Note

Inhibition of Myeloperoxidase-catalyzed Tyrosylation by Phenolic Antioxidants in vitro*

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We have developed an in vitro assay system for the evaluation of the inhibitory effects of phenolic antioxidants on myeloperoxidase (MPO) activity. The formation of dityrosine from the MPO/H2O2-L-tyrosine system was used as an indicator of the MPO activity. Because the buffer system used does not include chloride ion, this assay has the advantage of exclusion of direct reaction between an antioxidant and HOCl. In this assay, ferulic acid, gallic acid, and quercetin strongly inhibited the dityrosine formation, and curcumin and caffeic acid were also effective.

Key words: myeloperoxidase; dityrosine; phenolic antioxidants; polyphenol

Active oxygen species may contribute to the development of diseases. Among these species, oxidants derived from activated neutrophils or macrophages have been of significant interest. One of the oxygen species, hypochlorite (HOCl), is generated by the neutrophil myeloperoxidase (MPO, EC 1.11.1.7) in the presence of hydrogen peroxide (H2O2) and chloride ion, and attacks bacteria to protect our lives (Equation 1).

\[ \text{H}_2\text{O}_2 + \text{Cl}^- + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O} \]  

(Eq. 1)

On the other hand, the excess activation of the immune system during inflammation possibly causes host cell/tissue damage via harmful oxidants. Some specific products like 3-chlorotyrosine, which may become fingerprints of neutrophil-derived damage, are formed by the treatment of proteins with HOCl and its related chlorination species. MPO can also catalyze tyrosyl radical (Tyr') formation and two tyrosyl radicals can interact and then, as the major fluorescent product, dityrosine (Tyr-Tyr) is formed (Equation 2).

\[ \text{Tyr'} + \text{Tyr'} \rightarrow \text{Tyr} - \text{Tyr} \]  

(Eq. 2)

The tyrosyl radical formed can cause lipid peroxidation, tyrosylation of high density lipoprotein (HDL), and the cross-linking of proteins. The contribution of MPO to some diseases, like inflammatory diseases, has been assumed. The inhibition of MPO activity from excess activation of neutrophils during acute and/or chronic inflammation might prevent tissue damage. In our study, we examined the inhibitory effects of phenolic antioxidants based on the formation of dityrosine by the novel method.

Reaction conditions. The protocols using dityrosine generation as MPO activity are shown in Fig. 1. Almost all the antioxidants were obtained from commercial sources. Epigallocatechingallate (EGCG) and epicatechingallate (ECG) were gifts from Mitsui Norin Co., Ltd. (Tokyo, Japan). Curcumin was prepared from turmeric, which was a gift from the Daiwa Kasei Co., Ltd. (Saitama, Japan), as described previously. MPO from human sputum was purchased from Elastin Products Co., Inc. The assay was done as follows. MPO (1.1 units/ml), H2O2 (0.2 mM), and L-tyrosine (0.2 mM) were incubated in 0.1 M phosphate buffer (pH 7.4) containing 0.1 mM DTPA in the absence or presence of antioxidants (final concentration, up to 0.2 mM). The antioxidants were dissolved in ethanol, and 100 µl of the ethanol solution was added to 900 µl of the MPO/H2O2/L-tyrosine solution. Instead of ethanol, dimethyl sulfoxide is also available for a vehicle (solvent of antioxidants). In the case of β-carotene, intact emulsified ethanol solution was added to the reaction.

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Abbreviations: MPO, myeloperoxidase; HOCl, hypochlorite; DTPA, diethylenediaminetetraacetic acid; HPLC, high pressure liquid chromatography
mixture. The reaction mixture was incubated at 37°C for 1 h. In addition, the incubation of l-tyrosine with H₂O₂, in the absence of MPO, hardly generated the dityrosine (0.4% generation compared to the complete MPO/H₂O₂/l-tyrosine system). For the purpose of the decomposition of residual H₂O₂, catalase (final conc. 0.025 mg/ml) was added to the reaction mixture. The solution was further incubated at room temperature for 10 min, transferred to a centrifugal filtration tube, Ultrafree MC (Millipore), and filtration was then done at 8000 rpm for 5 min at 4°C. The filtrate was directly injected into a high pressure liquid chromatograph (HPLC) connected to a fluorescence detector (Shimadzu RF-535). Develosil ODS-HG-5 (4.6 × 150 mm) was used as the column, which was equilibrated with 0.5% acetic acid/methanol (29/1, v/v) at a flow rate of 0.8 ml/min. The detection of dityrosine was done by measuring the fluorescence intensity (ex. 300 nm, em. 400 nm). The amount of dityrosine was estimated by comparison with authentic dityrosine, which had been already prepared and identified by previous data. The spectral data are as follows: ¹H-NMR (D₂O) (ppm) 2.95 (dd, 2H, J = 14.66 & 7.72), 3.10 (dd, 2H, J = 14.66 & 5.20), 3.83 (dd, 2H, J = 7.72 & 5.20), 6.86 (d, 2H, J = 8.29), 7.00 (d, 2H, J = 1.89), 7.09 (dd, 2H, J = 8.29 & 1.89); LC/MS (ESI⁺), m/z 361 (M + H)⁺; FAB/MS, m/z 361 (M + H)⁺.

**MPO-inhibition assay.** As an example of a typical phenolic antioxidant, p-coumaric acid was first used (Fig. 2). In the absence of p-coumaric acid, the fluorescent compound was detected and the retention time agreed with that of the synthetic dityrosine. The dityrosine formation decreased with the increasing concentration of p-coumaric acid. Under our conditions, 50% inhibition was observed at about a 0.1 mM concentration (data not shown). Next, using the concentration of 0.1 mM (50% inhibition by p-coumaric acid), the inhibitory effects of various antioxidants were examined. As shown in Fig. 3, ferulic acid, quercetin, and gallic acid completely inhibited the dityrosine formation. The IC₅₀s are approximately 30 μM (quercetin) and 40 μM (ferulic acid and gallic acid). On the other hand, endogenous radical scavengers, including α-tocopherol, β-carotene, glutathione, ascorbic acid, and lipoic acid, could not inhibit the dimerization. Since many strong antioxidants, like α-tocopherol, reduced glutathione, and ascorbic acid could not inhibit the dityrosine generation, the “antioxidative” activity of the effective polyphenols such as gallic acid and quercetin has little relation to the inhibition of the MPO-derived tyrosylation.

The modification of tyrosine by MPO produces dityrosine, 3-chlotyrosine, 3-nitrotyrosine, and p-hydroxyphenylacetic acid. In this study, for the evaluation of the inhibitory effect of the myeloperoxidase activity, we measured the dityrosine formation using the HPLC-fluorescence detector. This method excludes counts of the modified antioxidant-derived fluorescence as dityrosine generation. It has been reported that raloxifene, a selective estrogen receptor modulator, inhibited MPO-dependent tyrosyl radical

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**Fig. 1.** Assay Protocol for Evaluation of Dityrosine Formation from Myeloperoxidase/H₂O₂/l-Tyrosine System.

**Fig. 2.** HPLC Chart for Dityrosine Generated by Myeloperoxidase/H₂O₂/l-Tyrosine System.
Fig. 3. Effects of Various Antioxidants on Dityrosine Generation from Myeloperoxidase/H$_2$O$_2$/L-Tyrosine System.

The experimental diagram is shown in Fig. 1. The antioxidant at a concentration of 0.1 mM was added before the hydrogen peroxide application. The data were calculated and expressed as a relative ratio by comparison of the amount of dityrosine in the presence of the sample to that in the absence of an antioxidant. The index (relative ratio) below 1.0 means the suppression of MPO activity.

formation, which was evaluated by fluorometry. However, the raloxifene-derived fluorescence might interfere with the assay using a fluorometer.

In our system, HOCl or its related species can't be generated because of the omission of chloride ion from the reaction buffer. This omission of chloride ion gives an advantage of exclusion of direct reaction/quench between an antioxidant and HOCl. On the other hand, the MPO-inhibitory activity evaluated by the assay may not reflect its physiological performance. Because HOCl can't contribute to dityrosine generation in our system, the "targets" of inhibitory action by antioxidants are a MPO itself and/or generated tyrosyl radicals. Though the concentration of L-tyrosine (0.2 mM) is higher than that of an antioxidant, some antioxidants inhibited the dityrosine formation completely (Fig. 3). This result indicates that the simple competitive inhibition (substrate replacement) and/or quenching of generated tyrosyl radicals can't explain the inhibition mechanism. This suggests that the polyphenols may react directly with the enzyme. The mechanism remains to be identified.

MPO has two transformations (compounds I and II) and the changes are important for enzyme activity. Phenolic antioxidants may interact with the transformation. The active site for tyrosyl radical generation in a MPO molecule may be occupied by an antioxidant.

The inhibition mechanism is unknown and, moreover, the in vivo action of plant polyphenols after food intake is still unclear. We have also reported that cyanidin 3-O-β-D-glucoside (C3G) attenuates the hepatic ischemia-reperfusion injury. The in vivo model showed that a tissue MPO activity during reperfusion periods was suppressed by C3G administration before ischemia. To make clear the inhibitory actions of phenolic antioxidants, more detailed studies are needed. In summary, we estimated a novel MPO inhibition assay and found that some phenolic antioxidants are better inhibitors than the internal (endogenous) antioxidants, at least, at the same dose.

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References


9) Sampson, J. B., Ye, Y.-Z., Rosen, H., and Beckman, J. S., Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and...
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Myeloperoxidase Inhibition Assay


