Indication of the Maillard Reaction during Storage of Protein Isolates

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In a previous study, feeding an isolated soy protein (ISP)-based diet to rats was found to reduce colon cancer risk as assessed by a reduced number of colonic precancerous lesions. However, this same ISP, after storage at room temperature for >2 years, increased the number of precancerous lesions (Gallaher *et al.*, 1996). We hypothesize that this increase was due to the development of Maillard reaction products in the ISP during storage. Thus, the objective of this study was monitor development of the Maillard reaction during storage of ISP and delactosed whey protein concentrate. Proteins were stored at different water activities (0.22, 0.33, 0.55) and temperatures (22, 30, 45 °C) with and without glucose (5% w/w) and increases in browning (A = 420 nm) and fluorescence (λ_{ex} 365 nm/λ_{em} 475 nm) determined. In the absence of glucose, only soy protein underwent browning; otherwise the rate of browning and fluorescence increased with increasing temperature and water activity. To investigate why ISP underwent browning in the absence of glucose while whey protein concentrate did not, the reaction of genistein was investigated. Genistein is an isoflavone with putative chemoprotective properties found in ISP but not in whey. Genistein (2 mM) was incubated alone or with lysine (2 mM) in buffer. The absorbance (A = 420 nm) of the reaction mixtures and genistein concentration was measured over time. It was found that genistein underwent reaction both alone and in the presence of lysine. The rate of browning was found to parallel the rate of genistein loss, suggesting that genistein plays a role as a reactant in nonenzymatic browning reactions. This suggests that long-term storage of ISP will lead to the loss of genistein and potentially result in the development of carcinogens.

Keywords: *Maillard reaction; isolated soy protein; storage; genistein; browning; fluorescence*

INTRODUCTION

Currently there is considerable and increasing interest in the health benefits of soy-containing foods, in particular in the role of soy in lowering the incidence of certain cancers. It has been suggested that the high intakes of soy may explain, in part, the lower incidence of certain cancers in Asian countries, where soy consumption is high, when compared to Europe or America (Setchell et al., 1984; Adlercreutz, 1990). Two recent epidemiological studies have reported an inverse association between reproductive cancers and intake of soy foods, supporting this suggestion (Goodman, 1997; Witte, 1997). As reviewed by Messina et al. (1994) the majority of animal studies have found a protective effect of dietary soy products against a variety of cancers. A possible mechanism by which soy may exert an anticancer effect is its high isoflavone content. Isoflavones have a number of properties that make them putative chemopreventive agents. For example, genistein, the major isoflavone in soybeans, is a powerful inhibitor of tyrosine kinase activity in vitro (Akiyama et al., 1987) and, thus, inhibits cell growth proliferation. It has also been shown to inhibit cell cycle progression and normalizes transformed cells, both of which are consistent with a protective effect against cancer. Isoflavones also have antioxidant activity (Wei et al. 1995), which may act to reduce cancer risk. The anti-estrogenic activity of isoflavones may play an important role in reducing risk of hormone-dependent cancers (Adlercreutz et al., 1987).

The Maillard reaction has been studied in great detail because of its role in the development of colors and flavors during food processing and storage. Several Maillard reaction products (e.g. heterocyclic amines), however, have been implicated as carcinogens, and many more have been identified as mutagens by the Ames test (Skog, 1993). Most of these studies involve meat (beef, chicken, pork, lamb) cooked at high temperatures (>120 °C). The progression of the Maillard reaction, in a food or model system, is most commonly measured by following the formation of brown pigments. However, due to the insolubility of protein pigments, color development cannot be easily measured for these reactions so the reaction is also followed by measuring the formation of fluorescence compounds.

The available lysine content of proteins is closely related to their tendency to react. Milk proteins that are rich in lysine tend to be more reactive than proteins low in lysine, such as soy proteins (Schnicles et al., 1976; Wolf et al., 1977). Warren and Labuza (1977) observed a significant loss of lysine before brown pigments could be observed. The two major physicochemical factors

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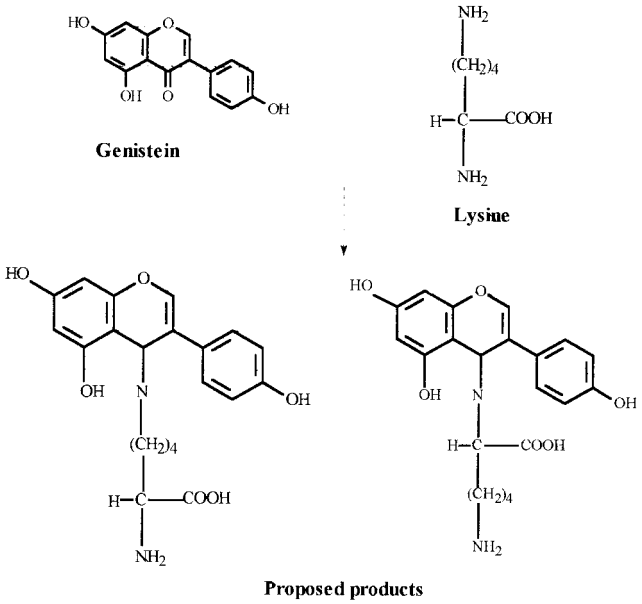

Figure 1. Proposed mechanism for the reaction of genistein with lysine, with 2 possible products.

that alter the rate of the Maillard reaction are temperature and water content as a function of water activity (a_w). The Maillard reaction has an activation energy of 25−40 kcal/mol suggesting that the reaction rate increases by 3−8-fold for every 10 °C rise in temperature (Q_{10}) (Labuza and Saltmarch, 1981). Above the monolayer moisture content ($a_w \sim 0.2$) the rate of browning increases approximately 2−3 times for every 0.1 a_w increase. Thus, storage at a room humidity of 50% RH, giving an $a_w \sim 0.5$ for the product, would have the potential effect of increasing the rate by as much as 27 times as compared to the optimal a_w of 0.2. If temperature and a_w effects are combined, storage at 35 °C and 50% RH could increase the rate up to 600-fold compared to more favorable conditions of 20 °C and 20% RH. Thus, conditions that can occur in many food storage environments may be detrimental to the product. However, in many feeding studies the storage conditions of the diet components are not given consideration as a factor that could influence the outcome.

Since there are few studies on the Maillard reaction in aged food proteins, there have been even fewer studies on the role of stored proteins on the incidence of cancer. A preliminary experiment in this laboratory found that carcinogen-treated rats fed a diet containing isolated soy protein (ISP) had fewer precancerous colonic lesions compared to rats fed a casein-based diet. However, when the experiment was repeated using the same ISP stored at ambient temperature and humidity for more than 2 years, rats fed the aged ISP had a greater number of colonic precancerous lesions (Gallaher *et al.*, 1996). Since the aged ISP had a higher fluorescence (λ_{ex} 365 nm/λ_{em} 475 nm) than recently processed ISP, we hypothesized that products are formed during by the Maillard reaction which may have carcinogenic properties. These Maillard products counteract the beneficial effects of the soy proteins and genistein.

Since it is well-known that plant flavonoids such as anthocyanins, catechins, and flavonols undergo reactions with amine groups (Singleton, 1972; Jurd, 1972), it is possible that isoflavones (i.e. genistein) act as Maillard reactants. Figure 1 shows a suggested mechanism, based on the well-known reaction of carbonyl groups with the amino groups of amino acids. Thus, genistein and other isoflavones in soy have the potential to react with the free amino groups present in the protein. This has important implications in the anticancer affects of soy. First, during storage there would be an overall loss of genistein, a putative anticancer agent, and second, the products of a Maillard reaction involving genistein would be unknown, potentially carcinogenic products. Consequently, the role of genistein in the Maillard reaction is important to study.

Thus, the objectives of this study were to determine whether the Maillard reaction takes place in ISP and other proteins under several defined storage conditions. We monitored the formation Maillard reaction products by measuring changes in fluorescence and browning. We also determined the role of isoflavones in the Maillard browning reaction.

Table 1. Water Activity and Moisture Content of Protein Systems Used for Storage Study

	nominal water activity	equilibrium moisture content, (g H_2O/100 g solids)		nominal water activity	equilibrium moisture content, (g H_2O/100 g solids)
ISP	0.33	6.16	whey	0.33	7.69
	0.54	9.64		0.54	10.04
	0.75	12.60		0.75	15.69
ISP + 5% glucose	0.33	6.62	whey + 5% glucose	0.33	6.77
	0.54	8.72		0.54	10.25
	0.75	14.21		0.75	14.82

MATERIALS AND METHODS

All chemicals were obtained from Fisher Scientific unless otherwise stated. Recently processed commercial isolated soy protein (Supro 970, Protein Technologies Int., St. Louis, MO) and delactosed whey protein isolate (BiPro, Davisco Foods Int., LeSuer, MN) served as model proteins. Genistein was obtained from Toronto Research Chemicals (Toronto, Canada). Proteins were stored frozen (−20 °C) over dry desiccant until utilized for the storage study.

Protein Storage Study. Model systems consisted of the protein isolates alone or with glucose (5% w/w, dry basis). These protein systems were equilibrated in open pans in desiccators at the desired a_w (0.33, 0.54, or 0.76). After 2 weeks storage at room temperature, a_w was measured using an Aqualab II (Pullman, WA) to test for equilibration. Sorption isotherms were measured for the protein systems according to the method described by Labuza (1985). The nominal a_w and moisture conditions of all samples are listed in Table 1.

For the storage study, samples of proteins (~25 g) alone or with glucose previously equilibrated to different a_ws were stored in sealed aluminum foil laminated pouches (~ 4 in. × 5 in.) at 22, 30, or 45 °C. The progression of the Maillard reaction was measured weekly for samples stored with high a_w and held at the higher temperatures (i.e. 0.76 a_w, 30 and 45 °C) and every 2 weeks for the other samples.

To measure fluorescence accurately, it was necessary to solubilize the protein powders with NaOH. Approximately 40 mg of the stored protein mixture was dissolved in NaOH (10 mL, 0.05 M) and heated at 45 °C for 45 min. A protein blank fluorescence reading was made by preparing a fresh protein sample in the same way. Fluorescence was measured using a Perkin-Elmer 650-10S fluorescence spectrophotometer (λ_{ex} 365 nm/λ_{em}475 nm). Quinine sulfate solution (3 μg/mL) was used as a standard.

For browning measurements, due to the insoluble nature of the brown pigments in proteins, a sample (0.75 g) was digested by the proteolytic method described by Saltmarch and Labuza (1981). Protein samples were dissolved in water and digested by pancreatin (1 mL, 50 mg/mL) by shaking at 45 °C. After 2 h, trichloroacetic acid (50% w/v) was added to stop the enzymatic reaction and the samples were centrifuged (8000*g* for 10 min) and filtered (Whatman no. 1 filter paper).

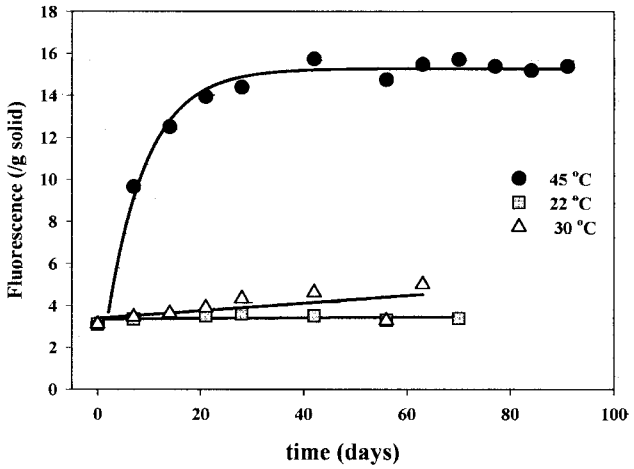

Figure 2. Fluorescence as a function of time in isolated soy protein + glucose (5% w/w) stored at a_w 0.75 and 22, 30, and 45 °C.

A blank was prepared as above but in the absence of protein. Browning was analyzed by measuring the absorbance at 420 nm in a spectrophotometer, and the results were converted to absorbance/gram of solids.

Genistein as a Maillard Reactant. The role of genistein as a Maillard reactant was investigated by measuring the rate of change in absorbance and by measuring the genistein concentration of reaction mixtures containing genistein (<2 mM) or genistein + lysine (both <2 mM). Concentrated solutions of genistein or lysine (20 mM) were prepared in borate or Tris buffer (0.4 M, pH 9 adjusted with NaOH) and then diluted (and mixed for the genistein + lysine mixture) to the correct concentration in buffer and incubated at 60 °C. To aid dissolution, genistein was first dissolved into a small volume of methanol and made up to volume with buffer and sonicated for 10 min and then, before incubation, the reaction mixtures were filtered to remove any undissolved crystals and finally degassed with nitrogen.

At timed intervals, two aliquots were removed. One (~3−5 mL) was cooled, and after appropriate dilution, absorbance (A = 420 nm) was measured. The second aliquot (100 μL) was stored at −20 °C for HPLC analysis of genistein concentration.

Genistein concentration was determined by HPLC using a 5 mm Novapak C18 radial compression column. The buffers, A and B, were 15% and 65% methanol (HPLC grade), respectively. The mobile phase was isocratic 20% B for 5 min and then increased linearly to 70% B over 20 min and isocratic at 70% B for a further 20 min (45 min in total). The flow rate was 1 mL/min. Absorbance was measured at 263 nm (Spectroflow 757, Kratos Analytical). The concentration of genistein was determined by comparison to standard genistein solutions of known concentration. Samples from the reacted mixtures were diluted 1:25 with methanol solution (80% v/v) containing butyrophenone (25 μL/100 mL) as an internal standard. Peak areas were calculated using a chromatography software program (712 System Controller, Gilson).

RESULTS AND DISCUSSION

Progress of the Maillard Reaction in Stored Proteins. When proteins were stored in the absence of glucose, at 30 and 45 °C and all a_w, fluorescence increased linearly with time. Browning of proteins stored without glucose at 45 °C and a_ws of 0.54 and 0.75 also increase linearly with time.

When glucose was added, the protein−glucose samples stored at 45 °C and 0.75 a_w showed an initial increase in browning and fluorescence that quickly reached a plateau (Figures 2 and 3). This suggests that one of the reactants, i.e., lysine, became limiting. However, only storage at high temperatures and higher a_ws showed a significant increase in fluorescence (30 and 45 °C) or in browning (45 °C only). Thus, the early Maillard reaction occurs in stored isolated proteins at temperatures as low as 30 °C in as short a time as 2 months (Figure 2).

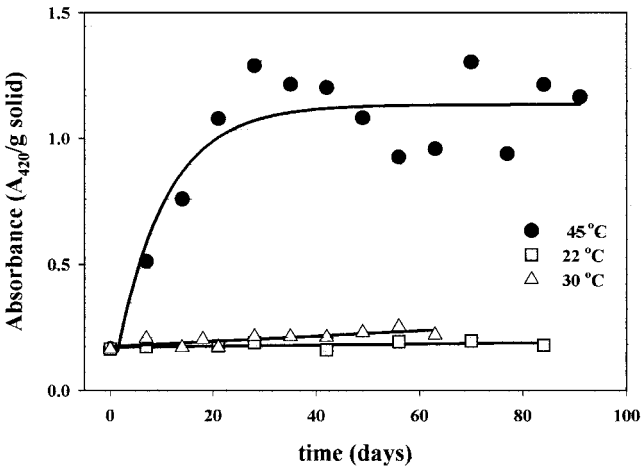

Figure 3. Browning development in isolated soy protein + glucose (5% w/w), stored at a_w 0.75 and 22, 30, and 45 °C.

Table 2. Pseudo-Zero-Order Rate Constants for the Formation of Fluorescence in Protein Systems Containing Soy Protein Isolate (ISP), ISP + Glucose (5% w/w), Whey Protein Isolate (Whey), and Whey + Glucose (5% w/w)

		fluorescence (/g solid/day) × 10^3	
system	a_w	30 °C	45 °C
ISP	0.33	3.1	13.0
	0.54	4.2	14.4
	0.75	8.8	69.8
ISP + glucose	0.33	0	7.9
	0.54	10.3	33.6
	0.75	19.7	433.7
whey	0.33	2.4	16.4
	0.54	3.4	27.9
	0.75	3.5	31.6
whey + glucose	0.33	2.8	21.2
	0.54	3.5	82.3
	0.75	16.3	710.6

Table 3. Pseudo-Zero-Order Rate Constant for Browning of Protein Isolates

system	a_w	absorbance (A_{420}/g solid/day) × 10^3 at 45 °C	system	a_w	absorbance (A_{420}/g solid/day) × 10^3 at 45 °C
ISP	0.33	<0.5	whey	0.33	<0.5
	0.54	0.7		0.54	<0.5
	0.75	1.7		0.75	<0.5
ISP + glucose	0.33	<0.5	whey + glucose	0.33	0.6
	0.54	3.2		0.54	6.0
	0.75	40.2		0.75	34.5

Zero-order rate constants for changes in fluorescence (g of solid/day) and browning (A_{420}/(g of solid/day)) for these protein mixtures were calculated from the initial linear portion of the curve after the method of Baisier and Labuza (1992) and are shown in Tables 2 and 3, respectively. The pseudo-zero-order reaction rates for ISP + glucose and whey + glucose (0.75 a_w and 45 °C) were calculated using data from the first 21 days of storage due to the formation of the plateau mentioned above. As seen in Table 2, glucose increased the rate of fluorescence formation by more than 4 times as compared to no glucose in the whey and in the ISP system, and the rate of fluorescence formation at 45 °C

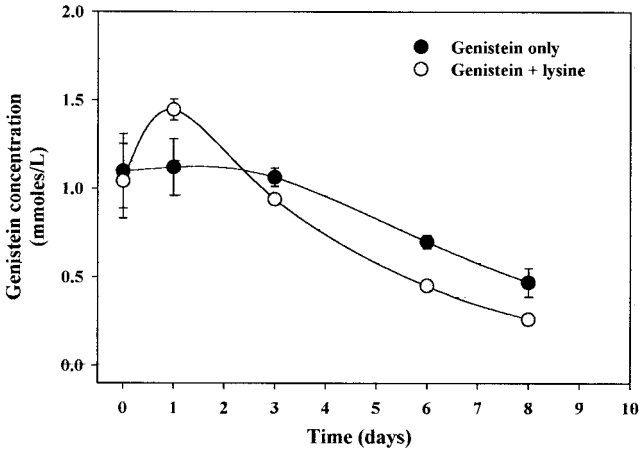

Figure 4. Change in genistein concentration (mM) with time for solutions containing genistein (2 mM) or genistein + lysine (2 mM), pH 9 (0.4 M Tris), 60 °C.

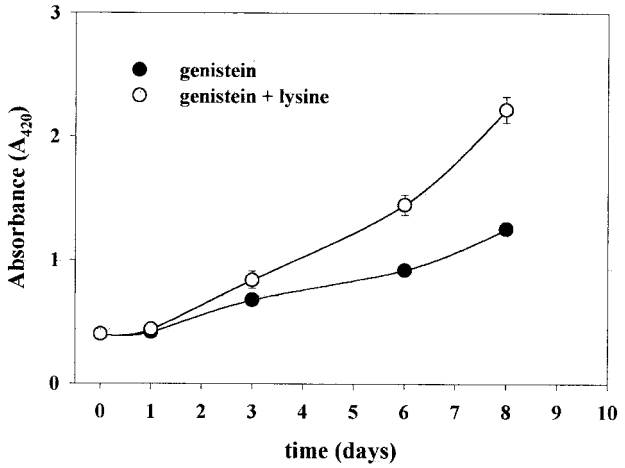

Figure 5. Change in absorbance (A_{420}) with time for solutions containing genistein (2 mM) or genistein + lysine (2 mM), pH 9 (0.4 M Tris), 60 °C.

was up to 9 times faster with the addition of glucose. The rate of browning in ISP was 24 times faster (Table 3) with the addition of glucose compared to browning in ISP without glucose, while essentially no browning occurred in the whey system when glucose was absent. These higher rates are expected since it is known that an excess of reducing sugars promotes the rate of Maillard browning (Labuza and Baisier, 1992). Since the fluorescence peak precedes the increase in pigment formation, it has been suggested that fluorescent compounds are precursors of the brown pigments. In the present study, however, plots of formation of brown pigments and fluorescence over time for ISP and whey showed that fluorescence and brown pigments were formed during the same period of time. Baisier and Labuza (1992) observed similar behavior for a glucose−glycine solution.

Comparison between Soy and Whey Protein Isolates. A comparison of the reaction rates between the two proteins (at 45 °C and 0.75 a_w) shows that the rate of formation of fluorescence is slightly higher in the whey + glucose system than in the ISP + glucose system. This agrees with the results of Labuza and Schmidl (1986), who found that browning was ~1.5 times faster in whey protein than in soy protein in model systems containing glucose (10%), protein (30%), fat (20%), and cellulose (20%) at a a_w of 0.7. Since milk proteins are richer in available lysine, they would be expected to brown more readily than proteins low in lysine such as soy (O'Brien and Morrisey, 1989). However, in the present study browning is slightly faster in the ISP + glucose system compared to whey + glucose. When glucose is absent, the rate of fluorescence formation at 0.75 a_w was nearly twice as fast in ISP than in whey, and browning only occurred in the ISP system. Two reasons ISP undergoes the Maillard reaction in the absence of reducing sugar are that, first, the products formed by ISP are more soluble than those formed by whey and, second, ISP contains other reactive compounds. Isoflavones in soy, such as diadzein and genistein, could act as Maillard reactants.

Role of Genistein in the Maillard Reaction. There were problems with the solubility of genistein. Our attempt to make a 2 mM solution of genistein led to a saturated solution with a final concentration of 1 mM (Figure 4). This problem occurs when working with sparingly soluble organic compounds (Grant and Higuchi, 1990). We found little data on the solubility of genistein. Walter (1941) stated that genistein is soluble in the "usual organic solvents" but did not qualify this statement with any data. The solubility of genistein does not seem to have been studied further. During the course of the present investigation it was found that genistein was soluble in dimethyl sulfoxide but has limited solubility in methanol and ethanol solutions. Solubility was also pH dependent, being greater at pH > 8.5. Unfortunately, we found that genistein precipitated out of solution after apparent dissolution. Interestingly, during the initial 24 h of incubation, lysine increased the genistein concentration. This was most likely due to formation of a complex between lysine and genistein, leading to an apparent increase in genistein solubility. Such complexes commonly form when sparing soluble organic compounds are dissolved into aqueous solutions (Grant and Higuchi, 1990).

Table 4. Pseudo-Zero-Order Rates of Browning of Solutions Containing Genistein (2 mM) Alone and with Lysine (2 mM) Incubated at 60 °C and pH 9

	zero-order rate constant (A_{420}/day)	
reaction mixture	Tris buffer	borate buffer
genistein	0.10	0.10
genistein + lysine	0.20	0.17

Despite the limited genistein solubility, the disappearance of genistein with time is clearly evident (Figure 4). It is also evident that genistein loss after 24 h was faster in the presence of lysine. Browning was observed in all systems containing genistein and genistein + lysine (Figure 5). Thus, genistein acts as a Maillard reactant with lysine and also reacts alone. The browning reaction followed zero-order kinetics (Table 4). The addition of lysine increased the rate of browning by ~2 times. Thus, at least half the reaction with amino acid was due to genistein reacting with itself. To eliminate the possibility that the amine group of the Tris buffer might increase browning, the reaction rates were determined in borate buffer as well. The reaction rate was similar in both Tris and borate buffer, showing that the reaction was independent of the type of buffer. Genistein could be involved in the Maillard browning reaction in two ways: (1) autodegradation and (2) reaction with available amino groups. How these reactions would effect the antioxidant and putative anticancer properties of genistein is unknown but most

likely would lead to their loss. It is also possible that genistein reacts to form products that may have deleterious effects of their own.

The results above indicate that the Maillard reaction occurs in isolated proteins which contain no obvious source of reducing sugar, even under relatively mild storage conditions. Given the potential of certain Maillard reaction products to have detrimental biological effects, appropriate steps should be taken to properly store protein isolates in studies examining the health benefit of these products. Failure to do so may lead to erroneous results. In addition, in studies with soy isolates, genistein appears to increase the Maillard reactivity of soy. Thus, long-term storage of soy isolates could lead to the loss of the health benefits of genistein. The present results, coupled with our previous studies showing that aged ISP increases the incidence of colon cancer, suggests that genistein reacts to form new compounds with potentially detrimental biological effects. It is suggested that protein isolates, particularly soy isolates, should be stored under conditions which would prevent the formation of Maillard reaction products, i.e., $a_w < 0.3$ and < 4 °C.

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