	6.89	0.324	0.158
Galangin	Not detectable	7.78	0.500	0.388

positioned to explore the application of HE as an ultrasensitive probe in quantifying the pro-oxidant activity of common beverages that are known to generate hydrogen peroxide as observed.[20]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.08.044.

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