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Research Article

Albumin stabilizes (–)-epigallocatechin gallate in human serum: Binding capacity and antioxidant property

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(–)-Epigallocatechin gallate (EGCg) is the major component of green tea and is known to show strong biological activity, although it can be easily oxidized under physiological conditions. In this study, we indicate that EGCg is stable in human serum and that human serum albumin (HSA) stabilizes EGCg under aerobic condition. Although EGCg is usually decomposed within 1 h in aqueous solution at neutral pH, EGCg in serum and phosphate buffer (pH 7.4) containing HSA was stable over 1 h, even at neutral and slightly alkaline pH. Under these conditions, EGCg binds to HSA non-covalently. The sulfhydryl group acts as an antioxidant for EGCg oxidation. Incubation of EGCg with HSA is accompanied by the oxidation of a free sulfhydryl group in HSA. These results suggest that the antioxidant property and the binding capacity of HSA contribute to the stabilization of EGCg in human serum.

Keywords: Antioxidant property / Binding capacity / (–)-Epigallocatechin gallate / Human serum albumin / Stability

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1 Introduction

The disease-preventive effects of green tea and its main constituent polyphenols have been identified by epidemiological findings and established through *in vivo* and *in vitro* experimental and clinical studies over the past decade, as reviewed recently [1]. In particular, (–)-epigallocatechin gallate (EGCg), the most abundant catechin in green tea, has been found to show the strongest biological effects for antioxidation, anti-carcinogenicity, and anti-obesity in numerous *in vivo* and *in vitro* experiments [2, 3]. However, as this catechin is not stable under typical experimental conditions such as cell culture media and buffer solutions, studies on the EGCg action mechanisms have been difficult.

In general, EGCg has poor stability in neutral or alkaline solutions. In solutions at high pH, EGCg undergoes autoxidation to form hydrogen peroxide, resulting in dimerization and decomposition [4, 5]. Several other factors, including temperature, oxygen levels, antioxidant levels, metal ions, concentration of EGCg, and other ingredients in tea, also affect the stability of EGCg [6]. Further, *in vitro* studies indicated that EGCg is unstable in several cell culture media [4, 7, 8].

Some evidence indicates that EGCg is stable in the neutral or slightly alkaline pH environment of the body, as EGCg and its metabolites have been detected in biological fluids after green tea ingestion [9, 10]. Sang *et al*. [6] reported that oxidative products of EGCg could not be detected using the sensitive mass spectrometric method in plasma samples of mice after treatment with 50 mg/kg EGCg. Under normal conditions, oxygen partial pressure in the internal organs and body fluids is much lower than under cell culture conditions (about 40 *versus* 160 mmHg, respectively) [11]. Although EGCg has been thought to be stable under anaerobic conditions *in vivo*, the mechanism of EGCg stabilization has not yet been characterized.

In the present study, to better understand the EGCg stabilization *in vivo*, the stability of EGCg in human serum was examined under aerobic conditions.

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Abbreviations: **EGCg**, (–)-epigallocatechin gallate; **GSH**, reduced glutathione; **GSSG**, oxidized glutathione; **HRP**, horseradish peroxidase; **HSG**, human serum γ-globulin

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2 Materials and methods

2.1 Materials

EGCg was kindly provided by the Tokyo Food Techno Co. Ltd. (Shizuoka, Japan). Human serum albumin (HSA), human serum γ-globulin (HSG), reduced glutathione (GSH) and oxidized glutathione (GSSG) were purchased from Sigma (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated streptavidin and enhanced chemiluminescence Western blotting detection reagents were obtained from GE Healthcare UK Ltd. (Buckinghamshire, UK). Iodoacetyl-LC-biotin and biotin-LC-hydrazide were purchased from Pierce (Rockford, IL, USA). All other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2 Preparation of protein-removed human serum

Fresh protein-removed human serum was prepared from whole blood. After allowing whole blood to clot for 1 h at 25°C in sterile, silicone-coated tubes, the clear supernatant (serum) was withdrawn by pipette and subjected to centrifugal filtration using Microcon-3 (molecular mass cut-off of 3000 Da; Millipore, Bedford, MA, USA) to obtain protein-removed human serum. After collecting the protein-removed serum, the protein solution was collected from the filter by centrifugation. The removed protein was then subsequently added to 100 mM sodium phosphate buffer (pH 7.4).

2.3 HPLC analysis

Stability of EGCg was evaluated by an RP-HPLC analysis as previously reported [12]. Briefly, separation of EGCg was carried out on a nanospace Gulliver system (Jasco, Tokyo, Japan) with a UV detector, using a Capcell Pak C18 UG120 column (Shiseido Co. Ltd., Tokyo, Japan) with a mobile phase consisting of 25% methanol with water with 0.1% TFA. The flow rate was 1 mL/min, and the elution profiles were monitored by absorbance at 285 nm.

2.4 Stability of EGCg in serum and buffer solutions with serum proteins

The stability of EGCg in human serum (70.3 mg/mL protein) was examined. EGCg (100 μM) was dissolved in serum or diluted with 100 mM sodium phosphate buffer (pH 7.4) as a control to produce final protein concentrations of 0.7 or 7.0 mg/mL, and then incubated at 37°C for 1 h. EGCg (100 μM) was also incubated in 100 mM sodium phosphate buffer (pH 7.4) without (control) or with 30 mg/mL serum proteins (HSA or HSG) at 37°C for 1 h. The amount of EGCg was determined by HPLC analysis as described above.

2.5 Native-PAGE/Western blotting and redox-cycling staining

The samples (20 μg) were separated by native-PAGE using 4–16% gel (NativePAGE-NOVEX-Bis-Tris Gel System, Invitrogen, USA). The proteins were stained with Simply Blue SafeStain (Invitrogen). For redox-cycling staining, a gel was transblotted onto a PVDF membrane. The EGCg-HSA complexes were detected by staining with NBT (240 μM) in potassium glycinate (2 M, pH 10) [13–15].

2.6 Peptide mass fingerprinting

Peptide mass fingerprinting was performed as previously reported [16]. Briefly, peptide extracts from gel pieces were analyzed with an UltraFLEX MALDI-TOF MS (Bruker Daltonics, Ltd., Bremen, Germany). A few microliters of the sample were mixed with equal volumes of a saturated solution of sinapinic acid or a-cyano-4-hydroxycinnamic acid (Bruker Daltonics) in ACN/0.1% TFA; the mixture (1 μL) was deposited on the MALDI-TOF MS target. Proteins were identified with MASCOT (Matrix Science, London, UK) searching algorithms using the nonredundant database.

2.7 Biotin-labeling of a free sulfhydryl group and protein carbonyls in HSA

Biotin labeling of EGCg-treated HSA was performed as previously reported [14]. Briefly, HSA (0.3–30 mg/mL) were incubated with EGCg (100 μM) in 100 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 h. After dilution to 0.1 mg/mL, the samples were treated with 5 mM iodoacetyl-LC-biotin or 5 mM biotin-LC-hydrazide at room temperature for 1 h in the dark. The biotin-labeled proteins were subjected to 10% SDS-PAGE and Western blotting with HRP-streptavidin. The bands were visualized by a lumino-image analyzer (FUJIFILM Co., Tokyo, Japan).

2.8 Stability of EGCg in buffer solutions with amino acids

EGCg (100 μM) was incubated in 100 mM sodium phosphate buffer (pH 7.4) without (control) or with 1 mM amino acids (Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, and Val) at 37°C for 1 h. The amount of EGCg was determined by HPLC analysis as described above.

2.9 Stability of EGCg in buffer solutions with glutathione and measurements for GSH levels

EGCg (100 μM) was incubated with 1 mM glutathione (GSH or GSSG) in 100 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 h. The amount of EGCg in the mix-

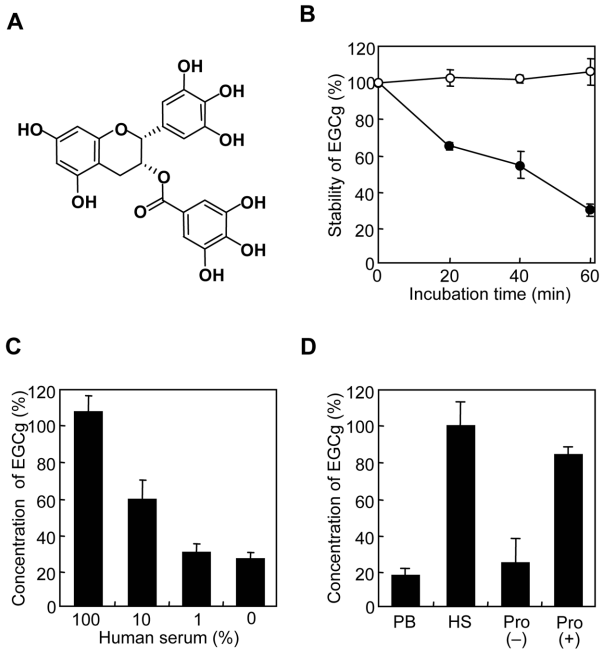

Figure 1. Stabilization in human serum. (A) Chemical structure of EGCg. (B) Stability of EGCg in human serum. EGCg (100 μM) was incubated in human serum at 37°C for 1 h. The amount of EGCg in the serum was measured by HPLC. Symbol: human serum (○); 100 mM phosphate buffer (pH 7.4) (●). (C) Effect of diluted-human serum on the stability of EGCg. (D) Effect of serum protein on stability of EGCg. Human serum was subjected to ultrafiltration using a protein-cut-off filter, and EGCg (100 μM) was incubated with the protein-removed serum or serum protein-added buffer at 37°C for 1 h. PB, 100 mM phosphate buffer; HS, human serum; Pro (–), protein-removed serum; Pro (+), serum protein added to 100 mM phosphate buffer (pH 7.4).

ture was measured by HPLC as described above. To determine the influence of incubation with EGCg on redox states of glutathione, EGCg (0–500 μM) was incubated with GSH (500 μM) in 100 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 h. The samples were mixed with a solution of potassium phosphate containing diethylenetriamine pentaacetic acid and lubrol. Solutions of chromogenic reagent in HCl and 30% NaOH were added to the mixtures and incubated at 25°C for 10 min in the dark. Concentrations of GSH in the reaction of mixtures were analyzed quantitatively by colorimetric assay with the final absorbance at 400 nm using a microplate spectrophotometer (Molecular Devices Co., Sunnyvale, USA).

3 Results

3.1 Albumin stabilizes EGCg in human serum

To better understand the stability of the green tea catechin, EGCg (Fig. 1A) *in vivo*, we examined EGCg stabilization in human serum. While incubation of EGCg in sodium phosphate buffer resulted in a time-dependent decrease in the EGCg concentration, EGCg in human serum was stable for over 1 h (Fig. 1B). Although a previous study reported that EGCg was stable in acidic conditions, but not in neutral and slightly alkaline conditions [4], our results showed that the stability was weakened by dilution of slightly alkaline serum with a neutral pH buffer (Fig. 1C). This weakened stability of EGCg in the serum suggests that factors other than the pH are involved.

To assess the effects of other possible factors such as serum protein on the stability of EGCg, ultrafiltration of human serum using a protein-cut-off filter was performed to remove the protein from the serum. After EGCg incubation at 37°C for 1 h, we found that the relative values of the EGCg concentration compared to standard human serum was 24% in protein-removed serum and 83% in serum protein-added buffer (Fig. 1D). These results suggest that serum protein contributes to the stability of EGCg in human serum.

To further examine the stability of EGCg in the presence of serum protein, EGCg (100 μM) was incubated with major blood proteins, HSA or HSG (30 mg/mL), in 100 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 h. EGCg was unstable in the slightly alkaline buffer with HSG, but stable in the buffer with HSA (Fig. 2A). In addition, the stabilization was weakened by dilution of the HSA with the buffer (Fig. 2B). These results strongly suggest that abundant amounts of HSA stabilize EGCg in human serum.

3.2 Interaction of EGCg with albumin in human serum

Previous reports indicated that EGCg binds to a wide variety of proteins including serum albumin, vimentin, and the 67-kDa laminin receptor [17–19]. To identify the interacting proteins with EGCg in human serum, EGCg-treated serum was analyzed by native-PAGE/Western blot followed by redox-cycling staining with NBT/glycinate. At an alkaline pH, the catechol moiety catalyzes the redox cycling in the presence of glycinate, and the generated superoxide reduces NBT to the blue-purple insoluble formazan on the membrane, allowing for the detection of catechin-bound proteins [20]. As shown in Fig. 3A (upper panel), the EGCg-treated human serum produced positive bands around 66 kDa when assayed by redox-cycling staining. For proteomic identification, these bands were excised from the native-PAGE gels (Fig. 3A, lower panel), subjected to trypsin digestion, and analyzed by MALDI-TOF MS (data not shown). The 66-kDa protein was identified as HSA by peptide mass fingerprint analysis. Using MASCOT, the provability based MOWSE score was 306 for HSA ($p < 0.05$), with 28 peptide matches (error ± 0.05%), which represents 51% sequence coverage. This approach provides direct evidence that EGCg binds to HSA in human serum.

To determine whether EGCg binds to HSA in aqueous solution, HSA (30 mg/mL) was incubated with EGCg (0–

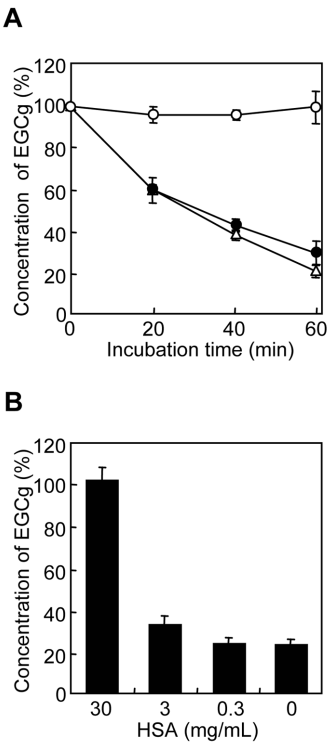

Figure 2. Stabilization of EGCg by HSA. (A) Stability of EGCg in solution with human serum proteins. EGCg (100 μM) was incubated in 100 mM sodium phosphate buffer with serum proteins (30 mg/mL). The amount of EGCg in the serum was measured by HPLC. Symbol: with HSA (○); with HSG (●); without serum protein (△). (B) Effect of HSA on the stability of EGCg. EGCg (100 μM) was incubated in 100 mM sodium phosphate buffer with 0, 0.3, 3, and 30 mg/mL HSA.

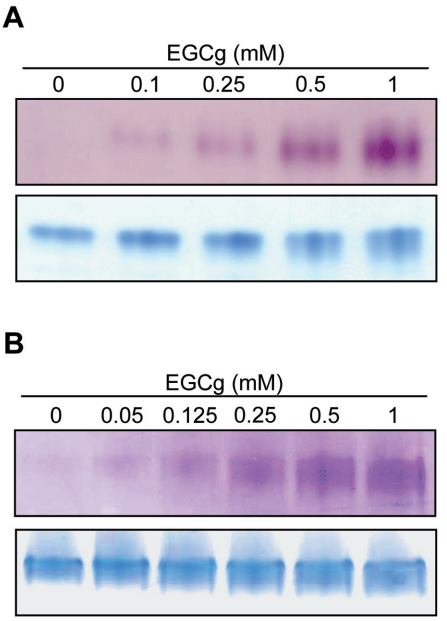

Figure 3. Interaction of EGCg with HSA. (A) Interaction of EGCg with HSA in human serum. Human serum was incubated with EGCg (0–1 mM) at 37°C for 1 h. The samples were analyzed by native-PAGE followed by redox-cycling staining (upper) and CBB staining (lower). (B) Interaction of EGCg with HSA in buffer solution. HSA (30 mg/mL) was incubated with EGCg (0–1 mM) in 100 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 h. The samples were analyzed by native-PAGE followed by redox-cycling staining (upper) and CBB staining (lower).

1 mM) in 100 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 h and analyzed by native-PAGE/Western blot followed by redox-cycling staining. As shown in Fig. 3B, accompanied by addition of EGCg, the expanse and mobility shift of the albumin band (66 kDa) were observed. The 66-kDa bands were concentration-dependently apparent by redox-cycling staining. This result indicates that EGCg interacts with albumin in buffer solution. When EGCg was stable (Fig. 1), EGCg was detected without albumin in human serum and the buffer solution by HPLC analysis. Reversibility of EGCg-HSA complex was confirmed by MALDI-TOF MS, which showed no peak corresponding to the EGCg-HSA complex (data not shown). Previous studies proposed that EGCg binds reversibly to serum albumin in buffer solution through a hydrophobic interaction. In the present study, we have directly demonstrated the interaction of EGCg with albumin in human serum, and indicated that EGCg non-covalently binds to HSA.

3.3 Stabilization of EGCg by sulfhydryl groups

To evaluate the effect of HSA on the stability of EGCg, the stability was examined in the presence of all 20 amino acids. When EGCg (100 μM) was incubated with the amino acids (1 mM) in 100 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 h, only cysteine containing the sulfhydryl group stabilized EGCg (Fig. 4A). To examine the effect of the sulfhydryl groups on the stability of EGCg, EGCg was incubated in the presence of GSH. Incubation of EGCg (100 μM) with GSH (1 mM) in 100 mM sodium phosphate buffer (pH 7.4) stabilized EGCg as well as cysteine (Fig. 4B). However, the improved stabilization was weakened by dilution of the GSH with the buffer. The ratio of the two redox forms of glutathione, the reduced (GSH) and the oxidized (GSSG) forms, is crucial for its antioxidant role. To determine the influence on the redox states of glutathione during the EGCg incubation, GSH (500 μM) was added to various concentrations of EGCg (0–500 μM) at 37°C for 1 h and GSH concentration was analyzed quantitatively by colorimetric assay. While about 88% EGCg remained during the incubation (Fig. 4B), approximately 40% of the GSH was lost after 1 h of incubation with 50 μM EGCg (Fig. 4C). On the other hand, oxidized cysteine and oxidized glutathione did not stabilize EGCg (Fig. 4D). Sulfhydryl groups have been shown to have an antioxidant role by direct scavenging of reactive oxygen species such as hydrogen peroxide [21]. In addition, free

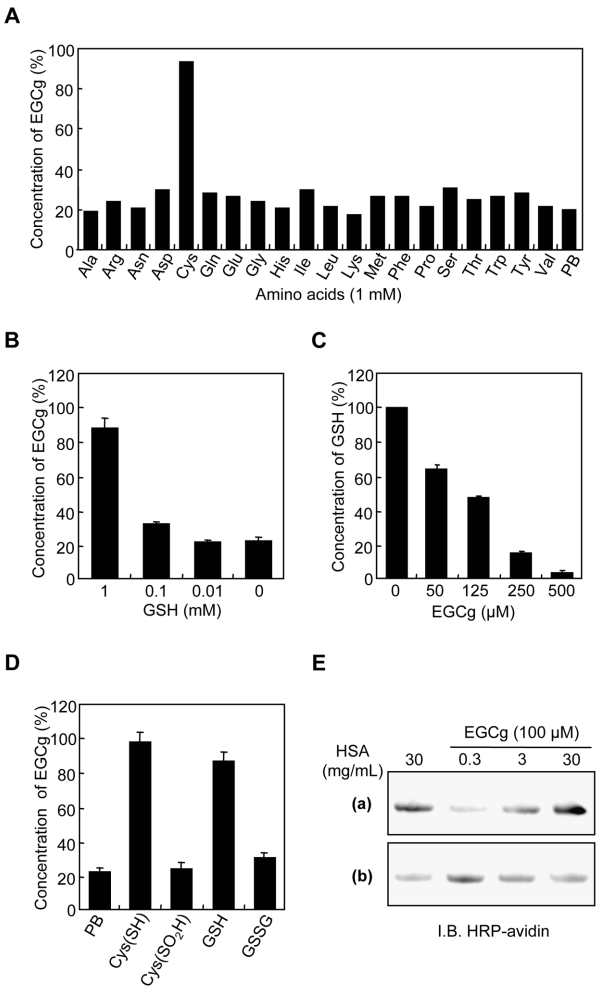

Figure 4. Stabilization of EGCg by sulfhydryl groups. (A) Stability of EGCg in the buffer solution with amino acids. EGCg (100 μM) was incubated in 100 mM sodium phosphate buffer (pH 7.4) with amino acids (1 mM) at 37°C for 1 h. The amount of EGCg in the serum was measured by HPLC. (B) Effect of GSH on stability of EGCg. EGCg (100 μM) was incubated in 100 mM sodium phosphate buffer with 0, 0.01, 0.1, and 1 mM GSH. (C) Measurement for GSH level. EGCg (0–500 μM) was incubated with GSH (500 μM) in 100 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 h, and analyzed by colorimetric assay for glutathione. (D) Effect of oxidized sulfhydryl groups on stability of EGCg. EGCg (100 μM) was incubated in 100 mM sodium phosphate buffer (pH 7.4) with cysteine, cysteine sulfinic acid, GSH, and GSSG (1 mM) at 37°C for 1 h. (E) Loss of sulfhydryl group in HSA (upper) and concomitant formation of protein carbonyls (lower). HSA (0.3–30 mg/mL) were incubated with 100 μM M EGCg in 100 mM sodium phosphate buffer (pH 7.4) at 37°C. The reaction mixtures were incubated with iodoacetyl-LC-biotin or biotin-LC-hydrazide and resolved by SDS-PAGE. Biotin-labeled proteins were detected by Western blot analysis with HRP-conjugated NeutrAvidin.

sulfhydryl groups directly prevent catechol oxidation by reduction from quinone to catechol [22]. These results indicate that GSH prevents EGCg oxidation by this antioxidant role of the sulfhydryl group. To assess the oxidation of the free sulfhydryl group of HSA, different volumes of HSA (0.3, 3, and 30 mg/mL) were incubated with EGCg (100 μM) in 100 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 h. Free sulfhydryl groups in HSA were labeled with iodoacetyl-LC-biotin and the biotin-labeled HSA was analyzed by SDS-PAGE/Western blot probed with streptavidin-HRP. Under this condition, stability of EGCg was weakened by a decrease in HSA concentration (Fig. 2B). As shown in Fig. 4E (upper panel), the free sulfhydryl group of HSA was remarkably decreased in the unstable condition (0.3 or 3 mg/mL HSA) of EGCg (Fig. 4E, upper panel). The loss of the free sulfhydryl group was accompanied by an increase in the carbonyl protein due to the oxidation of HSA (Fig. 4E, lower panel). These results indicate that albumin stabilizes EGCg in human serum due to the antioxidant role of the sulfhydryl group.

4 Discussion

Several lines of evidence indicate that EGCg has poor stability in neutral or alkaline solutions [4–6]. In addition, previous reports show that EGCg in several cell culture media is unstable [4, 8]. In general, EGCg is stable in acidic solution and it is suspected that EGCg is stable in the neutral or slightly alkaline environment of the body. Previous reports indicate that EGCg appears in blood and urine after oral administration to mammals including humans [23–25] and measurements of EGCg concentrations in human, rat, and mouse blood show a half-life of about 5 h [26, 27]. However, the mechanism behind the EGCg stability in blood samples remains unclear. This study provides direct evidence that albumin stabilizes EGCg in human serum under aerobic condition (Figs. 1 and 2). Moreover, EGCg non-covalently binds to HSA in human serum (Fig. 3), and the sulfhydryl group acts as an antioxidant for EGCg oxidation (Fig. 4).

Previously, HSA, the most abundant protein in plasma, has been proposed to have an antioxidant role due to its single sulfhydryl at Cys34, which comprises approximately 80% of the total free sulfhydryl groups in plasma and reacts preferentially with reactive oxygen and nitrogen species [28–30]. Cys34 in HSA is believed to act as an important scavenger of reactive species as the sulfhydryl content of HSA decreases with oxidation. This is corroborated by our observation that the free sulfhydryl group in HSA is remarkably decreased in the unstable condition of EGCg (Fig. 4E).

The reversible protein-binding ability of HSA in blood is also an important factor in the transport and release of various endogenous and exogenous compounds. Interaction with albumin directly influences the duration of the effectual compound, and consequently contributes to their magnitude of biological actions *in vivo* [31, 32]. Moreover, a recent study revealed that dietary flavonoids can be

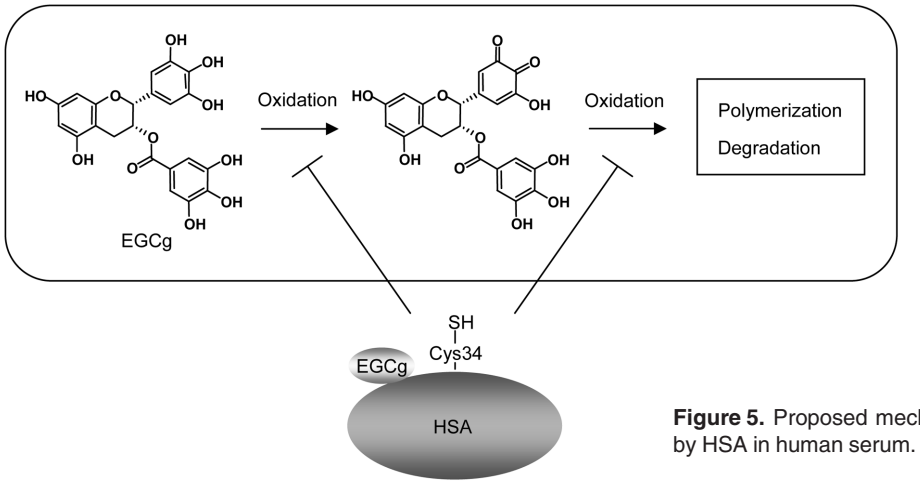

Figure 5. Proposed mechanism for the stabilization of EGCg by HSA in human serum.

detected in plasma as serum albumin conjugates and the binding is believed to modulate the bioavailability of the flavonoids [33]. In this regulation, a change of the structure transitions is important. HSA is known to undergo different pH-dependent structural transitions. The binding capacity of ligand binding domains such as site II on HSA increases at pH 6.5 – 8.2 where albumin undergoes a conformational change, a N-B transition, at slightly alkaline pH [34]. In addition, the N-B transition causes exposure of Cys34 on the HSA molecule [35]. Therefore, the environment of slightly alkaline pH in serum may contribute to stability of EGCg by increasing the binding capacity and antioxidant property, in contrast with its pH effects.

Based on these findings and observations, we propose a mechanism for the stabilization of EGCg by albumin in human serum (Fig. 5). EGCg generally has poor stability in neutral or alkaline solutions. At high pH, the presence of a hydroxide ion that can easily attack the proton of the phenol group leads to generation of the phenoxide anion. This anion is much more reactive toward electrophilic agents, and forms a semiquinone radical, which can undergo further dimerization or other reactions [4 – 6]. However, Cys34 in HSA directly prevents the EGCg oxidation by scavenging reactive oxygen species or reducing catechol from quinone. The binding capacity may be effective for the expression of antioxidant property. However, the EGCg binding site is not completely characterized. Therefore, discussion of the relationship between the binding site and the stability is difficult.

In conclusion, this study provides fundamental information on the stability of EGCg under *in vivo* and *in vitro* conditions, including plasma or culture medium, and encourages further investigation into the biological activities of EGCg. Under normal conditions, the oxygen partial pressure in the internal organs is much lower than that under *in vitro* conditions. It is believed that EGCg acts as antioxidant under oxidative stress. The antioxidant property and the binding capacity of HSA may contribute to the improvement of bioavailability of EGCg as an antioxidant *in vivo*. Our results provide a basis for future studies of EGCg and other compounds that can undergo auto-oxidation.

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The authors have declared no conflict of interest.

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