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Enzymatic synthesis and antioxidant property of gelatin-catechin conjugates

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Abstract

Gelatin-catechin conjugate was synthesized by the laccase-catalyzed oxidation of catechin in the presence of gelatin. The conjugate had a good scavenging activity against superoxide anion radicals. Moreover, the conjugate showed an amplified inhibition effect on human low density lipoprotein oxidation initiated by 2,2′-azobis(2-amidinopropane)dihydrochloride as a radical generator.

Introduction

Catechins are one of main classes of flavonoids and present in tea, wine, chocolate, fruits, etc. They are potentially beneficial to human health as they are strong antioxidants, anti-carcinogens, anti-inflammatory agents, and inhibitors of platelet aggregation in *in vivo* and *in vitro* studies (Jovanovic *et al.* 1994, Rice-Evans *et al.* 1996, Jankun *et al.* 1997, Heim *et al.* 2002). Many of these activities have been attributed to antioxidant actions of catechins.

We have synthesized polymers of flavonoids and polymer-flavonoid conjugates to amplify the physiological properties of the flavonoids (Kurisawa *et al.* 2003a,b, Chung *et al.* 2003). We have reported that poly(catechin), as one of the strategic molecular designs, was synthesized by peroxidase-catalyzed oxidative coupling and exhibited great improvement in radical scavenging activity, protection effects against human low-density lipoprotein (LDL) oxidation and inhibited xanthine oxidase activity, compared with a catechin monomer (Kurisawa *et al.* 2003a). Also, laccase-catalyzed polymerization of rutin produced a water-soluble flavonoid polymer that had greatly amplified scavenging activity against superoxide anion, compared with the rutin monomer (Kurisawa *et al.* 2003b).

This enzyme-catalysed oxidation has also been developed for conjugation of phenols on polymers. Tyrosinase catalyzed a coupling of several phenols including catechin with chitosan to produce functional materials based on the biopolymer (Kumar *et al.* 1999, Chen *et al.* 2000, Wu *et al.* 2002). The formation of a Michael-type adduct and/or Schiff base was proposed during the tyrosinase-catalyzed conjugation of catechin with chitosan. We have found that laccase efficiently induces the conjugation of catechin on polyamines (Chung *et al.* 2003). Furthermore, the enzymatically synthesized poly(allylamine)-catechin conjugate showed a good antioxidant property against LDL oxidation induced by a free radical.

Gelatin is the most widespread water-soluble protein in the body, resulted from partial degradation of water-insoluble collagen. Gelatin has been widely used in food, pharmaceutical, and photographic industries (Veis 1964). Characteristic properties of gelatin including typically low level of immunogenicity and cytotoxicity, great capacity for modification at the level of amino acids, and good biodegradability, have expanded its applications for biomaterials such as hard and soft capsules, sealants for vascular prostheses, and

matrices for drug carrier and three-dimensional tissue regeneration (Nimni *et al.* 1988, Einerson *et al.* 2002). Of the lysine residues in gelatin, 2 or 3% may be available for the enzymatic conjugation of catechin. We have focused our interest on conjugation of catechin onto gelatin using enzymatic oxidation, which may produce a high-functionalized natural biopolymer.

This study deals with enzymatic synthesis and antioxidant properties of a gelatin-catechin conjugate. The conjugate inhibited LDL oxidation initiated by 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) as a radical generator with amplified effect on the catechin molecular level, compared to unconjugated catechin.

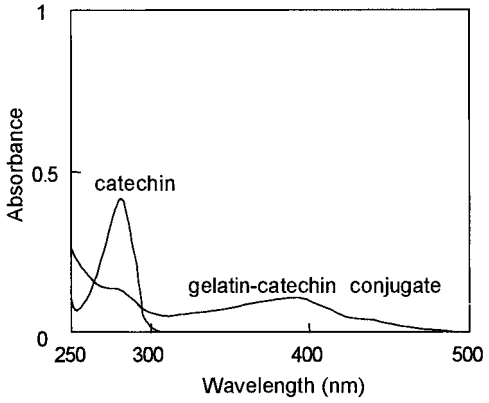

Fig. 1. UV-vis spectra of catechin and the gelatin-catechin conjugate. Catechin molar fraction = 1.7%, [conjugate] = 5×10^{-3} wt %.

Materials and methods

Materials

Catechin was purchased from Tokyo Kasei Co., Japan. Gelatin (PA-100) and laccase were gifts from Nippi Inc., Japan and Novozymes Japan Ltd., Japan, respectively. Low-density lipoprotein (LDL) from human plasma was purchased from Sigma. Xanthine, xanthine oxidase (from butter milk) and 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) were obtained from Wako Pure Chemical Industries, Japan. Diphenyl-1-pyrenylphosphine (DPPP) was purchased from Dojindo, Japan. Other reagents and solvents are commercially available and used as received.

Conjugate synthesis

In air, gelatin (2 g) was dissolved in 40 ml 0.1 M phosphate buffer (pH 7). Catechin (0.12 g) in 5 ml methanol was added. The reaction was started by the addition of laccase solution (10 units) and the mixture was kept at 20 °C with gentle stirring. After 24 h, the reaction mixture was dialysed (cut-off molecular weight = 500 Da). The dialysis solution was changed four times. The remaining solution was lyophilized to give 1 g conjugate.

Superoxide anion scavenging activity

Superoxide anion was generated by xanthine/xanthine oxide and measured by the cytochrome *c* reduction method (McCord *et al.* 1969). Test samples were incubated in a 67 mM phosphate buffer solution (pH 7.6) containing 0.1 mM EDTA, xanthine (66 μM) and cytochrome *c* (0.13 mg ml^{-1}) at 37 °C. The measurement was started with adding xanthine oxidase (6.6×10^{-3} units ml^{-1}). Production of superoxide anion was followed spectrophotometrically at 550 nm for 1 min. Superoxide scavenging activity was calculated according to the following equation.

$$\text{Superoxide scavenging activity (\%)} = \frac{\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}}}{\text{absorbance}_{\text{control}}} \times 100.$$

Determination of oxidized LDL phospholipids

Immediately before oxidation, LDL was dialysed against 1000 times the volume of the degassed 10 mM phosphate buffered saline solution (pH 7.4) containing 2.7 mM KCl and 0.137 M NaCl for 24 h at 4 °C in the dark, with continuous N_2 sparging. The dialysis solution was changed four times. LDL (100 μg ml^{-1}) was incubated with 200 μM (final concentration) of DPPP for 5 min at 37 °C in the dark, under N_2. Before LDL oxidation, the DPPP-labelled LDL was preincubated in the presence or absence of various concentrations (0–80 μM) of an antioxidant sample for 1 h at 37 °C in the dark. Oxidation of LDL preincubated in the presence or absence of a sample was carried out by further incubation with AAPH (1 mM) for 96 h at 37 °C. Oxidation of DPPP was measured at the indicated times using a 1420 $ARVO_{SX}$ multilabel counter (Wallac, Perkin Elmer). Wavelengths of excitation and emission were set at 355 nm and 405 nm, respectively.

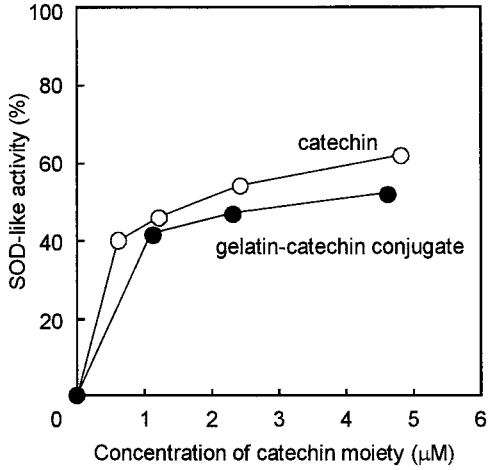

Fig. 2. SOD-like activity of catechin and the gelatin-catechin conjugate. Superoxide anion was generated by xanthine/xanthine oxide and measured by the cytochrome *c* reduction method. Test samples were incubated in a phosphate buffer solution (67 mM, pH 7.6) containing EDTA (0.1 mM), xanthine (66 μM) and cytochrome *c* (0.13 mg ml^{-1}) at 37 °C. The measurement was started with adding xanthine oxidase (6.6×10^{-3} units ml^{-1}). Production of superoxide anion was followed spectrophotometrically at 550 nm for 1 min.

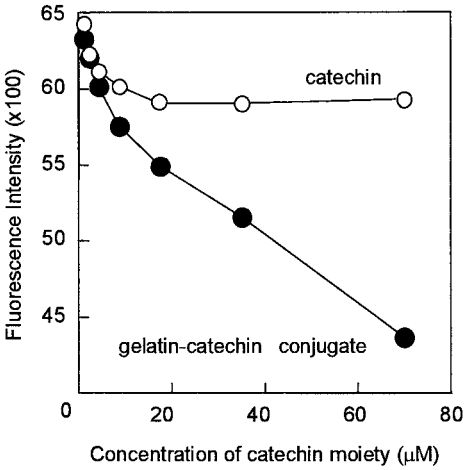

Fig. 3. Inhibition effect of catechin and the gelatin-catechin conjugate against LDL oxidation induced by AAPH (1 mM), $n = 8$. Immediately before oxidation, LDL was dialysed against 1000 times the volume of the degassed phosphate buffered saline solution (10 mM, pH 7.4) containing 2.7 mM KCl and 0.137 M NaCl for 24 h at 4 °C in the dark, with continuous nitrogen sparging. LDL (100 μg ml^{-1}) was incubated with 200 μM (final concentration) of DPPP for 5 min at 37 °C in the dark, under N_2. Before LDL oxidation, the DPPP-labelled LDL was preincubated in the presence or absence of various concentrations (catechin moiety: 0–70 μM) of an antioxidant sample for 1 h at 37 °C in the dark. Oxidation of LDL preincubated in the presence or absence of a sample was carried out by further incubation with AAPH (1 mM) for 1 h at 37 °C.

Results and discussion

The conjugation of catechin on to gelatin successfully proceeded in the presence of the laccase catalyst under air at room temperature to give a pale orange water-soluble conjugate. The ratio of catechin on gelatin was determined by elemental analysis as 1.7 mol%. The conjugate formation was confirmed by UV-vis spectroscopy (Figure 1); there was a characteristic peak due to the conjugated catechin moiety at 390 nm. A similar peak was observed in the catechin conjugate on chitosan or poly(allylamine) (Wu *et al.* 2002, Chung *et al.* 2003). The conjugate displayed water-soluble properties, while catechin has low solubility in water.

The superoxide radical anion is a reactive oxygen species, which is formed during normal aerobic metabolism and by activated phagocytes (Fantone & Ward 1985). Reduction of O_2 to superoxide anion by xanthine oxidase (XOD), generating hydroxyl radicals and uric acid, is an important physiological pathway (Halliwell & Gutteridge 1989). However, superoxide anion damages biomacromolecules both directly and indirectly by forming hydrogen peroxide or highly reactive hydroxyl radicals (Floyd 1990).

A mixture of xanthine and XOD generates superoxide anion, which reduces cytochrome *c* to give the reduced form of cytochrome *c* and increases UV absorbance at 550 nm (McCord & Fridovich 1969). Compounds capable of scavenging superoxide anion, such as superoxide dismutase (SOD), inhibit the reduction of cytochrome *c*. We found that the conjugate showed a good concentration-dependent SOD-like activity, although the activity was slightly inferior to that of intact catechin (Figure 2). Gelatin did not scavenge superoxide anion at all (data not shown). These data clearly indicate that the present enzymatic conjugation confers the SOD-like activity on gelatin.

A good example of the significance of oxidative modification of protein *in vivo* has been reported for LDL, where modification by oxidation was associated with the pathogenic process of atherogenesis (Steinberg *et al.* 1989). Although the mechanism for *in vivo* oxidation of LDL has not been established, free radical autoxidation may be a factor. We examined the antioxidant effect of the conjugate against LDL oxidation initiated by the free radical generator 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH). In order to evaluate antioxidant effect against phospholipid oxidation 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

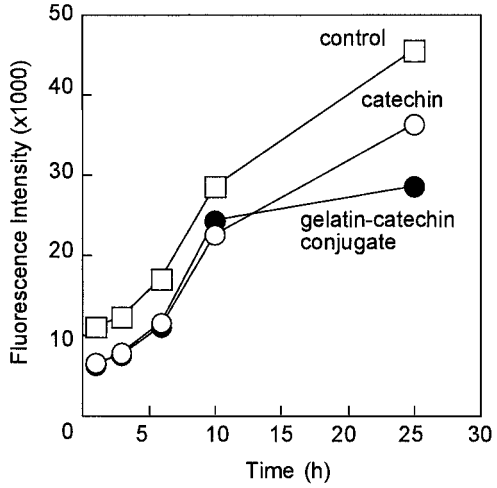

Fig. 4. Long-term inhibition effects against LDL oxidation by catechin and the gelatin-catechin conjugate, [catechin moiety] = 1.1 μM, $n = 8$. Immediately before oxidation, LDL was dialysed against 1000 times the volume of the degassed phosphate buffered saline solution (10 mM, pH 7.4) containing 2.7 mM KCl and 0.137 M NaCl for 24 h at 4 °C in the dark, with continuous nitrogen sparging. LDL (100 μg ml^{-1}) was incubated with 200 μM (final concentration) of DPPP for 5 min at 37 °C in the dark, under N_2. Before LDL oxidation, the DPPP-labelled LDL was preincubated in the presence or absence Before LDL oxidation, the DPPP-labelled LDL was preincubated in the presence or absence of an antioxidant sample for 1 h at 37 °C in the dark. Oxidation of LDL preincubated in the presence or absence of a sample was carried out by further incubation with AAPH (1 mM) for 25 h at 37 °C.

a sample of antioxidant, prior to oxidation by addition of AAPH. Incubation of AAPH with LDL generates peroxyl radicals, leading to a chain reaction which produces peroxidation products such as hydroperoxides and aldehydes (Niki 1990). DPPP, a non-fluorescent molecule, reacts stoichiometrically with hydroperoxide to give diphenyl-1-pyrenylphosphine oxide (DPPP = O), which is strongly fluorescent (Akasaka *et al.* 1987). The conjugate showed greater inhibitory activity against LDL oxidation in a catechin moiety-concentration dependent manner, compared to unconjugated catechin (Figure 3). Interestingly, the inhibition effect of the conjugate against the AAPH induced-oxidation more effectively lasted for a long-term oxidation, compared to unconjugated catechin (Figure 4). Gelatin itself did not inhibit LDL oxidation in this system (data not shown). These results suggest that the gelatin-catechin conjugate is more capable of inhibiting oxidation of LDL than unconjugated catechin.

Conclusion

The gelatin-catechin conjugate was synthesized by the laccase-catalyzed oxidation of catechin with gelatin in an aqueous medium. The resulting conjugate showed the amplified activity to inhibit oxidation of LDL. The conjugate is highly expected as biodegradable, biocompatible polymeric antioxidant to offer protection against a wide range of free radical-induced diseases.

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