行政院國家科學委員會專題研究計畫 成果報告

大豆異黃酮代謝物致甲狀腺過氧化氫酵素失活現象---共價結合研究

研究成果報告(精簡版)

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行政院國家科學委員會補助專題研究計畫 v 成 果 報 告
□期中進度報告

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---共價結合研究

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執行單位: 亚洲大学生物科技与生物资讯系

中 華 民 國 95 年 01 月 22 日

I. 中文摘要

由於大豆異黃酮與人類及豬甲狀腺的功能有關,在推薦大豆食品作為保健食品的同時,必需考慮它對人類可能產生的不良影響.大豆異黃酮代謝物,如染料木黃酮(genistein)及大豆甘原(daidzein)已被證明對甲狀腺功能有不良的影響.本研究發現染料木黃酮,大豆甘原及雌馬酚(equol)對泌乳過氧化氫酵素(LPO),豬甲狀腺過氧化氫酵素(pTPO)的活性的致失活效果隨著作用時間而增加.酵素動力學的參數說明LPO及pTPO被大豆異黃酮代謝物導致失活的抑制為一種"機制因子型的致失活作用(mechanism-based inactivation). 染料木黃酮及大豆甘原對 LPO 的解離常數(*Ki*)為 0.2 及 0.5 mM,分配係數(partition ratio)均 為 2 比 1。以放射性同位素氚標定的染料木黃酮與 LPO 作用,在 LPO 失去活性失去後檢測染料木黃酮與 LPO 的共價結合率.結果顯示大約 500nmole 的染料木黃酮可與 40nmole 的 LPO 結合(約 12.5:1). 以液相二極質譜儀(LC-MS-MS)檢測 LPO 活化中心亞鐵紅素(heme)的變化,結果顯示,雖然 LPO 或大鼠 TPO 的活性降低,總亞鐵紅素卻沒有減少,也沒有被修飾的現象. 以液相二極質譜儀(LC-MS-MS)檢測 LPO活化中心亞鐵紅素(heme)的變化,結果顯示,雖然LPO或大鼠TPO 的活性降低,總亞鐵紅素卻沒有減少,也沒有被修飾的現象. 以放射性同位素稀釋法檢測染料木黃酮與 LPO 的共價結合現象, 以氚---染料木黃酮與 LPO 作用,再以 HPLC 分析經過胰蛋白酵素分解的蛋白胜(peptide),發現約有 5¯6 個分離峰有氚---染料木黃酮.再以不具放射性同位素的染料木黃酮與 LPO 作用, 根據氚---染料木黃酮的分離峰保留時間(retention time),收集對應峰的分離液,濃縮後以 MALDI-TOF MS 檢測分子量,5¯6 個對應峰分離液(保留時間=24.5, 32, 34, 36 or 37.5 分)的分子量介於 820-3,500 Da. 保留時間為 24.5 分的分離峰可以檢測出 2,259, 2,663 及 3,350 Da 等代謝物.經由計算可以推測 2,259 = LVGYLDEEGVLDQNR (1,719) + 2 個染料木黃酮 (270); 2,667(分子量計算值; MALDI-TOFMS 分子量檢測值為 2,263) = TPDNIDIWIGGNAEPMVER (2,127 為分子量計算值; MALDI-TOFMS 分子量檢測值為 2,123) + 2 個染料木黃酮 (270); 及 3,060 (為分子量計算值; MALDI-TOFMS 分子量檢測值為 3,063) =WLPAEYEDGLALPFGWTQR (2,250 為分子量計算值; MALDI-TOFMS 分子量檢測值為 2,253) + 3 個染料木黃酮 (270). .保留時間為 36 分的分離峰可以檢測出 1,222 及 1,792 Da 等代謝物.經由計算可以推測: 1,792 為 CDENPYR (982) + 3 分子的染料木黃酮 (270). 推論染料木黃酮可與上述蛋白胜片段中的 組氨酸(H), 苯丙氨酸(F), 色氨酸(W) 或酪氨酸 (Y)產生共價結合. 然而,在保留時間為 32, 34 或 37.5 分的分離峰分離液中無法檢測出蛋白胜. 以 LC-MS-MS 檢測的結果發現,保留時間為 32, 34 或 37.5 分的分離峰含有染料木黃酮的多聚合體,這個結果可以解釋這三個分離峰的放射性,基本上是由氚---染料木黃酮的多聚合體所反應出來的結果.本研究進一步將比對 LPO 及其他甲狀腺過氧化氫酵素的氨基酸序列,探討染料木黃酮與其他甲狀腺過氧化氫酵素共價結合的關係.

關鍵詞: 大豆異黃酮, 泌乳過氧化氫酵素, 甲狀腺過氧化氫酵素, 共價結合, 蛋白胜片段, 氨基酸序列

Inactivation of thyroid peroxidase by isoflavone metabolites- a covalent binding study

II. ABSTRACT

The soybean has a long association with goiter in animals and humans and current promotion by soy as a nutritional aid requires a full understanding of potential adverse effects. It was previously identified that isoflavone metabolites, genistein and daidzein, as the only anti-thyroid compounds present in soy. In this study, it was showed that genistein, daidzein and equol caused irreversible, time-dependent inactivation of bovine lactoperoxidase (LPO) and porcine thyroid peroxidase (TPO) that was dependent on H_2O_2-induced turnover. The kinetics was consistent with a mechanism-based inactivation and apparent dissociation constant for genistein and daidzein were 0.2 and 0.5 μM, respectively. The partition ratios were estimated to be 2:1 for genistein:LPO and daidzein:LPO, respectively. Radiolabeled genistein was applied as a model to study the covalent bonding of genistein and LPO, which was concomitant to the loss of enzymatic activity and the binding was not recovered by gel filtration. The heme prothetic group of LPO and TPO was released by proteolysis and analyzed using LC with electrospray MS. Although it was observed that total heme decreased after isoflavone-mediated inactivation, no evidence for modification of LPO and rat TPO (rTPO) in which radical products derived from oxidative processing of genistein and daidzein inactivate the peroxidases by destruction of the prosthetic heme and/or effects of soy and prompted further examination of isoflavone-induced thyroid effects *in vivo.* Covalent binding study of ^{3}H-genistein and LPO showed that the covalent binding of genistein and LPO reached to 40 nmole/nmole in the presence of 500 nM genistein.

Peptide analysis showed that radilabeled genistein bound to 5~6 peptide fragments with molecular weights between 820 Da to 2,700 Da. MALDI-TOF MS analysis showed that fragments with molecular weight at 2259, 2663 and 3350 Daltons (peak at 24.5 min) were putatively recognized as: 2259= LVG**Y**LDEEGVLDQNR (1719) + 2 genistein (270); 2667 (observed MW=2263) = TPDNIDI**W**IGGNAEPMVER (2127; OMW=2123) + 2 genistein (270); and 3060 (OMW=3063) =**W**LPAE**Y**EDGLALP**F**G**W**TQR (2250; OMW = 2053) + 3 genistein (270). Molecular weight at 1222 and 1792 Daltons were observed in the peak sample of retention time at 36 min. The fragment of 1792 Daltons was putatively recognized as: 1792 = CDENP**Y**R (982) + 3 genistein (270). These results concluded that genistein might covalently bound to the aromatic amino acids such as histidine (H), phenylalanine (F), tryptophan (W) or tyrosine (Y). However, no major peptide related fragments were observed in the pooled samples collected from retention times at 32, 34, 36 or 37.5 min. LC-MS-MS analysis showed that these peaks might be the fractions containing genistein polymers for covalent binding study of ^{3}H-genistein and LPO showed that the these peaks were highly radioisotope labeled fractions due to the response from ^{3}H-genistein.

Key words: soy isoflavones, lactoperoxidase, thyroid peroxidase, covalent binding, peptide, amino acid sequence

III. INTRODUCTION

Previous studies considered soybean as goitrogenic in human and animals (1-6). The consumption of soy products in infant formula and vegetarian diets may become potential problems causing goiter and hypothyroidism (1-3). It was reported that genistein and daidzein inactivated TPO-catalyzed iodination and coupling reactions (6). The kinetics for inactivation of LPO by isoflavone metabolites was still not clarified. In this study, bovine LPO was used as a model to elucidate the inactivation mechanisms of bovine LPO by genistein, daidzein and equol. Thee results were applied to study the inactivation of porcine TPO and rat TPO microsomes by soy isoflavone metabolites.

IV. MATERIALS AND METHODS

Chemicals.

Lactoperoxidase (LPO) (A_{412}/A_{280}=0.80; ε_{412}= 112 $mM^{-1}cm^{-1}$), genistein, hydrogen peroxide, guaiacol, iodoacetamide, potassium iodide were purchased from Sigma. Tris-(2-carboxyethyl)-phosphine HCl (TECP-HCl) was purchased from PIERCE (Rockford, IL). Pronase, trypsin, endoproteinasews 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). Daidzein was purchased from Toronto Research Chemical Co. (North York, Ontario, Canada), and equol was purchased from Indofine Chemical Co. (Somerville, NJ). Thyroid peroxidase was prepared with modification as described (6).

Inactivation of LPO by isoflavones and H_2O_2.

LPO (0.2 μM) was incubated with various concentrations of genistein (10 nM to 1 μM), daidzein (5 nM to 500 nM) or equol (10 nM to 1 μM) in the presence of 100 μM H_2O_2. This reaction was initiated by H_2O_2 and incubated for 12 minutes. Aliquots of 100 μl were removed at 15 seconds and 2 minutes intervals and were assayed for remaining guaiacol oxidation activity. The guaiacol assay contained 5 mM guaiacol, 500 μM H_2O_2 in 0.1 M potassium phosphate buffer, pH 7.0. Assays were monitored at 470 nm using HP 8452A diode array spectrophotometer (Hewlett Packard). LPO with same concentrations were also incubated with 1 μM genistein, daidzein or equal for 12 minutes to compare the % remaining guaiacol oxidation activity in the presence of 100 μM H_2O_2. KI (100 μM) was also added to the mixtures contained 1 μM genistein and 100 μM H_2O_2 to study the recovery of oxidation rate by KI.

Inactivation of microsomal TPO by isoflavones and H_2O_2.

Microsomal TPO (0.2 μM) was incubated with 1 μM genistein, daidzein or equol. The reactions were initiated by 250 nM H_2O_2. Percent of remaining activity was monitored by using guaiacol assay as mentioned above.

Determination of kinetic parameters for inactivation of LPO by genistein, daidzein or equol

LPO (0.2 μM) was incubated for 12 min at room temperature with 100 μM H_2O_2 and various concentrations of genistein (10 nM ~ 1 μM) or daidzein (5 nM ~ 500 nM) in a final volume of 1 ml to assess inactivation of LPO by genistein or daidzein during turnover. Aliquots of 100 μl were removed at 15 seconds and 2-minute intervals and were assayed for

remaining activity using the guaiacol oxidation assay. Guaiacol assay mixtures contained 5 mM guaiacol, 500 μM hydrogen peroxide in 0.1 M phosphate buffer, pH 7.0. Assays were monitored at 470 nm using a HP 8452A diode array spectrophotometer. Extrapolation of the linear portion to the abscissa gives the approximate value of partition ratio. Half times were plotted against the reciprocal of genistein, daidzein or equol concentration. K_i is the negative reciprocal of the intercept on the abscissa; $t_{1/2}$ is the intercept on the ordinate.

Covalent binding of ^{3}H-labeled genistein to LPO.

LPO (0.26 μM) was incubated with 10 μM 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 minutes at room temperature. Aliquots of 100 μl were removed to measure radioactivity after gel filtration. The incubation mixtures were filtered by using PD-10 G 25 columns. Fractions from 1~6 ml were collected in 20 ml liquid scintillation vials. Twenty ml of scintillation fluid (Ultra Gold, Packard) was added and radioactivity was measured by liquid scintillation spectrometry. Radioactivity present was used to calculate the amount of ^{3}H-genistein bound covalently bound to LPO.

Determination of heme by HPLC and LC/MS.

One mg of LPO was dissolved in 0.1 M potassium phosphate buffer, pH 7.0, with or without 650 μM genistein. 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 mixture was incubated at room temperature for 30 minutes. The remaining activity of each sample was monitored by guaiacol assay after gel filtration. Following chromatography on a PD-10 column equilibrated in potassium phosphate buffer, pH 7.0, pronase (0.25 mg/mg enzyme) was added to protein containing fractions which were then incubated at 37 ^{O}C overnight. Heme and heme derivatives were analyzed by HPLC using a Vydac C18, 4.6 x 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 20% A and 80% B for 2 minutes followed by a linear gradient to 50% A and 50% B. Isocratic elution in 50% A and 50% B continued for 3 minutes. The flow rate was 1 ml/min. Elution was monitored at 400 nm.

Samples were also analyzed by LC/MS using the same column and mobile phase. Mass determinations were performed using a platform single quadrupole mass spectrometer (Micromass, Altrincham, U.K.) equipped with an APCI interface. The mass spectrometer was operated at capillary voltage 3.50 kV, HV lens voltage 0.45 kV, cone voltage 40~110 V. The source temperature was 150°C and scanning was performed over the range of *m/z* 300-1150 at a rate of 1.49 sec/scan for positive ion acquisition.

LPO (0.26 μM) was also incubated with 10 μM genistein (with 0.5 nmol ^{3}H-genistein), and various concentrations of H_2O_2 (H_2O_2 /LPO = 0, 2.5, 5, 10, 25, 50, and 100) in a total volume of 1 ml for 30 minutes at room temperature. Aliquots were removed to measure radioactivity and guaiacol oxidation activity after gel filtration. Radioactivity present was used to calculate the amount of ^{3}H-genistein bound covalently bound to LPO. Inactivated LPO was calculated according to the loss of guaiacol oxidation activity.

Covalent binding of heme and ^{3}H-genistein were also analyzed by incubating 1 mg LPO with 100 μM genistein and ~60 nM ^{3}H-genistein in the presence of 750 μM H_2O_2 for 30 minutes at room temperature. The mixtures were then digested with pronase for overnight at 37°C after gel filtration by using PD-10 G 25 column. Samples were analyzed by HPLC mentioned

above. Fractions were collected at one-minute intervals in scintillation vials containing 8ml scintillation fluid (Ultra Gold, Packard) and radioactivity was determined by Liquid Scintillation Spectrometry.

Peptide digestion and covalent binding of ^{3}H-genistein with peptides.

One mg of LPO was incubated with or without 650 μM genistein in the presence of 650 μM H_2O_2 in 0.1 M phosphate buffer, pH 7.0 at room temperature for 30 minutes. The reaction mixtures were passed through PD-10 columns equilibrated with the same buffer. One-ml fractions were collected. Fractions from 4~6 ml containing the large molecules, were collected and pooled. Two ml of a buffer containing 0.1 M Tris-HCl, 6M guanidine, 0.2 mM EDTA, pH 8.0 and 1.0 mg TECP-HCl were added to the collected fractions which were then incubated at 45°C. After one hr incubation, 100 mg iodoacetamide was added to the mixture and incubation was continued for another one hr at 45°C. The mixture was then dialyzed (Spetra/Por MWCO: 6-8,000; The Spectrum Co., Gardena, CA) against double deionized water for 4 hr and then against 0.1 M Tris-acetate buffer, pH 8.0 overnight. The dialyzed solution was incubated with one unit of deglycosidase F at 37°C for 4 hr then with trypsin (trypsin: protein 1:30), 0.5 units endoproteinase Lys-C, and 5 units Glu-C at 37°C overnight. After enzymatic digestion, the peptide solution was analyzed by HPLC.

HPLC of digested native or inactivated enzyme (injection volume = 200 μl alquots) was achieved on an Aquapore BU-300 7 μ, 0.4 x 25 cm column (Perkin Elmer). The solvent system contained 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 minutes followed by a linear gradient to 25% A and 75% B over 50 minutes. This was followed by isocratic elution for 5 minutes in 25% A and 75% B. The flow rate was 1.0ml/min. Elution was monitored at 215 nm and 255 nm.

LPO (1 mg) was incubated with 650 μM genistein, ~60 nM ^{3}H-genistein and 650 μM H_2O_2 in 0.1 M phosphate buffer, pH 7.0 at room temperature for 30 minutes as described above. The mixture was digested and analyzed by HPLC also as described above. 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

MALDI-TOF MS analysis for genistein-bound LPO fragments after digestion

LPO (1mg) was incubated with 650 μM genistein and 650 μM H_2O_2 in 0.1 M phosphate buffer, pH 7.0 at room temperature for 30 minutes as described above. The mixture was digested and then aliquots of 200 μl sample preparations were analyzed by HPLC and the digested fragments were collected according to the co-elution peaks showed on previous study. The peaks with retention times at 24.5, 32, 34, 36, 37.5 and 40 min were collected and pooled after 20 injections. The pooled samples were then dried with a speed vacuum system. The dried samples were applied in a Vestec model YM200 (Vestec Inc., Houston, TX, USA) MALDI-TOF MS for analysis (7). Mass assignments were made using a commercial software package (Gram 386, Galactic, Salem, NH). The mass accuracy was estimated to be $\pm$ 5 Da. Only the major peaks that corresponded to peptide fragments were assigned. Not all peaks were interpreted.

V. RESULTS AND DISCUSSION

The inactivation of bovine lactoperoxidase (LPO) by genistein, daidzein and equol were

time-dependent (Fig. 1). The inactivation of LPO mediated by 1 μM genistein was ~90% recovered by 100 μM KI after 12 min incubation (Fig. 2). These data showed that in the presence of KI which might be a competitor for genistein for LPO inactivation. In the presence of 250 μM H_2O_2, LPO activity was reduced to ~60% by 10 nM genistein, 1 μM daidzein or μM equol after 12 min incubation. However, 1 μM genistein had only ~50% inactivation for porcine TPO under the same condition (Fig. 3). Partition ratio studies for genistein, daidzein and bovine LPO showed that the Ki of genistein and daidzein for LPO were 200 nm and 500 nM. The k_{inact} of genistein and daidzein for LPO were 1.38 sec^{-1} and 0.92 sec^{-1} respectively. The heme prothetic group of LPO and TPO was released by proteolysis and analyzed using LC with electrospray MS. Although it was observed that % of total heme did not decrease after isoflavone-mediated inactivation, no evidence for modification of LPO (Fig. 4) in which radical products derived from oxidative processing of genistein and daidzein inactivate the peroxidases by destruction of the prosthetic heme and/or effects of soy and prompted further examination of isoflavone-induced thyroid effects *in vivo* (7-10). Titration covalent binding study of ^{3}H-genistein and LPO showed that the covalent binding of genistein and LPO reached to 40 nmole/nmole in the presence of 500 nM genistein (data not shown). These results implied that inactivation of LPO by genistein was not in the heme moiety as other LPO inactivator (12)

Peptide fragmentation analysis by HPLC and radioactivity showed that after enzymatic digestion with trypsin, endoproteinase Lys-C, and Glu-C at 37°C overnight, five peaks (RT=24.5, 32, 34, 36, 37.5 and 40 min) after peptide fragmentation were found with ^{3}H-genistein bound (Fig. 5). LC/MS/MS analysis also confirmed that various molecules of genistein were found in these peaks (data not shown). MALDI-TOF MS analysis showed that fragments with molecular weight at 2259, 2663 and 3350 Daltons (peak at 24.5 min). These fragments were putatively recognized as: 2259= LVG**Y**LDEEGVLDQNR (1719) + 2 genistein (270); 2667 (observed MW=2263) = TPDNIDI**W**IGGNAEPMVER (2127; OMW=2123) + 2 genistein (270); and 3060 (OMW=3063) =**W**LPAE**Y**EDGLALP**F**G**W**TQR (2250; OMW = 2053) + 3 genistein (270). Molecular weight at 1222 and 1792 Daltons were observed in the peak sample of retention time at 36 min. The fragment of 1792 Daltons was putatively recognized as: 1792 = CDENP**Y**R (982) + 3 genistein (270) (Table 1). However, no major peptide related fragments were observed in the pooled samples collected from retention times at 32, 34, 36 or 37.5 min. LC-MS-MS analysis showed that these peaks might be the polymers of genistein (data not shown). From these results observed in this study, it is concluded that genistein and daidzein are suicide inactivators for bovine LPO and porcine TPO. Genistein is more potent than daidzein and equol for the inactivation of bovine LPO and porcine TPO. KI is able to reverse genistein-inactivated LPO guaiacol oxidation activity. Loss of LPO guaiacol oxidation activity is due to covalent binding of genistein to LPO. Bis-hydroxymethyl heme of LPO was not affected by genistein during turnover, also no evidence to show the covalent binding of genistein adducts with heme adducts. ^{3}H-genistein covalently bound to peptide fragments of LPO during turnover, thus the covalent binding of genistein to LPO is responsible for LPO inactivation. Polymers of genistein may be formed in the incubation of genistein and LPO in the presence of hydrogen peroxide.

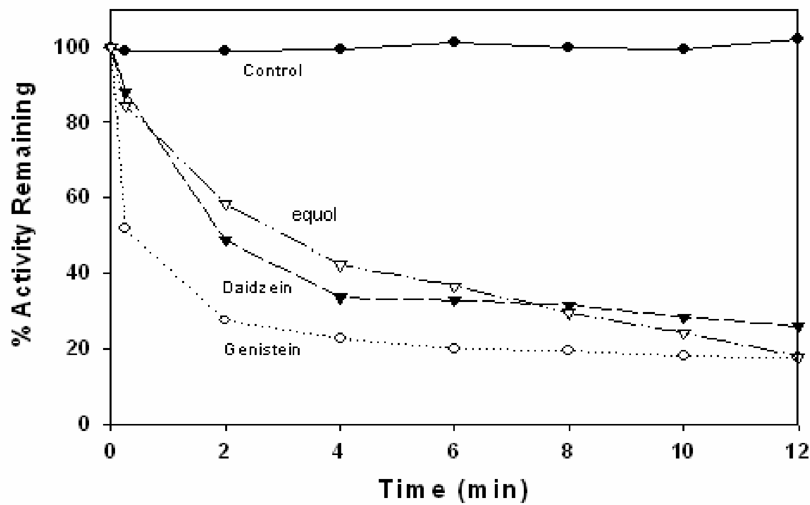

Figure 1 . Time-dependent inactivation of bovine LPO by isoflavones. LPO (0.2 µM) were also incubated with 1 µM genistein, daidzein or equal in 0.1 M potassium phosphate buffer, pH 7.0 for 12 minutes in the presence of 100 µM H_2O_2. Aliquots of 100 µl were removed at 15 seconds and 2 minutes intervals and were assayed for remaining guaiacol oxidation activity The guaiacol assay contained 5 mM guaiacol, 500 µM H_2O_2 in 0.1 M potassium phosphate buffer, pH 7.0. Assays were monitored at 470nm.

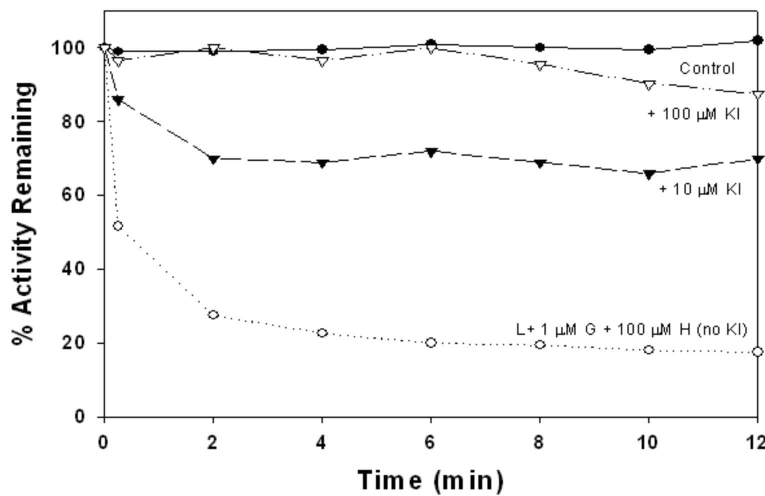

Figure 2. Recovery of genistein-inactivated bovine LPO activity. KI (10 µM or 100 µM) was added to the mixtures contained 0.2 µM LPO, 1 µM genistein and 100 µM H_2O_2 in 0.1 M potassium phosphate buffer, pH 7.0. Aliquots of 100 µl were removed at 15 seconds and 2 minutes intervals and were assayed for remaining guaiacol oxidation activity to study the recovery of LPO oxidation activity by KI.

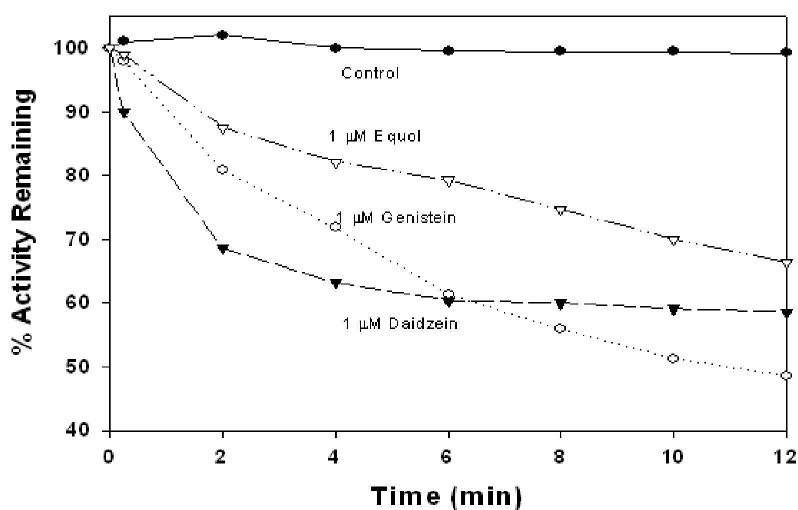

Figure 3. Inactivation of porcine TPO (pTPO) by isoflavones. pTPO (0.2 μM) was incubated with 1 μM genistein, daidzein or equol, and 250 nM H_2O_2. Aliquots of 100 μl were removed at 15 seconds and 2 minutes intervals to analyze the inactivation rate of porcine TPO by isoflavones. Percent of remaining LPO oxidation activity was monitored by using guaiacol assay as mentioned in Figure 1.

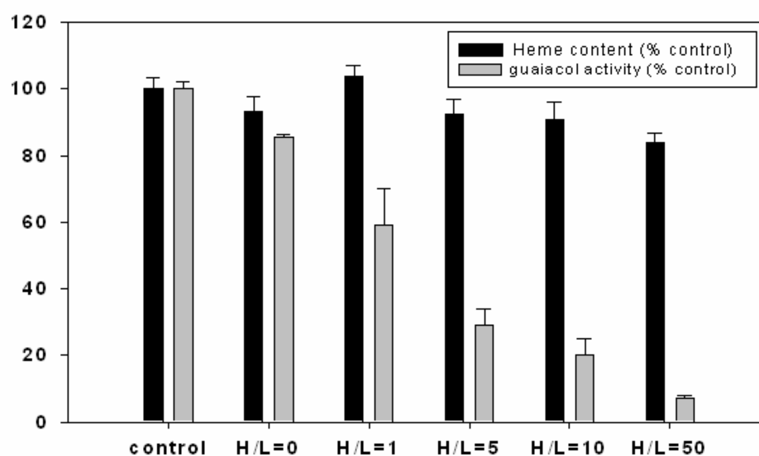

Figure 4. Analysis of percent heme content, and percent guaiacol activity for genistein-inactivated LPO. LPO (13 μM) was incubated with or without 650 μM genistein, and various concentrations of H_2O_2 (H_2O_2/ LPO = 0, 1, 5, 10, and 50). After incubation at room temperature for 30 minutes, reaction mixtures were analyzed for guaiacol oxidation activity (as mentioned in Figure 1) after gel filtration. Following gel filtration, protein fractions were digested by pronase, and heme and heme derivatives were analyzed by HPLC

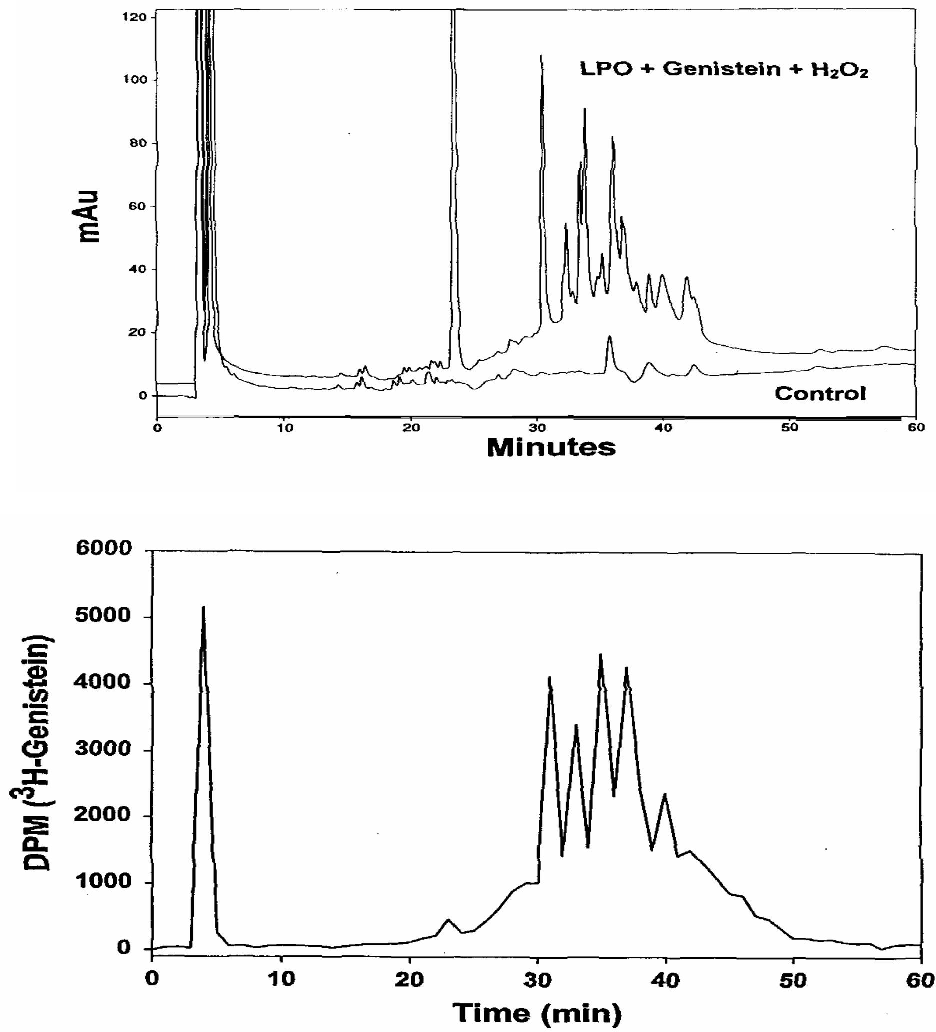

Figure 5. Peptide fragmentation analysis by HPLC and radioactivity.

Upper plot: One mg of LPO was incubated with or without 650 μM genistein in the presence of 650 μM H_2O_2 in 0.1 M phosphate buffer, pH 7.0 at room temperature for 30 minutes.

Lower plot: LPO (1 mg) was incubated with 650 μM genistein, ~60 nM ^{3}H-genistein and 650 μM H_2O_2 in 0.1 M phosphate buffer, pH 7.0 at room temperature for 30 minutes. The mixture was digested, and analyzed by HPLC as described above.

Table 1. Assignments of peptide fragments from MALDI-TOF MS analysis

Retentiom time (min)	Peptide fragments (Da)	Assignments (amino acid sequences + genistein)
24.5	2,259	LVG**Y**LDEEGVLDQNR (1719) + 2 genistein (270)
	2,267 (OMW=2,263)	TPDNIDI**W**IGGNAEPMVER (2,127; OMW=2,123) + 2 genistein (270)
	3,060 (OWM=3,063)	**W**LPAE**Y**EDGLALP**F**G**W**TQR (2,250; OMW = 2,053) + 3 genistein (270)
36	1,222	N/A
	1,792	1792 = CDENP**Y**R (982) + 3 genistein (270)
32, 34 or 37.5 min.	N/A	N/A

OWM：Molecular weight observed under MALDI-TOF MS analysis

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