Collagen-Catechin Interactions: a NMR Approach

Lucarini M. [1], D'Evoli L. [1], Delfini M. [2], Sciubba F.[2], Capitani D.[3], Lombardi Boccia G [1]

[1] National Institute for Food and Nutrition Research, Roma, Italy
[2]University of Rome La Sapienza, Department of Chemistry, Rome, Italy
[3] Magnetic Resonance Laboratory Annalaura Segre, Institute of Chemical Methodologies (IMC), Rome, Italy

Abstract—**This work focuses on understanding at molecular level the mechanism of interaction between collagen and catechin. Interactions were investigated by NMR measurements both in solution and in solid state. We also explored the possibilities offered by NMR to characterize the effect of catechin on the stability of collagen to oxidation. Collagen type I fibres were used throughout the study. Collagen was treated with two different concentration of catechin . Oxidation was carried out by incubation of collagen solution with three different oxidation system s ($Fe(II)/H_2O_2$; $Cu(II)/H_2O_2$; $NaOCl/H_2O_2$). High resolution 1-H 1D and 2D spectroscopy were recorded at 30°C on Bruker Avance 400. Solid state NMR experiments were performed by a Bruker spectrometer equipped with a CPS MAS accessory. Data obtained from 1D and 2 D proton NMR and ^{13}C CP MAS spectroscopy pointed out that interactions between collagen and catechin preferentially occurred between catechin B ring and the amino acids proline and hydroxyproline. Oxidation studies carried out by metal/H2O2 systems on collagen showed that both iron and copper were able to interact with collagen by site specific attak.**

A strong effect of catechin in collagen complex was shown. Catechin was able to protect collagen proline from oxidation by metal/H_2O_2 systems. The protective effect of catechin towards collagen oxidation was markedly evident for the copper oxidation system.

Keywords— **Collagen, Catechin, NMR.**

I. INTRODUCTION

Meat storage generates free oxygenated radicals that react with lipids and proteins; these oxidative processes affect colour, flavour and nutritional value of meat. Proteins are the main target of free radical attack [1] and their oxidation products are responsible for many biological modification affecting meat quality and meat technology. The addition of natural antioxidants extracted from plants are used to slow down oxidation processes and increase the shelf life of meat [2]. Among antioxidants, flavonoids, ubiquitous in plants, are a large class of compounds usually occurring as glycosides. They contain several phenolic hydroxyl functions attached to ring structure [3] .

The present study aims to characterize, at molecular level, the interaction between meat proteins and flavonoids. Collagen was chosen as the target protein of oxidation. This protein is characterised by triple helical structure with a repeating sequence of Gly-X-Y where the amino acids X and Y are often Pro and 4-hydroxyproline, HyPro, respectively [4]. Catechin was chosen as flavonoid antioxidant; it belongs to Flavan-3-ol families and is one of the most active antioxidants in the hierarchy of flavonoids. Collagen oxidation has been studied using different oxidative systems including metal ion/H_2O_2 and singlet oxygen.

II. MATERIALS AND METHODS

Collagen type I fibres from Bovine Achille Tendon (BAT) and (±) catechin, was sourced from Sigma (Sigma Chemicals Co., USA) and were used without further purification. The commercial products were used without any preliminary purification.

A. Sample preparation

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 agitation. After treatment, samples were dialyzed against bidistilled water and then freeze-dried. Dialysis was conducted for 24 h at +4°C.

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: $Fe(II)/H_2O_2$, $Cu(II)/H_2O_2$ and $NaOCl/H_2O_2$ systems following the

experimental protocols suggested by Penkova et al. [5] Hawkins and Davies [4] and Fujimori [6]. The oxidation procedures were also applied to collagen samples treated with catechin. Incubations were carried out at 22°C for 1 h. Oxidation was stopped by adding 10 mM EDTA, and samples were dialyzed against bidistilled water at +4°C. For dialysis, the membranes (Spectrapor membranes) were used with the Molecular Weight cut-off of 12000 Dalton.

After dialysis all the samples were lyophilized and analysed by high resolution NMR.

1-H 1D and 2D (NOESY and ROESY) NMR experiments were recorded at 30°C on Bruker Avance 400 spectrometer equipped with triple resonance probes incorporating shielded z-axis gradient coils.

^{13}C CP-MAS (Carbon-13 Cross-Polarization Magic Angle Spinning) NMR spectra were recorded by a Bruker AMX-200 spectrometer equipped with a CP-MAS accessory.

III. RESULTS

Figure 1 shows high resolution ^{1}H NMR spectra related to native and oxidized collagens.

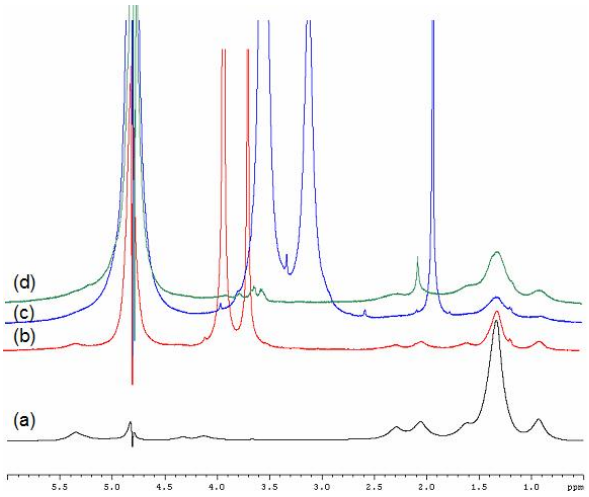

Fig. 1 1D 1H NMR spectra of collagen (a), collagen oxidized by Fe(II)/H_2O_2 system (b), collagen oxidized by Cu(II)/H_2O_2 system (c) and collagen oxidized by NaClO/H_2O_2 system (d).

From comparison of native collagen with collagen oxidized by Fe/H_2O_2 and Cu/H_2O_2, it was observed that both oxidation systems triggered an increased mobility of Glycine and Alanine. Thus, these amino acids are most likely the main oxidation sites of metal based oxidation systems. In fact, Peak Half Height Widths (PHHW), which were about 60 Hz in non-modifed collagen, were greatly reduced in oxidized samples. Strong chemical shift variation was observed for the proton resonances of Gly α CH e Ala α CH (about 0.5 ppm for Fe(II)/H_2O_2 system and about 1 ppm for Cu(II)/H_2O_2 system) in the oxidised collagen samples. These paramagnetic shifts can be divided into two components, a scalar (through-bond) *contact shift*, which propagates no further than four or five bonds from the metal site, and a dipolar (through space) *pseudocontact shift*. Both shift involve the formation of a complex between protein and the paramagnetic ions. Also the singlet oxygen (fig. 3.17 d) caused damages to the polymer (from the literature mostly attributed to cross-link actions), but its attack followed a radical and random mechanism.

In fig. 2 typical ^{13}C CP MAS spectra of dry collagen fibrils (a) and oxidized collagen fibril (b-d) are plotted. CP MAS spectra demonstrated that collagen was mostly sensitive to be damaged by the Cu(II)/H_2O_2 system respect to the other ones.

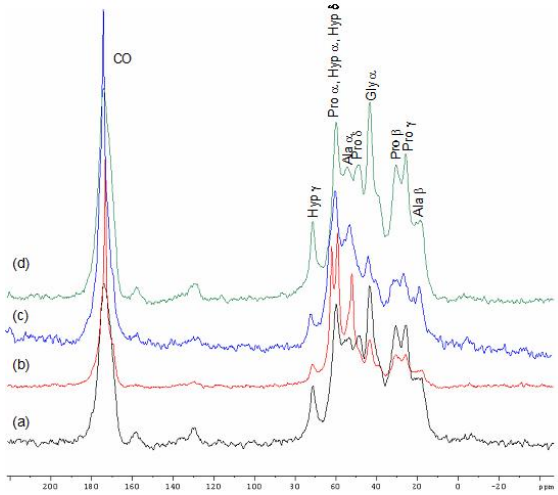

Fig. 2 Solid state ^{13}C MAS NMR spectra of non-modified collagen (a), collagen oxidized by Fe(II)/H_2O_2 system (b), collagen oxidized by Cu(II)/H_2O_2 (c) and collagen oxidized by NaClO/H_2O_2 system (d).

Comparing spectra from native collagen with those obtained from collagen oxidized by singlet oxygen system (fig 2 d), little changes were observed. A change in the Pro γ/Pro β ratio was observed respect to the non-modified collagen. This means that proline isomerization is strongly involved in the collagen cross-link process.

Cu(II)/H_2O_2 oxidation system mainly destroyed glycine and proline amino acids identified between 20 and 40 ppm. Also, the CO signal exhibits little changes in peak shape when collagen was oxidized by metal/H_2O_2 systems.

Regarding Fe(II)/H_2O_2 system (fig 2 b) the Hyp α, Hyp δ and Pro α signal resolution, at about 60 ppm, was improved by oxidation and additional resonance line could be resolved.

Concerning the interaction between collagen and catechin, data from 1H, 2D and ^{13}C CP MAS spectroscopy experiments (data not shown) pointed out that interactions between collagen molecule and catechin preferentially occurred between OH groups of the B catechin ring (figure 3) and collagen proline residue.

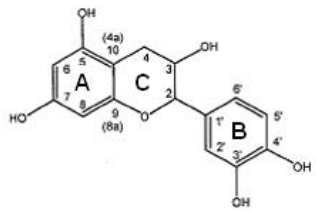

Fig. 3 Structure and atom numbering of catechin.

In figures 4 shows spectra of collagen modified with 0.001 M catechin after treatment with the three oxidation systems.

A noticeable effect of catechin on modified collagen was evident (figure 4) compared to the native collagen (figure 3).

These effects were markedly evident for the Cu(II)/H_2O_2 oxidation system. According to previous considerations, this could be ascribed to an increased copper distance from collagen attack sites compared to the results from the non-modified collagen.

^{13}C CP MAS spectra (data not shown) confirmed results obtained from 1H NMR analysis. Cu(II)/H_2O_2 oxidation, as well as Fe(II)/H_2O_2 one, led to the Hyp α, Hyp δ and Pro α signal resolution, at about 60 ppm. The protective effect against oxidation exhibited by catechin was much stronger in the case of copper oxidations.

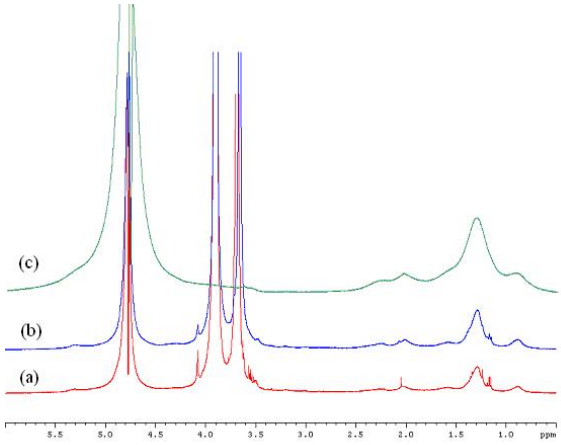

Fig. 4 1D 1H NMR spectra of collagen modified with catechin 0.0O1M oxidized by Fe(II)/H_2O_2 system (a), collagen oxidized by Cu(II)/H_2O_2 (b) and collagen oxidized by NaClO/H_2O_2 system (c).

IV. DISCUSSION AND CONCLUSIONS

Oxidation studies carried out by metal/H_2O_2 systems on non modified and catechin modified collagen showed that both iron and copper were able to interact with collagen by site-specific attacks. Also it was demonstrated, from considerations related to the chemical shift found after the oxidation experiments, that copper is closer than iron to the collagen attack site. These results were consistent with findings from literature that pointed out the major specificity of Cu(II)/H_2O_2 oxidation system respect to the iron system.

The main interaction sites between collagen and catechin were highlighted too. Also it has been shown that collagen modified with catechin has a greater stability to oxidation processes that may occur during refrigerated storage of meat.

The main effect of catechin in the collagen complex was to protect proline from oxidation preventing copper and iron approach to collagen molecule.

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