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Oxidative coupling of epigallocatechin gallate amplifies antioxidant activity and inhibits xanthine oxidase activity

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Oxidative coupling of epigallocatechin gallate resulted in great improvement in antioxidant activity such as radical scavenging activity against superoxide anion and in activity to inhibit xanthine oxidase, offering high potential as a therapeutic agent for prevention of xanthine oxidase-induced diseases such as gout.

Hydrogen peroxide, hydroxyl radicals, peroxide anions and superoxide anions are generally known as reactive oxygen species (ROS) inducing aging and many kinds of diseases such as atherogenesis and carcinogenesis.[1] Xanthine oxidase (XO) is not only an important biological source of ROS but also the enzyme responsible for the formation of uric acid associated with gout leading to painful inflammation in the joints.[2] Thus, antioxidation and XO inhibition are an important pharmacological action. Antioxidants possessing both ROS scavenging and XO inhibition activity could be beneficial as protective agents in a number of diseases related to ROS and/or XO. Green tea catechins have been recognized as attractive functional compounds due to their pathologically beneficial bioactivity;[3] they belong to the flavonoids, one of the most numerous and best-studied groups of plant polyphenols, and are found in the most extensively consumed beverage worldwide. Epigallocatechin gallate (EGCG) (Fig. 1) is a major ingredient of green tea possessing powerful antioxidant activity and cancer-chemopreventive activity due to the actions of radical scavenging, enzyme inhibition and metal chelation.[4] These activities are attributed to the many hydroxyl groups of the EGCG molecule and/or hydrophobic interaction between EGCG and enzymes. Moreover, high-molecular fractions of extracted polyphenols from natural plants including procyanidin were demonstrated to exhibit greater antioxidant activity and anticancer activity correlating with the degree of polymerisation, as well as no pro-oxidation effect.[5] Therefore, we believe that one promising way to achieve the enhanced antioxidant activity and the inhibitory effect against enzymes is by construction of a polymeric structure of flavonoid compounds. We have previously reported that the enzyme-catalyzed polymerization of flavonoids such as catechin and rutin improved their abilities to scavenge superoxide radical and to inhibit the peroxidation of human low density lipoprotein induced by a free radical generator, compared to each monomer.[6,7] In addition, the poly(catechin) showed great amplification of XO inhibition compared to the monomeric catechin, although the effect is not yet enough for a useful therapeutic agent.

We report herein the amplification of antioxidant and XO inhibitory activities of an enzymatically synthesized oligomer of EGCG,[8]† aiming at offering the opportunity of a therapeutic agent for prevention of various free radical and/or enzyme-induced diseases.

The antioxidant activity of oligo(epigallocatechin gallate) (OEGCG) was evaluated in terms of superoxide anion scavenging ability.‡ Superoxide anions are well known to be generated by a xanthine/XO system. They can damage biomacromolecules both directly and indirectly by forming hydrogen peroxide or reactive hydroxyl radical.[9] The superoxide scavenging activity of EGCG, OEGCG, catechin, poly(catechin) (PC),[6] dibutylhydroxytoluene (BHT) and Trolox (a hydrophilic analogue of α-tocopherol) was expressed by IC_{50} (the concentration of each monomeric unit needed to scavenge superoxide by 50%) as shown in Table 1. OEGCG showed greatly amplified scavenging activity on an EGCG unit level, compared with monomeric EGCG. The activity was also much higher than those observed for the commercial antioxidants (BHT and Trolox), catechin and PC, even though PC also showed amplified scavenging activity compared with monomeric catechin.

Fig. 2 shows XO inhibition activity assessed by evaluating uric acid formation from XO§ and calculated on a base of the monomeric unit. The XO inhibition effect of EGCG monomer was quite low with inhibition less than about 5% over a range of tested concentrations. In contrast, OEGCG showed a greatly amplified XO inhibition effect in a concentration dependent manner. Moreover, the inhibition of OEGCG was higher than that of allopurinol, a frequently used commercial inhibitor for gout treatment.[10] These results demonstrate that the EGCG oligomer is one of the leading candidates as a therapeutic molecule against various diseases induced by free radicals and/or enzymes including gout. Although the XO inhibitory effect of OEGCG was smaller

Table 1 Superoxide anion scavenging activity[a] (n = 3)

Sample	Superoxide scavenging activity IC_{50}/μM
EGCG	59.0 ± 4.7
OEGCG	12.7 ± 1.4
Catechin	>> 200
PC	92.7 ± 8.7
BHT	>> 200
Trolox	155 ± 7.8

[a] Superoxide scavenging activity was evaluated by a chemiluminescence method.

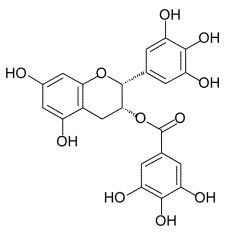

Fig. 1 Chemical structure of epigallocatechin gallate.

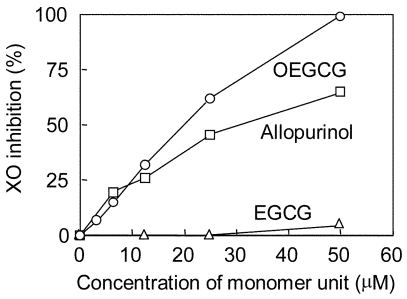

Fig. 2 XO inhibition of EGCG oligomer.

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than that of superoxide anion scavenging in the low concentration of OEGCG, the inhibition effect of OEGCG on the chemiluminescence growth may result from both superoxide anion scavenging and XO inhibition. This suggests that the IC_{50} value of superoxide anion scavenging of OEGCG may be underestimated, even though the capacity of OEGCG to scavenge superoxide anion is still much larger than that of EGCG.

To elucidate the mechanism of XO inhibition by OEGCG, steady-state analysis of XO was performed in which the concentrations of xanthine and OEGCG were varied systematically (Fig. 3). It was found that OEGCG is an uncompetitive inhibitor of XO with respect to xanthine as a substrate because the lines at different concentrations of OEGCG in the Lineweaver–Burk plot are parallel. Similar behaviors were observed for other inhibitors of XO.[11] The detailed mechanism of inhibition by OEGCG will be examined for elucidation of the high inhibition activity for XO.

Formation of nano-structured complexation between XO and OEGCG was revealed by dynamic laser scattering (DLS) measurements. Fig. 4 shows the particle size changes of EGCG and OEGCG in a mixture with XO as an increasing concentration of each sample. The particle size in the mixture of OEGCG with XO increased with increasing concentration of a monomeric unit due to formation of complexes between OEGCG and XO. In contrast, the particle size in the mixture of EGCG with XO was consistent with that in the solely XO solution without a change in a range of tested concentrations. Khan *et al.* have reported that a complex formed through hydrophobically driven binding of EGCG with peptides in mM concentration.[12] From our results, EGCG was unable to form complexes at low concentrations of μM level. However, the effective chain length of OEGCG facilitated the complex formation with XO in even such a low concentration maybe because of multivalent interaction between the oligomeric chain and XO. It is considered that the strong complex formation by OEGCG primarily results in powerful XO inhibition.

In conclusion, an enzymatically synthesized oligomer of EGCG showed much greater superoxide scavenging and XO inhibitory activity than EGCG monomer. Also, these activities are higher than those of commercial antioxidants or a therapeutic agent for gout treatment. These results implied that OEGCG is a highly potent therapeutic agent to prevent a wide range of free radical-induced and/or enzyme-related diseases.

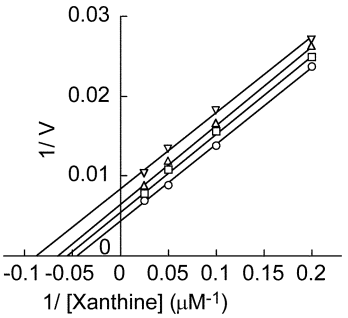

Fig. 3 Steady-state analysis of XO inhibition by oligo(EGCG). The results are presented as a Lineweaver–Burk plot and show an uncompetitive type of inhibition. Oligo(EGCG) concentrations used were (from bottom to top) 0, 1.25, 2.5, and 4 μM.

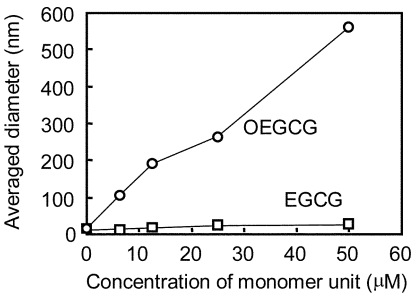

Fig. 4 Diameter of complexes between OEGCG and XO.

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Notes and references

† OEGCG was synthesized and characterized by slight modification of a method used for poly(rutin).[7] EGCG (50 mg) and laccase derived from *Myceliophthora* (5 units) in 5 mL of 0.1 M acetate buffer solution (pH 5) were placed in a 50 mL flask. The polymerization reaction was carried out at room temperature under air. After 24 h, the mixture was subjected to purification by dialysis (cut-off molecular weight = 1×10^3). The dialysis solution was changed three times. The remaining solution was lyophilized to give the oligomer quantitatively. Molecular weight was estimated by size exclusion chromatography (SEC, Tosoh GPC-8020 equipped with RI-8020 detector) with two TSKgel α-M columns using DMF containing 0.10 M LiCl as eluent, after acetylation: M_n = 4200; M_w/M_n = 1.8. The calibration curves for SEC analysis were obtained using polystyrene standards.

‡ Superoxide anion was generated by xanthine/xanthine oxidase (XO) and measured by a chemiluminescent superoxide probe method.[13] The chemiluminescence (CL) intensity of 2-methyl-6-*p*-methoxyphenylethynylimidazopyrazinone (MPEC) triggered by superoxide anion was measured in a 100 mM potassium phosphate buffer solution (pH 7.5) containing 0.05 mM EDTA, 0.04 unit ml^{-1} of XO from butter milk, MPEC (10 μM, ATTO Co. Ltd., Japan) and a test sample. Light emission was started by the addition of 0.3 mM of xanthine. CL spectra were monitored for 30 s using a Corona Microplate Photoncounter, MTP-700CL (Corona Electric Ltd., Japan). Superoxide anion scavenging activity was calculated according to the following formula:

$$\text{Superoxide scavenging activity (\%)} = \frac{CL_{control} - CL_{sample}}{CL_{control}} \times 100 \quad (1)$$

where $CL_{control}$ and CL_{sample} represent chemiluminescent intensity in the absence and presence of sample, respectively.

§ The activity of XO was measured spectrophotometrically by monitoring the formation of uric acid from xanthine at 295 nm for 30 min by a UV–visible spectrometer (Hitachi U-2001, Japan).[14] The assay was carried out under the same conditions as the superoxide anion assay mentioned above, and the percentage of activity was calculated.

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