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Thermal Stability of Genistein and Daidzein and Its Effect on Their Antioxidant Activity

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Soy isoflavones, present in many processed soy foods, are known for their phytoestrogenic and antioxidant activities. The aim of this work was to study the kinetics of genistein and daidzein degradation at elevated temperatures and to follow changes in their antioxidant activity. Daidzein and genistein in model solutions (pH 7 and 9) were thermally treated at 120 °C or incubated at 70, 80, and 90 °C. Isoflavone degradation was observed at all temperatures, with apparent first-order kinetics at 70−90 °C, and E_a = 8.4 and 11.6 kcal/mol at pH 9, respectively. Microcalorimetric stability tests showed a similar pattern of degradation, however, with higher E_a (genistein, 73.7 kcal/mol; daidzein, 34.1 kcal/mol) that may be attributed to the anaerobic conditions. The antioxidant activity of incubated isoflavone solutions, followed by the ABTS test, decreased rapidly at pH 9 for genistein, whereas only moderate reduction was observed for daidzein (pH 7 and 9) or genistein at pH 7. This may indicate different degradation mechanisms for genistein and daidzein.

KEYWORDS: Genistein; daidzein; stability; microcalorimetry; antioxidant

INTRODUCTION

Phenolic compounds found in soy and soy products are considered to be potential phytoestrogens because of their ability to interact with estrogen receptors (*1*). Soy isoflavones, the most common estrogenic compounds found in plants, have been shown to reduce risks of cancer (*2*−*4*) and inhibit the activity of tyrosine kinase (*5*). In addition, isoflavones may have a role in decreasing the risk of cardiovascular diseases by reducing the level of total cholesterol as well as low-density lipoprotein (LDL) cholesterol (*6*). Other health benefit claims include reductions in postmenopausal symptoms and risk of osteoporosis in women (*7*). Their ability to reduce cancer and cardiovascular risks is partially explained by their antioxidant activity (*8*, *9*).

The main isoflavones found in soybeans are genistein, daidzein (**Figure 1**), and glycitein, each of which exists in four chemical forms, as an aglycon (genistein, daidzein, and glycitein), a *β*-glycoside (genistin, daidzin, and glycitin), an acetylglycoside (6″-*O*-acetylgenistin, 6″-*O*-acetyldaidzin, and 6″-*O*-acetylglycitin), and a malonylglycoside (6″-*O*-malonylgenistin, 6″-*O*-malonyldaidzin, and 6″-*O*-malonylglycitin). The isoflavone content and composition vary in different soy foods and change as a result of food manufacturing processes (*10*). For example, the isoflavone content increased when soy sauce was produced from whole soybeans rather than defatted beans (*11*). Loss of isoflavones occurred also in soy protein isolate (SPI), a widely distributed food source, manufactured by extraction of soy flour in an alkaline environment (*12*). Wang et al. (*13*) found that a 26% reduction in total isoflavone concentration occurred during the process of SPI washing. In addition, only very small amounts of isoflavones were detected in SPI that was washed in an alcohol solution. Interestingly, although thermal treatments altered the profile of isoflavone conjugates, the total isoflavone concentration did not change (*14*). These findings are supported by other studies showing insignificant changes in total isoflavones during extrusion, baking, and frying, accompanied by a decrease in the content of malonyl derivatives (*15*, *16*).

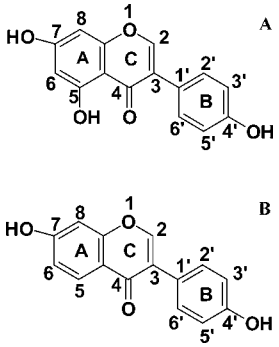

Figure 1. Chemical structures of genistein (A) and daidzein (B).

Very few studies have looked into the mechanism of isoflavone modifications during processing and storage. Wang et al. (*17*) reported that heating daidzein and genistein conjugates under acid condition released free isoflavones and that genistein was further degraded. In addition, genistein formed conjugates with very high UV absorption when mixed with dextrose, fructose, maltose, or sucrose, proportional to the amount of sugar added (*17*). Davies et al. (*18*) reported that genistein forms

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Maillard browning products during its autodegradation or reaction with lysine at 60 °C. Thermal treatments of tofu reduced its daidzein and daidzein conjugate content (*7*).

Isoflavone degradation and modifications during food processing and storage may cause changes in their bioactivity. This was concluded in Singletary's research (*19*), where the inhibition of breast cancer cell line proliferation by extracts from blends of soy protein and cornmeal was reduced by extrusion. Gallaher et al. (*20*) reported that long-term storage of SPI changed its bioactivity from anti- to procarcinogenic agent. Therefore, the stability, profile, and biological activity of isoflavones are strongly related and affected by processing and storage conditions. However, very little information is available on the kinetics and stability of isoflavone standards. The aim of this research is to study the thermal stability of genistein and daidzein, the kinetics of their degradation reactions, and the antioxidant activity of the reaction products.

MATERIALS AND METHODS

Chemicals. Genistein, daidzein, Trolox, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) were obtained from Sigma-Aldrich Chemical Co. (Rehovot, Israel). All other reagents were of analytical grade.

Thermal Stability of Isoflavones. The thermal stability and kinetics of isoflavone degradation were determined by incubating isoflavone solutions under isothermal conditions and monitoring their concentration by HPLC. For stability experiments, genistein and daidzein were dissolved in methanol or DMSO, respectively, and then diluted to final concentrations of 0.25 mM in borate buffer (pH 9, 0.1 M) or 0.1 mM in phosphate buffer (pH 7, 0.1 M). Samples were autoclaved for 20 min at 120 °C and analyzed by HPLC.

Studies of isoflavone degradation kinetics were conducted on genistein and daidzein solutions. Isoflavones were dissolved in methanol or DMSO and then diluted to a concentration of 30 μM in borate buffer (pH 9, 0.1 M) or phosphate buffer (pH 7, 0.1 M). Isoflavone samples were incubated at three temperatures (70, 80, 90 °C), and samples were withdrawn in triplicates at predetermined times for isoflavone analysis. Loss of isoflavones was detected by HPLC. The reaction rate was determining at three temperatures, and the Arrhenius relationship was used to model the temperature effect on the reaction rate constants.

HPLC Analysis. Isoflavone analysis was conducted by reverse-phase high-performance liquid chromatography (RP-HPLC), HP 1100, equipped with a diode array detector, and controlled by the ChemStation software package (Hewlett-Packard, Wilmington, DE). HPLC analyses were carried out on a reverse-phase C_{18} column (25 cm × 4.6 mm SupelcoSil column, Supelco). Samples were eluted at a flow rate of 1 mL/min by a gradient of 25% methanol (A) in citrate solution (pH 3.5) (B) at time 0 up to 50% B at 20 min and an isocratic eluent to the end of the program. Genistein and daidzein were detected at 262 and 254 nm, respectively, and quantified by external standards.

Microcalorimeter Stability Testing. Isoflavone microcalorimeter stability analyses were performed by a VP-DSC microcalorimeter (VP-DSC expanded range microcalorimeter, Microcal Inc., Northhampton, MA). Genistein and daidzein stock solutions were prepared by dissolving the isoflavones in methanol or DMSO. Solutions with final concentrations of 1.0 mM isoflavone were prepared by diluting stock solutions in borate buffer (pH 9, 0.1 M). The samples were filtered using a 0.45 μm filter, followed by 8 min of deaeration (Thermovac-sample degassing and thermostat, Microcal Inc., vacuum = 28.4 in. of mercury), and 1 mL was injected to the microcalorimeter cell. Samples were scanned by temperature ramping from 50 to 120 °C at a scanning rate of 3.8 °C/h, followed by a rescan. Analysis of the data was performed by the Origin 5.0 software package (Microcal), and the heat capacities (ΔC_p) were obtained for every temperature (kcal/mol °C). Enthalpies (ΔH) of the degradation reactions were calculated after subtraction of the rescan from the first scan.

The degradation rate constants were calculated for a temperature range, in which exponential increase in the decomposition rate was observed. Kinetic constants at each temperature were calculated as follows:

A. A constant temperature for short time intervals was assumed, and Δt was calculated on the basis of the scan rate.

$$\Delta t = \Delta T/3.8$$

B. Because the final isoflavone concentration (ΔA_T) was measured by HPLC, the change in isoflavone concentration at each time interval was calculated by

$$\Delta A = (\Delta H/H_T) \times \Delta A_T$$

where ΔH is the change in enthalpy within the time interval (kcal/mol) and H_T is the total enthalpy of the measured degradation reaction.

C. The average isoflavone concentration (A_{av}) during each time interval was determined by

$$A_{av} = \left[1 - \frac{\left(\frac{H_2 + H_1}{2}\right)}{H_T}\right] \times \Delta A_T$$

where H_1 and H_2 are the partial isoflavone decomposition enthalpies of up to the beginning or end of each time interval, respectively.

D. Once Δt, ΔA, and A_{av} were calculated, k_{av} was determined by

$$k_{T,av} = -\frac{\Delta A/\Delta t}{A_{av}}$$

Activation energies of the degradation reaction (E_a) were then calculated by using the Arrhenius relation.

Antioxidant Assay (ABTS). The antioxidant activities of genistein and daidzein were evaluated using the Trolox equivalent antioxidant capacity (TEAC) method (*21*). This method estimates the total antioxidant activity of genistein and daidzein samples relative to that of a standard solution of Trolox. ABTS radical cation was prepared by reacting a 7mM aqueous solution of ABTS with 24.5 mM potassium persulfate, followed by dilution to an absorbance of 0.7 (±0.1) at 734 nm. Absorbance readings (734 nm) were taken immediately after the addition of 0.5 mL of this diluted solution to 0.5 mL aliquots of antioxidant samples. Appropriate solvent blanks were run in each assay. The antioxidant activity was determined as the percent of decolorization of the radical cation. The antioxidant potential of the samples is presented as Trolox equivalents.

Data Analysis. Initial isoflavone concentration was determined by using four to six replicates, and all kinetics experiments were performed in triplicates. The results are presented as the means ± the standard deviation (SD). Microcalorimeter studies were performed in duplicates, and the results represent the means ± SD of the overall calculated reaction rates. Statistical analysis was performed by using the data analysis tool pack of Microsoft Excel 2000 software.

RESULTS AND DISCUSSION

The overall goal of this study was to evaluate the stability of isoflavones in high temperatures, to study the reaction kinetics, and to examine the effect of isoflavone degradation on its antioxidant potential. Initially, genistein and daidzein stability was studied in conditions simulating a commercial sterilization process. The results indicate that genistein and daidzein are degraded when exposed to high temperature (**Figure 2**). The pH effect on the stability of genistein and daidzein at 120 °C was studied at alkaline (pH 9) and neutral environments (pH 7). In alkaline solution the concentration of genistein was reduced by 60%, whereas daidzein was less affected by the thermal treatment and a minor 15% reduction was observed. Interestingly, in the neutral pH, daidzein was less stable than genistein, and its concentration decreased by 40%, as compared

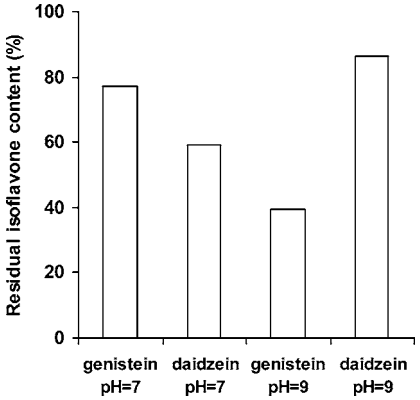

Figure 2. Residual isoflavone content in model solutions following thermal treatment at 120 °C for 20 min. Standard deviation was <1% for all samples.

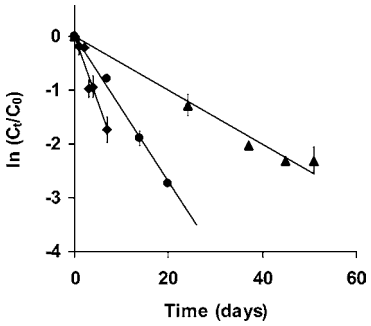

Figure 3. Degradation of daidzein in a model solution at 70 (■), 80 (●), and 90 °C (◆) (pH 7, 30 μm).

to 22% for genistein. These results are consistent with a study of Mahungu et al. (*15*) in which high temperatures (110, 130, and 150 °C) decreased the overall content of the isoflavones in soy protein isolates and corn mixtures. In their study, the loss of the daidzein and its conjugates (44%) was higher than the loss of genistein (33%). It should be noted, however, that most of the isoflavone degradation in the study of Mahungu et al. (*15*) was attributed to the malonyl derivatives and only a small change in the aglycons was observed.

Kinetics of Isoflavone Degradation Reaction at High Temperatures. In the first part of the study, we have shown that genistein and daidzein degrade at elevated temperatures. Therefore, we carried out a series of experiments to study the kinetics of genistein and daidzein degradation and its temperature dependence. Solutions of genistein and daidzein were incubated at temperatures ranging from 70 to 90 °C, and the reduction in their concentration was monitored by HPLC. The results of the kinetic experiments show that both isoflavones degrade at pH 9 and 7 and that the degradation reaction is apparently a first-order reaction. A typical degradation curve is shown in **Figure 3**. The rates that were calculated for the reactions at all temperatures are presented in **Table 1**. Genistein degradation was accelerated in an alkaline environment and yields higher rate constants than in neutral pH. For example, the rate for 90 °C at pH 9 was 0.222 (1/day) ($p < 0.001$) and at pH 7 it was only 0.030 (1/day) ($p < 0.001$). Higher stability at pH 7 was also detected in daidzein solutions. The pH dependence of the degradation rate was reported for other polyphenols, such as green tea catechins (*22*, *23*). Their degradation accelerated significantly when the pH was elevated from 5 to 7.4. Green tea catechins, however, degraded at a much higher rate, losing ~75% after 0.5 h of incubation at pH 7 and 37 °C (*23*). The degradation rates of daidzein at both pH values

Table 1. Kinetics Constants for Genistein and Daidzein Degradation

compound	pH	temp (°C)	k (1/day)	r^2	P [a]	E_a (kcal/mol)
genistein	9	90	0.222	0.945	<0.001	11.57 ± 2.31
		80	0.102	0.891	0.004	
		70	0.087	0.775	<0.001	
	7	90	0.030	0.870	<0.001	3.88 ± 0.25
		80	0.025	0.932	<0.001	
		70	0.022	0.729	0.003	
daidzein	9	90	0.547	0.911	<0.001	8.38 ± 1.40
		80	0.323	0.967	<0.001	
		70	0.277	0.802	0.016	
	7	90	0.262	0.909	<0.001	21.69 ± 1.48
		80	0.091	0.865	<0.001	
		70	0.045	0.907	<0.001	

[a] Significant correlations.

were higher than for genistein (**Table 1**). Apparently, daidzein is more labile to degradation than genistein at high temperatures.

The degradation of isoflavones at elevated temperatures is in agreement with earlier observations in soy foods and model systems. Thermal treatments of tofu decreased the content of daidzein and genistein and their conjugates (*7*). During the thermal treatment of tofu, genistein derivatives were only slightly affected, and most of the isoflavone decomposition is attributed to the loss of free daidzein. These results support our observation that daidzein is generally more labile to heat treatments. The calculated rates for isoflavone loss in heated tofu (5−13 1/day at 80−100 °C) are 1 order of magnitude higher than the values calculated in the present work. In addition, research conducted on soy milk showed rate constants for genistin degradation similar to those found in the present study (0.06−0.11 1/day at 70−90 °C) (*24*). In model systems, however, the stability measured for genistein is in good agreement with the values obtained in the present research. The study of Davies et al. (*18*) using model genistein solutions at pH 9 reported a half-life of 6 days at 60 °C, compared to ~8 days at 70 °C in the present work.

Measuring the reaction rates at three temperatures enabled the calculation of the activation energies for genistein and daidzein degradation. Using the Arrhenius equation, plotting [ln(k) vs 1/T], the activation energies (E_a) of the degradation reaction were calculated and are presented in **Table 1**. The activation energies for daidzein degradation are similar to activation energies reported previously for the degradation of epigallocatechin gallate (*25*). The E_a for the catechins was 18.7 kcal/mol as compared to 8.38 and 21.69 kcal/mol for pH 9 and 7 for daidzein, respectively. The activation energy for genistin loss at elevated temperatures in soy milk was 17.6 kcal/mol (*24*). In addition, analysis of the data for daidzein loss during thermal treatments of tofu showed an activation energy of 19.5 kcal/mol (*7*).

The results obtained in the isothermal kinetic experiments indicate that isoflavone degradation at elevated temperatures can be at least partially explained by auto-decomposition. Another explanation may be that the loss of daidzein and genistein is a result of an oxidation reaction, because all experiments were performed under atmospheric oxygen pressure.

Microcalorimeter Stability Tests. Differential scanning calorimetry (DSC) can be used as a quick stability screening method in combination with chromatographic techniques. The advantage of VP-DSC compared to conventional stability testing is the speed of the measurements and the small amount of sample that is required. In the present study, a DSC-microcalo-

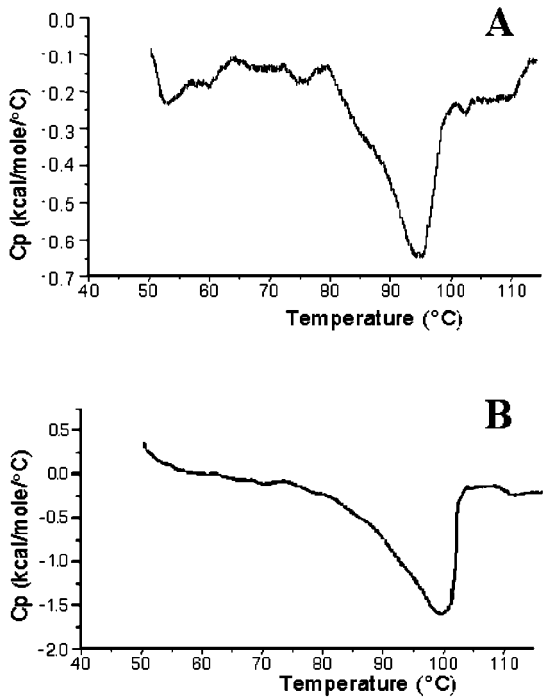

Figure 4. Decomposition of genistein (A) and daidzein (B) during microcalorimetric stability tests (VP-DSC expanded range microcalorimeter). Genistein and daidzein model solutions (1 mM, pH 9) were scanned at a heating rate of 3.8 °C/h.

Table 2. Enthalpy Change and Activation Energies of Genistein and Daidzein Degradation As Determined by Microcalorimetric Thermal Analysis (VP-DSC Expanded Range Microcalorimeter; Concentration = 1 mM; Heating Rate = 3.8 °C/h)

	genistein	daidzein
degraded concn (mM)	0.29 ± 0.08	0.58 ± 0.07
ΔH (kcal/mol)	17.96 ± 1.53	25.04 ± 10.3
E_a (kcal/mol)	73.70 ± 1.26	34.14 ± 0.86

rimeter was used to evaluate the stability of the isoflavones and to assess the enthalpies of the degradation reactions. In addition, kinetic constants and activation energies of each reaction were calculated.

Genistein and daidzein in the concentration of 1.0 mM at pH 9 were deaerated and scanned from 50 to 120 °C at the scanning rate of 3.8 °C/h. In all scans, genistein and daidzein exhibit a large exothermic peak, indicating that indeed a degradation reaction occurs under these scanning conditions (**Figure 4**). The degradation reactions reached their maximum at ~95 °C for genistein and at ~98 °C for daidzein. At the end of each thermal analysis, samples were analyzed by HPLC, and the final isoflavone concentration was determined. Using the HPLC results, measured enthalpies were normalized for the actual amount of isoflavone being degraded. The results of the microcalorimetric measurements are presented in **Table 2**.

The HPLC results show that more daidzein was degraded than genistein. Daidzein concentration had decreased by 58% as compared with genistein, for which the reduction was only 29%. These results support the findings from the isothermal stability experiments, showing higher stability of genistein. The calculated enthalpy for isoflavone degradation in 1 mM solutions was higher for the decomposition of daidzein (25.05 kcal/mol) as compared to that of genistein (17.96 kcal/mol) (not significant statistically). The degradation rate constants were calculated for a temperature range in which exponential increase in the

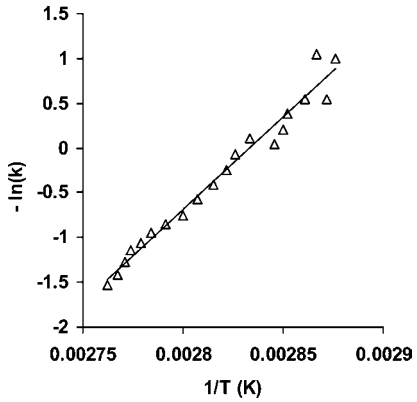

Figure 5. Arrhenius plot of daidzein decomposition during microcalorimeter scanning (1 mM, pH 9).

decomposition rate was observed (from 82 to 91 °C). The calculated reaction rates were used to plot $-\ln(k)$ vs $1/T$ (see example in **Figure 5**), and the activation energies (E_a) were calculated by using the Arrhenius equation (**Table 2**), yielding significant and highly correlated values of $\ln(k)$ vs $1/T$ ($r^2 > 0.965$, $P < 0.001$). The activation energies are of the same order of magnitude as those found for epicatechin (EC) from green tea (*22*). The activation energy for the degradation reaction of epicatechin (EC) at temperatures >82 °C was 38.1 kcal/mol (*22*).

From a comparison of the results of the isothermal and microcalorimetric stability tests, a number of interesting findings can be noted. First, daidzein was less stable than genistein despite the fact that it contains one fewer hydroxyl group. Various stability studies on polyphenols showed that increased stability is associated with a reduced number of hydroxyl groups (*23*). It was suggested that the increased number of hydroxyls in the aromatic ring B has a destabilizing effect. The results of the present study, however, show that the elimination of a hydroxyl group from ring A reduced the stability of daidzein as compared to genistein. This difference between daidzein and genistein may be explained by the location of this hydroxyl group (e.g., on carbon 5 of ring A) and by its proximity to the 4-oxo moiety in ring C, which may stabilize the hydroxyls in ring A of genistein.

A second noteworthy observation is that the degradation in deaerated solutions during the microcalorimetric tests was faster than that during aerobic isothermal experiments, yielding different activation energies. The higher degradation rate of isoflavones under anaerobic conditions is somewhat unusual, as previous studies showed an opposite observation. The study of Markis and Rossiter (*26*) showed an increased degradation rate of quercetin and rutin in an aerobic environment. Their corresponding degradation rates under aerobic conditions were 16.1 and 8.9 (1/day) at 97 °C compared to 3.7 1/day for quercetin and 5.4 1/day under anaerobic conditions. A model developed by Zimeri and Tong (*25*) for epigallocatechin gallate predicts an increase of its degradation rate with the increase in dissolved oxygen. The unusual increase in degradation rate of isoflavones under anaerobic conditions may be explained by a different degradation reaction. This may be supported by the fact that >90% of isoflavones were lost during aerobic isothermal degradation, whereas only 58% were lost under anaerobic microcalorimetric testing. Furthermore, different activation energies were measured for aerobic and anaerobic conditions. The activation energies for genistein, for example, were 11 kcal/mol in aerobic solution and 73.70 kcal/mol in anaerobic conditions (**Tables 1** and **2**). The differences in the

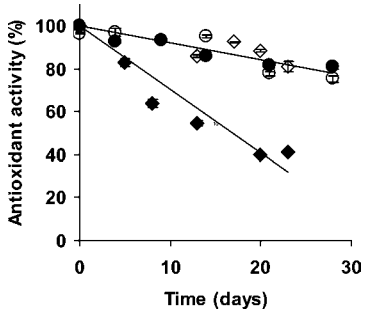

Figure 6. Antioxidant activity of genistein and daidzein in model solutions (30 μM) incubated at 90 °C, expressed as a percentage of Trolox antioxidant activity. Genistein: pH 7 (●) and pH 9 (◆). Daidzein: pH 7 (○) and pH 9 (◇).

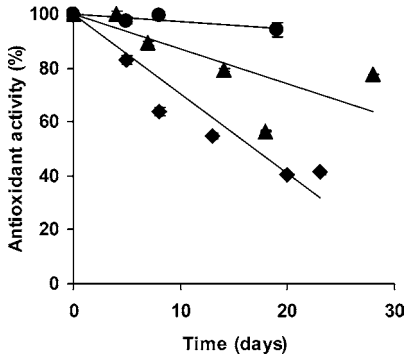

Figure 7. Antioxidant activity of genistein in model solutions (30 μM, pH 9), expressed as a percentage of Trolox antioxidant activity: 70 (●), 80 (▲), and 90 °C (◆).

degradation rates and the activation energies under aerobic and anaerobic conditions suggest that two or more reactions exist. It is also very likely that these reactions differ in their mechanism, their products, and thus also their temperature dependence.

Antioxidant Tests. Genistein and daidzein are known for their antioxidant activity in biological systems with an emphasis on the inhibition of LDL oxidation (*27*). These polyphenols can scavenge a peroxyl radical and protect against iron-induced free radical reactions (*28*). The previously described kinetic study showed that genistein and daidzein are lost due to long-term thermal treatments. To establish whether the degradation of these isoflavones was also affecting their antioxidant activity, we followed the antioxidant potential of genistein and daidzein model solutions during their incubation. The antioxidant activities of genistein and daidzein solutions were compared to that of Trolox, and the results are presented as percent of ABTS radical cation inhibition by Trolox. This assay was shown to be comparable to the LDL oxidation assay for isoflavones (*27*). The antioxidant activity of genistein and daidzein model solutions during thermal treatment at 90 °C is presented in **Figure 6**. The antioxidant ability of genistein was reduced by 60% at pH 9.0 as compared with daidzein that was less affected, showing only a 20% decline in its activity. In pH 7.0, both genistein and daidzein maintained their antioxidant abilities at >70% for at least 30 days. It is therefore suggested the products of genistein degradation at pH 9.0 are different from its degradation products at pH 7.0. This difference is expressed in the higher antioxidant activity of pH 7.0 degradation products. Antioxidant activity of genistein during thermal incubation at pH 9 is presented in **Figure 7**. Although genistein loses most of its antioxidant activity at 90 °C, at 70 °C a genistein solution was hardly affected as an antioxidant despite a decline of 80% in genistein concentration. This may indicate that lowering the temperature affected the type of degradation products being formed or inhibited the further degradation of these products to non-antioxidant compounds.

Previous studies suggested that the 4′-hydroxyl group in ring B and the 5,7-dihydroxy structure of ring A are responsible for the activity of isoflavones as scavengers of the aqueous-phase radicals (*27*). That study and an ESR study conducted by Mitchell et al. (*29*) suggested that genistein had a higher antioxidant capacity than daidzein. The study of Cao et al. (*30*), however, showed higher antioxidant activity for daidzein using a method similar to the one used in the present study. Here, too, daidzein had an initial antioxidant activity similar to that of genistein. Interestingly, it was daidzein degradation products (at pH 9) that had antioxidant potential, whereas genistein products showed only minor antioxidant activity. Because daidzein showed higher sensitivity to thermal treatments, it is likely that the relatively high antioxidant activity of its degradation products, as compared to that of genistein products, indicates a different degradation mechanism.

Conclusion. This study evaluated the stability of genistein and daidzein during incubation at elevated temperatures. The degradation rate of genistein and daidzein was higher at pH 9 than at pH 7. Both isoflavones showed apparent first-order degradation kinetics, with temperature dependence that fit the Arrhenius model. Higher activation energies were calculated for degradation reactions under anaerobic conditions. It was therefore suggested that in the presence of oxygen, a different mechanism governs the degradation of genistein and daidzein. The antioxidant activity of daidzein solutions showed only a moderate decrease, whereas genistein solutions at pH 9.0 lost their antioxidant potential. It is likely that the genistein degradation mechanism differs from that of daidzein. Studies are currently underway to measure the stability of additional isoflavone derivatives and to assess the bioactivity and bioavailability of the degradation products.

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