Original Article

The Covalent Binding of Genistein to the Non-prosthetic-heme-moiety of Bovine Lactoperoxidase Leads to Enzymatic Inactivation*

Hebron C. CHANG[1,#], Daniel R. DOERGE[2], ChengHong HSIEH[3], LIN YingJu[4], and FuuJen TSAI[4]

1.Department of Biotechnology, Asia University, Taichung 41354, Taiwan, China; 2.Division of Biochemical Toxicology, National Center for Toxicological Research, FDA, Jefferson, AR 72079, USA; 3.Department of Health and Nutritional Biotechnology, Asia University, Taichung 41354, Taiwan, China; 4. Genetic Center China Medical University, Taichung 40402, Taiwan, China

Abstract

Objective Genistein, a major soy isoflavone metabolite (SIF), inactivates oxidation activity of bovine lactoperoxidase (LPO). Modification of the heme moiety of LPO by nitrogen-containing compounds has been shown to inactivate LPO. In contrast, SIF mediated inactivation of LPO does not involve a heme modification and the mechanism of SIF inhibition is poorly understood.

Methods After inactivation of LPO by genistein in the presence of H_2O_2, trypsin-digested LPO peptide fragments were collected and analyzed by MALDI-TOF-MS to characterize the chemical binding of genistein(s) to LPO.

Results The heme moiety of LPO was not modified by genistein. A covalent binding study showed that ^{3}H-genistein bound to LPO with a ratio of ~12 to 1. After HPLC analysis and peak collection, trypsin-digested peptide fragments were analyzed by MALDI-TOF-MS. The 3H-genistein co-eluted peptide fragments (RT=24 min) were putatively identified as 199IVGYLDEEGVLDQNR214 with two bound genistein molecules or a genistein dimer (2 259 Da), 486TPDNIDIWIGGNAEPMVER504 with two bound genistein molecules or a genistein dimer (2 663 Da), and 161ARWLPAEYEDGLALPFGWTQR182 with three bound genistein molecules or a genistein trimer (3 060 Da). The fragment with a mass of 1 792 Da (RT=36 min) was identified as 132CDENSPYR139 with three genistein molecules or a genistein trimer.

Conclusions The results suggest that LPO was inactivated by irreversible covalent binding of genistein or genistein polymers to particular peptide fragments constituting regions of the outward domain. No genistein interaction with the prosthetic heme moiety of LPO was observed.

Key words: Lactoperoxidase; Genistein; Modification of heme; Covalent binding; Peptide fragments

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INTRODUCTION

Soybean has a long association with goiter in animals and humans, and the promotion of soy as a nutritional aid requires a clear understanding of any possible adverse effects. Previous studies have shown soybean as goitrogenic in human and animals[1]. The consumption of soy products in infant formula and vegetarian diets has caused several problems including goiter and hypothyroidism[2]. Duncan et al. reported that T3 levels decreased in premenopausal women but not in postmenopausal women consuming soy isoflavone supplements[3-4].

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#Correspondence should be addressed to Hebron C. CHANG. Tel: 886-4-23323456-1831. Fax: 886-4-23305834. E-mail: hebronchang@asia.edu.tw

Biographical note of the first author: Hebron C. CHANG, PhD, male, born in 1956, majoring in Biochemical Toxicology.

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Isoflavone metabolites, genistein and daidzein, have been shown to be anti-thyroid compounds present in soy[1]. Genistein and daidzein were also identified as anti-thyroid compounds for both human and porcine thyroid peroxidases (hTPO, and pTPO)[1,5,6] and were classified as suicide inactivators for rat microsomal TPO (rTPO), pTPO, and hTPO[7-8]. Genistein and daidzein have been reported to cause irreversible, time-dependent inactivation of bovine lactoperoxidase (LPO) and pTPO and this inactivation was dependent on H_2O_2-induced turnover. Previous results have shown that inactivation of peroxidase activities of bovine lactoperoxidase (bLPO), microsomal rat thyroid peroxidase (rTPO), human thyroid peroxidase (hTPO) and porcine TPO (pTPO) caused by genistein were 53%±3%, 66%±7%, 62%±3%, and 40%±6%, respectively. Thus, LPO was selected as a model enzyme to elucidate the inactivation mechanism of peroxidase activity mediated by soy isoflavone metabolites in this study. The kinetic parameters for covalent binding of genistein to LPO, and therefore inactivation of LPO peroxidase activity, were determined. The covalent incorporation of ^{3}H-genistein to LPO led to the irreversible inactivation of LPO. Modification of the heme prosthetic group of LPO by genistein was analyzed using LC with electrospray MS after proteolysis of genistein-inactivated LPO. MALDI-TOF mass spectrometry was also applied to study the covalent binding of genistein with LPO peptides. MALDI-TOF results were analyzed to putatively identify the genistein-bound peptide fragments.

MATERIALS AND METHODS

Reagents

Bovine lactoperoxidase (LPO), genistein, hydrogen peroxide, guaiacol, iodoacetamide, and potassium iodide were purchased from Sigma. Tris-(2-carboxyethyl) -phosphine HCl (TECP-HCl) was purchased from PIERCE (Rockford, IL, USA). Pronase, trypsin, endoproteinases Lys-C, Glu-C, and deglycosidase F were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). ^{3}H-genistein (25-30 Ci/mmol) was purchased from SibTech. Co. (Elmsford, NY, USA). Daidzein was purchased from Toronto Research Chemical Co. (North York, Ontario, Canada), and equol was purchased from Indofine Chemical Co. (Somerville, NJ, USA).

Inactivation of LPO by Isoflavones and H_2O_2, and the Determination of the Kinetic parameters

LPO (0.2 μmol/L) was incubated with various concentrations of genistein (10 nmol/L to 1 μmol/L) in the presence of 100 μmol/L H_2O_2. This reaction was initiated by the addition of H_2O_2 and incubated for 12 min. Aliquots of 100 μL removed at 15 seconds and 2 min intervals were assayed for the remaining guaiacol oxidation activity mediated by peroxidase. The guaiacol assay contained 5 mmol/L guaiacol, 500 μmol/L H_2O_2 in 0.1 mol/L potassium phosphate buffer, pH 7.0. Assays were monitored at 470 nm using a HP 8452A diode array spectrophotometer (Hewlett Packard) as previous described[9]. Extrapolation of the linear portion to the abscissa gives the approximate value of the partition ratio. Half-times were plotted against the reciprocal of genistein, daidzein or equol concentrations. K_i is the negative reciprocal of the intercept on the abscissa, and k_{inact} was derived from the ordinate intercept.

Inactivation of Bovine LPO and ^{3}H-genistein Incorporation

LPO (0.26 μmol/L) was incubated with 10 μmol/L genistein (with 0.5 nmol ^{3}H-genistein, ~60 *p*mol; 25-30 Ci/mmol), and various concentrations of H_2O_2 (H_2O_2/LPO = 0, 2.5, 5, 10, 25, 50, and 100) for 30 min at room temperature. Aliquots of 100 μL were removed to measure radioactivity after gel filtration. The incubation mixtures were then filtered using PD-10 G 25 columns. Fractions from 2-6 mL were collected in liquid scintillation vials to measure radioactivity for calculation of the covalent binding of ^{3}H-genistein to LPO.

Determination of Genistein Mediated Heme Modification of LPO by HPLC and LC-MS

One mg of LPO was dissolved in 0.1 mol/L potassium phosphate buffer, pH 7.0, with or without 200 μmol/L genistein and the reaction was initiated by adding various concentrations of H_2O_2 (H_2O_2/LPO = 0, 1, 5, 10, and 50) and the reaction mixtures were incubated at room temperature for 30 min. The remaining activity of each sample was monitored by a guaiacol assay after a gel filtration step. Following chromatography on a PD-10 column equilibrated in potassium phosphate buffer, pH 7.0, pronase was added to the filtrates and incubated overnight at 37 °C. Heme and heme derivatives were analyzed by HPLC using a Vydac C18, 4.6×250 mm column. The solvent system consisted of 0.1% formic acid in acetonitrile (solution A) and 0.1% formic acid in water (solution B). Elution was initiated using a mobile phase consisting of 20% A and 80% B for 2 min followed by a linear gradient to 50% A and 50% B. Isocratic elution in 50% A and 50% B continued for

3 min. The flow rate was 1 mL/min and the elution was monitored at 400 nm. Modified heme samples were also analyzed by LC-MS using the same column and mobile phase for HPLC analysis. Mass determinations were performed using a platform single quadruple mass spectrometer (Waters, MA, USA) equipped with an APCI interface. The mass spectrometer was operated at a capillary voltage of 3.50 kV, a HV lens voltage of 0.45 kV and a cone voltage of 40-110 V. The source temperature was 150 °C and scanning was performed over the range of *m/z* 300-1 150 at a rate of 1.49 s/scan for positive ion acquisition. Covalent binding of heme and ^{3}H-genistein were also analyzed by incubating 1 mg LPO with 100 μmol/L genistein and ~60 nmol/L ^{3}H-genistein in the presence of 750 μmol/L H_2O_2 for 30 min at room temperature. The mixtures were then digested with pronase overnight at 37 °C after gel filtration using a PD-10 G25 column. Samples were analyzed using the above HPLC protocol. Fractions were collected at one-minute intervals in scintillation vials containing 8 mL scintillation fluid (Ultra Gold, Packard) and radioactivity was determined by liquid scintillation spectrometry.

Peptide Digestion and Covalent Binding of ^{3}H-genistein with LPO

One mg of LPO was incubated with or without 1 mmol/L genistein in the presence of 100 μmol/L H_2O_2 in 0.1 mol/L phosphate buffer, pH 7.0 at room temperature for 30 min. After enzymatic digestion following the previously mentioned protocol, the peptide solutions were analyzed by HPLC. HPLC analysis of digested native or inactive enzyme (injection volume = 200 μL) was achieved using an Aquapore BU-300 7 μm, 0.4×25 cm column (Perkin Elmer) with a solvent system containing solvent A (0.1% formic acid in H_2O) and solvent B (0.1% formic acid in acetonitrile). Chromatography was initiated with a mobile phase of 90% A and 10% B for 2 min followed by a linear gradient to 25% A and 75% B over 50 min following isocratic elution for 5 min in 25% A and 75% B. The flow rate was 0.5 mL/min. The elution profile was monitored at 215 nm and fractions were collected at retention times of 24.5, 32, 34, 36, 37.5, and 40 min. The fractions at particular retention times were pooled after 20 injections, dried with a speed vacuum system and then stored at −80 °C for further use. LPO (1 mg) was also incubated with 1 mmol/L genistein, ~60 nmol/L ^{3}H-genistein with or without 1 mmol/L H_2O_2 in 0.1 mol/L phosphate buffer, pH 7.0 at room temperature for 30 min[9]. The incubation mixtures were digested and analyzed by HPLC, as described above. Fractions were collected at one-min intervals in scintillation vials containing 8 mL scintillation fluid and radioactivity was determined by liquid scintillation spectrometry. For the different aliquot studies described above, various concentrations of LPO and genistein were used to have adequate amounts for these experiments. However, all conditions examined were in the saturation status and the percentages of the remaining activities of LPO for each study were analyzed.

MALDI-TOF MS Analysis for Genistein-bound LPO Fragments after Digestion

The dried fraction samples were applied in a Vestec model YM200 (Vestec Inc., Houston, USA) for MALDI-TOF-MS analysis. The mass assignments were made using a commercial software package (Gram 386, Galactic, Salem, NH, USA). The mass accuracy was estimated to be ±5 Da. Only the major peaks that corresponded to peptide fragments were assigned.

RESULTS

Kinetic Parameters Describing the Inactivation of LPO Activity by Isoflavone Metabolites

Inactivation of bovine LPO mediated by isoflavone metabolites was time-dependent. The activity was reduced to ~20%, ~30%, or ~25% by 1 μmol/L genistein, daidzein or equol, respectively, in the presence of 100 μmol/L H_2O_2 (Figure 1). The K_i of genistein and daidzein for LPO were found to be 200 and 500 nmol/L. The k_{inact} of genistein and daidzein for LPO were 1.38 sec^{-1} and 0.92 sec^{-1}, and the partition ratios for LPO were estimated as two and one for genistein and daidzein, respectively (Table 1). However, the incorporation of ^{3}H-genistein and inactivated LPO was about 3 to 1 in the presence of various concentrations of H_2O_2 (Figure 2).

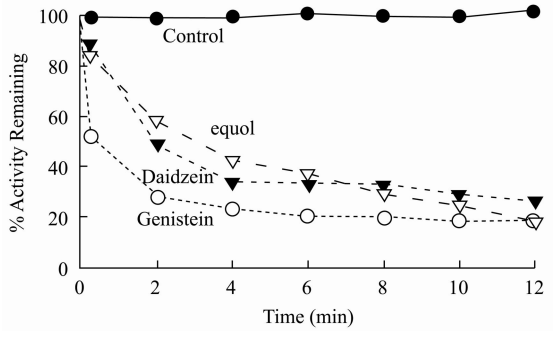

Figure 1. Time-dependent inactivation of bovine LPO by isoflavones.

Table 1. Kinetic Parameters for the Inactivation of LPO by Genistein and Daidzein

Isoflavone Metabolite	K_i (nmol/L)	k_{inact} (min^{-1})	k_{inact} /Ki($nmol \cdot L^{-1} \cdot min^{-1}$)	Partition Ratio
Genistein	200 (50*)	1.38 (0.28*)	6.90×10^{-3} ($5.60 \times 10^{-3*}$)	2
Daidzein	500 (143*)	0.92 (0.31*)	1.84×10^{-3} ($2.17 \times 10^{-3*}$)	1

Note. *Data taken from the kinetic parameters of rat TPO inactivated by soy isoflavone metabolites[8].

Analysis of Heme Modification Mediated by Genistein and Covalent Binding of Genistein and LPO

Following the inactivation of LPO guaiacol oxidation activity by genistein in the presence of H_2O_2, heme derivatives and the percentage of modified heme were analyzed by HPLC. Results showed that the heme content was not modified following gel filtration (Figure 3). A titration covalent binding study of ^{3}H-genistein and LPO showed that the covalent binding of genistein and LPO was ~12 to1 in the presence of 500 nmol/L genistein (data not shown). These results implied that the inactivation of LPO mediated by genistein did not involve the heme moiety, as shown for other nitrogen-compounds[9].

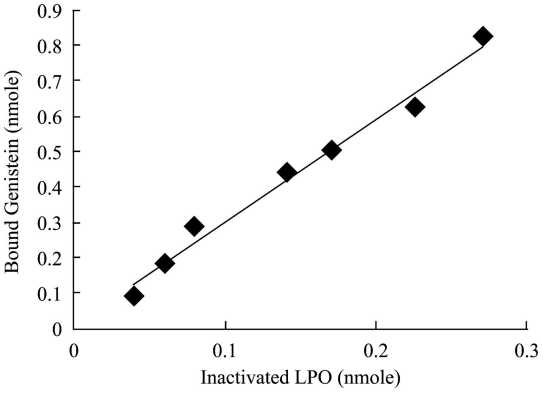

Figure 2. The inactivation of bovine LPO and the incorporation of genistein.

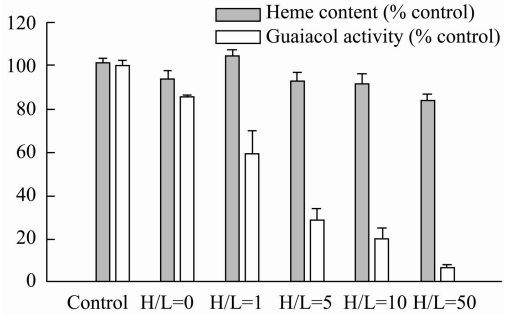

Figure 3. Analysis of the percent heme content, and the percent guaiacol activity for enistein-inactivated LPO.

MALDI-TOF MS Analysis for Genistein-bound LPO Peptides after Enzymatic Digestion

Genistein-bound LPO peptide analysis using HPLC and liquid scintillation spectrometry gave six peaks (retention times at 24, 32, 34, 36, 37.5, and 40 min) representing six peptide fragments that co-eluted with bound ^{3}H-genistein (Figure 4). LC/MS/MS analysis also confirmed that various molecules of genistein, which may have polymerized, were found in these peaks (data not shown). Non-radioactive MALDI-TOF-MS analysis showed that the species eluting at 24 min contained peptides with masses of 2 259, 2 663, and 3 060 Da (data not shown). The peptide fragment 2 259 Da was identified as ^{199}IVGYLDEEGVLDQNR^{214} (1 719) with two bound genistein molecules (540). The peptide fragment 2 663 Da was identified as ^{486}TPDNIDIWIG GNAEPMVER^{504} (2 123) with two bound genistein molecules (540). The peptide fragment 3 060 Da was identified as ^{161}ARWLPAEYEDGLALPFGWTQR^{182} (2 250) with three bound genistein molecules (810). The species that eluted at 36 min contained peptides with masses of 1 222 and 1 792 Da. The peptide fragment of 1 792 Da was identified as ^{132}CDENSPYR^{139} (982) with three bound genistein molecules (810) (Table 2). No major peptide related fragments were identified in the fractions collected at the retention times of 32, 34, 37.5, or 40 min. The observed radioactivity from these fractions may have been caused by ^{3}H-genistein or the formation of genistein polymers (as observed in LC-MS-MS analysis). In conclusion, the results indicate that 10-12 genistein monomers may have formed poly mers that are incorporated into peptide sequences of LPO and therefore inactivate the enzyme.

DISCUSSION

From previous results and results presented in this study, genistein or daidzein represent suicide inactivators for LPO, porcine TPO, and rat TPO[8]. Genistein is more potent than daidzein and equol for the inactivation of bovine LPO and porcine TPO. This

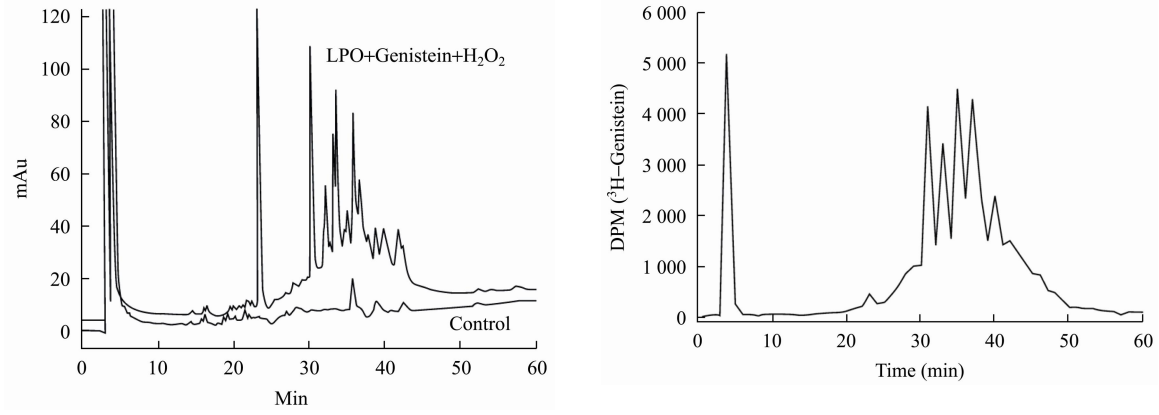

Figure 4. Peptide fragmentation analysis by HPLC and radioactivity. Upper plot: LPO was incubated with or without genistein in the presence of H_2O_2. Lower plot: LPO was incubated with genistein, 3H-genistein and H_2O_2. The mixture was digested and analyzed by HPLC as previously described [9].

Table 2. Assignments of Peptide Fragments from MALDI-TOF MS Analysis

RT (min)	Peptide Fragments (Da)	Assigned Amino Acid Sequences + Genistein Metabolite
	2 259	^{199}IVGYLDEEGVLDQNR^{214} (1 719) + 2 genistein (540)
24	2 667 (2 263*)	^{486}TPDNIDIWIGGNAEPMVER^{504} (2 127; 2 123*) + 2 genistein (540)
	3 060 (3 063*)	^{161}ARWLPAEYEDGLALPFGWTQR^{182} (2 250; 2 253*) + 3 genistein (810)
36	1 222	N/A
36	1 792	^{132}CDENSPYR^{139} (982) + 3 genistein (810)
32, 34, 37.5, or 40 min	N/A	N/A

Note. *Observed Mass.

phenomenon was also reported by others for human recombinant TPO[10]. Interestingly, a previous report showed that genistein also reduced oxidative activity of rat TPO and LPO by 80%[8]. However, the K_i of genistein and daidzein for rat TPO were 50 and 143 nmol/L, and the k_{inact} of genistein and daidzein for rat TPO were 0.28 and 0.31 sec^{-1}, respectively[8]. Both results showed that genistein was more potent against LPO and rat TPO oxidase activities. Although the loss of LPO guaiacol oxidation activity mediated by genistein or daidzein involved a mechanism based inactivation with a turnover number of 1-2 and a covalent binding phenomenon of genistein to LPO, the possible covalent modification of the bis-hydroxymethyl heme of LPO by genistein was not observed during the turnover. This result did not agree with the report published by Singh et al. in which aromatic products covalently bound to the heme moiety of LPO and caused inhibition of LPO activity[10]. A previous report showed that genistein polymers were possibly formed during the incubation of genistein with rat TPO in the presence of hydrogen peroxide, and the polymer(s) may play a role in rat TPO inactivation. Based on the observed turnover number of genistein to LPO (2:1), genistein polymers may also be produced in this reaction. It was also proposed that the heme moiety of peroxidase was the active center for rat TPO compound I formation, and this may be affected by genistein; however, heme modification of rat TPO was not observed[8]. Loss of oxidative activity of LPO was caused by the covalent binding of genistein to LPO, but no evidence was found that covalent modification caused by genistein was due to this compound binding into the active site of LPO. Interestingly, a titration covalent binding study of ^{3}H-genistein and LPO showed that the covalent binding of genistein to LPO was ~12 to 1 in the presence of 500 nmol/L genistein during turnover. This result indicates that the inactivation of LPO activity is caused by the covalent binding of genistein to a structural peptide(s) that may lead to a conformational change rather than the modification of the heme moiety.

The genistein modified peptide fragments after trypsin digestion included ^{132}CDENSPYR^{139} and ^{161}ARWLPAEYEDGLALPFGWTQR^{182} that were found to putatively bind three molecules of genistein. The

peptide fragments ^{199}IVGYLDEEGVLDQNR^{214} and ^{486}TPDNIDIWIGGNAEPMVER^{504} were found to putatively bind two molecules of genistein. Ten molecules of genistein to one molecule of LPO was calculated from the digest experiments, and this finding is concomitant with the results of the titration covalent binding study (ca. 12 to 1). The binding sites of LPO are Asp225 and Glu375, as shown in previous reports[11-12]. The active site residue is His226, and the residues involved in coordinating the metal are Lys227, Thr301, Phe303, Asp305, Ser307, and His468[12]. Singh et al. showed that aromatic ligands such as acetyl salicylic acid (ASA), salicylhydroxamic acid (SHA) and benzylhydroxamic acid (BHA) bound to LPO at the substrate binding site on the distal His side with hydrogen bond formation and this binding mediated the inhibition of LPO; however, no covalent binding was reported[12]. LPO, myeloperoxidase (MPO), eosinophil peroxidase (EPO) and thyroid peroxidase (TPO) were recently reconstructed as the peroxidase-cyclooxygenase superfamily[13]. These peroxidases transform to compound I and compound II in the presence of hydrogen peroxide during turnover, resulting in the generation of free radicals[8,14-16]. Genistein radicals produced[8] by peroxidase in the presence of hydrogen peroxide may be polymerized or covalently bound to peptide fragments of peroxidases. Such covalent attachment leads to enzymatic inactivation and most likely differs from the inhibition of peroxidases caused by non-covalent binding of inhibitors in the active site[12]. Singh et al. reported that the substrate channel of LPO contain aromatic residues including Phe113, Phe239, Phe254, Phe380, Phe381, Phe422, and Pro424, and a phosphorylated Ser198 was at the surface of the calcium binding channel[10]. These results revealed that the heme moiety was covalently modified by $OSCN^-$ and caused the inhibition of LPO activity. The properties of $OSCN^-$, ASA, SHA and BHA studies by Singh et al might not be the same as soy metabolites. Singh et al. reported significant findings on the inhibition mechanism of LPO by these compounds. Sisecioglu et al. also reported that the phenolic hydroxyl group of norepinephrine bound to the heme moiety of LPO in the same manner[17]. Huang et al. mentioned that oxidation of SCN^-, Br^-, and Cl^- (X^-) by horseradish peroxidase (HRP) and other plant and fungal peroxidases resulted in the modification of the heme moiety by HOX. However, by using a LPO heme moiety mutant (D225E375), it was proposed that the heme moiety of LPO was protected from vinyl group modification by the heme protein covalent bonds[18]. The covalent binding between the heme and the protein residues of LPO protected the heme from modification by halide ions[18-19]. This hypothesis supported the concept that the heme moiety of LPO was protected and was not modified by genistein or its polymer as proposed in this study.

The phenomenon of covalent binding of genistein to particular amino acids in the identified peptide fragments was not performed. Amino acids in the active site moiety of LPO that contain hydroxyl groups have previously been reported to form hydrogen bonds with aromatic compounds[10]. The peptide fragments that contain amino acids with hydroxyl groups may represent the target moieties for the genistein radical to form a covalent modification with LPO. The substrate channel found in LPO[10] may impede the genistein polymer(s), if it is indeed formed, to bind with the active site and to generate a modified active site due to the biophysical properties of the substrate channel of LPO. This possible obstruction may cause covalent binding of genistein polymer(s) with other amino acids other than the active site moiety that might affect the conformation of LPO and result in LPO inactivation. The results of molecular modeling have shown that the covalent binding of genistein and the heme moiety do not occur (data not presented). It was also interesting that the peptide fragments found covalently bound to genistein in this study are located in the outward domain of LPO (data not shown). Molecular modeling analysis to study the binding mechanism of genistein and/or genistein polymers with aromatic amino acid(s) in the assigned peptide fragments represents an interesting future study.

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