Inhibitory mechanism of sinapinic acid against peroxynitrite-mediated tyrosine nitration of protein in vitro

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Abstract The peroxynitrite-scavenging ability of some phenolic antioxidants, *p*-coumaric acid, caffeic acid and sinapinic acid, was examined and compared with ascorbic acid and tocopherol using 3-nitrotyrosine formation as a marker. Among these, caffeic acid and sinapinic acid strongly inhibited the formation of 3-nitrotyrosine in protein. The treatment of protein with peroxynitrite in the presence of sinapinic acid, but not caffeic acid, produced a novel product determined by reversed-phase high performance liquid chromatography (HPLC). The product formed was purified and then identified as a mono-lactone type dimer (ML) of sinapinic acid by nuclear magnetic resonance (NMR) and liquid chromatography-mass spectrometry (LC-MS). This ML was converted from a di-lactone type dimer, obtained from sinapinic acid with peroxidase/hydrogen peroxide, in neutral buffer. In this report, we have proposed that the ML of sinapinic acid is generated via one-electron oxidation by peroxynitrite treatment.

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Key words: Peroxynitrite; Sinapinic acid; Adduct; Nitrotyrosine; Phenolic antioxidant

1. Introduction

Much attention has focused on the oxidation of biomolecules in vivo concerning several diseases [1]. However, it is still not clear which oxidants are related to these phenomena. Peroxynitrite, produced from nitric oxide and superoxide, is one plausible oxidant in vivo and its marker, 3-nitrotyrosine, has been detected in the tissues of patients with numerous diseases [2–4]. There have been many reports concerning the inhibitory activity of endogenous antioxidants, such as ascorbic acid, glutathione and tocopherol(s), against this powerful oxidant [5–7]. In addition, some natural antioxidants other than these endogenous compounds exhibit inhibitory activity against peroxynitrite [8,9]. In contrast to the inhibitory activities of these antioxidants, inhibition by phenolics against peroxynitrite-induced modification has been partly elucidated [7,9,10]. In this study, we examined the inhibitory effect of some phenolic compounds on the nitration of protein by peroxynitrite. We found that sinapinic acid, widely prevalent in the plant kingdom [11], was a strong inhibitor of peroxynitrite. During the inhibition of protein nitration by sinapinic acid, a novel phenolic compound, mono-lactone type dimer (ML), was detected. An intermediate of this compound is considered to be a di-lactone type dimer (DL) and the inhibitory effect of sinapinic acid on the peroxynitrite-induced protein modification is probably due to its one-electron oxidation.

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Abbreviations: NMR, nuclear magnetic resonance; LC-MS, liquid chromatography-mass spectrometry; ML, mono-lactone type dimer; DL, di-lactone type dimer

2. Materials and methods

2.1. Chemicals

p-Coumaric acid and caffeic acid were purchased from Nacalai Tesque. Cinnamic acid and ferulic acid were obtained from Tokyo Kasei Kogyo. Sinapinic acid was purchased from Aldrich Chemical. *d,l*-α-Tocopherol was obtained from Sigma. L-(+)-Ascorbic acid and collagen (type I) were purchased from Wako Pure Chemical. Peroxynitrite was synthesized [12] and quantified prior to use as previously described [13]. Briefly, an acidic solution (10 ml, 0.6 M HCl) containing H_2O_2 (0.7 M) was vigorously mixed with $NaNO_2$ (10 ml, 0.6 M) in an ice cold bath and the reaction mixture was quenched with ice cold NaOH (20 ml, 1.5 M). The solution was frozen for several hours and the yellowish top layer was collected.

2.2. Instrumentation

^{1}H-NMR spectra were recorded on a JNM GSX-270 (JEOL). Two-dimensional NMR spectra were measured with a Brucker AM-400 spectrometer. High performance liquid chromatography (HPLC) analysis was done using a Wakosil-II 5C18HG column (i.d. 4.6×250 mm, Wako Pure Chemical) on a Shimadzu CLASS-LC10 series HPLC system equipped with a photo diode array detector (SPD-M10Avp, Shimadzu). The solvent mixture, H_2O:MeOH:TFA (600:400:1), was used at a flow rate of 1.0 ml/min at 40°C. Liquid chromatography-mass spectrometry (LC-MS) was performed using the same HPLC column connected to a PLATFORM II (VG Biotech) with a negative electrospray interface.

2.3. Detection of 3-nitrotyrosine by an enzyme-linked immunosorbent assay (ELISA)

The detection of 3-nitrotyrosine in collagen treated with peroxynitrite was performed following the previously described method [9]. Briefly, 90 μl of protein (final 0.5 mg/ml) dissolved with 0.1 M phosphate buffer (pH 7.4) was placed in a 1.5 ml microtube and 10 μl of antioxidant solution (final 0.3 mM) dissolved in dimethyl sulfoxide (DMSO) was added and this mixture was then treated with peroxynitrite (final 1.0 mM) with vigorous vortex mixing. To avoid any pH effect, as little peroxynitrite solution as possible was used by employing a concentration greater than 150 mM. The modified collagen was diluted 50-fold with phosphate buffer and 50 μl aliquots were pipetted into 96 hole plates. The plates were kept at 4°C overnight and then blocked at 37°C for 1 h with gentle shaking. The coated protein was successively treated with antiserum against 3-nitrotyrosine that was prepared by a previously described method [3] and then with peroxidase-labelled anti-rabbit IgG goat antibody. *o*-Phenylenediamine was added as a substrate for peroxidase with hydrogen peroxide and the amount of 3-nitrotyrosine was evaluated by UV absorption at 492 nm using a multiplate reader (SPECTRA MAX 250, Molecular Devices Corporation).

To evaluate the residual impurities other than peroxynitrite, this modification was compared using 'decomposed peroxynitrite' in which peroxynitrite was added to the buffer prior to the collagen. In practice, peroxynitrite (final 1.0 mM) was added to 0.1 M phosphate

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buffer (pH 7.4) in 1.5 ml microtubes and stored for about 30 s. The corresponding collagen was then added with vigorous vortex mixing, followed by the successive procedures already described above.

Under the conditions described above, 10% DMSO, dissolving reagent for lipophilic samples, had little effect on the determination of the inhibitory activity against peroxynitrite [9].

2.4. Isolation of the adduct from sinapinic acid and peroxynitrite

Sinapinic acid solution (2.0 mg/0.5 ml MeOH) was added to 4.5 ml of 0.1 M phosphate buffer (pH 7.4) in a 50 ml round bottom flask. Two equivalents of peroxynitrite solution were then added dropwise with vigorous mixing and the reaction was cooled with an ice cold bath if needed. The combined crude reaction mixture was then temporarily placed in vacuo for the evaporation of MeOH and concentrated by freeze-drying. This sample was dissolved and purified by HPLC using a Wakosil-II 5C18 column (i.d. 20×250 mm, Wako Pure Chemical: UV at 340 nm). A solvent mixture of H_2O:MeOH:TFA (600:400:1) was used as the mobile phase with a flow rate of 5.0 ml/min at ambient temperature. To this fraction, a small amount of *p*-hydroquinone was added to maintain a stable condition. The sample was then concentrated by evaporation in vacuo and freeze-drying, which yielded the adduct (0.6 mg).

2.5. Peroxidase-catalyzed oxidation of sinapinic acid

The reaction was performed as previously described [14]. Sinapinic acid (50 mg) was dissolved in 25 ml of water–acetone (80/50 (v/v)). Two mg of horseradish peroxidase (Merck) was then suspended and 28 μl of 30% hydrogen peroxide (Wako Pure Chemical) was added dropwise. The reaction mixture was stirred for 45 min at ambient temperature and a small amount of catalase (Wako Pure Chemical) was then added and stirred for about 15 min to decompose the remaining hydrogen peroxide.

2.6. Quantitative analysis of sinapinic acid-derived adduct by peroxynitrite

Reaction conditions were similar to those described in Section 2.4. Briefly, 450 μl of 0.1 M phosphate buffer (pH 7.4) was placed in a 1.5 ml microtube and 50 μl of sinapinic acid dissolved in MeOH was added (final 0.2 mM) and this mixture was then treated with two equivalents of peroxynitrite (360 mM) with vigorous vortex mixing. This reaction mixture was stored at −80°C just before HPLC analysis. The peak area of the adduct was compared with the standard sample solution at UV 340 nm.

3. Results

We examined the activity of some natural antioxidants that inhibit the peroxynitrite-induced protein modification using the anti-3-nitrotyrosine antibody. In these results, *p*-coumaric acid and its derivatives, sinapinic and caffeic acids, exhibited stronger inhibitory activities against peroxynitrite than α-tocopherol and ascorbic acid (Fig. 1). At the lower concentration, caffeic acid and sinapinic acid were more effective than *p*-coumaric acid (data not shown). We analyzed the reaction mixture of peroxynitrite-exposed collagen in the presence of sinapinic acid and caffeic acid by reversed-phase HPLC and a novel peak was detected with sinapinic acid but not with caffeic acid. Next, we analyzed the modification of sinapinic acid itself exposed to peroxynitrite by HPLC equipped with a photodiode array detector and a novel peak, named X, was then detected at 7.6 min (Fig. 2A, filled peak). This compound X exhibited a λ_{max} at 342 nm which was about 15 nm longer than that of sinapinic acid. Moreover, this peak X had a corresponding signal at *m/z* 445 based on LC-MS analysis, which was considered to be a dimerization product of sinapinic acid. In a previous study, we hypothesized that the nitration of *p*-coumaric acid was one possible inhibitory mechanism [9]. However, no nitrated compound was detected in this reaction mixture by LC-MS. To identify the structure of X, isolation was carried out as described in Section 2 and the ^{1}H-NMR spectra were then measured in acetone-d_6 (Table 1). In previous studies, the detection of many dimerization products of sinapinic acid and its related compounds has been reported [15–17]. Comparing these structures, it was suggested

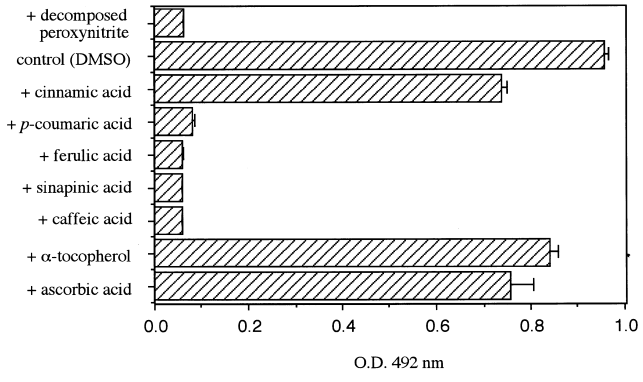
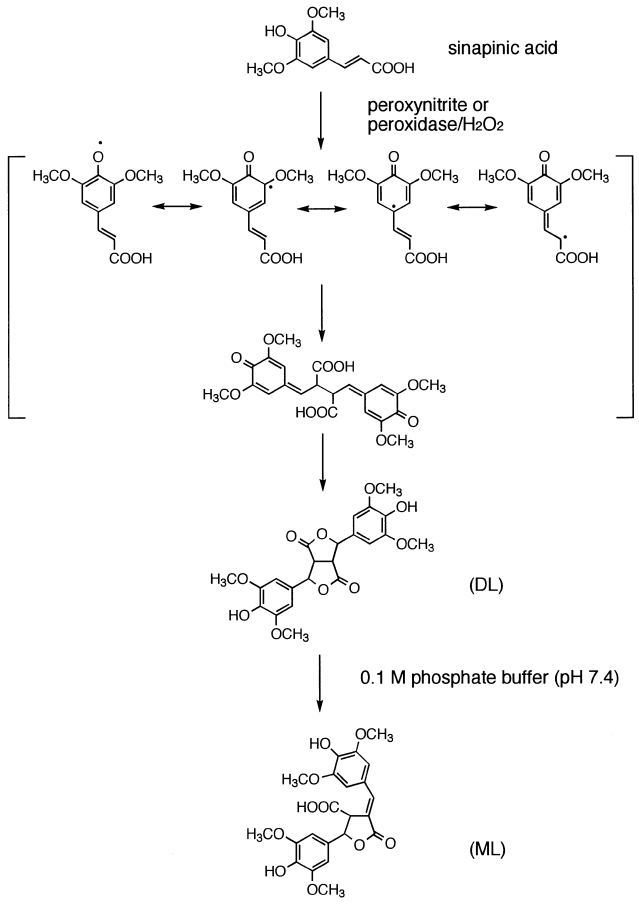


Fig. 1. Effect of some natural antioxidants on protein nitration by peroxynitrite. Protein solutions that contained 0.3 mM each samples were treated with 1.0 mM peroxynitrite and the nitration was evaluated by ELISA as described in Section 2. Control means the peroxynitrite-exposed collagen in the presence of 10% DMSO without antioxidants. Data represent the mean ± S.D. of three measurements.



Scheme 1. Proposed mechanism for the formation of ML via one-electron oxidation of sinapinic acid.

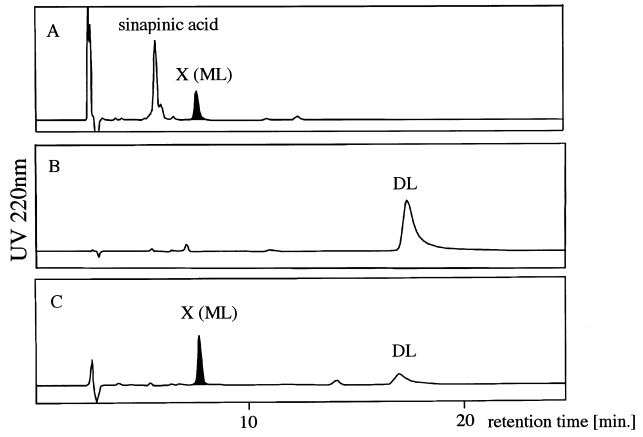


Fig. 2. Typical HPLC chromatogram of peroxynitrite-treated sinapinic acid in 0.1 M phosphate buffer (pH 7.4) (A), horseradish peroxidase/H_2O_2-treated sinapinic acid in water-acetone (B), authentic DL dissolved in 0.1 M phosphate buffer (pH 7.4) in a few hours (C). Analytical conditions are described in Section 2. Peaks eluted at 5.6 and 7.6 min on (A) represent sinapinic acid and its dimer (ML), respectively.

that this compound X had a ML structure (Scheme 1) as described by Ralph et al. [16], since no olefin protons with *trans* coupling were detected by ^{1}H-NMR even though its λ_{max} was shifted to longer wavelengths. This suggests that the sinapinic acid radical, the reaction intermediate with peroxynitrite, did not react at its aromatic ring because of steric hindrance and/or radical orientation, but was dimerized at the side chain via a one-electron oxidation as exhibited in Scheme 1. To confirm this hypothesis, we treated sinapinic acid with horseradish peroxidase and hydrogen peroxide as described in Section 2 (Fig. 2B). This reaction product was different from X, but coincident with a DL obtained from the previous method [18], though its ^{1}H-NMR spectrum was somewhat different (Table 1). However, a spontaneous conversion of DL to X in 0.1 M phosphate buffer (pH 7.4) (Fig. 2C) suggested that the parent molecule of X was DL. This similarity was also indicated by the methylated compounds obtained by the treatment of X with excessive trimethylsilyl(diazomethane) having *m/z* 488 and 502 by EI-MS, respectively, which were also produced from DL by the same agent as by-products of the dimethylated DL. Moreover, di-*t*-butyldimethylsilyl DL, obtained by the conventional method [19], gave di-*t*-butyldimethylsilyl X with its corresponding alcohol by alkaline hydrolysis (data not shown). From these conversions of DL (and its silylether) and spectral observations of compound X as described above, we concluded that the adduct X is a ML, though its stereochemistry has been ambiguous. Based on the results described above, it was suggested that peroxynitrite converted sinapinic acid to ML via a one-electron oxidation as described by Lacki and Duvnjak [20].

Table 1
^{1}H-NMR spectrum of X (ML) and DL

X (acetone-d_6)	DL (acetone-d_6)
7.86 (brs, 1H)	7.47 (s, 2H)
7.56 (d, J = 2.0, 1H)	6.69 (s, 4H)
7.33 (brs, 1H)	5.71 (s, 2H)
7.02 (s, 2H)	4.07 (d, J = 0.5, 2H)
6.63 (s, 2H)	3.79 (s, 12H)
5.69 (d, J = 2.4, 1H)	
4.28 (m, 1H)	
3.81 (s, 6H)	
3.75 (s, 6H)	

In our previous study, a nitrated product was obtained with a poor yield from *p*-coumaric acid [9]. On the other hand, treatment of 200 μM sinapinic acid with two equivalents peroxynitrite, as described in Section 2, gave 61.8 ± 3.4 μM ML with 36.7 ± 2.6 μM of unreacted sinapinic acid. Furthermore, peroxynitrite addition (> 400 μM) caused a decrease in the ML formation (data not shown).

4. Discussion

We have previously described the inhibitory activity of *p*-coumaric acid and caffeic acid against peroxynitrite-mediated 3-nitrotyrosine formation [9] and a similar result was also reported more recently [10]. In these previous studies, ferulic acid and caffeic acid demonstrated stronger activities than *p*-coumaric acid. Moreover, many reports have described the activities of ferulic acid and caffeic acid, including the phenethyl ester as an active component of propolis [21–23], whereas sinapinic acid has not received much attention. In this study, sinapinic acid exhibited inhibitory activity against peroxynitrite-mediated tyrosine nitration, which was stronger than that of the other well known endogenous antioxidants such as α-tocopherol and ascorbic acid (Fig. 1). In addition, we found a dimerization product of sinapinic acid by peroxynitrite treatment and its structure was identified as a ML by NMR and LC-MS. To elucidate the reaction mechanism, we synthesized a DL and observed the rapid conversion of this synthetic DL to ML in neutral buffer. This result suggests that DL was a plausible intermediate of the ML formation as shown in Scheme 1.

Many reports have described the peroxynitrite-mediated reactions and the predominant reactions are considered to be the hydroxylation or nitration of aromatic compounds [9,24]. To resolve these reaction mechanisms, many experiments have been done using antioxidants. However, previous results did not obviously establish whether a one- or two-electron oxidation occurred as competing results were obtained, e.g. the oxidative product of α-tocopherol [7,25] and the effect of the hydroxyl radical scavenger [5,24]. Recently, the reactions of *p*-coumaric acid and its derivatives with peroxynitrite have been described. In these studies, mono-phenolic groups such as *p*-coumaric acid and ferulic acid were nitrated [9,10], whereas catecholate, caffeic acid and chlorogenic acid were converted to *o*-quinone derivatives [10,26]. In the present study, we were able to isolate the dimerization product from sinapinic acid and peroxynitrite. These results suggest that certain kinds of radical species as represented by 'ONOO*' were present in the peroxynitrite-derived reaction. Recent studies [27,28] are gradually revealing its chemistry. However, the peroxynitrite-derived reactant is still ambiguous. Our results, using sinapinic acid, suggest the occurrence of a one-electron oxidation by peroxynitrite treatment similar to the recent study that used melatonin [29], although further study is needed to better elucidate the radical species.

In summary, sinapinic acid strongly inhibits the peroxynitrite-mediated tyrosine nitration of protein in vitro. In this inhibitory reaction, a novel ML was produced by a one-electron oxidation of sinapinic acid, possibly via a di-lactone intermediate.

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