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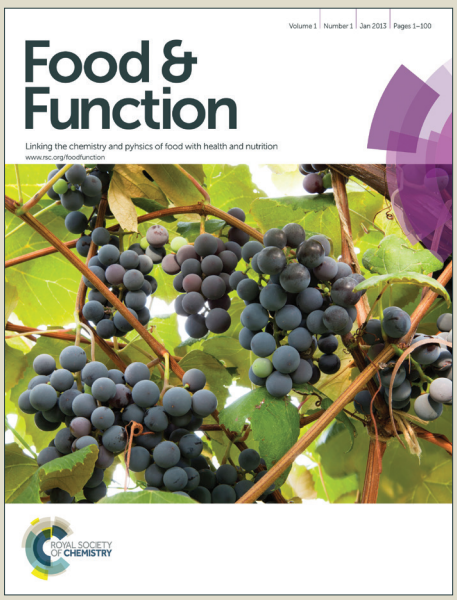

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GLYCATION IS REGULATED BY ISOFLAVONES

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Abstract

The effect of soy isoflavones on the Maillard reaction (MR) was investigated. Model systems composed of the soy protein glycinin (10 mg mL^{-1}) and fructose (40 mg mL^{-1}) under basic pH (~12) conditions were employed for testing the anti-glycative effect of the major antioxidant soy isoflavones (genistin and genistein at 10 μg mL^{-1}) and a soy isoflavone-rich extract. The contents of total phenols (TPC) and total flavonoids (TFC) of the isoflavone-rich extract were determined. Glycinin was pre-incubated with isoflavones for 1h and 16h at 60ºC prior to MR. The progress of MR was estimated by analysis of free amino groups by OPA assay; carbohydrate covalently bound to protein backbone using phenol-sulfuric acid assay, protein-bound N^{ε}-(carboxymethyl)lysine (CML) by UPLC-MS and spectral analysis of fluorescent protein-bound AGEs. Genistin (10 μg mL^{-1}, 23 μM) and its aglycone genistein (10 μg mL^{-1}, 37 μM) did not prevent protein glycation (p>0.05). The soy isoflavone-rich extract containing 2.5 mg mL-1 of TFC efficiently decreased bound of carbohydrate to the protein skeleton (20%) (p<0.05) and formation of advanced glycation end products (AGEs) (> 80%) (p<0.05). The anti-glycative mechanism of isoflavones may be related to its conjugation to glycation sites of the protein structure (free amino groups), their antioxidant character and trapping of dicarbonyl intermediates. Extracts based on mixtures of isoflavones may be useful for producing glycated conjugates avoiding the substantial formation of AGEs bound to protein.

1. Introduction

Glycation, also known as Maillard reaction (MR), is a non-enzymatic reaction between carbonyl groups of reducing sugar and free amino groups of proteins. Non-oxidative and/or oxidative modifications of the early MR products (MRPs) (Amadori and Heyns

products) result in the formation of a variety of advanced glycation endproducts (AGEs). AGEs can be fluorescent crosslinked structures such as pentosidine, or non-fluorescent structures such as N^{ε}-(carboxymethyl)lysine (CML)[1]. AGEs are formed in foods and in the human body and they are considered to be undesirable compounds[2]. The search for natural inhibitors of AGEs formation is of great interest and a priority research line[3-4]. Compounds with antioxidant properties may be powerful inhibitors of the formation of AGEs.

Soy isoflavones are phytochemicals that exist in two chemical forms, glucosidic conjugates (daidzin, genistin, and glycitin), and the unconjugated forms, or aglycones (daidzein, genistein, and glycitein)[5]. Isoflavones are powerful natural antioxidants[6]. In a previous study, the relationship between the effect on AGEs formation and radical scavenging activity of 62 flavonoids, including 7 isoflavones (daidzein, daidzin, genistein, genistin, tectoridin, puerarin and biochanin), was examined[7]. AGEs formation was assessed solely by measuring characteristic fluorescence at an excitation wavelength of 370 nm and an emission wavelength of 440 nm. Soy isoflavones (at 200 μM) inhibited formation of fluorescent AGEs by 12-34% (genistin, daidzein, genistein) or lacked the effect (daidzin). To the best of our knowledge no previous studies regarding the inhibitory effect of soy isoflavones on the formation of non-fluorescent AGEs, such as CML, have been published.

Formation of MRPs derived from the interaction of soy proteins and fructose may be inhibited by isoflavones. As a consequence, isoflavones may be used to form particular glycoconjugates with specific and improved functional properties. Other phytochemicals, such as ferulic acid, have been successfully used to achieve this aim[8].

The present work aimed to gain new knowledge on the anti-glycation capacity of soy isoflavones (pure and soy isoflavone-rich extract) and their potential for the

formation of novel glycoconjugates avoiding tedious and high cost purification processes for removing contaminants (AGEs and unreactive fructose and proteins). Fructose is commonly used as a sweetener in processed foods and beverages, such as soy milk[9-10]. As a consequence, fructose was selected for glycation of soy glycinin. Nowadays, soy based foods are very popular because their health promoting properties, which are mainly associated with the proteins[11] and isoflavones[12]. Therefore, their presence as contaminants in glycoconjugates preparations is not considered a disadvantage. On the other hand, the glycation process is considered an effective method to reduce soy protein allergenicity, because it can be expected that sugars will cover the epitopes of the allergens and reduces soy protein immunoreactivity[13]. The glyconjugates formed under the conditions described in the present manuscript may be hypoallergenic, free of AGEs and with potential to be used as functional food ingredient. The effect of soy isoflavones on the formation of early MRPs and AGEs, both fluorescent and non-fluorescent (CML), in reaction mixtures composed by soy proteins and fructose under basic conditions was investigated. A reaction medium with alkaline pH (0.2% KOH) was used to enhance the solubility of soy proteins, to catalyse the MR and to promote the interaction between soy isoflavones and glycinin.

2. Materials and methods

2.1 Materials

All chemicals and solvents were of analytical grade. Fructose, genistin, genistein, lysine, N^{α}-acetyl-L-lysine, nonafluoropentanoic acid (NFPA), phenol, sodium bisulfite, sodium borohydride, sodium dodecyl sulfate (SDS), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Biuret reagent, Folin-Ciocalteu's phenol reagent, hydrochloric acid 37% (HCl), methanol, sodium hydroxide (NaOH),

sulfuric acid 96%, potassium hydroxide (KOH), and trichloroacetic acid (TCA) were purchased from Panreac Química S.A. (Barcelona, Spain). Other chemicals and their suppliers were as follows: β-mercaptoethanol (Merck, Hohenbrunn, Germany), acetonitrile HPLC grade (ACN) (Chromanorm, Leuven, Belgium), CML (NeoMPS, Strasbourg, France), d_4-lysine (Cambridge Isotopes, Andover, MA, USA), *o*-phthaldialdehyde (OPA) (Fluka, Buchs, Switzerland), and sodium tetraborate (Acros-Organics, Geel, Belgium). d_4-CML was kindly provided by Professor S.R. Thorpe (Department of Chemistry and Biochemistry, University of South Carolina, SC, USA). Microtest 96-well plates were purchased from Sarstedt AG & Co. (Nümbrecht, Germany). The Amicon® Ultra-4 centrifugal filter unit fitted with an Ultracel-50 cellulose membrane (50 kDa cut-off) were from Millipore Co. (Billerica, MA, USA). Soybean flour (from organically grown; 35% protein, 3.1% carbohydrates, and 18% fat macronutrient composition is provided by manufacturer El Granero, Spain) and commercial isoflavone preparation (capsules active of ethanolic extract 117.85 mg 60% v/v dry hypocotyls *Glycine max* (L) Merr. (Soybean) containing 30% of total isoflavones) were purchased from a local supermarket in Spain (Phyto Soya Forte, Arkopharma, Spain).

2.2 Isolation of soy glycinin protein

Soy glycinin was extracted from defatted soy flour as described by Wu *et al*[14]. Briefly, defatted soybean flour (150 mg) was resuspended in deionized water, 1:15 (w/v), the pH was adjusted to 7.5 with 2 M NaOH, and the resulting slurry was mechanically stirred for 1 hour at room temperature. After centrifugation at 14,250*g* for 30 min at 20ºC, the insoluble residue was discarded. Sodium bisulfite was added to the supernatant to achieve a final concentration of 10 mM of SO_2 (0.98 g L^{-1}). Glycinin was precipitated by decreasing the pH to 6.4 with 2 M HCl and incubating at 4ºC overnight. Then the

protein solution was centrifuged at 7,500*g* for 20 min at 4ºC. The precipitated glycinin was resuspended in deionized water, freeze-dried and stored at -20ºC until use. Protein content was determined by Biuret assay and the purity of the glycinin fraction was checked by SDS-PAGE electrophoresis (data not shown).

2.3 Preparation of soy isoflavone-rich extract

The commercial preparation containing a mixture of daidzein, glycitein and genistein (600 mg) was dissolved in 10 mL of reaction medium (0.2% KOH solution) and stirred for 1 hour at room temperature. The preparation was centrifuged at 4,000*g* for 15 min and the supernatant (soluble fraction) was collected. Finally, the concentration of soluble phenolic compounds was estimated by the Folin-Ciocalteu micromethod assay[15] using genistein (0.5-5 mg mL^{-1}) for obtaining the calibration curve. The analysis of total flavonoid content (TFC) of the soy isoflavone-rich extract was determined according to López-Vargas *et al* (2013)[16]. Briefly, 1 mL of sample was mixed with 0.3 mL $NaNO_2$ (5%) and 0.3 mL $AlCl_3$ (10%) were added after 5 min. The samples were mixed in a vortex for 2 min., incubated at room temperature for 6 min. and added with 2 mL NaOH (1M). The absorbance was read at 510 nm. A calibration curve of genistein (0.05-5 mM) was used for quantification of TFC. The results were expressed as mg genistein equivalent/g of product. All measurements were performed in triplicate. Concentrations of compounds able to react with Folin-Ciocalteu of 5 mg mL-1 and flavonoids of 2.5 mg mL-1 were detected in the isoflavone-rich extract.

2.4 Model system preparation

To prepare the glycated model systems, soy glycinin (10 mg mL^{-1}) was mixed with genistin (10 µg mL^{-1}), genistein (10 µg mL^{-1}) or soy isoflavone-rich extract (2.5 mg mL^{-1}) in 0.2% KOH solution (pH 12) and pre-incubated for 1 h or 16 h at 60°C. Fructose (40 mg mL^{-1}) was then added and incubation continued for a further hour. Glycation

was stopped by cooling the samples on ice. The pH of the model glycation systems at the beginning and end of the reaction was 12. Some samples were also prepared in the absence of fructose. All samples were prepared in triplicate.

2.5 Sample fractionation

Incubated samples were fractionated by ultrafiltration in order to recover unmodified and glycated proteins for further analysis. Briefly, sample (2 mL) was placed in the sample reservoir of an Amicon® Ultra-4 centrifugal filter unit fitted with an Ultracel-50 cellulose membrane (50 kDa cut-off) and centrifuged at 7,500*g* for 20 min at room temperature. The filters were washed with distilled water (4 mL). The concentrated samples were recovered, dissolved in water (2 mL) and stored at -20ºC until analysis. Recovery of protein (RMM > 50 kDa) was determined by the Biuret method in microplate format. Free amino groups, incorporation of sugar into the protein backbone, CML, and fluorescecent AGEs were determined as described below.

2.6 Determination of free amino groups: available lysine

Free amino groups of samples were determined by the *o*-phthalaldehyde (OPA) method[17]. OPA reagent was prepared fresh before use by mixing 0.1 M sodium tetraborate (pH 9.5, 50 mL), 20% (w/v) SDS (5 mL), β-mercaptoethanol (0.2 mL) and OPA (80 mg dissolved in 2 mL of methanol), and adjusting the final volume to 100 mL with distilled water. An aliquot of protein solution containing 25 μg protein was mixed with OPA reagent (3 mL). After incubation for 5 min at room temperature, the fluorescence was read against a blank containing the OPA reagent using a Shimadzu spectrofluorometer RF-1501 (Shimadzu Co., Kyoto, Japan). The wavelength of maximum excitation was 340 nm and the wavelength of maximum emission was 455 nm. Calibration curves were constructed using standard solutions of N^{α}-acetyl-L-lysine (10-1000 μM). All measurements were performed in triplicate and data were expressed

as a % of free amino groups. Untreated glycinin (control) was assumed to have 100% amino groups available.

2.7 Estimation of carbohydrate covalently bound to the protein backbone

Carbohydrate bound to the protein backbone was analysed by the phenol-sulfuric acid method in microplate format[18]. An appropriate dilution of sample (100 µL) was pipetted into a glass vial, to which was added concentrated sulfuric acid (300 µL) and 5% phenol in water (60 µL). After incubating at 90ºC for 5 min, samples were cooled to room temperature for 5 min. Finally, 200 µL aliquots were placed in the wells of a 96-well microplate and the absorbance was measured at 490 nm by employing a microplate reader BioTek PowerWave™ XS (BioTek Instruments, Inc., Winooski, VT, USA). A calibration curve of fructose (5-40 µg per well) was constructed and employed for quantification. Data were expressed as µg of fructose/100 mg of protein. All measurements were performed in triplicate.

2.8 CML analysis

Protein-bound CML was determined by ultra-performance liquid chromatography (UPLC)-MS/MS according to the procedure recently described by Assar *et al*[19]. Prior to analysis, samples were reduced with sodium borohydride, protein was isolated by TCA precipitation and hydrolysed with 6M HCl. The protein hydrolyzate was purified by solid phase extraction prior to CML analysis by UPLC-MS/MS. Samples were analysed in triplicate.

2.9 Fluorescence measurement

Fluorescent protein-bound AGEs were measured as previously reported by Wang *et al*.[20] by measuring the fluorescence intensity of samples using an excitation wavelength of 337 nm and emission wavelengths ranging from 350 nm to 550 nm with a Shimadzu spectrofluorometer RF-1501 (Shimadzu Co., Kyoto, Japan). Glycinin samples at a

concentration of 10 mg mL^{-1} in distilled water were positioned in a cuvette of 1 cm path length. All emission spectra were recorded at 0.5 nm wavelengths intervals.

2.10 Statistical analysis

Statistical analyses were performed using the SPSS program (SPSS 21.0 for Windows; SPSS Inc., Chicago, IL, USA) to look at the influence of the addition of isoflavones in the progress of the glycation reaction (decrease of sugar and amino acids and formation of fluorescent AGEs and CML). Data were expressed as the mean value ± SD of triplicates from three independent experiments. Analysis of variance (ANOVA) and the Duncan test were applied to determine differences between mean values obtained for model systems with different composition. Differences were considered to be significant at $p < 0.05$.

3. Results

The loss of available primary amino groups is an indicator used to estimate the extent of the MR[21]. Fig. 1 shows amino groups availability obtained by OPA assay. The percentage of available amino groups in all samples was determined as the relative difference between the percentage of reactive amino groups in the unheated soy glycinin protein sample and that in the glycated models. Heating of soy glycinin protein (heated control) did not significantly ($p > 0.05$) affect the availability of free amino groups. A significant decrease ($p < 0.05$) in free amino groups availability of soy glycinin protein was observed by heating in presence of fructose (glycation model system) suggesting the occurrence of the MR. Values of availability of free amino groups in the samples containing the soy isoflavone-rich extract and glycation model system did not significantly differ ($p > 0.05$). Interestingly, the addition of pure genistin and genistein

together with fructose caused a significant decrease in free amino groups' availability ($p < 0.05$).

Monitoring sugar conjugation to protein allows evaluation of the degree of formation of the earliest MRPs[22]. Sugar conjugation was detected by the phenol-sulfuric acid method. As shown in Fig. 2, fructose was successfully bound to soy glycinin protein (glycation model system) indicating the formation of early MRPs (Heyns compounds) ($p < 0.05$). As expected, controls (unheated soy glycinin and protein heated in absence of fructose) proved that reactivity could not be attributed to possible release of carbonyl functions from the protein. Similar levels of early MRPs were formed in the inhibition model systems containing genistin and genistein and the glycation model system (absence of isoflavones). The content of protein-bound early MRPs was significantly ($p < 0.05$) lower (20%) in samples treated with isoflavone extract compared to that found in the sample corresponding to positive control of the Maillard reaction (glycation model).

CML, a non-fluorescent AGE, is an important specific biomarker that increases during the MR and correlates with the severity of the reaction. We evaluated whether isoflavones could inhibit CML formation during soy glycinin glycation by fructose. Fig. 3 shows the effect of genistin, genistein and soy isoflavone-rich extract on protein-bound CML formation. CML was readily formed under the experimental control conditions (soy glycinin/fructose) and in the presence of genistin and genistein, but its generation was significantly inhibited ($p < 0.05$) by 87% and 92% in glycation models treated with the soy isoflavone-rich extract for 1 and 16 hours, respectively. The level of CML inhibition was greater than that observed for the formation of early MRPs (20%). This is a novel result.

Fig. 4 illustrates the effect of genistin, genistein and soy isoflavone-rich extract on the formation of fluorescent AGEs. The fluorescence spectrum of glycinin treated with carbohydrate and isoflavones showed modifications with respect to the control and heated glycinin spectrum. Glycinin reaction with fructose caused formation of fluorescent compounds with emission maxima between 420-425 nm and intensity of 70.1 arbitrary units of fluorescence. The addition of genistin and genistein in the reaction mixtures did not inhibit the formation of fluorescence compounds, identical spectra to that obtained for the glycation control being observed. These results support the lack of inhibitory activity in the formation of AGEs at naturally occurring concentrations of these compounds in soy proteins. The addition of the soy isoflavone-rich extract inhibited fluorescence formation. The resulting emission spectrum showed a maximum at 470 nm with an intensity of 15.8 arbitrary units of fluorescence. This intensity value was similar to that detected for the unheated and heated protein; however, the shape of the spectra of these samples differed.

4. Discussion

Results on availability of free amino groups (Fig. 1) and level of sugar bound to the proteins (Fig. 2) suggest that the MR is the major chemical reaction involving free amino groups of the protein polypeptide chain in the model systems**.** Although other reactions, e.g., cross-linking of proteins, may also decrease the level of free amino groups, no evidence was obtained for such chemical events being important under our experimental conditions. Pure isoflavones (genistin and genistein) at the concentrations tested in the present research did not significantly inhibit the progress of the Maillard reaction (Fig. 2-4). However, the soy isoflavone-rich extract (composed of a mixture of soy isoflavones) significantly decreased the formation of early MR products (Fig. 2) and

also the progress of the reaction to the advanced stage. Thus, the formation of both non-fluorescent (CML) (Fig. 3) and fluorescent AGEs (Fig. 4) was significantly inhibited.

In the present study, the availability of free amino groups on soy glycinin protein was reduced in all glycation models at 60°C (Fig. 1). This suggests that amino groups on soy glycinin were progressively bound to the carbonyl moiety of fructose and/or isoflavones. In addition, lower levels (20%) of sugar bound to the protein were found in samples treated with the isoflavone enriched extract compared to the glycation control or samples containing the pure isoflavones. These data suggest that isoflavones are able to decrease the reactivity of amino groups on the soy glycinin with fructose molecules in the reaction mixture during the early steps of the MR. In addition, our findings seem to indicate the possible reaction of phenolic compounds with soy glycinin and consequent formation of glycinin-isoflavone complexes. Although the mechanisms of inhibition by polyphenolic compounds of glycation are not completely understood, it is possible that some of these natural compounds bind to protein inhibiting Amadori product generation and subsequent AGEs formation[23]. Further analysis employing advanced analytical tools for the identification of the novel structures is needed to confirm this hypothesis.

Several *in vivo* and *in vitro* studies have indicated that dietary phenolic compounds could inhibit the formation of AGEs[7,8,24]. The inhibitory effects of flavonoid compounds on AGEs formation are mainly thought to involve their potent antioxidant activity, leading to scavenging of free radicals formed during glycation, and thus inhibiting the subsequent formation of AGEs. However, limited effort has been devoted to understanding the underlying mechanisms of action of effective natural AGE inhibitors. To inhibit AGEs formation, anti-AGE agents, such as flavonoids, may act through different mechanisms.

Reactive dicarbonyl intermediates, such as MGO, play an important role in the chemistry of AGEs formation. Wu *et al.*[25] found that protein co-incubated with genistein and MGO could inhibit MGO-induced reactive oxygen species. Lv *et al.*[26] have hypothesized that dietary flavonoids such as genistein can inhibit the formation of AGEs by trapping reactive dicarbonyl intermediates under neutral and alkaline conditions *in vitro*. Dicarbonyl intermediates may be produced by degradation of fructose. Our data for sugar bound to protein also suggest a major formation of early Maillard reaction products in all samples containing fructose (Fig. 2). In agreement, the trapping of dicarbonyl intermediate seems to be not the only anti-AGEs pathway by which the isoflavones enriched extract operates.

It has been proposed that no oxidation reaction is involved in the formation of Amadori or Heyns rearrangement products, whereas oxidation plays a role in the formation of AGEs. Flavonoids with antioxidant properties, such isoflavones, may protect against glycation-derived free-radical-mediated oxidation by acting as transition metal ion chelators, and preventing the self-oxidation of reducing sugars, Amadori products and reactive carbonyl species[27]. It has been reported by Jang *et al.*[28] that daidzein and genistein isoflavones, obtained from *P. lobata* root extracts, possess significant inhibitory activity against fluorescent AGE formation with IC_{50} values of 12.0 μg mL^{-1} and 70.1 μg mL^{-1}, respectively. We found no anti-AGEs activity with genistin and genistein at 10 μg mL^{-1} concentration; however, the soy isoflavone-rich extract was an effective anti-glycation agent (2.5 mg mL^{-1}).

The isoflavone enriched extract greatly inhibited the formation of non-fluorescent (Fig. 3) and fluorescent (Fig. 4) AGEs. The characteristic fluorescence spectrum of AGEs changed in samples containing the isoflavone extract. This is attributed to the absorption of the AGE glycophore, formed by the linking of protein

and glucose molecules, in accordance with data obtained by Rondeau *et al.*[29] These results agree with those obtained by Wang *et al.*[20] using ferulic acid and feruloyl-oligosaccharides as glycation inhibitors. They obtained a progressive decrease in fluorescence with increasing concentration of inhibitor.

The formation of CML and fluorescent AGEs like pentosidine is catalysed under oxidative conditions. In agreement with this, our data seem to indicate that although part of the AGEs formed in our particular system can come from oxidative sugar degradation (CML), they are also being generated from early MR products (Heyns rearrangement products).

The primary structure of isoflavones is three benzene rings with one or more hydroxyl groups; this structure is the key factor that determines their anti-oxidant activity. Matsuda *et al.*[7] examined several flavonoids for inhibitory activity towards AGE formation. Compared to the well-known AGE inhibitor, aminoguanidine, flavonoids showed stronger inhibitory effects. Nevertheless, isoflavones only weakly inhibited AGEs formation (by 25-46% at 200 μM). In the current study, the isoflavones enriched extract showed strong inhibitory activity, therefore, the antioxidative effects of isoflavones are apparently, at least in part, involved in AGEs inhibition mechanisms.

In the present *in vitro* study, we demonstrate that a mixture of isoflavones (soy isoflavone-rich extract) is an effective inhibitor of the formation of early MR products and AGEs. Our data suggest that the formation of early MR products may be inhibited by conjugation of isoflavones to the active site of glycation, while AGEs formation may be modulated by trapping of dicarbonyl intermediates and oxygen radical species. In addition, our results suggest that a soy isoflavone-rich extract might be useful for the generation of particular glycoconjugates with improved functional properties. Further

research should be performed to confirm this hypothesis. Phytochemomics[30] may be an appropriate tool for the generation of this necessary knowledge.

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Figure 1. Free amino group content of unheated and heated protein (control), glycation model (soy glycinin protein-fructose) and isoflavones inhibition model in 0.2% KOH solution in presence of genistin, genistein and soy isoflavone-rich extract (60ºC for 60 min). Data are expressed as a percentage of the control (unheated protein) value. Data are means of triplicate analyses. Error bars denote the relative standard deviation. Values with different letters are significantly different (p<0.05).

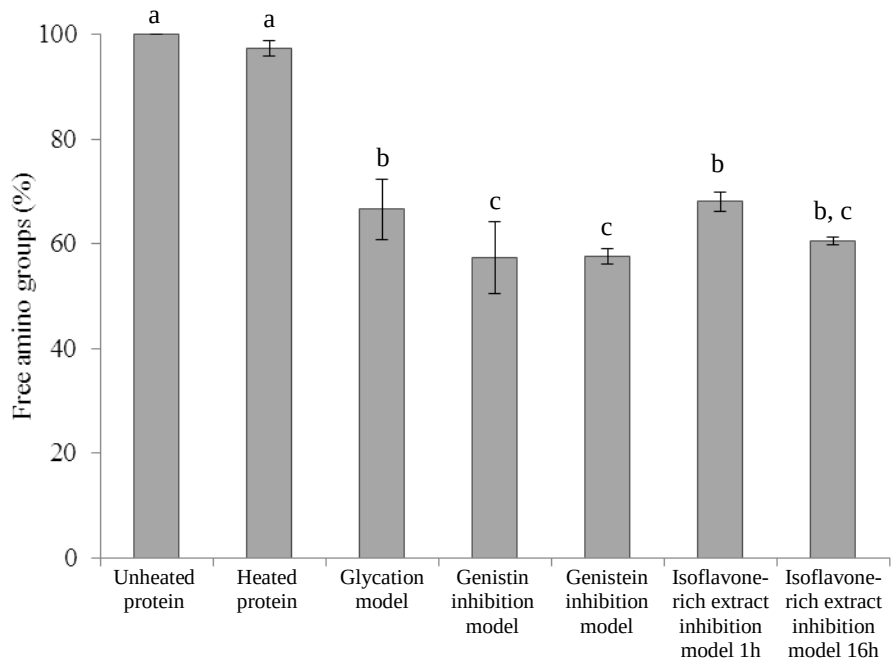

Figure 2. Sugar content of unheated and heated protein (control), glycation model (soyglycinin protein-fructose) and isoflavones inhibition model in 0.2% KOH solution in presence of genistin, genistein and soy isoflavone-rich extract (60ºC for 60 min). Data are expressed as µg sugar/100 mg protein. Data are means of triplicate analyses. Error bars denote the relative standard deviation. Values with different letters are significantly different (p<0.05).

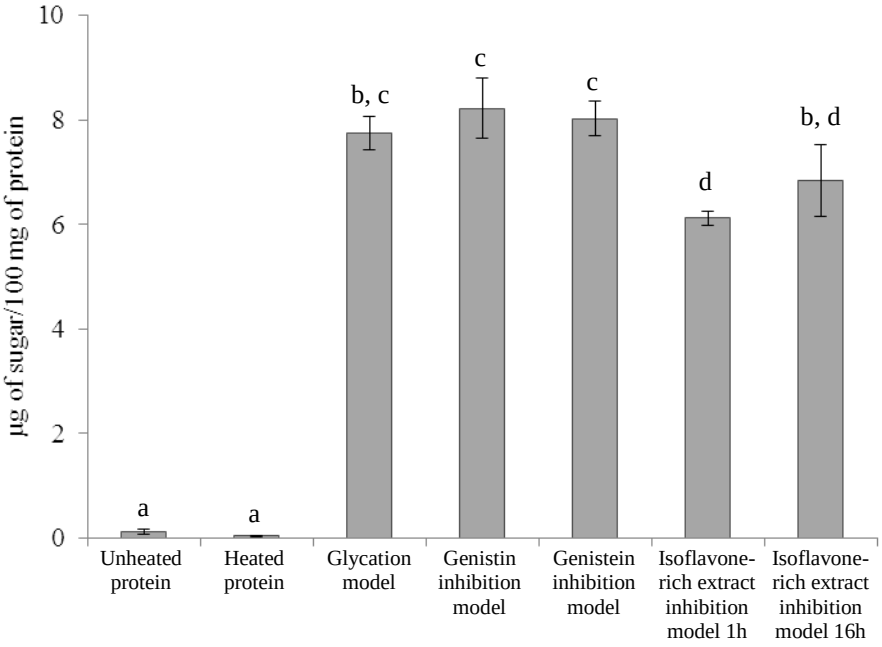

Figure 3. Content of protein-bound CML in unheated and heated proteins (control), glycation model (soy glycinin protein-fructose) and isoflavones inhibition model in 0.2% KOH solution in presence of genistin, genistein and soy isoflavone-rich extract (60ºC for 60 min). Data are expressed in millimol of CML/mol of lysine. Values are means of triplicate analyses. Data are means of triplicate analyses. Error bars denote the relative standard deviation. Values with different letters are significantly different (p<0.05).

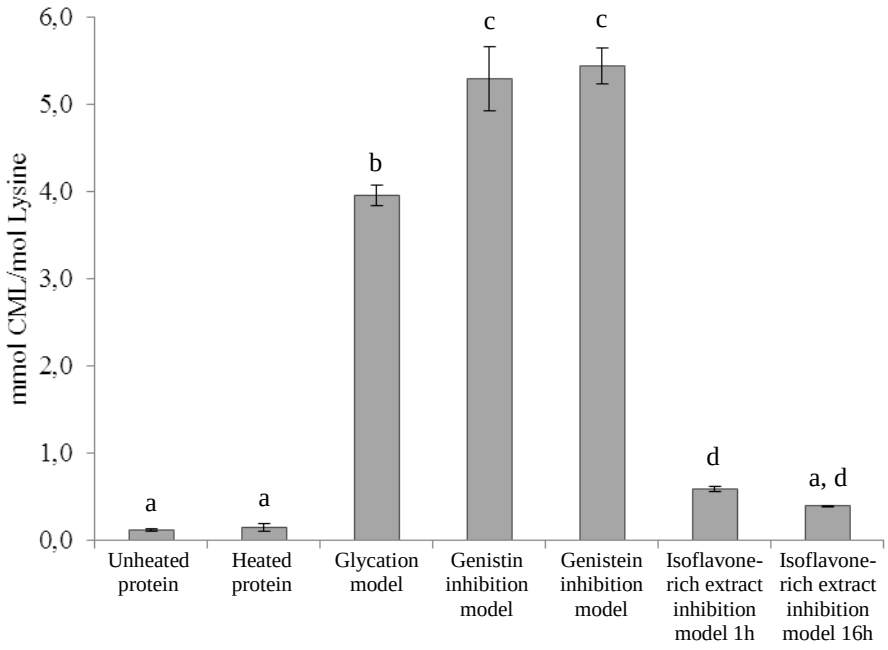

Figure 4. Fluorescence spectra of glycation model systems in 0.2% KOH solution in presence of genistin, genistein and soy isoflavone-rich extract.