SUPPLEMENTARY MATERIAL

Role of catechin on collagen type I stability upon oxidation: a NMR approach

Massimo Lucarini\textsuperscript{a*}, Fabio Sciubba\textsuperscript{b}, Donatella Capitani\textsuperscript{c}, Maria Enrica Di Cocco\textsuperscript{b}, Laura D’Evoli\textsuperscript{a}, Alessandra Durazzo\textsuperscript{a}, Maurizio Delfini\textsuperscript{b}, Ginevra Lombardi Boccia\textsuperscript{a}

\textsuperscript{a}CREA Research Centre for Food and Nutrition, Via Ardeatina, 546, 00178 Rome, Italy; \textsuperscript{b}Department of Chemistry, “Sapienza” University of Rome, Piazzale Aldo Moro, 5, 00185 Rome, Italy; \textsuperscript{c}Institute of Chemical Methodologies, CNR, Research Area of Rome, M.B.10, 00016 Monterotondo Stazione, Rome, Italy

*Corresponding author: Dr. Massimo Lucarini, CREA – Research Centre for Food and Nutrition, Via Ardeatina, 546, 00178 Roma, Italy.
Tel. +39 06 51494446 Fax: +39 06 51494550; e-mail: massimo.lucarini@crea.gov.it
Role of catechin on collagen type I stability upon oxidation: a NMR approach

The study focuses on the understanding, at molecular level, of the mechanism of interaction between protein and flavonoids. Collagen and catechin interactions were investigated by NMR in solution and solid state. The effect of catechin on the stability of collagen to oxidation was also explored. Collagen was treated with two concentrations of catechin solutions. Oxidation was carried out by incubation of collagen solution with three oxidation systems: Fe(II)/H₂O₂, Cu(II)/H₂O₂, NaOCl/H₂O₂. The effects of oxidation systems were evaluated by high resolution 1D and 2D proton spectroscopy and solid state NMR (¹³C CP MAS) experiments. Interactions between collagen and catechin preferentially occur between catechin B ring and the amino acids Pro and Hyp of collagen. Results showed that both iron and copper oxidation systems were able to interact with collagen by site specific attack. Moreover, catechin protects collagen proline from oxidation by metal/H₂O₂ systems, this was notable for the copper system.

Experimental section

Chemicals
Collagen type I fibers from Bovine Achilles Tendon (BAT) and (±) catechin, was sourced from Sigma (Sigma Chemicals Co., USA) and were used without any preliminary purification. Double-distilled water (Millipore, Milan, Italy) was used throughout the study.

Catechins treated collagen
Collagen (2.5 mg/mL) was treated with two concentrations of catechin (0.01 M and 0.001 M) for 24 h at room temperature (25°C) in the dark and without any shaking. Collagen treated sample were dialyzed against distilled water and then freeze-dried. Dialysis was conducted for 24 h at +4°C. After catechin treatment collagen became slightly pink. Lyophilized samples were characterized by NMR experiments in solution and in solid state.

Treatment with protein oxidizing agents
Oxidation was carried out by incubation of collagen solution (1 mg/ml in 0.05 M acetic acid, pH 4.3) with three different oxidant systems:
i) Fe(II)/H₂O₂ system: 50 μM Fe(II)/ 5 mM. H₂O₂ (Penkova et al. 1999; Hawkins and Davies, 1997);

ii) Cu(II)/H₂O₂ system: 50 μM Cu(II)/ 5 mM. H₂O₂ (Penkova et al. 1999; Hawkins and Davies, 1997);

iii) NaOCl/H₂O₂ system: 10 mM NaOCl/10 mM. H₂O₂ (Fujimori, 1989);

The oxidation procedures were also performed on the collagen samples treated with catechin. The incubations were carried out at 22°C for 1 h, and at the end of the oxidation samples were dialyzed against ultra-pure water at +4°C, using a Spectrapor membranes with a molecular weight cut-off of 12000 Da. Dialysis was conducted for 24 h at +4°C. After that sample were freeze-dried.

**High resolution 1-H 1D and 2 D NMR spectroscopy**

All NMR experiments were recorded at 30°C on Bruker Avance 400 spectrometer equipped with triple resonance probes incorporating shielded z-axis gradient coils. Instrumental standard pulse sequences were used for one and two-dimensional homonuclear spectra. Water suppression was achieved with presaturation. Spectra were acquired with 64 scans with a relaxation delay of 6 s. All homonuclear two-dimensional experiments (NOESY and ROESY) were acquired with 128 scans per t1 increment, 4 K complex points in t2 and 512 t1 increments. The NMR data were processed by Topspin software (Bruker, Karlsruhe Germany). Proton chemical shifts were referenced to Sodium Trimetil Sylil Propionate (TSP).

**Solid state NMR studies of collagen and collagen-treated samples**

¹³C CP-MAS NMR spectra were recorded with a Bruker AMX-200 spectrometer equipped with a CP-MAS accessory. Chemical shift were calibrated through external benzene and converted to the values of tetramethylsilane (Me₄Si). Sample was ground into a fine powder and packed into a cylinder (4 mm external diameter). Then, it is placed in a special probe/rotor so that it will be oriented at 54.74° to the magnetic field and spun.
Figure S1 Structure and atom numbering of catechin.

Figure S2. A) 1H NMR spectra of collagen B) Solid state 13C MAS NMR spectra of collagen.

Figure S3. NOESY spectrum of collagen modified with 0.001M catechin.
Figure S4. Enlargement of figure S5B. Spectra of collagen (a) and collagen oxidized by Fe(II)/H₂O₂ system (b).

Figure S5. A) 1D 1H NMR spectra of collagen modified with catechin 0.001M oxidized by Fe(II)/H₂O₂ system (a), collagen oxidized by Cu(II)/H₂O₂ (b) and collagen oxidized by NaClO/H₂O₂ system (c); B) Solid state 13C MAS NMR spectra of collagen modified with catechin 0.001M (a), collagen modified with catechin 0.001M oxidized by Fe(II)/H₂O₂ system (b), collagen modified with catechin 0.001M oxidized by Cu(II)/H₂O₂ (c) and collagen modified with catechin 0.001M oxidized by NaClO/H₂O₂ system (d).
Table S1. Chemical shift and peak half height widths for non-modified collagen, collagen treated with catechin (0.01M and 0.001M) and the respective sample oxidized by metal based systems.

| Catechin concentration | Oxidation System | Ala Chemical Shift $\delta$ (ppm) | PHHW (Hz) | Gly Chemical Shift $\delta$ (ppm) | PHHW (Hz) |
|-----------------------|------------------|----------------------------------|-----------|----------------------------------|-----------|
| No Treated            | No Treated       | 4.32                             | 57.20     | 4.13                             | 60.57     |
| No Treated            | Fe(II)/H$_2$O$_2$| 3.90                             | 8.18      | 3.67                             | 9.10      |
|                       | Cu(II)/H$_2$O$_2$| 3.54                             | 26        | 3.11                             | 20.6      |
| 0.001 M               | Fe(II)/H$_2$O$_2$| 3.9                              | 3.28      | 3.7                              | 3.60      |
|                       | Cu(II)/H$_2$O$_2$| 3.9                              | 5.24      | 3.7                              | 5.69      |
| 0.01M                 | Fe(II)/H$_2$O$_2$| 3.9                              | 3.16      | 3.7                              | 3.44      |
|                       | Cu(II)/H$_2$O$_2$| 3.9                              | 7.00      | 3.7                              | 7.86      |
References of Supplementary Material

Fujimori E. 1989. Cross-linking and fluorescence changes of collagen by glycation and oxidation. Biochim Biophys Acta. 998:105-110.

Hawkins CL, Davies M.J. 1997. Oxidative damage to collagen and related substrates by metal ion-hydrogen peroxide systems: random attack or site-specific damage? Biochim Biophys Acta. 1360:84-96.

Penkova KR., Koynova R, Kostov G, Tenchov B. 1999. Discrete reduction of type I collagen stability upon oxidation. Biophys Chem. 83:185-195.