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# Assessment of the Reactivity of Selected Isoflavones against Proteins in Comparison to Quercetin

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Selected isoflavones (genistein, daidzein, formononetin, prunetin, biochanin A, and two synthetic isoflavones) were allowed to interact with soy and whey proteins. The reaction products were analyzed in terms of covalent binding at the nucleophilic side chains of proteins. Changes in molecular properties of the proteins derivatives were documented by SDS-PAGE, IEF, and SELDI-TOF-MS. The structural changes induced were studied using circular dichroism. The in vitro digestibility was assessed with trypsin. The results show that the occurrence of the catechol moiety, that is, the two adjacent (ortho) aromatic hydroxyl groups on ring B of the flavonoid structural skeleton appears to be perquisite condition for covalent binding to proteins. The catechol moiety on ring A was less reactive. Its absence lead to a slight or no significant reaction, although noncovalent interactions may still be possible, even when lacking this structural element. A comparison of the data is also made with quercetin representing the flavonols.

**KEYWORDS: Isoflavones; quercetin; soy proteins; whey proteins; protein−isoflavone interactions; protein derivatization; structural changes; in vitro proteolytic degradation**

## INTRODUCTION

We know comparatively little about the interactions of the minor phenolic components in plants with the main constituents of foods (e.g., proteins), although they occur very frequently together in the same food matrix. One such group of phenolic compounds is represented by isoflavones having in common the 1,2-diphenylpropane skeleton (**Figure 1**). Isoflavones are taxonomically narrowly distributed, being mainly restricted to the subfamily of Papilionaceae of Leguminosae, with soy beans as the principle human dietary source (*1*−*3*). As recently reviewed, studies have shown that there is a large variability in concentration and composition of the isoflavones among different soybeans or soy-protein products and that this is a function of species differences, geographic and environmental conditions, and extent of industrial processing of the soybeans (*1*). These compounds have a wide range of hormonal and nonhormonal activities, as shown in vitro and/or in animals, and these suggest plausible mechanisms for potential biological effects of diets rich in these compounds in humans (*2*). Isoflavones are present in bioactive concentrations in food products such as soy protein isolates (*4*). It is generally accepted that consuming the phytochemical components of soy, particularly the isoflavones, in pure form, as in supplements, may pose some health concerns due to their high concentrations, but that consumption of more whole foods containing these is natural (low concentration, embedded in food matrix) and, as such, safe (*4*). Some of these health concerns may be directed to the possibility of interactions with body proteins including enzymes, hormones, receptors, etc. with physiological and in some cases toxic consequences, most of which are still unknown (*5*). Phenolic compounds and polyphenol oxidases such as tyrosinase can be expected to be present simultaneously in diets including fruits and vegetables (*6*). Therefore, it seems very plausible, to expect phenol oxidation to *o*-quinone (a very reactive electrophilic compound) and isomerization to *p*-quinone methide derivatives, as shown for quercetin in biological systems (*6*, *7*). The reaction pathway of the quinone formation could be explained on the basis of the occurrence of the catechol moiety of quercetin, that is, the two adjacent (ortho) aromatic hydroxyl groups and the resulting *p*-quinone methide derivatives are then in position to undergo covalent binding to nucleophilic reaction partners. In this context, it was shown that the presence of quercetin leads to quinone methide glutathione conjugate formation in a cellular in vitro model system (*7*). Recently, we also demonstrated the covalent attachment of phenolic acids and flavonoids to the nucleophilic side chains of proteins in model systems (*8*−*11*). Such reactions are likely to take place during harvesting, storage, and processing of plant foods as shown for chlorogenic acid and coffee proteins (*12*, *13*). Interactions between phenolic compounds and the main food constituents cannot be excluded during the daily preparation of meals and beverages. Additionally, in some cases, the functional properties of foods may also be influenced by the interaction of individual food components with phenolic com-

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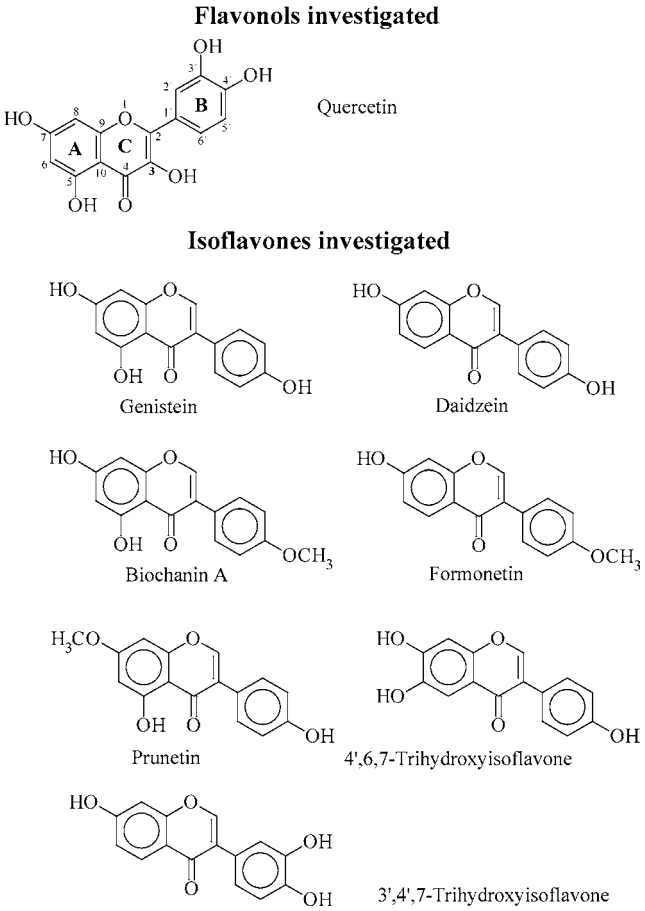


**Figure 1.** Structures of flavonol and isoflavones applied.

pounds. Therefore, the objective of this study was to allow interactions of selected isoflavones (genistein, daidzein, formononetin, prunetin, and biochanin A, as well as two synthetic isoflavones) with soy and whey proteins (**Figure 1**) and to demonstrate, when present, the possibility of their covalent binding. Soy glycinin was chosen, as it is found in the same food matrix as the isoflavones. Whey proteins, especially $\beta$-lactoglobulin, which in itself is a well-described and well-characterized protein, which simplifies its analysis, were applied because they are often used in composite foods with soy products. The intention was to determine the effect of different structural elements in the listed isoflavones on their reactivity toward proteins. With reference to **Figure 1**, it can be observed that the main structural differences can be classified in the following subgroups: (I) presence of two adjacent (ortho) aromatic hydroxyl groups on the ring B (**Figure 1**; 3′,4′,7 trihydroxyisoflavone (3′,4′,7 THI), (II) presence of two adjacent (ortho) aromatic hydroxyl groups on the ring A (**Figure 1**; 4′,6,7 trihydroxyisoflavone (4′,6,7 THI)), and (III) absence of catechol structure elements in both rings A and B (genistein, daidzein, formononetin, prunetin, biochanin A)

In the last case (absence of catechol moiety in the isoflavone structure), a possible reaction via a semiquinone intermediate formation as proposed for covalent binding of *m*-dihydroxybenzene (resorcinol) and ferulic acid to proteins (*14*) should theoretically provide a legible mechanism for the covalent binding. Further, a comparison of the reactivity will also be made with quercetin, which has an additional hydroxyl group at the position C3 in the ring C (**Figure 1**) and which differs from isoflavones by having a 1,3-diphenylpropane skeleton.

## MATERIALS AND METHODS

**Materials.** Soy glycinin (SG) was prepared from defatted unheated soy flour (type 1, protein content ca. 52%, Sigma Chemicals Co., St. Louis, MO) according to the method of Thanh and Shibasaki (*15*). Kjeldahl determination of the extracted SG gave a protein content of 99.8%. Glycinin purity was determined by SDS-PAGE and was estimated to be about 95% by densitometric analysis of the gel. Other proteins investigated were whey protein isolate (WP, New Zealand milk products, DSE 1591) and $\beta$-lactoglobulin ($\beta$-Lg, Fluka Chemie AG, Buchs, Switzerland). Trypsin was from porcine pancreas (EC 3.4.21.4, protein content 98%, Sigma Chemicals Co.).

The following phenolic compounds were applied: genistein (4′,5,7-trihydroxyisoflavone) and daidzein (4′,7-dihydroxyisoflavone), both from Sigma Chemicals Co.; biochanin A (5,7-dihydroxy-4′-methoxy-isoflavone), formononetin (7-hydroxy-4′-methoxy-isoflavone), prunetin (4′,5-dihydroxy-7-methoxy-isoflavone), and 3′,4′,7-trihydroxyisoflavone, 4′,6,7-trihydroxyisoflavone, all from Fluka Chemie AG; and quercetin (Riedel-deHaën Laborchemikalien GmbH & Co. KG, Seelze, Germany).

Due to the poor solubility of the isoflavones in the aqueous media, the following solvent mixtures were found to be applicable: ethanol/acetone (Et/Ac; 1:1; v:v) or pure ethanol (Et). The derivatives were prepared by mixing 0.09 mmol of the isoflavones dissolved in 12 mL of ethanol/acetone with 0.3 g of protein suspended in 18 mL of deionized water. This amount of solvent was necessary to keep the isoflavones and proteins in solution, taking in account its denaturing effect on protein and underlining the model character of this investigations. After the addition of the isoflavones, the pH was adjusted to 9. After 24-h reaction time under continuous stirring at room temperature (24 °C) with free exposure to air, the samples were dialyzed for 18−20 h against water (pH 9) and finally lyophilized. The dried samples were washed four times with Et/Ac (1:1; v/v), with a final washing step with only acetone to remove the free unbound isoflavones. The nonderivatized protein (control) was prepared under the same conditions but without addition of the isoflavones.

In a similar way, derivatives of $\beta$-Lg were also prepared with 3′,4′,7 THI, 4′,6,7 THI, and quercetin, as described in ref *11* using ethanol as solvent. The final concentration of ethanol in the reaction mixture was kept below 10%.

**Methods.** Changes in the content of free amino groups were analyzed using trinitrobenzenesulfonic acid (TNBS) according to Adler-Nissen (*16*). The derivatives were dissolved in 1% SDS for this purpose. Tryptophan (samples dissolved in 8 M urea) was estimated using a Jasco fluorescence detector FP 920 (Gross-Umstadt, Germany; Tokyo, Japan) as described in ref *14*. The change in thiol content (cysteine) of the derivatives was determined by gel permeation liquid chromatography of fluorescently labeled proteins with monobrombimane (Fluka Chemie AG), as described in ref *17*. For this purpose, the samples were dissolved in 200 mM Tris-HCl pH 8, containing 1% SDS, 3 mM EDTA, and 3 mM dithiothreitol. HPLC conditions applied were as follows: eluent, 0.1 M Tris-HCl with 0.1% SDS, pH 8; flow rate, 0.8 mL $min^{-1}$; column, TSK−Gel G3000 PWXL 7.8-mm ID × 30-cm (Tosohaas GmbH, Stuttgart, Germany); column temperature, 25 °C; detection, UV, 220 nm; fluorescence, excitation, 380 nm; emission, 480 nm; injection volume, 10 $\mu$L. A Jasco HPLC system (Gross-Umstadt, Germany, Tokyo, Japan) was used.

SDS-PAGE was applied for molecular weight determination (*14*). The change in the band intensity was estimated using densitometer scanning software (Bio-Rad, Fluor-S MultiImager, Hercules, CA).

The covalent nature of the binding of the isoflavones was also confirmed by applying surface-enhanced-laser-desorptions-ionization-time-of-flight-mass spectrometry (SELDI-TOF-MS). These experiments were performed by dissolving 1 mg of the protein sample in 1 mL of distilled water. A 40-$\mu$L aliquot of this solution was mixed with 10 $\mu$L of acetonitrile and 20 $\mu$L of sinapic acid as matrix (5 mg in 125 $\mu$L of acetonitrile + 125 $\mu$L of 1% trifluoroacetic acid, v/v; mixed, short centrifugation and using the supernatant). A 1.5-$\mu$L aliquot was then brought twice on to a target (a gold chip from CIPHERGEN Seldi Protein biology system, Fremont, CA). After crystallization of the sample by air-drying, measurements were carried out on CIPHERGEN

Seldi Protein biology system−SELDI-TOF-MS instrumentation (CIPHERGEN) by use of the following spot protocol: set high mass to 25 000 Da, optimized from 5000 to 25 000 Da; set starting laser intensity to 205; set starting detector sensitivity to 8; focus lag time at 1892 ns; set Seldi acquisition parameters delta to 4. transients; set warming positions with two shots at intensity 250 and do not include warming shots; identify peaks using auto identify from 10000 Daltons to 25000 Daltons.

Isoelectric focusing (IEF) of the samples was carried out in a pH range from 3 to 10 (PAGE, total acryl amide concentration $T = 10\%$), as described in ref*14*.

The solubility of the lyophilized samples at pH 4.5 was determined in a 0.05 M sodium phosphate buffer system by removing the insoluble material through centrifugation at 9088*g*, 10 min (Megafuge 2.0R, Heraeus, Hanau, Germany). The precipitate was solubilized in electrophoresis incubation buffer, and SDS-PAGE was performed as described above.

Far-UV circular dichroism (CD) of the $\beta$-Lg samples were recorded in the range of 178−260 nm in 0.1 M sodium phosphate buffer pH 7 at a protein concentration of 0.2 mg/mL using a Jasco J 710 spectropolarimeter (Gross-Umstadt, Germany) as described in ref *9*. The CD spectra were analyzed by a curve-fitting software CDPro using CONTIN methods as described reported in ref *18* to obtain the secondary structural contents of the proteins. The estimation was performed by use of a 43-protein reference set (*18*).

In vitro tryptic digestion (100 $\mu$L of enzyme, 1 mg/mL) of the samples (final concentration 4 mg/mL) dissolved in 0.1 M Tris-HCl pH 8 containing 0.02 M $CaCl_2$ was investigated by incubating at 35 °C (enzyme/substrate ratio, E/S = 1:60). Undigested protein samples for SDS-PAGE were prepared by removing 100 $\mu$L from the incubation mixture prior to the addition of trypsin. After different durations of hydrolysis (5−60 min), 100 $\mu$L was removed from the incubation mixture. The degradation was stopped by addition of the denaturation buffer (100 $\mu$L 0.05 M Tris-HCl pH 6.8 containing 4% SDS (w/v), 5% mercaptoethanol (v/v), 12% glycerin (w/v), and 0.01% Coomassie Brilliant blue R 250 (w/v)) and heating at 100 °C for 5 min. The samples were then cooled, and 10−20 $\mu$L was loaded in the slots for the SDS-PAGE analysis.

**Statistical Analysis.** Generally, the analyses were repeated 10 times and evaluated by standard deviation. Student *t*-tests were performed for the results shown in **Table 1**. The values were compared to that of the control proteins and termed significant for $p < 0.05$. The other analyses were repeated thrice and a maximum of ± 5% standard deviation from the averaged values was generally tolerated. The averaged values are documented in the respective figures.

## RESULTS AND DISCUSSION

In preliminary experiments, the potential nucleophilic covalent binding of isoflavones to proteins was monitored by following the change in the content of tryptophan, thiol, and amino groups. The results obtained are summarized in **Table 1**. The measured concentration of tryptophan was effected slightly but significantly by genistein, when allowed to react with SG. In the case of WP isolate, besides genistein, also daizein and biochanin were found to cause a decrease in the amount of measured tryptophan, whereas for $\beta$-Lg, both prunetin and formononetin reacted. These results indicate that there is no clear trend, and the reaction, although to a small extent (5−24% decrease of tryptophan), is significant and seems to depend on both the protein as well as on the isoflavone applied. With regard to the reactivity of the two synthetic isoflavones (3′,4′,7 THI and 4′,6,7 THI) toward proteins, the former was found to be the most reactive, its reactivity being as high as that obtained by applying quercetin (*11*) (**Table 1**). 3′,4′,7 THI caused a decrease of tryptophan amounting to 43−53%, whereas 4′,6,7 THI caused a significant depletion of 10−16%. In both cases, the effect of the solvent (Et/Ac or Et) applied to solubilize the isoflavones during derivatization is distinctly detectable (**Table 1**).

**Table 1.** Changes in the Content of Tryptophan, Thiol, and Amino Groups in Protein-Isoflavone and Protein-Quercetin-Derivatives

| sample[a] | tryptophan [%] | thiol groups [%] | free amino groups [%] |
|---|---|---|---|
| | 1. soy glycinin, control | | |
| | **100.0** ± 3.9 (44.2 nmol/mg P)[b] | **100.0** ± 1.6 (128.2 nmol/mg P) | **100.0** ± 4.0 (529.4 nmol/mg P) |
| **2.** + genistein | 86.4 ± 3.8[c] | 88.7 ± 5.6[c] | 90.6 ± 2.5[c] |
| **3.** + daidzein | 99.3 ± 4.1 | 92.0 ± 3.6[c] | 100.5 ± 1.8 |
| **4.** + biochanin A | 94.7 ± 2.7 | 95.5 ± 2.8 | 94.8 ± 1.5 |
| **5.** + formononetin | 97.0 ± 4.5 | 108.2 ± 8.3 | 98.5 ± 3.5 |
| **6.** + prunetin | 100.4 ± 2.4 | 102.0 ± 8.7 | 107.6 ± 1.7 |
| | 1. whey proteins, control | | |
| | **100.0** ± 4.8 (65.9 nmol/mg P) | **100.0** ± 1.0 (300.7 nmol/mg P) | **100.0** ± 2.9 (628.0 nmol/mg P) |
| **2.** + genistein | 76.3 ± 4.8[c] | 105.0 ± 3.3 | 86.4 ± 0.4[c] |
| **3.** + daidzein | 82.2 ± 8.0[c] | 91.6 ± 4.1[c] | 88.0 ± 3.5[c] |
| **4.** + biochanin A | 79.6 ± 9.8[c] | 94.2 ± 4.1 | 93.5 ± 4.6 |
| **5.** + formononetin | 94.4 ± 9.8 | 102.9 ± 1.9 | 89.3 ± 2.1[c] |
| **6.** + prunetin | 92.9 ± 4.7 | 104.8 ± 0.2 | 88.3 ± 2.6[c] |
| | 1. $\beta$-lactoglobulin, control | | |
| | **100.0** ± 8.6 (65.0 nmol/mg P) | **100.0** ± 10.0 (282.2 nmol/mg P) | **100.0** ± 7.5 (681.8 nmol/mg P) |
| **2.** + genistein | 101.1 ± 9.4 | 100.3 ± 7.4 | 99.9 ± 2.4 |
| **3.** + daidzein | 90.6 ± 7.8 | 107.2 ± 6.9 | 97.9 ± 0.9 |
| **4.** + biochanin A | 91.0 ± 7.4 | 106.0 ± 5.6 | 100.2 ± 2.0 |
| **5.** + formononetin | 82.8 ± 5.5[c] | 99.7 ± 2.2 | 98.7 ± 2.0 |
| **6.** + prunetin | 86.4 ± 3.4[c] | 108.9 ± 0.9 | 91.7 ± 2.4 |
| **7.** + 3′,4′, 7 THI | 47.3 ± 6.9[c] | 75.4 ± 9.5[c] | 64.1 ± 1.5[c] |
| **8.** + 4′, 6, 7 THI | 84.5 ± 7.3[c] | 101.5 ± 8.9 | 75.1 ± 6.4[c] |
| **9.** + 3′,4′, 7 THI (Et)[a] | 56.7 ± 2.7[c] | 68.2 ± 9.5[c] | 67.4 ± 2.6[c] |
| **10.** + 4′, 6, 7 THI (Et)[a] | 90.2 ± 4.3[c] | 104.2 ± 9.8 | 79.3 ± 6.0[c] |
| **11.** + quercetin (Et)[a] | 49.0 ± 3.1[c] | 76.8 ± 2.8[c] | 68.0 ± 2.7[c] |

[a] The derivatization was performed with the isoflavones solubilized in ethanol/acetone (1:1) or in ethanol (Et). [b] P = protein. [c] $p < 0.05$.

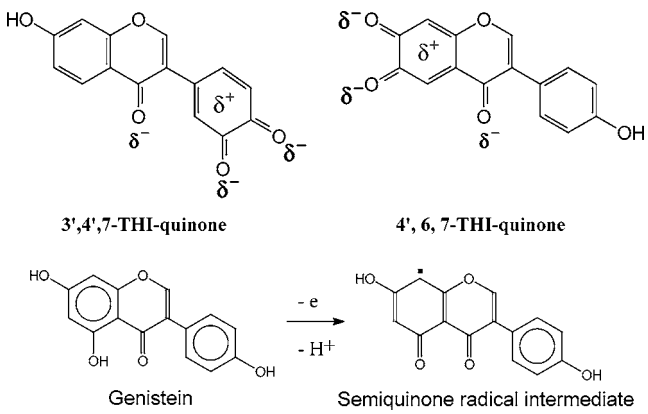


**Figure 2.** I-effect of 3′,4′,7 THI quinone and 4′,6,7 THI quinone; formation of a semiquinone radical intermediate from genistein.

A possible reaction mechanism to explain this decrease can be given by a nucleophilic addition of the heterocyclic nitrogen of the tryptophan moiety to an oxidized species of a phenolic compound (*14*). Only quercetin, 3′,4′,7 THI, and 4′,6,7 THI are in position to undergo oxidation leading to formation of the corresponding quinones. This being the case due to the presence of the structural element composed of two adjacent hydroxyl groups (catechol moiety) in them. The reaction of other isoflavones may take place via a semi-quinone (**Figure 2**) and as discussed in detail elsewhere (*14*). Further, there is a statistically significant difference (*t*-test) in the reactivity of 3′,4′,7 THI and 4′,6,7 THI (**Table 1**). The catechol moiety in 3′,4′,7 THI is situated on the ring B of the isoflavone structure, which can rotate freely due to the single bond connecting ring B and C, being mainly responsible for increasing the reactivity of 3′,4′,7 THI in addition to the induction effect (I-effect)

induced by the electron distribution in the respective quinone (**Figure 2**), whereas those in 4′,6,7 THI are located on the inflexible ring A. Therefore, the reactivity of 4′,6,7 THI is lower compared to that of 3′,4′,7 THI and is governed only by the I-effect (**Figure 2**).

The reactivity of isoflavones against thiol groups, documented by the corresponding decrease in their content, is given in **Table 1**. Genistein and daidzein both cause a significant deviation from the corresponding SG control, whereas for WP only daidzein is effective (**Table 1**). The reaction with $\beta$-Lg is limited to 3′,4′,7 THI and quercetin, both exhibiting a similar degree of reactivity (**Table 1**). 4′,6,7 THI does not react at all with the thiol groups. Two reaction mechanisms can be postulated for the observed decrease in the amount of the thiol groups (Supporting Information); the first one being by undergoing reactions with oxidized phenolic compounds as postulated (Supporting Information) and documented in ref *19*. Since the analysis was performed under reducing conditions and in the presence of SDS, it can be deducted that the thiol groups were not converted to disulfide bridges, but had reacted with the phenolic compounds. The second reaction mechanism can be postulated by the redox interaction between the phenol and the disulfide bridges in an initial step (Supporting Information), whereby the phenol is oxidized to the corresponding quinone and the disulfide bridges reduced to thiol groups. The latter can then undergo a nucleophilic addition with each other in a second step. The proof that such a reaction does take place was given by investigating the reaction of lysozyme, which does not contain any free thiol groups, with phenolic acids (*14*). In this context, it has been recently reported that an *o*-quinone trapping method by glutathione appeared to be an accurate method for quantification of *o*-quinone metabolites (*6*, *7*, *20*−*22*). In these studies, the method proved useful for analyzing and characterizing glutathione adducts via thiol groups with flavonoids, which can be formed at C2′, C5′, C6′, C6, and C8 (*6*, *20*).

The reaction of the isoflavones with the free amino groups is documented in **Table 1**. A significant depletion in the content of these groups was noted in the presence of genistein during the derivatization of SG. Whereas in the case of WP, besides genistein, also daizein, prunetin, and formononetin caused a significant reduction in the measured amount of the amino groups. Only quercetin, 3′,4′,7 THI, and 4′,6,7 THI reacted with $\beta$-Lg. Once again, the effect of the solvent (Et/Ac or Et) applied to solubilize the isoflavones during derivatization is distinctly detectable (**Table 1**). Further, there is no statistically significant difference in the reactivity of 3′,4′,7 THI and 4′,6,7 THI (**Table 1**). The reaction mechanism for free amino groups (see also Supporting Information) with phenolic compounds similar to that of tryptophan is given in detail in (*14*, *19*).

This estimation of the reactivity of the protein side chains is incomplete, since it has been theoretically and experimentally observed that further amino acid residues such as proline, methionine, histidine, and tyrosine also have the potential to react with the phenolic compounds (*19*, *23*).

Upon further oxidation of such addition products with nucleophilic side chains of proteins to form its quinone, a second addition may occur, which leads to formation of cross-linked protein polymers (*19*, *23*). This can be confirmed by SDS-PAGE analysis as documented in **Figure 3**, which shows the $\beta$-Lg derivatives generated in the presence Et/Ac with genistein, daidzein, 3′,4′,7 THI, and 4′,6,7 THI. Only the latter two reagents (3′,4′,7 THI being more reactive) caused a dimerization of $\beta$-Lg, thereby producing a molecular weight fraction of ca. 38 kDa (**Figures 3** and **4**). A similar observation was also made

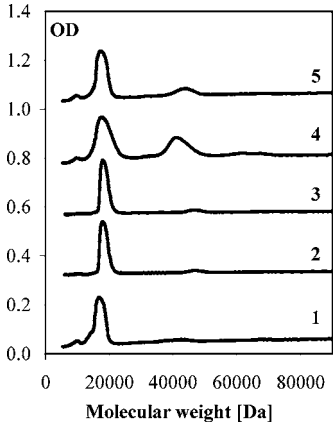


**Figure 3.** SDS-PAGE of the $\beta$-Lg derivatives (generated in the presence Et/Ac). Code: 1 = $\beta$-Lg, control; 2 = genistein–$\beta$-Lg derivative; 3 = daidzein–$\beta$-Lg derivative; 4 = 3′,4′,7 THI–$\beta$-Lg derivative; 5 = 4′,6,7 THI–$\beta$-Lg derivative.

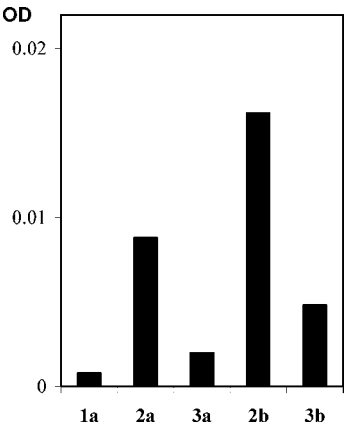


**Figure 4.** Amount of $\beta$-Lg dimer formed during derivatization with synthetic isoflavones (SDS-PAGE)–influence of solvent applied. Code: 1a = $\beta$-Lg, control (Et); 2a = 3′,4′,7 THI–$\beta$-Lg derivative (Et); 3a = 4′,6,7 THI–$\beta$-Lg derivative (Et); 2b = 3′,4′,7 THI–$\beta$-Lg derivative (Et/Ac); 3b = 4′,6,7 THI–$\beta$-Lg derivative (Et/Ac).

when applying quercetin as reported elsewhere (*11*). No polymerization was found with the isoflavones genistein, daidzein, formononetin, prunetin, and biochanin A, independent of the protein used (results not shown), although they partly invoked a significant decrease in the amount of the reactive side chains of the corresponding proteins (see **Table 1**). Further, a recognizable effect of the solvent (Et/Ac or Et) applied to dissolve the isoflavones during derivatization can also be illustrated (**Figure 4**). The reactivity in Et/Ac being higher, as also observed by comparison of the results listed in **Table 1**. An explanation of this behavior is given later in context of the discussion on the corresponding structural studies.

Since the above-mentioned results give only an indirect clue to the covalent binding of isoflavones, a direct method was sought after and found partly in applying SELDI-TOF-MS to evaluate detailed changes in molecular weights of the derivatized monomer protein molecules. Generally, the mass spectra show peaks, which are separated by an increments of the molecular weight of the reacting molecules (isoflavones). SELDI-TOF-MS was performed for all the derivatives listed in **Table 1**. The control proteins (also subjected to derivatization conditions) showed a marked declination in resolution when compared to their respective starting materials as illustrated for $\beta$-Lg in **Figures 5** and **6** (curves 1 and 2, respectively). This change in resolution is induced by the solvent applied to dissolve the

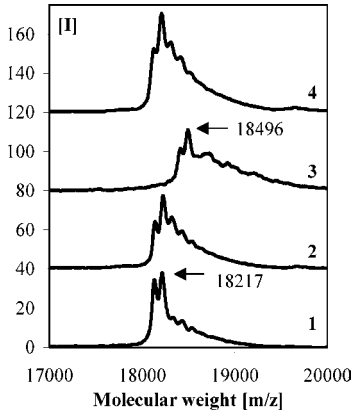


**Figure 5.** SELDI-TOF-MS of the $\beta$-Lg derivatives (generated in the presence Et). Code: 1 = $\beta$-Lg, starting material; 2 = $\beta$-Lg, control (Et); 3 = 3′,4′,7 THI–$\beta$-Lg derivative (Et); 4 = 4′,6,7 THI–$\beta$-Lg derivative (Et).

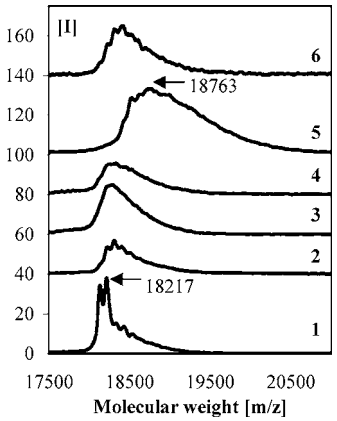


**Figure 6.** SELDI-TOF-MS of the $\beta$-Lg derivatives (generated in the presence Et/Ac). Code: 1 = $\beta$-Lg, starting material; 2 = $\beta$-Lg, control (Et/Ac); 3 = genistein–$\beta$-Lg derivative (Et/Ac); 4 = daidzein–$\beta$-Lg derivative (Et/Ac); 5 = 3′,4′,7 THI–$\beta$-Lg derivative (Et/Ac); 6 = 4′,6,7 THI–$\beta$-Lg derivative (Et/Ac).

isoflavones during derivatization. Further, the influence of Et/Ac mixture is more pronounced. Even an incubation in urea and dithiothreitol (denaturation reagents) did not improve the resolution. For this purpose, a special chip (H4, CIPHERGEN) with an aliphatic hydrophobic surface was applied to facilitate an on-chip removal of the denaturing reagents prior to the mass analysis (results not shown). Therefore, the original protein material was compared only with the controls, and the latter with the corresponding derivatives. For example, the mass spectra of the $\beta$-Lg derivatives illustrating such changes are shown in **Figures 5** and **6**. Genistein, daidzein, and 4′,6,7 THI induced no visible changes (curves 2, 3, and 6, respectively, **Figure 6**). Generally, no distinct changes in the mass spectra of SG, WP, and $\beta$-Lg derivatives with genistein, daidzein, formononetin, prunetin, and biochanin A were observed (results not shown). A recognizable derivatization was noted only for 3′,4′,7 THI (curve 3, **Figure 5**; and curve 5, **Figure 6**). In the presence of Et as solvent, an increase in molecular weight of 270 Da arising from one molecule of 3′,4′,7 THI added to $\beta$-Lg was witnessed (**Figure 5**). The mass of the main peak increased from 18226 Da in the corresponding control to 18496 Da in the derivative. In contrast, the derivatization in the presence of Et/Ac led to an addition of at least two molecules of 3′,4′,7 THI (**Figure 6**) with a parallel increase in mass from 18316 Da in control to 18763 Da in the derivative. Similar results were also obtained for the derivatization products of quercetin (using Et as solvent) and $\beta$-Lg. Here also, addition of at least one molecule of quercetin was analyzed (*11*).

The mass spectra also gave a possibility to observe the changes occurring in the dimer of the $\beta$-Lg (results not shown). The mass increased from 36587 Da of the control (Et) to 37129 Da in the 3′,4′,7 THI derivative (Et), showing the binding of at least two molecules of the isoflavone. Similarly, a rise in molecular weight from 36718 Da (Et/Ac) to 37817 Da in the 3′,4′,7 THI derivative (Et/Ac) was also recorded, accounting for 4 molecules of the isoflavone added. These results could mean that either "2m+H" (two molecules with one charge) were analyzed or actually the dimer existed in this form. Either way, the mass analysis in this form is not yet satisfactory, and further methods such as LC/MS coupled with specific proteolytic analysis and NMR will be performed to characterize this product better.

Besides the molecular weight, other characteristic properties of the proteins may also be effected. The covalent attachment of the isoflavone to proteins, on one hand, would cause the blocking of the hydrophilic groups such as amino and thiol groups as discussed above, but on the other hand, there is also an increase in the amount of apolar groups (benzene ring) and polar groups (hydroxyl groups) being introduced. All these changes affect the hydrophilic/hydrophobic character of the derivatized proteins, which can be illustrated by RP-HPLC (*8*, *14*, *19*). The RP-HPLC of the SG after treatment with the isoflavones did not induce any change in the chromatographical behavior, whereas in the case of WP and $\beta$-Lg, alterations in the chromatograms were noted (results not shown). In this context, the strongest change was brought by the treatment of $\beta$-Lg with 3′,4′,7 THI (results not shown). The peak formation was thereby adversely affected, indicating possible denaturation and molecular interactions.

As a result of the covalent linkages of the phenolic compounds with the proteins, there is a change in the net charge of the protein molecules, which is reflected in the corresponding change of the isoelectric point of the derivatives (*8*). Isoelectric focusing of SG, WP, and $\beta$-Lg samples treated with genistein, daidzein, formononetin, prunetin, biochanin A, and 4′,6,7 THI did not show any change in the distribution of the isoelectric points (results not shown). Again, only 3′,4′,7 THI effects this property as shown, for example, for $\beta$-Lg in **Figure 7**. A shift of isoelectric points of the fractions toward a lower pH can be documented (**Figure 7**, trace 4).

The changes in molecular weight including cross linking together with changes induced in hydrophilic/hydrophobic character as well as on isoelectric points of the 3′,4′,7 THI derivative of $\beta$-Lg also leads to an alteration of the solubility behavior as documented in **Figure 8**. Generally, whey proteins are nitrogenous fractions remaining soluble in the supernatant at pH 4.6 after precipitation of casein. Thus, the loss in solubility at this pH is commonly used to access the extent of protein denaturation (*24*). Therefore, it was possible to illustrate the degree of denaturation in $\beta$-Lg caused by solvent system applied during the derivatization and by the reacting isoflavones (**Figure 8**). The comparison of the lanes 1 and 4 here, corresponding to the two differently treated controls of $\beta$-Lg, shows that Et/Ac mixture denaturates the protein more strongly than only Et. Further, both 3′,4′,7 THI and 4′,6,7 THI seem to intensify this denaturation (lanes 2−3 and lanes 5−6, **Figure 8**).

To clarify these denaturating effects of the isoflavones, selected samples of $\beta$-Lg were analyzed further by means of far-UV-CD experiments. The corresponding spectra are docu-

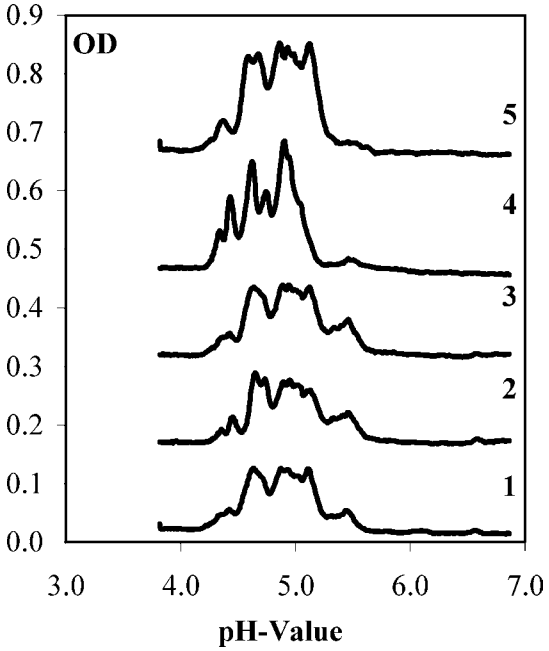


**Figure 7.** IEF of the $\beta$-Lg derivatives (generated in the presence Et/Ac). Code: 1 = $\beta$-Lg, control; 2 = genistein–$\beta$-Lg derivative; 3 = daidzein–$\beta$-Lg derivative; 4 = 3′,4′,7 THI–$\beta$-Lg derivative; 5 = 4′,6,7 THI–$\beta$-Lg derivative.

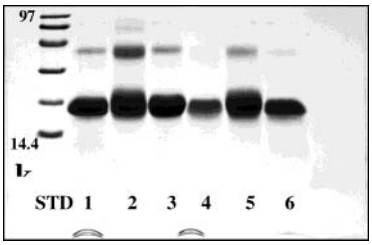


**Figure 8.** Solubility behavior/SDS-PAGE of the $\beta$-Lg derivatives precipitated at pH 4.5. Code: 1 = $\beta$-Lg, control (Et/Ac); 2 = 3′,4′,7 THI–$\beta$-Lg derivative (Et/Ac); 3 = 4′,6,7 THI–$\beta$-Lg derivative (Et/Ac); 4 = $\beta$-Lg, control (Et); 5 = 3′,4′,7 THI–$\beta$-Lg derivative (Et); 6 = 4′,6,7 THI–$\beta$-Lg derivative (Et); STD = standard proteins.

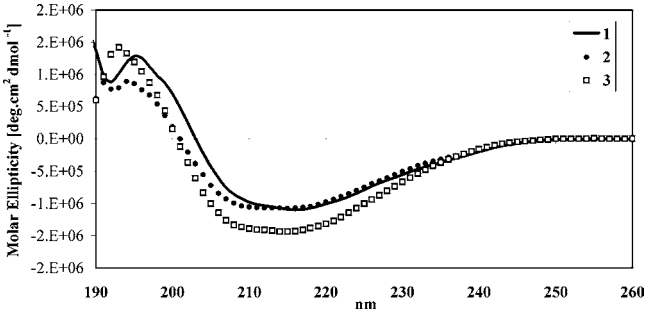


**Figure 9.** Far-UV-CD spectra of $\beta$-Lg–influence of the solvent applied to solubilize the isoflavones. Code: 1 = $\beta$-Lg, starting material; 2 = $\beta$-Lg, control (Et); 3 = $\beta$-Lg, control (Et/Ac).

mented in **Figures 9**−**12**. The influence of the solvent system used to solubilize the isoflavones during the derivatization is recognizable in **Figure 9**. The Et/Ac mixture induces a stronger alteration of the CD-spectra, which, when analyzed for the distribution of the secondary structure elements results in an increase of the α-Helix content (**Table 2**). In contrast, Et causes only a slight change in the composition of the secondary structure elements (**Table 2**) and the corresponding CD-spectra

**Table 2.** Assignment [%] of the Secondary Structures to $\beta$-Lactoglobulin ($\beta$-Lg) Derivatives According to Sreerama and Woody (*18*) by Applying 43/48-protein Reference Set (190–240 nm) and CONTIN Method

| sample[a] | α-helix | $\beta$-strand | $\beta$-turn | random coil |
|---|---|---|---|---|
| 1. $\beta$-Lg, starting material | 14.8 | 35.4 | 21.3 | 28.6 |
| 2. $\beta$-Lg, control (Et/Ac) | 20.8 | 28.4 | 23.2 | 27.6 |
| 3. $\beta$-Lg + genistein (Et/Ac) | 21.8 | 28.9 | 22.2 | 27.1 |
| 4. $\beta$-Lg + daidzein (Et/Ac) | 18.6 | 30.8 | 21.4 | 29.2 |
| 5. $\beta$-Lg + 3′,4′,7 THI (Et/Ac) | 9.1 | 28.1 | 18.7 | 44.1 |
| 6. $\beta$-Lg + 4′,6,7 THI (Et/Ac) | 14.8 | 29.7 | 20.6 | 35.0 |
| 7. $\beta$-Lg, control (Et) | 15.4 | 32.1 | 22.3 | 30.1 |
| 8. $\beta$-Lg + 3′,4′,7 THI (Et) | 12.2 | 28.9 | 20.7 | 38.2 |
| 9. $\beta$-Lg + 4′,6,7 THI (Et) | 13.6 | 30.0 | 20.4 | 35.9 |

[a] The derivatization was performed with the isoflavones solubilized in ethanol/acetone (Et/Ac, 1:1) or in Ethanol (Et).

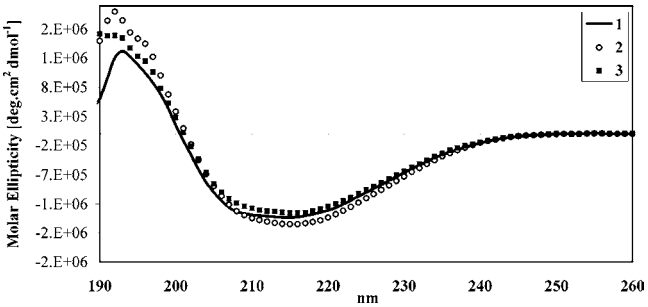


**Figure 10.** Far-UV-CD spectra of $\beta$-Lg-derivatives (generated in the presence Et/Ac). Code: 1 = $\beta$-Lg, control; 2 = genistein–$\beta$-Lg derivative; 3 = daidzein–$\beta$-Lg derivative.

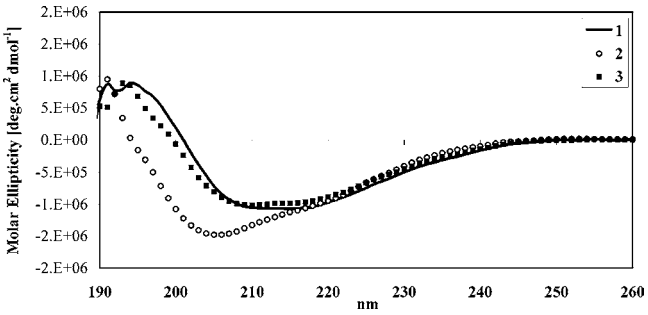


**Figure 11.** Far-UV-CD spectra of $\beta$-Lg-derivatives (generated in the presence of Et). Code: 1 = $\beta$-Lg, control (Et); 2 = 3′,4′,7 THI–$\beta$-Lg derivative (Et); 3 = 4′,6,7 THI–$\beta$-Lg derivative (Et).

(**Figure 9**). This would mean that the presence of Et/Ac during derivatization would cause a stronger structural change of the involved protein, thereby facilitating a better access for the reacting isoflavones as documented above for 3′,4′,7 THI. Due to these observed changes in the secondary structure, the original protein material was compared only with the controls and the latter with the corresponding derivatives. In this respect, the derivatization experiment with genistein and daidzein produced no significant change in the CD-spectra (**Figure 10**) and in the distribution of the secondary structure elements (**Table 2**). These data agree with the corresponding little or no effect achieved by the other analysis described above. With regard to the reactivity of 3′,4′,7 THI, and 4′,6,7 THI, both cause a significant change in the structure (**Figures 11** and **12** and **Table 2**), the reactivity being higher for the derivatives generated in the presence of Et/Ac. In all cases, there is a loss of α-Helix components accompanied by an increase in random coil elements, as documented in **Table 2**. The 3′,4′,7 THI derivatives show the strongest influence on the secondary structure (**Figures**

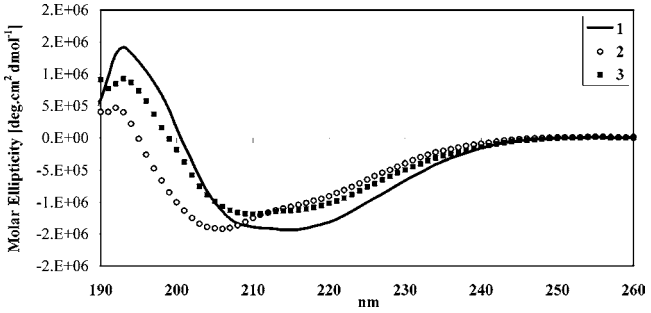


**Figure 12.** Far-UV-CD spectra of $\beta$-Lg derivatives (generated in the presence Et/Ac). Code: 1 = $\beta$-Lg, control (Et/Ac); 2 = 3′,4′,7 THI–$\beta$-Lg derivative (Et/Ac); 3 = 4′,6,7 THI–$\beta$-Lg derivative (Et/Ac).

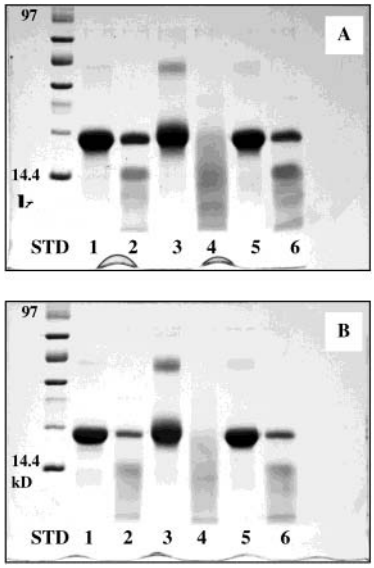


**Figure 13.** Tryptic digestion of $\beta$-Lg derivatives (E/S = 1:60, SDS-PAGE analysis). Code: A = derivatives generated in the presence of Et; B = derivatives generated in the presence of Et/Ac; 1–2 = $\beta$-Lg, control, 0 and 5 min digestion; 3–4 = 3′,4′,7 THI–$\beta$-Lg derivative, 0 and 5 min digestion; 4–6 = 4′,6,7 THI–$\beta$-Lg derivative, 0 and 5 min digestion; STD = standard proteins.

**11** and **12** and **Table 2**) in agreement with the other results described above. The slight but significant reactivity of 4′,6,7 THI is also in accord with the results documented in **Table 1** and **Figures 3** and **4**.

A change in the structure accompanying the derivatization of proteins as observed above may produce a change in the digestion properties. This was tested by in-vitro digestion of the 3′,4′,7 THI and 4′,6,7 THI derivatives and the corresponding controls, as documented in **Figure 13**. Two results can be filtered from these data: First, the comparison of the Gel A and B shows the influence of the solvents used for derivatization. The digestion of the preparations generated in the presence of Et/Ac (gel B, **Figure 13**) can be digested much more easily than those produced by using Et. This influence becomes more clear under consideration of the time dependent digestion of the corresponding controls, as documented in **Figure 14**. This faster digestion of the control prepared using Et/Ac would mean a significant change in the structural properties, as confirmed by the CD studies discussed above. The second interesting result is the similar behavior of 4′,6,7 THI derivatives to their corresponding controls and the complete disappearance of the $\beta$-Lg band (and its dimer) in the 3′,4′,7 THI sample after 5-min digestion. This again is in accordance with the strong denaturation noted for the 3′,4′,7 THI derivatives. Trypsin splits

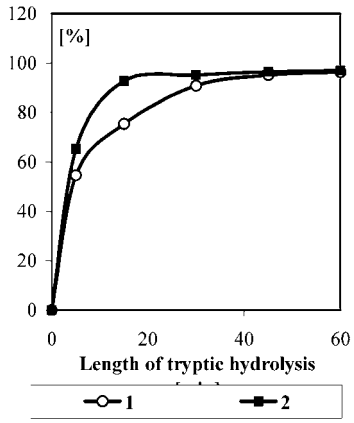


**Figure 14.** Time dependent tryptic digestion of $\beta$-Lg controls: influence of the solvent applied to solubilize the isoflavones. Code: 1 = $\beta$-Lg, control (Et); 2 = $\beta$-Lg, control (Et/Ac).

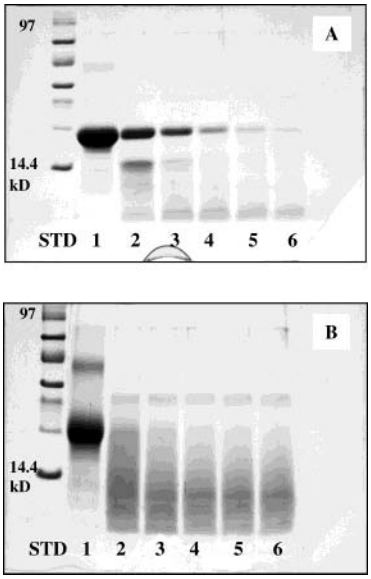


**Figure 15.** Time dependent tryptic digestion of $\beta$-Lg derivative (E/S = 1:60, SDS-PAGE analysis). Code: A = $\beta$-Lg, control (Et); B = 3′,4′,7 THI–$\beta$-Lg derivative (Et); 1–6 = are corresponding digestions after 0, 5, 15, 30, 45, and 60 min; STD = standard proteins.

preferentially those peptide linkages, which contain either lysine or arginine as amino side chains. Since it has already been shown, that 3′,4′,7 THI reacts with $\epsilon$-amino groups of lysine side chains (**Table 1**), we can conclude that their derivatization by isoflavone prevents or at least makes tryptic degradation difficult. This is not the case here, as shown by the results documented in **Figure 13**. So, a further experiment was started to monitor the time dependent tryptic hydrolysis of 3′,4′,7 THI derivative and the corresponding control. The intention was to find if at some stage the hydrolysis by trypsin is slowed or stopped by the derivatization. This indeed seems the case, as can be seen from **Figure 15**. After the breakdown to products around 14 kDa (and below) the further digestion of these peptides of 3′,4′,7 THI derivative seems to slow (**Figure 15**, gel B). This means that the further digestion may be prevented/slowed by the derivatized lysine side chains of the protein.

In summary, this model study shows that genistein, daidzein, formononetin, prunetin, and biochanin A may react with proteins; the estimation of the reactivity is limited to indirect methods as documented by **Table 1**. These results indicate that

there is no clear trend, and the reaction, although to a small extent (5−24% decrease of tryptophan), is significant and seems to depend on both the protein as well as on the isoflavone applied. With regard to 4′,6,7 THI, the reaction is more intensive with accompanying structural changes and dimerization. The latter have no influence on the characteristic protein properties such as solubility, chromatographic and isoelectric behavior, and in-vitro tryptic digestion. In case of the 3′,4′,7 THI derivative, a distinct addition of the isoflavone can be shown with corresponding changes in the different protein properties analyzed.

The results show that the highest reactivity can be attained with *o*-dihydroxyphenols, with the catechol group being located on the Ring B. The reason being in their capability of forming quinone and in the ability of the ring B to rotate freely. Distinct differences in reactivity between quercetin and 3′,4′,7 THI (hydroxyl groups in positions 3′ and 4′) were not found. The presence of the C3−OH group in quercetin appears to have no observable influence on the reactivity. Although the C3−OH group is postulated to be required for efficient quinone methide formation and the subsequent adduct formation in the A ring instead of in the B ring (*20*). In case the catechol moiety is located in ring A (4′,6,7 THI), the reactivity decreases when compared to those compounds with the *o*-dihydroxyl groups on ring B (3′,4′,7 THI and quercetin). The 4′,6,7, THI can also form quinone intermediates and the observed decrease in reactivity may be due to the absence of the possibility of the ring A to rotate freely. The other isoflavones tested (genistein, daidzein, formononetin, prunetin, and biochanin) are in turn, depending on the protein applied, further less reactive or in some cases do not react at all. These compounds are not in a position to be oxidized to quinones. They have either only one OH group on the ring A and/or B, or the dihydroxyl groups are located in *m*-position (genistein, biochanin A). The observed low reactivity may be explained by a reaction via a semiquinone intermediate formation as proposed for covalent binding of *m*-dihydroxybenzene (resorcinol) and ferulic acid to proteins (*14*). In presence of polyphenol oxidases, there is a further possibility of the conversion of the mono hydroxyl group on ring A and/or ring B to *o*-dihydroxyphenol. These can then react according to the same mechanism as described above for quercetin, 3′,4′,7 THI and 4′,6,7 THI.

The results further indicate that methods such as LC/MS coupled with specific proteolytic analysis and defragmentation studies (MS/MS) as well as NMR studies need to be established to characterize this reactions better. In this context, it has been recently reported that such methods are very useful in quantification of *o*-quinone metabolites of quercetin generated in the presence of tyrosinase and their subsequent scavenging by glutathione (*6*, *7*, *20*−*22*). In the presence of a suitable para substituent, *o*-quinones can isomerize to give *p*-quinone methides known to be even more reactive electrophiles (Supporting Information). According to the authors, for quercetin *o*-quinone and *p*-quinone methide, adduct formation at C2′, C5′, C6′, C6, and C8 is possible (*6*, *20*). Additional experiments of this study reveal the adduct formation (especially at C6 and C8) to be reversible, leading to interconversion between the quercetin glutathione adducts and possibilities for release and further electrophilic reactions of the quercetin quinone methide at cellular sites different from those of its generation (*6*).

The intention of this study was to show if principally covalent reactions between isoflavones and proteins in model studies are possible or not. With regard to the choice of the pH, we would like to point out, for example, that the pH of egg white amounts to 9 or higher; further, there are other food systems such as tortilla doughs that do use alkaline conditions. Further, the extraction of soy proteins is generally conducted at pH 8, where such reactions are likely (*15*). Our review (*8*) considers also the reaction of the phenolic compounds with proteins at different pH conditions 5−9 (Supporting Information). Covalent reactions as described here also took place and were identified, although at a lower rate. Therefore, the question of the relevance of such reactions of isoflavones remains interesting. Physiological interesting data from literature in this context should also be mentioned and discussed. More disturbing, however, is the plethora of dietary supplements of isoflavones that have flooded the market, with wide ranging claims and little regulation regarding their manufacture or efficacy. There is a paucity of information regarding the bioavailability, metabolism, and clinical effectiveness of such dietary isoflavone supplements (*25*). On the other hand, studies of soy isoflavones in experimental animals suggest possible adverse effects as well (e.g., enhancement of reproductive organ cancer, modulation of endocrine function, anti-thyroid effects) (*3*, *26*). Further, there is a very large variability in total isoflavone concentration of one of the key ingredients used by the food industry to manufacture many soy foods (*27*). These key ingredients are often highly purified proteins extracted from soy beans (*27*). Soy proteins isolated by isoelectric precipitation and the acid wash process do retain a large proportion of the isoflavones in association with the protein (*27*). Isolates prepared by an ethanol wash process generally do not carry significant amounts of isoflavones unless they are fortified with isoflavone extracts (*27*). These data suggest a possible interaction of proteins and isoflavones, although the larger part of these interactions may be governed by noncovalent binding. Also interesting is the fact that 4′,6,7 THI has been isolated from fermented soy products (tempeh) and termed generally as "György" isoflavone (*28*), which in turn was found by us to be quite reactive (**Table 1**). Further indication of protein/isoflavone interactions are given by studies that showed dose-dependent and significant inactivation of rat and human thyroid peroxidase by soy isoflavones (*26*, *29*). Recent studies demonstrate that exposure to genistein during gestation and lactation demasculinizes the reproductive system in rats, whereby genistein is shown to be capable of inhibiting Aromatase und 5α-reductase, both representing important enzyme systems (*30*). Further of interest, genistein is well-known to be genotoxic due to its ability to "poison" cellular DNA topoisomerase II, resulting in stable chromosome breakage and mutation and raising questions about the long term health effects associated with chronic flavonoid exposure. Interestingly, some isoflavones, such as biochanin and daidzein, are catalytic topoisomerase II inhibitors (not poisons) and actually antagonize the clastogenicity of topoisomerase II poisons (*31*). These examples indicate that many of the positive or negative biological effects of isoflavones or their metabolized forms (e.g., equol, 4′,7-dihydroxyisoflavane) may indeed have indirect or direct connection with their ability to interact with proteins in the form of hormone, enzymes of receptors. Detailed studies on such interactions would help give better insight and understanding of the observed biological effects.

## ABBREVIATIONS USED

β-Lg, β-lactoglobulin; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; E/S, enzyme/substrate ratio; Et, ethanol; Et/Ac, ethanol/acetone (1:1, v/v); IEF, isoelectric focusing; OD, optical density; RP-HPLC, reversed-phase high-performance liquid chromatography; SELDI-TOF-MS, surface

enhanced laser desorption/ionization time-of-flight mass spectrometry; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SG, soy glycinin; TCA, trichloracetic acid; THI, trihydroxyisoflavone; TNBS, trinitrobenzenesulfonic acid; Tris, Tris-(hydroxymethyl) aminomethane; UV, ultraviolet absorption; WP, whey protein isolate.

**Supporting Information Available:** Reaction mechanisms for phenolic compounds and references thereof. This material is available free of charge via the Internet at http://pubs.acs.org.

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