Mol. Nutr. Food Res. 2006, *50*, 373 – 377 DOI 10.1002/mnfr.200500187

Thermal degradation kinetics of isoflavone aglycones from soy and red clover

Florian Conrad Stintzing, Manuel Hoffmann and Reinhold Carle

Institute of Food Technology, Plant Foodstuff Technology, Hohenheim University, Stuttgart, Germany

Driven by their beneficial effects on human metabolism, isoflavonoids have gained considerable importance reflected by an increased number of isoflavone-rich foods, food supplements and pharmaceutical products on the market, mainly derived from soy and red clover. While it is well known that the genuine isoflavone pattern will be altered during processing, data on aglycone stability are rare. Therefore, a thorough study into the thermal sensitivities of biochanin A, daidzein, formononetin, flavone, genistein, glycitein and isoflavone was performed. Samples were heated at 150°C over a period of 7 h at three different pH values, and degradation of the aglycones was monitored by HPLC-DAD analyses. Therefrom, structure-related stability characteristics could be established. While virtually no decay was observed at pH 7.0 and 5.6, degradation was most prominent at pH 3. 1. Individual aglycone retention was further dependent on heating time with daidzein being the most labile compound after any time interval. Curve fitting of the data revealed first-order degradation kinetics for flavone and glycitein, while the remaining aglycones exhibited a sigmoidal degradation pattern.

Keywords: Aglycone / Isoflavone / Kinetics / Stability / Thermal degradation

Received: October 13, 2005; accepted: November 29, 2005

1 Introduction

Because of their multiple positive effects on human health such as prevention and alleviation of menopausal, diabetes and osteoporosis symptoms, prevention of breast, endometrial and prostate cancer as well as cardiovascular disease [1, 2], the interest in isoflavonoids has gained considerable importance as functional components in both foods and nutraceutical supplements [3–5]. While isoflavonoids may also be found in plants other than the Leguminosae [6, 7], their main intake from food is from soy and soy-based products. Total isoflavone concentrations are governed by cultivar, edaphic factors as well as storage and processing conditions [4, 8–11]. Furthermore, overall isoflavonoid contents and the respective compound pattern are subject to change upon thermal exposure [12]. This issue has been frequently addressed concluding that malonyl-derivatives would easily decarboxylate to generate acetyl-compounds, with the corresponding isoflavonoid-glucosides being rather stable [11, 13–17]. While it has been demonstrated that products obtained after microbial fermentation show a higher proportion of aglycones due to β-glucosidase activity [18, 19], the thermostability of isoflavone aglycones has not yet been investigated in detail. Very recent data [20, 21] suggest that isoflavone aglycones might be degraded at temperatures exceeding 150°C. Therefore, the present study was performed to get an insight into the structure-stability relationships of the most common isoflavone aglycones daidzein, genistein and glycitein from soy (*Glycine max* L. Merr.) and biochanin A and formononetin from red clover (*Trifolium pratense* L.). Daidzein, genistein, glycitein, formononetin and biochanin A, together with their related structures flavone and isoflavone (Fig. 1), were heated from 1 to 7 h at 150°C and three different pH values, and the resulting solutions were subsequently monitored by HPLC-DAD. While heating up to 100°C is crucial for genuine enzyme inactivation, the heating regimes applied in the present study are relevant for baking, frying, toasting and extrusion cooking of isoflavone-containing foods.

2 Materials and methods

2.1 Solvents and reagents

Daidzein, genistein and glycitein standards were purchased from LC-Laboratories (Woburn, MA, USA). Biochanin A, formononetin and flavone were from Sigma-Aldrich Chemie (Taufkirchen, Germany), and isoflavone was from Indofine Chemical Company (Hillsborough, NJ, USA).

Correspondence: Dr. Florian Conrad Stintzing, Institute of Food Technology, Plant Foodstuff Technology, Hohenheim University, August-von-Hartmann-Strasse 3, 70599 Stuttgart, Germany
E-mail: stintzin@uni-hohenheim.de
Fax: +49-711-459-4110

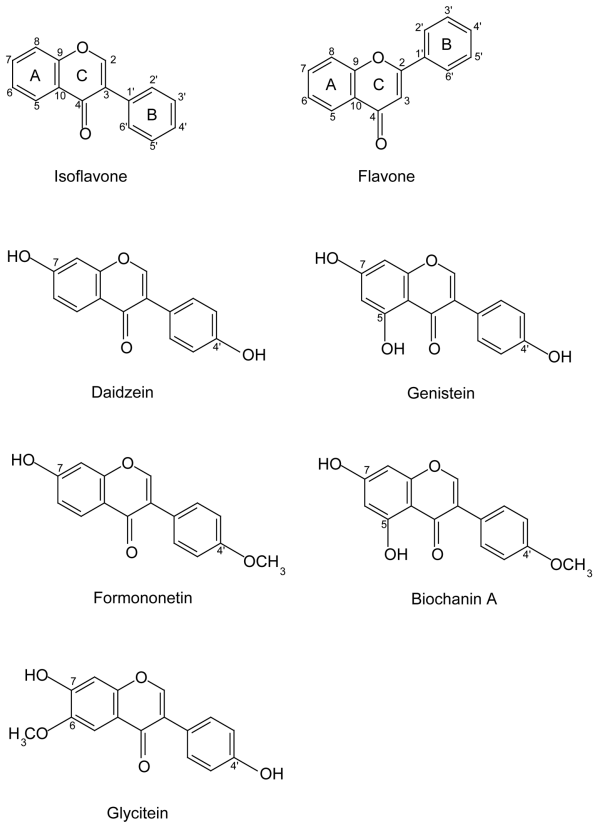

Figure 1. Chemical structures of flavone and six isoflavone aglycones.

Glacial acetic acid (analytical grade), hydrochloric acid (analytical grade) and methanol (HPLC grade) were purchased from VWR (Darmstadt, Germany). Deionised water was used throughout.

2.2 Sample preparation

Isoflavone aglycone standard solutions ranging from 0.3 to 0.6 mmol/L were prepared in aqueous methanol (80/20, MeOH/H_2O v/v). To assess the pH impact on thermal degradation, the aqueous proportion of the neutral sample (pH 7.0) was replaced by deionised water acidified to pH 4 and 2, resulting in pH values of 3.1 and 5.6 in the aqueous methanol solutions, respectively. Instead of using buffers, acidification was performed with hydrochloric acid to exclude any stabilizing effect. For adequate pH assessment of the pH 7 solvent, a small amount of sodium chloride was added to the aqueous methanol solution.

2.3 Heat treatment

For each isoflavone-pH combination, 2 mL was filled into two separate Pyrex culture tubes (100 mm × 14 mm id, Bibby Sterilin, Stone Staffs, England), respectively, closed tightly and heated at 150°C in a universal laboratory oven (Type U30ü, Memmert, Schwabach, Germany). After 1, 3, 5 and 7 h, two tubes of each sample were taken from the oven, immediately cooled in an ice-bath for 2 min to stop heat degradation, then membrane filtered (0.45 µm, polypropylene, VWR International, West Chester, PA, USA), transferred into a vial and subsequently used for HPLC-DAD analyses. Unheated samples served as blanks for duplicate analyses (0 h).

2.4 HPLC-DAD analyses

The HPLC-system was a Merck-Hitachi apparatus (Merck, Darmstadt, Germany) equipped with an auto sampler L-7200, an interface module D-7000, an L-7100 pump, a Jet-Stream column oven, and an L-7450A diode array detector. Optimum separation of isoflavones was achieved on an analytical scale (150 mm × 4.6 mm id) YMC-PACK ODS-AM 12S05 column with a particle size of 5 µm (YMC Europe, Schermbeck, Germany), fitted with a security guard C_{18} ODS column (4 mm × 3.0 mm id, Phenomenex, Torrance, CA, USA) at a flow rate of 1 mL/min, a constant temperature of 25°C and a pressure of 104 bar. Eluent A was 0.1% glacial acetic acid and B consisted of MeCN/glacial acetic acid (99.9/0.1 v/v). Separation was accomplished starting with 100% A for 2 min, followed by gradient elution steps to 3% B at 14 min, then 47% B at 45 min, and finally 100% B at 50 min before re-equilibration to starting conditions.

2.5 Data analysis

Based on four-point calibration curves for each isoflavone aglycone standard ($R^2 \geq 0.99$), the mean of peak areas of two separately heat-treated samples monitored at 260 nm were converted into molar concentrations and the latter was plotted against the heating time. Curve fitting was performed using Sigma Plot software (version 8.02, 2002, SPSS, Chicago, IL, USA).

3 Results and discussion

To assess the effect of the individual isoflavone substitution patterns on their thermostability, the five most common aglycones daidzein (7,4′-dihydroxyisoflavone), genistein (5,7,4′-trihydroxyisoflavone), glycitein (7,4′-dihydroxy-6-methoxyisoflavone), formononetin (7-hydroxy-4′-methoxyisoflavone) and biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) were subjected to thermal treatments at 150°C for up to 7 h. Furthermore, isoflavone and its position isomer flavone were included in this study (Fig. 1). In addition, the impact of pH on the respective compound

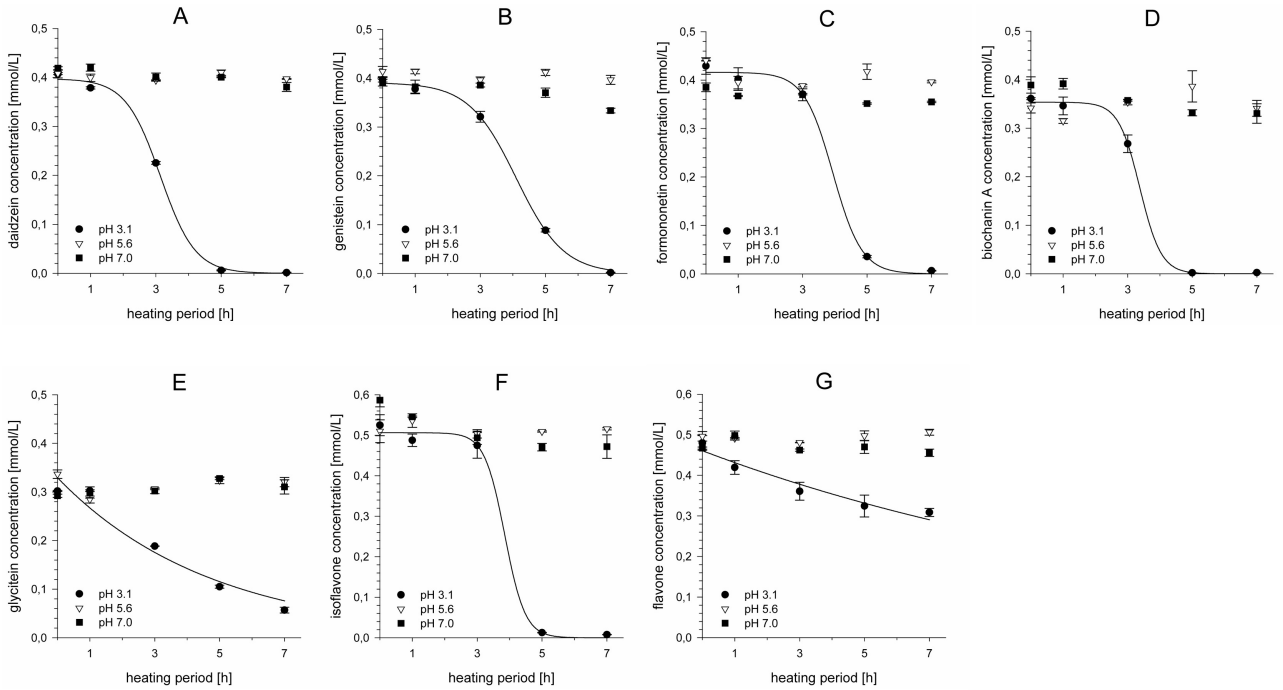

Figure 2. Time-temperature degradation profiles of seven isoflavonoid aglycone standards heated at 150°C for 7 h (A: daidzein, B: genistein, C: formononetin, D: biochanin A, E: glycitein, F: isoflavone, G: flavone). Data points are means of duplicate measurements.

Table 1. Regression fits and regression coefficients for flavone and isoflavone aglycone degradation at pH 3.1

	Coefficients[a)]				
	A[b)]	B[b)]	X_0[b)]	r[c)]	Type
Daidzein	0.3973	0.5318	3.138	0.998	Sigmoid
Genistein	0.3907	−0.7253	4.102	0.999	Sigmoid
Formononetin	0.4160	−0.4481	3.941	0.998	Sigmoid
Biochanin A	0.3537	−0.3308	3.377	0.999	Sigmoid
Glycitein	0.3294	0.2096	–	0.947	Exponential
Isoflavone	0.5064	−0.3140	3.853	0.997	Sigmoid
Flavone	0.4608	0.0658	–	0.941	Exponential

[a)] Coefficients for the terms $Y = A/[1 + \exp(-(X - X_0/B))]$, sigmoidal or $Y = A \times \exp(-B \times X)$, exponential.
[b)] Coefficients given are means for the duplicates.
[c)] Regression coefficient.

retention was monitored at pH 3.1, 5.6 and 7.0, respectively. Thermal stability was assessed by HPLC-DAD measurements. For baseline separation of all seven isoflavone aglycones, an HPLC gradient was established resulting in the following retention times: 36.1 min for daidzein, 36.7 min for glycitein, 40.7 min for genistein, 43.8 min for formononetin, 48.8 min for flavone, 48.9 min for biochanin A and 49.4 min for isoflavone, respectively.

While isoflavone aglycone contents were strongly affected at pH 3.1, degradation at 5.6 and 7.0 was generally moderate, slightly differing between the various compounds (Fig. 2).

According to the respective time course of aglycone retention two distinct groups could be distinguished at pH 3.1 (Fig. 2): The best curve fit for flavone (G) and glycitein (E) were obtained assuming an exponential rather than a linear decay representing a first-order degradation kinetics. Unexpectedly, the remaining aglycones (A–D, F) showed a sigmoidal decomposition pattern (Table 1).

First-order degradation kinetics have frequently been reported for food components such as anthocyanins [22], betalains [23], carotenoids [24], chlorophylls [25], catechin derivatives [26] and even daidzein and genistein [27]. Interestingly, a sigmoidal degradation course characterized by a

linear period, a period of fast conversion and a final intercept of retardation has hitherto exclusively been reported for ascorbic acid degradation [28] and autocatalytic processes such as radical reactions during carotenoid decomposition [29]. Since the experiments of the present study were conducted on a similar molar basis, varying compound concentrations cannot be made responsible for the differing degradation patterns observed. It is worth mentioning, however, that flavone was the only compound substituted at position 2 of the C-ring (Fig. 1), implying that the 3-substituted isoflavones exhibit different heat sensitivities. This assumption was supported by the findings that flavone was the most stable structure of all compounds after heating for 5 and 7 h. Its retention exceeded that of isoflavone, thus revealing a stabilizing effect of the 2→1′ (flavone-derivatives) *versus* the 3→1′ (isoflavone-derivatives) stereochemistry.

Furthermore, structure-stability relationships for isoflavone, daidzein, genistein, formononetin and biochanin A were established (Fig. 2). Glycitein featuring a methoxy-moiety at position 6 of the A-ring was the second most stable compound after 5 and 7 h. While after a heating period of 1 and 7 h, the retention of all aglycones showing sigmoid degradation was similar, notable differences were registered after 3 and 5 h. Highest contents were found for isoflavone, followed by formononetin, genistein, biochanin A and finally daidzein after 3 h, whereas genistein and then formononetin were the most stable structures after 5 h at 150°C. Obviously, among the 7,4′-isoflavone aglycones, the 4′-methoxy-substitution resulted in an increased stability (formononetin *vs.* daidzein). In contrast, heat resistance was improved in 5,7,4′-substituted structures when the 4′-position was a hydroxy- (genistein) instead of a methoxy-moiety (biochanin A). Daidzein was more heat sensitive than genistein, implying that additional hydroxylation at the A-ring enhances stability of 7,4′-dihydroxylated structures. In contrast, these findings did not hold true for 7-hydroxy-4′-methoxylated isoflavones, since biochanin A was almost completely degraded after 5 h, exhibiting less heat stability than formononetin. Interestingly, glycitein carrying a 6-methoxy moiety proved to be an exception, showing a first-order degradation as found for flavone.

The overall compound retention depended on duration of heat exposure. During the first 3 h, the order was daidzein< glycitein<flavone<biochanin A<formononetin<genistein< isoflavone, while after 5 and 7 h of heat exposure, daidzein and biochanin A were least stable followed by isoflavone, then formononetin, genistein, glycitein and finally flavone (Fig. 2).

Previously published data [14] noted a higher loss of daidzin derivatives *versus* genistin derivatives during tofu processing, both being ascribed to different solubilities and thermal sensitivities. These findings were later supported by Ungar *et al.* [27], when genistein was more stable than daidzein following first-order kinetics upon heating at 70, 80 and 90°C at pH 7, while at pH 9 the retention was inverse. However, contrasting data have been reported by Xu *et al.* [21] where daidzein was more stable than genistein and glycitein upon dry heating. Both aglycones were generated from their corresponding glucosides after 60 min at 200°C. These seemingly contradictory findings might be due to differing degradation kinetics depending on the respective temperature, the particular pH conditions, solid or liquid systems and finally stabilizing effects from buffer salts or food components.

4 Concluding remarks

The present study clearly corroborates that proneness of individual isoflavone aglycones to thermal decomposition strongly depends not only on their specific substitution pattern but also the respective pH value. Both exponential and sigmoidal decomposition were registered at pH 3.1, while degradation was negligible at pH 5.6 and 7.0. However, the chemical basis underlying the diverse degradation kinetics remains to be clarified. While in the present work the focus was directed towards model solutions, the practical relevance during frying, baking, toasting and also extrusion cooking of isoflavone-rich products should be considered in future investigations. Reduction of aglycone concentration might result in less bitter-tasting products with superior sensorial quality. Furthermore, the nutritional value of isoflavone-rich commodities might be altered through processing both with respect to their antioxidant potencies as well as to the bioavailability of their isoflavone-derived compounds. To get a deeper insight into these issues, the identification of the respective degradation products by HPLC-DAD-MS is currently underway.

The authors thank Rudolf Wild GmbH & Co. KG (Heidelberg-Eppelheim, Germany) for financial support.

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