Enzymatic Synthesis and Antioxidant Property of Poly(allylamine)–Catechin Conjugate

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(Received April 23, 2003; CL-030344)

A polymeric conjugate containing a catechin group has been synthesized by laccase-catalyzed oxidation of catechin in the presence of poly(allylamine). The resulting conjugate showed a good antioxidant property against LDL peroxidation induced by a free radical.

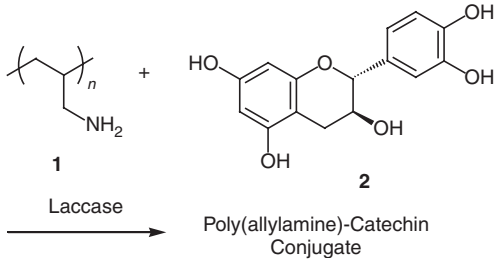
Flavonoids are one of the most numerous and best-studied group of plant polyphenols. Green tea catechins, belonging to the group of flavonoids, exhibit biological and pharmacological effects including antioxidant, anti-mutagenic, anti-carcinogenic, probiotic, anti-microbial and anti-inflammatory properties in numerous human, animal and in vitro studies.[1] These properties are potentially beneficial in preventing diseases and protecting the stability of the genome. Many of these activities have been attributed to antioxidant actions of green tea catechin.[2] However, the activities of flavonoids generally persist for limited short periods in vivo. In addition, several flavonoids have been shown to act as pro-oxidants and generate reactive oxygen species, such as hydrogen peroxide. Tea polyphenols were also reported to have pro-oxidant effects at lower dosages in the aqueous phase.[3] In contrast, a relatively high molecular fraction of extracted flavonoids has been reported to exhibit enhanced physiological properties such as antioxidant and anti-carcinogenic activity, and a relatively longer circulation time in vivo.[1c,4] High molecular weight plant polyphenols have also been reported to show no pro-oxidant effects.[4b,5]

We have designed polymerized flavonoids[6] and polymeric flavonoid-conjugates of various polyamines, in consideration of amplification of physiological properties of the flavonoids and an offer of opportunity of extravascular delivery at targeted solid tumor in vivo.[7] Many investigators have explored the antioxidant effects of low molecular weight flavonoids, but few have considered polymeric flavonoids and polymer conjugates of covalently bound flavonoids.

Recently, in vitro polymer production through enzymatic catalysis has received much attention as new methodology of polymer syntheses.[8] Tyrosinase catalyzed a coupling of various phenols on chitosan to produce functional materials based on the biopolymer.[9] During the reaction, unstable *o*-quinones are formed from phenols, followed by the reaction with amino group of chitosan to give the modified chitosan. However, the structure is very complicated; thus, the reaction mechanism and the actual linkages have not clearly been demonstrated.

We report herein laccase-catalyzed synthesis of a conjugate of catechin (**2**) onto poly(allylamine) (**1**), one of the most popular polyamines, and antioxidant properties of the resulting conjugate (Eq 1). Polyamines have demonstrated to inhibit growth of cancer cells and reduce tumors in animals and humans.[10] A hydrogel of crosslinked poly(allylamine) (RenaGel®) was found to be an effective and safe dietary phosphate binder in patients on hemodialysis. It also reduced total and low-density lipoprotein (LDL) cholesterols.[11] The present study is the first example that laccase catalyzed synthesis of a conjugate of flavonoid onto a polyamine by oxidative coupling. The antioxidant capacity of the conjugate was examined on inhibition effect against LDL peroxidation induced by a free radical. The conjugate of poly(allylamine)-catechin may offer improved physiological properties and/or controlled biodistribution of flavonoid.

In this study, laccase derived from *Myceliophthora* was used as a catalyst for the conjugation. The reaction of **1** having molecular weight $= 2 \times 10^4$ (10 mmol of monomer unit) with **2** (5 mol% for **1**) was carried out using the laccase catalyst (2×10^{-3} units) in an aqueous methanol (15 mL) at room temperature under air. By the addition of the laccase solution to colorless solution of the substrates, the mixture turned into light orange, and the color became dark brown as the reaction proceeded.



Changes in UV–visible absorbance of the reaction mixture at pH 7 were monitored. A progressive increase in the absorbance at 430 nm was observed over the time,[12] which was not seen in the laccase-catalyzed oxidative coupling of catechin under the similar reaction conditions. These data suggest the conjugate formation between **1** and **2**. After 24 h, the conjugate was purified by dialysis. The introduced ratio of **2** on **1** was determined by elemental analysis as 4.0 mol%, which was somewhat lower than the feed ratio. In ^{1}H NMR spectrum of the conjugate, a broad peak at δ 6.3-7.2 due to the aromatic proton derived from **2** was seen besides large peaks ascribed to **1**.

Figure 1 shows the absorbance increase of the peak at 430 nm in the reaction with different pH. At pH 7, the reaction proceeded the fastest. At pH 8, the absorbance increased till 6 h; afterwards the peak scarcely changed. This may be because the denaturation of the enzyme takes place in the higher pH solution. The slow reaction at pH 6 is probably owing to a very small amount of free amino groups of **1**. In the reaction without the enzyme at pH 7 (control experiment), the absorbance increase was very small, indicating that the present conjugation proceeded via the laccase catalysis.

Peroxidation of LDL leads to its enhanced uptake by macrophages, which is believed subsequently to result in foam cell formation, one of the first stages of atherogenesis. There-

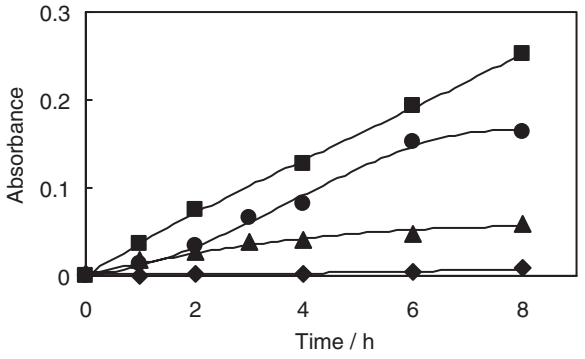


Figure 1. Absorbance changes of peak at 430 nm in the reaction of **1** and **2** using laccase catalyst in an aqueous methanol at room temperature under air: (▲) pH 6; (■) pH 7; (●) pH 8; (◆) pH 7 without laccase.

fore, antioxidants that protect LDL against peroxidation are potentially anti-atherogenic compounds. Although the mechanism for in vivo peroxidation of LDL has not been established, free radical autoxidation may be a factor. In order to evaluate antioxidant effect against peroxidation of LDL, LDL was labelled with diphenyl-1-pyrenylphosphine (DPPP), a fluorescent probe sensing hydroperoxide produced by lipid oxidation. The labelled LDL was preincubated with a sample of antioxidant, prior to oxidation by addition of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). Incubation of AAPH with LDL generates peroxyl radicals, leading to a chain reaction which produces peroxidation products such as hydroperoxides and aldehydes. DPPP, a non-fluorescent molecule, reacts stoichiometrically with hydroperoxide to give diphenyl-1-pyrenylphosphine oxide (DPPP=O), which is strongly fluorescent.[13]

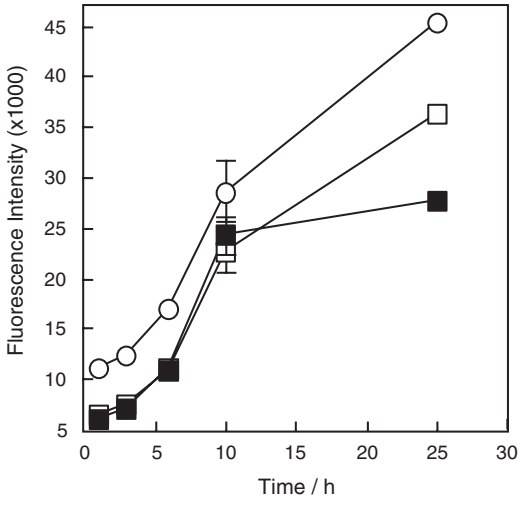


Figure 2. Long-term inhibition effects against LDL peroxidation by catechin (□), poly(allyamine)-catechin (4.0 mol%) conjugates (■) and negative control (○), [catechin]=1.1 μM. Peroxidation of DPPP labelled-LDL solutions containing samples was initiated by AAPH. Fluorescence intensity of DPPP oxide produced by peroxidation of LDL was measured by a spectrofluorophotometer. Wavelengths of excitation and emission were set at 351 nm and 400 nm, respectively.

Interestingly, the inhibition effect of the conjugate against the AAPH induced-peroxidation more effectively lasted for long-term oxidation, comparing to unconjugated catechin (Figure 2). The inhibition effect was in a concentration-dependent manner (data not shown). Poly(allyamine) itself exhibited no inhibitory effect on the LDL peroxidation in this system. This result would be attributed to the favorable molecular structure of the conjugate for preventing intermolecular aggregation of catechins. Moreover, the highly water-soluble poly(allylamine) chains would give catechin greater accessibility to radicals generated in an aqueous system.

In conclusion, poly(allylamine)-catechin conjugate obtained by laccase-catalyzed reactions showed more lasting antioxidant activity against LDL peroxidation induced by AAPH, comparing to unconjugated catechin. The conjugates are expected to be very potent as a therapeutic agent to offer protection against a wide range of free radical-induced diseases.

This work was supported by Program for Promotion of Basic Research Activities for Innovative Bioscience. We acknowledge the gift of laccase from Novozymes Japan Ltd. We are grateful to Professor Yasuhiro Aoyama for the use of the fluorescence spectrometer.

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Published on the web (Advance View) June 17, 2003; DOI 10.1246/cl.2003.620