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Anti-Thyroid Isoflavones from Soybean

ISOLATION, CHARACTERIZATION, AND MECHANISMS OF ACTION

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ABSTRACT. The soybean has been implicated in diet-induced goiter by many studies. The extensive consumption of soy products in infant formulas and in vegetarian diets makes it essential to define the goitrogenic potential. In this report, it was observed that an acidic methanolic extract of soybeans contains compounds that inhibit thyroid peroxidase- (TPO) catalyzed reactions essential to thyroid hormone synthesis. Analysis of the soybean extract using HPLC, UV-VIS spectrophotometry, and LC-MS led to identification of the isoflavones genistein and daidzein as major components by direct comparison with authentic standard reference isoflavones. HPLC fractionation and enzymatic assay of the soybean extract showed that the components responsible for inhibition of TPO-catalyzed reactions coeluted with daidzein and genistein. In the presence of iodide ion, genistein and daidzein blocked TPO-catalyzed tyrosine iodination by acting as alternate substrates, yielding mono-, di-, and triiodoisoflavones. Genistein also inhibited thyroxine synthesis using iodinated casein or human goiter thyroglobulin as substrates for the coupling reaction. Incubation of either isoflavone with TPO in the presence of H_2O_2 caused irreversible inactivation of the enzyme; however, the presence of iodide ion in the incubations completely abolished the inactivation. The IC_{50} values for inhibition of TPO-catalyzed reactions by genistein and daidzein were *ca.* 1–10 μM, concentrations that approach the total isoflavone levels (*ca.* 1 μM) previously measured in plasma from humans consuming soy products. Because inhibition of thyroid hormone synthesis can induce goiter and thyroid neoplasia in rodents, delineation of anti-thyroid mechanisms for soy isoflavones may be important for extrapolating goitrogenic hazards identified in chronic rodent bioassays to humans consuming soy products. BIOCHEM PHARMACOL **54**;10:1087–1096, 1997.

KEY WORDS. thyroid peroxidase; soybean; isoflavonoid; inhibitor; goitrogen; mechanism

The soybean and its products have been considered goitrogenic in humans and animals. Goiter and hypothyroidism were reported in infants receiving soy-containing formula [1–4], and such feedings in early life have also been associated with the development of autoimmune thyroid disorders [5]. Several investigators have reported induction of goiter in iodine-deficient rats maintained on a soybean diet [6–11]. Furthermore, Kimura *et al.* [9] reported the induction of thyroid carcinoma in rats fed an iodine-deficient diet containing 40% defatted soybean. Konijn *et al.* [10] showed that the anti-thyroid activity present in acidic acetone soybean extracts is water soluble, is dialyzable, and is not precipitated by either ammonium sulfate or trichloroacetic acid. The active ingredient was characterized partially by these workers as a small molecular compound of non-peptide origin, since it was not destroyed by either digestion with pancreatin or by boiling for 2 hr.

The function of the thyroid is synthesis of thyroid hormones, and TPO† catalyzes iodination of tyrosyl residues on Tg and the subsequent coupling of iodotyrosyl residues required for iodothyronine hormone formation. Inhibition of TPO-catalyzed reactions results in decreased levels of circulating thyroid hormones, which lead to increased secretion of TSH by the anterior pituitary. The increased levels of TSH provide a growth stimulus to the thyroid, and it has been proposed that a prolonged stimulus can select for clones of follicular cells with the potential for transformation [12]. This mechanism predicts that any compound that inhibits TPO-mediated thyroid hormone synthesis is a potential thyroid carcinogen.

The widespread use of soy products in infant food formulas and the significant consumption of soy products by people consuming a vegetarian diet require a closer evaluation and examination of the anti-thyroid activity of the soybean. This is important because of the current promotion of soy-based products as health foods possessing putative beneficial estrogenic and anti-carcinogenic properties. For example, genistein, but not daidzein, inhibits tyrosine kinase activity, and this property has been explored for

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† *Abbreviations:* APCI, atmospheric pressure chemical ionization; CcP, cytochrome c peroxidase; CID, collision-induced dissociation; DIT, 3,5-diiodotyrosine; LPO, lactoperoxidase; MIT, 3-iodotyrosine; PEG, polyethylene glycol; rT_3, 3,3′,5′-triiodothyronine; T_4, thyroxine; T_3, 3,5,3′-triiodothyronine; Tg, thyroglobulin; TPO, thyroid peroxidase; TSH, thyroid stimulating hormone; and MES, 2-[*N*-morpholine]ethanesulfonic acid.

potential anti-cancer potential [13]. Information in the scientific literature regarding the chemical nature of the active anti-thyroid component(s) from soybean, as well as the mechanism of action, is far from complete. In the present study, we report the chromatographic separation of the active anti-thyroid compounds of soybean, the elucidation of chemical structures, and the mechanisms for inhibition of TPO-catalyzed reactions.

MATERIALS AND METHODS

Reagents

Genistein, genistin, glucose, and glucose oxidase were obtained from the Sigma Chemical Co. (St. Louis, MO) and used as obtained. Daidzein was a gift from Dr. K. D. R. Setchell. TPO used in the present study was purified from porcine thyroid glands and quantified spectrophotometrically as previously described [14]. Human goiter Tg was a gift from Dr. Alvin Taurog, University of Texas Southwestern Medical School. The isoflavones were dissolved in either ethanol or DMSO that had been purified by distillation. A constant concentration of ethanol or DMSO (5%), which did not affect enzyme activity, was maintained in incubation mixtures.

Preparation of Soybean Extracts

Whole soybeans, advertised as not treated with herbicides or fungicides, were obtained from a local health food store and ground to a fine powder. The powdered sample (5 g) was extracted by stirring with 250 mL of acidic methanol (12 N HCl:CH_3OH, 10:90, v/v) with heating at reflux for 4 hr. The mixture was centrifuged at 20,000*g* for 20 min, the supernatant evaporated *in vacuo*, and the residue dissolved in 10 mL of 95% ethanol.

Liquid Chromatography

A fraction of the soybean extract was diluted 100-fold with methanol, and a 25-μL aliquot was injected into a reversed-phase HPLC column (NovaPak C18, Waters Associates, Milford, MA) using a GPM quaternary gradient pump (Dionex, Sunnyvale, CA). The column was eluted using a solvent system consisting of 30% solvent A and linearly increasing to 100% A in 20 min at a flow rate of 1.5 mL/min [A = acetonitrile; B = acetic acid:acetonitrile: water (0.5:5.0:94.5 by vol.)]. The peaks were monitored using a Spectra Focus forward-scanning optical detector (SpectraPhysics, San Jose, CA) at 260 nm and also by obtaining UV spectra between 200 and 350 nm. The same HPLC system was used to separate iodinated isoflavones. Elution was carried out with a mobile phase gradient starting with 50% A and increasing linearly to 100% A in 20 min at a flow rate of 1.5 mL/min. Peaks were detected using 275 nm absorbance.

For determination of TPO-inhibitory activity, individual identifiable peaks were collected after emerging from the detector, and when peaks were not present, fractions of 1 min were collected across the entire elution profile. The collected fractions were dried *in vacuo* and dissolved in 100–200 μL of ethanol. A 50-μL aliquot was then added to the tyrosine iodination assay to determine inhibition of TPO activity (see below).

HPLC was used to monitor TPO-catalyzed iodination of tyrosine to MIT and DIT because the UV absorbance of the isoflavones interfered with the usual spectrophotometric assay [14]. The same NovaPak C18 HPLC cartridge was used with solvent system consisting of A = acetonitrile; B = 0.2% trifluoracetic acid in 5% acetonitrile/water using a gradient of 10% A to 40% A in 20 min with a flow rate of 1.5 mL/min and UV detection at 230 nm. Retention times for tyrosine, MIT, and DIT were: 2.5, 3.6, and 4.8 min, respectively.

Mass Spectrometry

MS experiments were performed using a VG Platform single quadrupole instrument (Micromass, Altrincham, U.K.) equipped with an APCI interface. The total LC column effluent described above was delivered into the atmospheric pressure ion source (150°) through a heated nebulizer probe (500°) using nitrogen as the probe and bath gas (275 L/hr). Positive ions were acquired in full scan mode (m/z 100–600, 2.1 sec cycle time) in series with a UV detector set at 250 nm. Background-subtracted mass spectra were obtained by averaging spectra across the respective chromatographic peak and subtracting the average background immediately before and after this peak. At a low sampling cone-skimmer voltage (15 V), mass spectra for isoflavones and derivatives consisted predominately of the respective protonated molecule. At a higher voltage (50 V), in-source CID reactions produced numerous fragment ions. For on-line characterization of soy extracts, two separate scan functions were used to simultaneously obtain spectra at 15 and 50 V. The mass spectrometer was calibrated using a solution of PEGs [PEG 200 (25 μg/mL), 300 (50 μg/mL), 600 (75 μg/mL), and 1000 (250 μg/mL) obtained from Sigma Chemical Co.] in 50% acetonitrile in aqueous ammonium acetate (5 mM) over the range m/z 85–1200.

Inhibition of TPO-Catalyzed Reactions

Different amounts of soy extract, isolated HPLC fractions, or authentic isoflavones (2–80 μM) were added to reaction mixtures to determine the concentration dependence of TPO inhibition. The solution was incubated for 4 min and the reaction stopped by injection onto the HPLC column. Tyrosine (100 μM) and iodide (100 μM) or guaiacol (2.5 mM) were incubated with TPO (10 nM) plus inhibitor, and the reaction was initiated by the addition of H_2O_2 (100 μM) at 22 ± 0.1°. HPLC was used to follow the concentration of MIT/DIT and isoflavone present in incubations containing TPO, the H_2O_2-generating system consisting of

glucose (1.25 mM) plus glucose oxidase (10 nM), and genistein or daidzein.

Iodination of bovine casein (Sigma Chemical Co.) was carried out using Iodobeads (Pierce Chemical Co., Rockford, IL) using 10 beads in 5 mL MES buffer, pH 7.0, that contained 250 μM iodide to generate I_3^- for 1 min. Then a 10-mL aliquot of casein solution (1.25 mg/mL) in the same buffer was added. After incubation for 10–15 min at room temperature, the solution was dialyzed overnight. The degree of iodination was estimated spectrophotometrically using the change in absorbance at 290 nm as a measure of DIT formation (ΔA = 0.92/matom I, see Ref. 15). The content of MIT and DIT in iodinated casein was confirmed for one sample using HPLC analysis after proteolytic digestion of iodocasein; reasonable agreement with the spectrophotometric determination was seen (not shown).

Measurement of coupling, an *in vitro* assay of thyroid hormone synthesis, was carried out in the presence of TPO (20 nM) using chemically iodinated casein (1.25 mg/mL containing approximately 50–60 atom I/mol) as the source of iodotyrosines [16], various concentrations of isoflavone, and H_2O_2 (100 μM) for 1 hr in 0.05 M MES buffer, pH 6.5, at 37 ± 0.1°. Bovine mucosal alkaline phosphatase (10 units, Sigma Chemical Co.) was added to hydrolyze phosphate groups, including phosphotyrosines. This treatment increased yields of T_4 by approximately 25%. The reaction mixture was digested under a nitrogen atmosphere using pronase (250 μg/mL final concentration) for 1 hr and then with leucine-aminopeptidase (50 μg/mL) for 3 hr [17]. Thyroid hormones in the reaction mixture were extracted three times with ethyl acetate, and the extract was dried *in vacuo* and dissolved in a 100-μL aliquot of starting HPLC solvent. Extraction efficiency for a standard addition of T_4 was determined to be 92–95%. Thyroid hormones were measured by HPLC using a Hamilton PRP-1 reversed-phase column with a solvent system of A = acetonitrile, B = trifluoracetic acid:acetonitrile:water (0.2:5.0:94.8; by vol.) starting with 10% A in B and increasing linearly to 50% A in 20 min at a flow rate of 1.0 mL/min. The peaks were detected and iodothyronines quantified using 230 nm absorbance.

Measurement of TPO-catalyzed coupling was also carried out using human goiter Tg essentially as described by Taurog *et al.* [18] except that the proteolysis and HPLC analysis described above were used for quantifying iodothyronines. Samples were analyzed in triplicate for at least four different concentrations of isoflavone bracketing the IC_{50}.

Inactivation of TPO by Isoflavones

TPO was inactivated by isoflavones by incubating enzyme (1.0 μM) with 50 μM daidzein or 50 μM genistein and 200 μM H_2O_2 at 25 ± 0.1° in 0.1 M MES buffer (pH 7.0). After 4 min, aliquots were withdrawn and diluted 1000 to 2000-fold, and the remaining tyrosine iodination activity was measured. The activity was not restored by treatment of inactivated enzyme by centrifugal gel filtration or extensive dialysis as previously described [14]. In separate experiments to study its protective effect on inactivation, iodide (0.15 to 5.0 mM) was also included in the reaction mixture.

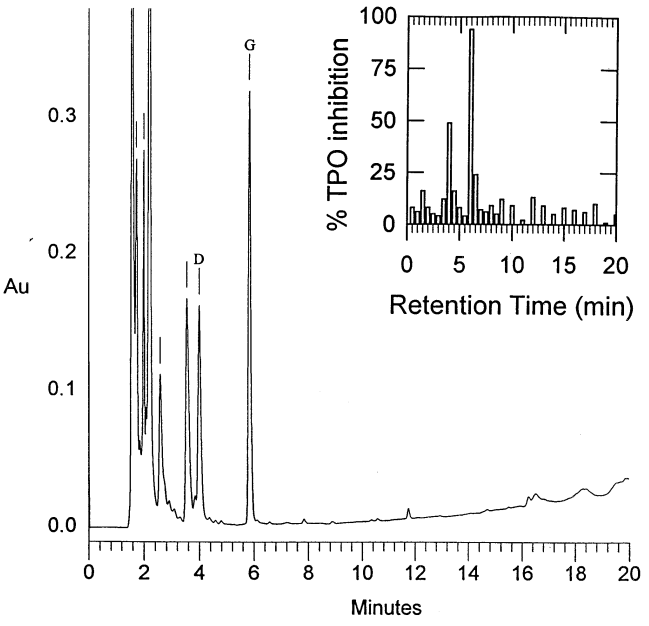


FIG. 1. HPLC fractionation of a soybean extract and inhibition of TPO-catalyzed tyrosine iodination. The soybean extract was fractionated using HPLC and UV detection (260 nm) as described in Materials and Methods, and the individually collected fractions were tested for inhibition of TPO activity (inset). The maximal TPO activity was 9 nmol MIT formed/min.

RESULTS

Characterization of Compounds Inhibiting TPO in Crude Soybean Extract

The presence of anti-thyroid components in soybean was investigated using a heated acidic methanol hydrolysis and extraction procedure. This procedure was selected to liberate the respective aglycones because glucoside conjugates are the predominant form in whole soybean [19, 20]. It was determined that this procedure completely converted genistein and daidzein conjugates to the respective aglycones (data not shown). When the extract was fractionated using HPLC, two distinct peaks of UV absorbance (retention times 4.0 and 5.8 min, see Fig. 1) were found to contain most of the inhibitory activity (Fig. 1 inset). Peaks labeled D and G showed UV absorption maxima at 251 and 259 nm, respectively (data not shown). These chromatographic and spectral properties were identical to those observed from authentic standards of daidzein and genistein, and coinjection of standards with the extract showed no evidence for inhomogeneity. The genistein and daidzein content based on HPLC analysis of the acidic methanol extract was determined to be 1.98 and 0.73 mg/g soybean, respectively, using external standard calibration.

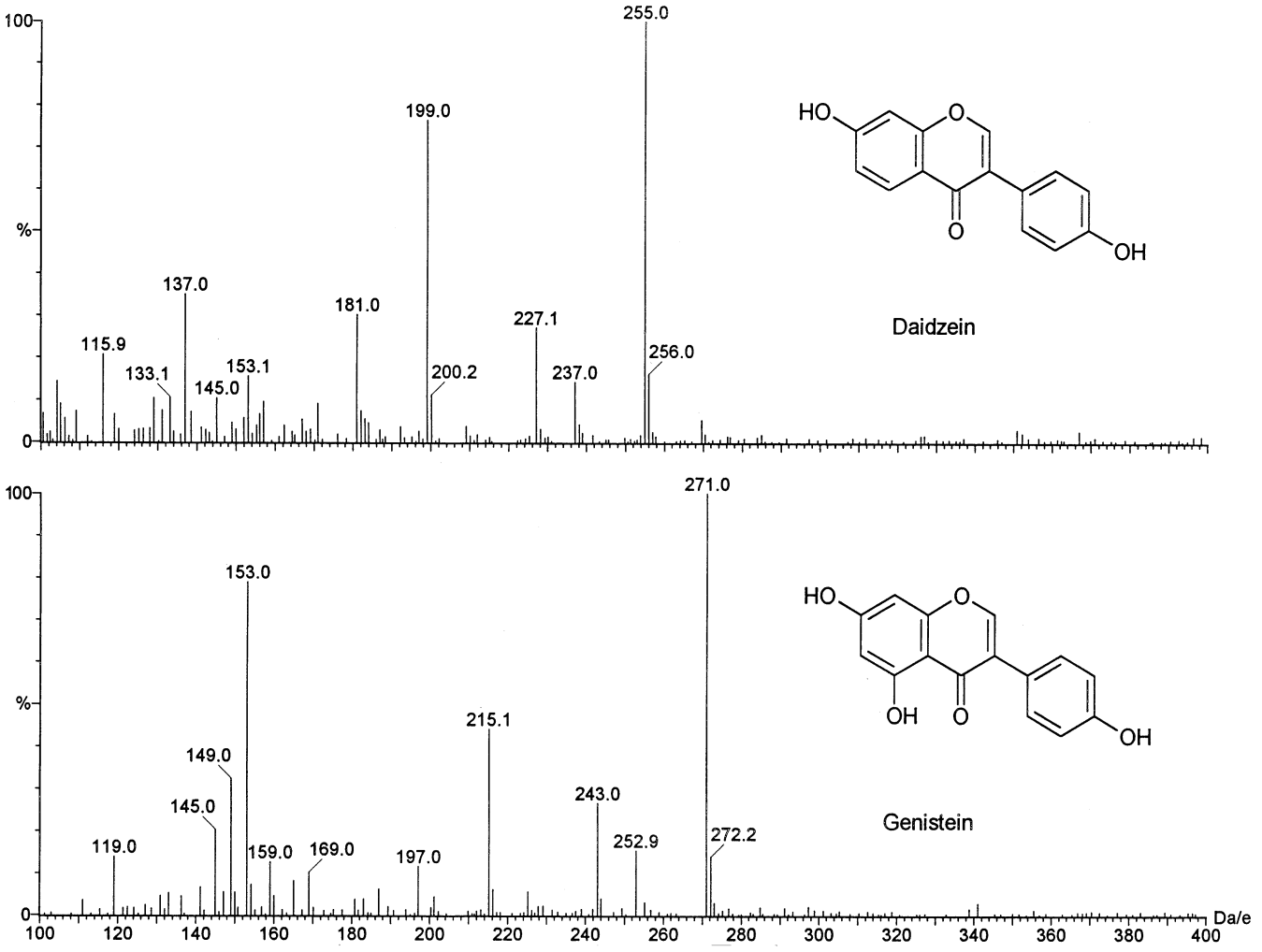


FIG. 2. On-line LC-APCI/MS analysis of genistein and daidzein from a soybean extract. Mass spectra were obtained following on-line LC separation of genistein (M + H^+ = 271 Da, bottom panel) and daidzein ((M + H^+ = 255 Da, top panel) in the soybean extract as described in Materials and Methods. The spectra were obtained using a sampling cone-skimmer potential of 50 V.

These values are consistent with the total isoflavone content in soybean reported previously [9, 20].

The soybean extract was analyzed further by on-line APCI/MS under conditions that produced mass spectra containing protonated molecules (M + H^+) and fragment ions (see Fig. 2). Mass spectra from peaks D and G contained protonated molecules corresponding to the masses predicted for daidzein and genistein (mol wt. = 254 and 270, respectively). Diagnostic fragment ions were also observed. Not only were the observed protonated molecules and fragment ions identical to those produced from authentic standards (not shown), but they were also very similar to the CID spectra previously reported for genistein and daidzein using thermospray ionization with tandem mass spectrometry [21, 22]. Since the chromatographic, spectroscopic, and TPO inhibitory properties were found to be identical with those exhibited by authentic isoflavones, subsequent mechanistic studies were carried out with pure isoflavones.

Inhibition of TPO-Catalyzed Iodination and Coupling by Isoflavones

Genistein and daidzein were found to inhibit TPO-catalyzed iodination of tyrosine. The IC_{50} values for these reactions were estimated from concentration-inhibition curves (not shown) to be 3.2 and 7.6 μM, respectively. These values were similar to those reported previously for related flavonoids [14]. The glycoside genistin was approximately 10-fold less potent than the aglycone with an IC_{50} value of 38 μM, and HPLC-UV analysis showed the commercial product to be devoid of the aglycone (<0.1%). A 25-μL aliquot of the crude extract (equivalent to 500 μg of soybean powder) produced 50% inhibition of TPO-catalyzed tyrosine iodination activity (data not shown). It was possible to compare the inhibition of TPO activity by the crude extract with that predicted from the measured isoflavone content. The extract aliquot contained 0.99 μg genistein and 0.37 μg daidzein, and these amounts are predicted to produce approximately 67% inhibition of

TABLE 1. Inhibition of TPO-catalyzed coupling in iodinated casein by genistein

Genistein (μM)	T_4 Residues (% control, average, N = 2)
0	100
1	93.8
2	57.0
4	32.7
8	23.8
20	26.3

TPO (20 nM) was incubated with chemically iodinated casein (1.25 mg/mL), alkaline phosphatase, various concentrations of genistein, and H_2O_2 (100 μM) for 1 hr at 37 ± 0.1°. Duplicate 1-mL reaction mixtures were digested with proteases, and the thyroid hormones were extracted and then quantified using HPLC as described in Materials and Methods. The content of iodothyronines present in iodocasein was below the detection limit (0.026 μg/ml casein or 16 ng), and the amount of T_4 formed in the control incubations was 1.02 μg/mg casein (0.85 newly formed residues per molecule).

TPO-catalyzed iodination in the 1.0-mL incubation. This compares closely with the 50% inhibition of TPO-catalyzed tyrosine iodination observed from addition of the soybean extract. These results also provide evidence that no major additional anti-thyroid compound is present in the soybean extract.

Genistein was also tested for the ability to inhibit iodothyronine formation by using iodinated casein, a model substrate that permits measurement of TPO-catalyzed coupling [16]. In this reaction, casein-bound iodotyrosyl residues are oxidatively converted by TPO in the presence of H_2O_2 to protein-bound T_4 and rT_3. The formation of prominent amounts of rT_3, but not T_3, is not observed *in vivo* or in model systems that use human goiter Tg as a coupling substrate [18, 23]. However, in all experiments, rT_3 formation mirrored T_4 formation, and inhibition by anti-thyroid chemicals was similar for both products. Bovine milk casein is a complex multimeric aggregate (>300 kDa) consisting of subunits with an average monomer molecular mass of 23.3 kDa [24] and contains 3.3 tyrosyl residues/mol [25]. Before treatment with TPO, the amount of total iodothyronines detected by HPLC in hydrolyzed iodocasein was below the detection limit (T_4 = 0.026 μg/mg casein or 16 ng on-column). In the absence of an inhibitor, TPO catalyzed the formation of approximately 1 μg T_4/mg casein (approximately 0.85 residues of T_4, 0.35 residues of rT_3 and *ca.* 0.17 residues of T_3 per mol of aggregated iodocasein assuming a molecular mass of 312 kDa, see Ref. 24) under the conditions described. Because the small amounts of T_3 observed were constant and unaffected by inhibitor concentration, we concluded that this resulted from artifactual deiodination of T_4 during sample preparation. Table 1 shows the concentration-dependent inhibition of T_4 synthesis in iodinated casein by genistein for duplicate experiments. Inhibition of rT_3 formation showed similar results (not shown). Using the combined data set for the two separate experiments, the IC_{50} value for genistein was approximately 3 μM.

Additional experiments were performed using human goiter Tg to measure the formation of thyroid hormones and its inhibition by isoflavones in a simultaneous iodination/coupling procedure [18]. When using Tg as the substrate, T_4 was the predominant product (1.06 ± 0.03 newly formed residue/molecule Tg), and only trace amounts of T_3 and rT_3 were observed. These studies confirmed the inhibition of coupling alone by genistein described above for iodinated casein. Genistein, daidzein, and genistin inhibited the formation of T_4 in a concentration-dependent manner (see Table 2), and the IC_{50} values for genistein, daidzein, and genistin were approximately 2.0, 8.8, and 40.6 μM, respectively. Genistein and daidzein also inhibited TPO-catalyzed oxidation of guaiacol. The IC_{50} values for these reactions were 0.7 and 12.4 μM, respectively.

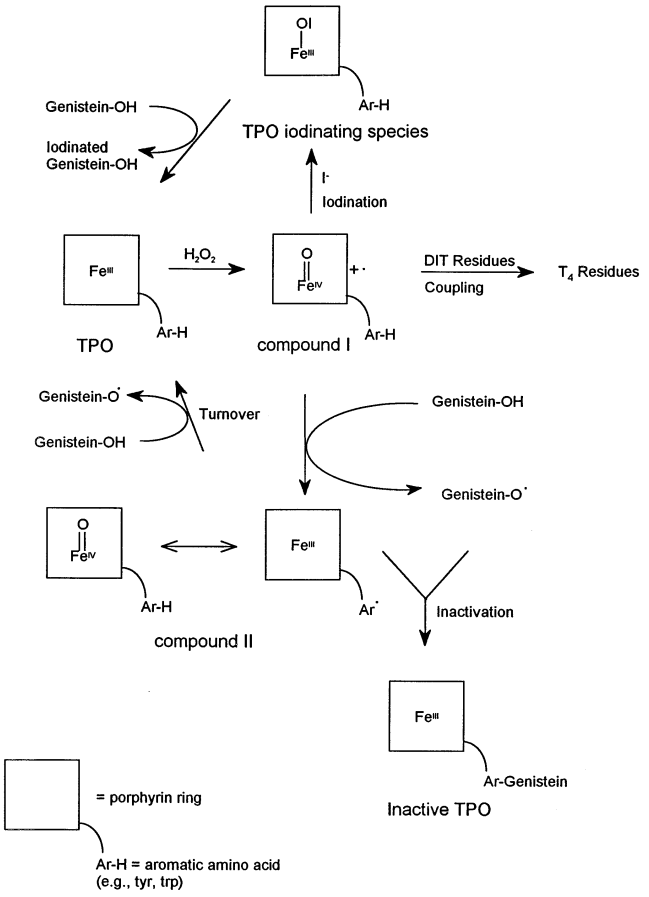
Characterization of Iodinated Isoflavones

Genistein and daidzein were potent inhibitors of TPO-catalyzed tyrosine iodination as described above. Genistein inhibition of TPO-catalyzed tyrosine iodination produced kinetics consistent with alternate substrate inhibition previously described for biochanin A [14]. In this mechanism (see Scheme 1), two-electron oxidation of iodide by TPO compound I produces an iodinating intermediate that is the equivalent of enzyme-bound hypoiodite [26]. Iodination of tyrosine by the enzymatic iodinating intermediate is blocked by isoflavones because of preferential iodination of

TABLE 2. Isoflavone inhibition of TPO-catalyzed coupling in human goiter Tg

Genistein (μM)	T_4 Residues (% control)	Daidzein (μM)	T_4 Residues (% control)	Genistin (μM)	T_4 Residues (% control)
0	100	0	100	0	100
1	52.4 ± 7.6	2.5	76.9 ± 6.5	10	98.3 ± 12.0
2.5	44.0 ± 8.0	5	58.5 ± 2.0	25	70.8 ± 10.8
5	19.8 ± 5.1	10	30.5 ± 4.8	50	46.9 ± 9.8
10	12.2 ± 2.4	20	12.2 ± 4.7	100	11.9 ± 0.7

TPO (24 nM) was incubated with KI (100 μM), 1.5 mM glucose, 5 mU/mL glucose oxidase, 0.76 μM Tg, and various concentrations of isoflavones for 1 hr at 37 ± 0.1°. Triplicate 1-mL reaction mixtures were digested with proteases, and the thyroid hormones were extracted and then quantified using HPLC as described in Materials and Methods (means ± SD). The content of iodothyronines present in the Tg was below the detection limit (0.02 newly formed residues or 16 ng), and the amount of T_4 formed in the control incubations was 1.06 ± 0.03 newly formed residue/molecule Tg.



SCHEME 1. Proposed mechanisms for genistein inhibition of TPO-catalyzed reactions.

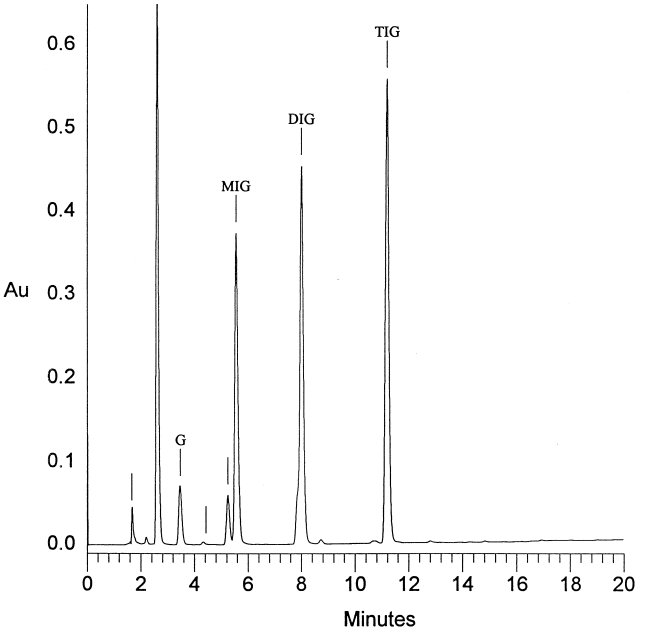


FIG. 3. TPO-catalyzed iodination of genistein. Genistein (25 μM) was incubated with TPO (50 nM) and iodide (100 μM) as described in Materials and Methods. The reaction was initiated by the addition of H_2O_2 (250 μM) and after 5 min the reaction mixture was analyzed using HPLC with UV 275 nm detection. Abbreviations: G, genistein; MIG, monoiodo-genistein; DIG, diiodo-genistein; and TIG, triiodo-genistein.

the flavonoid, presumably due to its greater reactivity with the electrophilic enzyme species.

Figure 3 shows the conversion of genistein to three products under such conditions. Analysis of reaction products using on-line LC-APCI/MS gave mass spectra consistent with derivatives containing either one, two, or three iodine atoms. The positive ion mass spectra are characterized by abundant protonated molecules (MH^+) as observed for the parent isoflavone (see Fig. 4). In addition, the iodination products show fragment ions corresponding to losses of successive iodine atoms ($-I^- + H^+$, $\Delta m = 126$ Da) including the parent isoflavone. For example, the triiodo-genistein spectrum consists of MH^+ (m/z 649) and fragment ions corresponding to the protonated diiodo (m/z 523) and monoiodo derivative (m/z 397) as well as genistein (m/z 271). Similar results were obtained with daidzein where mass spectra contained MH^+ of m/z 381, 507, and 633, consistent with formation of mono-, di-, and triiodo-daidzein derivatives by TPO-mediated iodination (not shown). These results are similar to those obtained from LC-APCI/MS analysis of TPO-catalyzed iodination of biochanin A to mono- and diiodinated derivatives. In that study, ^{1}H-NMR was also used to determine that the resorcinol moiety was the site for mono- and diiodination [14]. By analogy, we propose that genistein is mono- and diiodinated in the resorcinol ring (C6,8), and the third site for iodination is presumably the ortho position on the phenol ring (C3′).

Inactivation of TPO by Isoflavones

In the presence of H_2O_2, genistein and daidzein inactivated TPO by 60–70% (see Table 3). However, the glucoside genistin produced no loss of enzymatic activity under these conditions (data not shown). The inactivation was time dependent, increased with increasing concentrations of isoflavone or H_2O_2, and was enhanced when the incubation was conducted under anaerobic conditions. The inactivation was concomitant with a 60–80% loss of Soret absorbance and a red shift of the absorbance maximum from 412 nm (native TPO) to 415 nm (genistein-inactivated TPO) or 419 nm (daidzein-inactivated TPO) (Fig. 5). The presence of at least 150 μM iodide in the incubation mixtures completely protected against genistein- or daidzein-mediated TPO inactivation (see Table 3).

DISCUSSION

The results presented here demonstrate that the aglycones genistein and daidzein are the compounds contained in a hydrolyzed extract of soybean that inhibit TPO-catalyzed reactions. The hydrolytic procedure was required to convert the predominately conjugated isoflavones present in soybean to aglycones as previously described [19, 20]. The

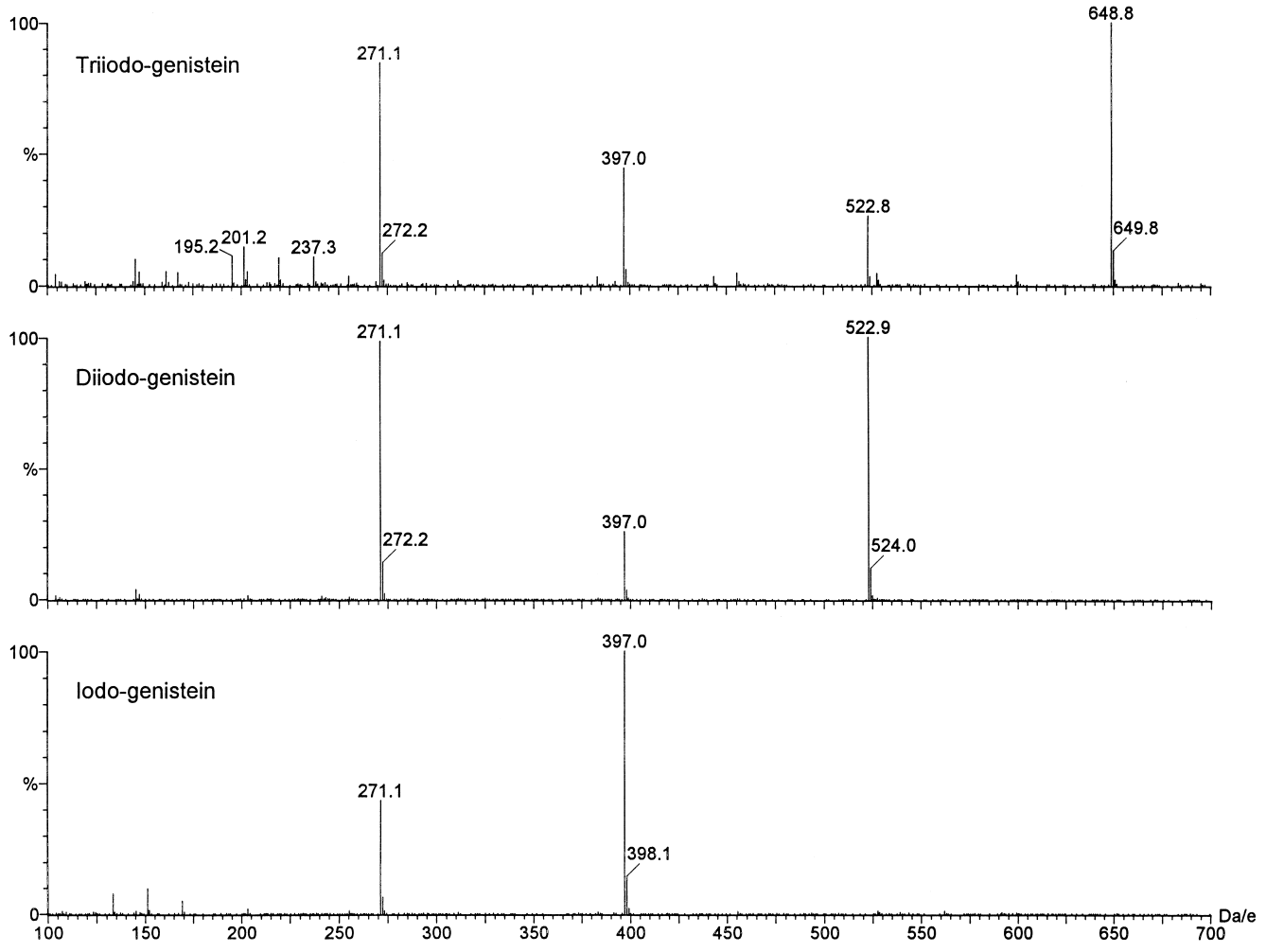


FIG. 4. Mass spectra of iodinated isoflavones. Incubations of TPO with genistein were conducted as described in Fig. 4 except LC-APCI/MS with a sampling cone-skimmer potential of 15 V was used to generate mass spectra of the genistein-derived products.

extract was fractionated by a reversed-phase HPLC separation (see Fig. 1), and the peaks containing inhibitory activity had chromatographic and spectroscopic properties (UV, APCI/MS) identical to authentic genistein and daidzein (see Fig. 2). All of the TPO inhibitory activity present in the extract was accounted for by the measured amounts of genistein and daidzein. Determining total isoflavones as the respective aglycone after hydrolysis can only give the maximum possible anti-thyroid potential of soy products because glucoside conjugates, which are the predominant forms in soybean, are weakly inhibitory. The mixture of glucoside conjugates and aglycones present in soybean have been shown to be bioavailable through identification of glucuronide and sulfate conjugates of isoflavones in plasma from humans consuming soy products [22]. This suggests that the conjugates are hydrolyzed during absorption from the gut as the aglycone. However, this does not give information about uptake into the thyroid, another critical factor for assessing goitrogenic potential that must be determined in future studies *in vivo*.

TABLE 3. H_2O_2-dependent inactivation of TPO by isoflavones

Reaction conditions	Activity (% of control)
TPO	99 ± 3
TPO + H_2O_2	96 ± 7
TPO + daidzein	97 ± 6
TPO + genistein	95 ± 5
TPO + daidzein + H_2O_2	38 ± 9
TPO + genistein + H_2O_2	29 ± 7
TPO + I^-	97 ± 5
TPO + daidzein + I^- + H_2O_2	94 ± 9
TPO + genistein + I^- + H_2O_2	95 ± 9

TPO (1.0 μM) was incubated with 50 μM daidzein or 50 μM genistein, 150 μM iodide ion, and 200 μM H_2O_2 in the combinations indicated at 25 ± 0.1° in 0.1 M MES buffer (pH 7.0). After 4 min, aliquots were withdrawn and diluted 1000 to 2000-fold, and the tyrosine iodination activity was measured. The values are means ± SD (N = 6). Under these conditions, maximal TPO activity was 9 nmol MIT formed/min.

Genistein also inhibited TPO-catalyzed phenolic oxidations including guaiacol oxidation and coupling of diiodotyrosyl residues in casein and thyroglobulin to form iodothyronines (see Tables 1 and 2). These reactions proceed by phenoxyl radical intermediates, and the pres-

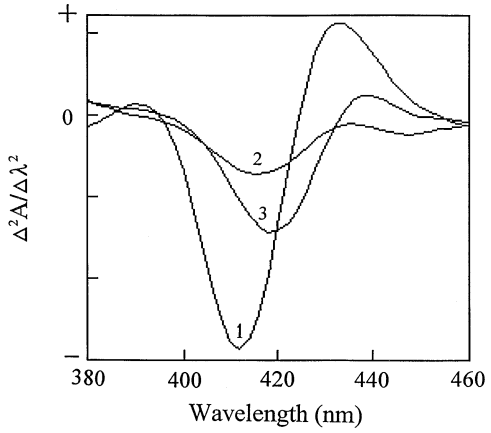


FIG. 5. Soret spectra for TPO and isoflavone-inactivated TPO. TPO (1 μM) was incubated with genistein or daidzein (50 μM) and H_2O_2 (200 μM) as described in Materials and Methods. After a 4-min incubation, second-derivative visible spectra were recorded. The spectra are shown for native TPO (1), genistein-inactivated TPO (2), and daidzein-inactivated TPO (3).

ence of phenolic groups in the isoflavones would be predicted to react with oxidized enzyme species, Tg radical intermediates, or both, to block coupling [14, 18, 27]. The IC_{50} for inhibition of coupling in a simultaneous iodination/coupling assay using Tg was also similar to that observed in a coupling-only assay system using previously iodinated casein as the substrate. Because of the ability of the thyroid both to produce Tg and to concentrate iodide, it is likely that coupling *in vivo* occurs in a manner more comparable to the simultaneous assay. Furthermore, the IC_{50} values obtained for isoflavones were similar for inhibition of iodination and coupling. This suggests a primary interaction between isoflavone and TPO that affects coupling and iodination activity in a similar manner. Furthermore, the levels of total isoflavones observed in human plasma following consumption of soy foods (*ca.* 1 μM, see Ref. 22) approach the concentrations required for inhibition of TPO-catalyzed reactions.

In the absence of iodide, genistein and daidzein caused time-dependent, irreversible inactivation of TPO concomitant to distinct changes in the visible spectrum of the heme prosthetic group (see Fig. 5). These observations are similar to those made for inactivation of TPO by other flavonoids (naringinen, quercitin, morin, and kaempferol; see Ref. 14) although the spectra of inactivated TPO have different long wavelength absorption maxima (428 nm for naringinen or kaempferol; 419 nm for daidzein; 415 nm for genistein). These data are consistent with the suicide inactivation mechanism for resorcinol and related flavonoids previously proposed for TPO, LPO, and CcP [14, 28]. Despite the dramatic changes in the heme visible spectrum, the results to date are consistent with enzyme inactivation through covalent binding to the polypeptide chain and not to the heme prosthetic group (see Scheme 1 and Ref. 29).

The inhibition of TPO-catalyzed iodination and coupling *in vitro* is consistent with the numerous reports of anti-thyroid effects in humans and animals from consumption of soy products, especially in cases of iodine deficiency. Many issues regarding the bioavailability of soy isoflavone conjugates and uptake into the thyroid remain unanswered. However, the demonstrated effects of the aglycones presented here, and the well-documented goitrogenic effects of soybeans in humans and animals, do provide a logical starting point from which possible anti-thyroid mechanisms can be examined. The different mechanisms reported for inhibition *in vitro* of the enzymatic reactions in thyroid hormone biosynthesis by isoflavones are useful for predicting potential anti-thyroid effects in animals and humans under several different dosing circumstances:

(A) In the normal case of iodine-sufficient individuals receiving intermittent or low doses of soy isoflavones, alternate substrate iodination would consume the inhibitory compounds after which Tg iodination and coupling reactions would resume unaffected. Since the normal thyroid contains significant amounts of iodide, its high substrate activity should prevent inactivation of TPO.

(B) In the case of iodine deficiency, low or intermittent doses of isoflavones could further deplete iodide levels by covalent incorporation of iodide into iodinated products. Also, enzymatic oxidation of the isoflavone would increase as the intrathyroidal iodide level decreased. Under these conditions, it is possible that inactivation of TPO could occur. This would produce a more long-lasting inhibition of hormone synthesis because enzymatic activity could be replaced only through *de novo* protein synthesis. However, either the alternate substrate inhibition or enzyme inactivation outcome is consistent with the anti-thyroid effects from soy observed in rodents maintained on an iodide-free diet [7, 9] and with the ability of added iodide to reverse the goitrogenic effect of a soybean diet in rats [7]. There are also reports of goiter and hypothyroidism in human infants receiving soy-based formulas [1–4] and evidence for elimination of such effects upon addition of iodide to the diet [3]. For this reason, it appears that iodide supplementation of formulas during manufacturing was implemented [1].

(C) In proposed rodent carcinogenicity bioassays, high doses of isoflavone will be administered chronically in a normal iodide-containing diet. Under these conditions, complete blockade of iodination and coupling is possible even with normal dietary iodide because alternate substrate inhibition would dominate. Since the body burden of isoflavone is replenished continually through feeding, the inhibition of thyroid hormone synthesis would persist throughout the lifetime of the animal. This hypothesis is consistent with observations of the hypothyroid condition that occurs in humans consuming principal foodstuffs (e.g. millet) that contain large amounts of anti-thyroid flavonoids [30, 31]. It is possible that these anti-thyroid effects could persist even if normal levels of iodide were present in the diet through universal iodination programs.

Under either condition B or C, the inhibition of thyroid hormone synthesis would increase TSH levels and could eventually induce thyroid follicular hyperplasia and tumors [12, 32]. Tumor formation through this nongenotoxic, hormonal process results from the growth stimulus provided by TSH, which can provide the selective environment for a transformed phenotype [12]. The observation of metastatic thyroid tumors in iodine-deficient rats, but not in iodide-supplemented rats, receiving a defatted soybean diet [9] is consistent with this proposal. The role of TSH in mediating thyroid tumors is well-documented in rats, but the importance in humans is unclear [32].

Finally, other possible toxicological consequences from ingestion of soy isoflavones come from the demonstrated estrogen receptor binding activity of genistein and daidzein [33, 34]. Although anti-carcinogenic properties from soybean isoflavones have been suggested [13, 35], the dose-response relationships that may separate toxic and beneficial responses are not clear. A possible consequence of TPO-mediated isoflavone iodination is modification of such estrogenic receptor binding activity or changing the pharmacokinetics for elimination. For example, McCague *et al.* [36] reported increased estrogen receptor binding affinity as well as a decrease in metabolism for 4-iodotamoxifen relative to the parent compound. Further experimentation will be required to assess the importance of TPO-mediated iodination in the biological activity and excretion of isoflavones.

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