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Inhibition of Non-enzymatic Glycation by Silk Extracts from a Mexican Land Race and Modern Inbred Lines of Maize (*Zea mays*)

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Non-enzymatic glycation and the accumulation of advanced glycation end products (AGEs) are associated with various disease states, including complications of diabetes and aging. Secondary metabolites from several plant species are known to inhibit non-enzymatic glycation and the formation of AGEs, including flavonoids found in the style (silk) of *Zea mays* (maize). Thirteen modern maize inbreds and one land race were tested for *in vitro* inhibition of non-enzymatic glycation of bovine serum albumin. Many of the tested extracts exhibited inhibitory activity, in particular the newest inbreds, which were bred for resistance to gibberella ear rot (*Fusarium graminearum*) and common smut (*Ustilago maydis*). The most active maize genotype (CO441), displaying an IC_{50} of 9.5 μg/mL, was more effective than aminoguanidine, a known inhibitor of glycation. Zapalote chico, a land race with high maysin content, showed only moderate inhibitory activity compared with the modern maize genotypes. Antiglycation activity was highly correlated with the total phenolic content of silk extracts and mildly correlated with resistance to certain fungal infections. The results identify modern resistant and high phenolic maize inbreds as promising candidates for the development of natural AGE inhibitors for the prevention and treatment of diabetic complications and the degenerative effects of aging.

Keywords: Zea mays; corn silk; advanced glycation end products; antiglycation; phenolics.

INTRODUCTION

In various biological settings, reducing sugars can react non-enzymatically with protein amino groups, forming Schiff bases and Amadori products, to produce advanced glycation end-products (AGEs). During AGE formation, Amadori rearrangements to more reactive intermediates induce protein cross-linking that not only affects protein function and half-life but also engages signaling cascades transduced by the AGE receptor (RAGE) (Ulrich and Cerami, 2001). AGEs and RAGE activation have been linked to the development of neurological, cardiovascular and renal complications of diabetes and aging (Ramasamy *et al*., 2005).

Consequently, inhibitors of AGE formation have recently regained attention as potential therapeutic agents. Aminoguanidine is a synthetic antiglycation agent that neutralizes reactive Amadori products to prevent crosslinking and AGE formation (Ulrich and Cerami, 2001). Eliciting promising results in numerous animal studies of diabetic complications, aminoguanidine has been tested in Phase III trials where it provided limited renoprotective benefits to diabetic patients (Bolton *et al*., 2004).

While synthetic analogs of aminoguanidine and other new AGE inhibitors continue to be investigated, a variety of plant-derived phenolic compounds have also demonstrated impressive antiglycation activity (Cervantes-Laurean *et al*., 2006; Matsuda *et al*., 2003; Lou *et al*., 2001; Kiho *et al.*, 2004). Considering the increasing global prevalence of diabetes, the development and validation of traditionally used medicinal and food plants would improve the treatment capacity in regions of limited access to pharmaceutical drugs and where compliance to native or natural products exceeds that with prescribed drugs.

In Chinese Traditional Medicine, the style of *Zea mays* (corn silk) is used as a diuretic and for the prevention of diabetic complications. Corn silk contains phenolic compounds of which flavonoids, such as luteolin and maysin, are abundant (Reid *et al.*, 1992; Snook *et al*., 1989). *In vitro* glycation studies have identified flavones as the most effective inhibitors of AGE formation among flavonoids with luteolin being exceptionally active (Wu and Yen, 2005; Matsuda *et al*., 2003). Suzuki *et al*. (2003) recently isolated and identified two novel flavone C-glycosides from corn silk with antiglycation activity comparable to aminoguanidine. To date, the activity of whole silk extracts has not been described and the effects of maize genotype on activity have not been characterized.

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Modern maize breeding has developed hybrid and inbred varieties that present early maturation, increased yield, and improved resistance to prevalent diseases and insects. Land races are traditional varieties rather than cultivars that have been developed using modern breeding methods. In addition to 13 inbred genotypes of maize, the study included a Mexican land race, Zapalote chico (ZC), whose maysin content in silk has been correlated with earworm resistance (Waiss *et al.*, 1979). Moreover, the concentration of phenolic compounds in silk invaded by fungal spores has been inversely correlated with the plant's susceptibility to disease (Reid *et al.*, 1992). For these reasons, it is hypothesized that antiglycation activity will vary among maize genotypes according to phenolic content and resistance levels of the silk, with genotypes of high phenolic content and resistance levels displaying the greatest antiglycation activity.

MATERIALS AND METHODS

Plant materials. Thirteen maize inbreds (CO266, CO272, CO325, CO354, CO359, CO388, CO389, CO430, CO431, CO432, CO433, CO441 and B73) and a Mexican land race (Zapalote chico) were planted in a two replicate trial at the Central Experimental Farm of Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada in 2004. Each genotype was planted in a single row of 15 plants per replicate (plot). Approximately 1 week after silk emergence, silk from 10 randomly selected plants was harvested and bulked per row. The silks were dried in an oven at 40 °C and stored in darkness at 4 °C until further processing. Prior to extraction, the silks were ground to a fine powder using a Wiley mill (mesh size 40).

Extract preparation. The powdered silk was extracted overnight in 10 mL 80% methanol/g dry material with agitation at 200 rpm. The samples were filtered through Whatman 70 mm filter paper and the filtrate subsequently dried under vacuum using a Speedvac System AE52010 (Savant, Halbrook, NY). Dried extracts were reconstituted in 80% methanol and serially diluted for testing in the glycation and phenolic assays.

Inhibition of AGE formation. The procedure was based on previous methods (Suzuki *et al.*, 2003; Lou *et al.*, 2001) with additional modifications. Bovine serum albumin (BSA; 1.0 mg/mL) (Sigma, St Louis, MO) was incubated in 100 mM sodium monophosphate solution (pH 7.4) with fructose (200 mM) and vehicle ($F_{control}$) or plant extract ($F_{extract}$), or aminoguanidine ($F_{positive}$). Aminoguanidine (Sigma, St Louis, MO), a known AGE formation inhibitor, was used as a positive control. To determine whether the extracts themselves interfered with fluorescence (quenching), each extract was tested at 40 μg/mL in the absence of BSA.

For each trial, 5 μL of vehicle, extract or aminoguanidine was added to sterile glass test tubes containing 1 mL of reaction solution. All treatments were sterilized by filtration through 0.2 μm membrane filters (Chromatographic Specialties Inc., Brockville, ON) into autoclaved tubes and sealed with cotton and parafilm. The tubes were then agitated and incubated at 37 °C for 7 days. After incubation, all treatments were transferred to opaque 96-well plates with clear bottoms (VWR, Mississaugua, ON) to quantitatively assess the formation of fluorescent AGEs using a SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 355 nm and 460 nm, respectively.

F_{blank} (fructose and vehicle) was subtracted from all results and the percent inhibition then calculated as: % inhibition = $[1 - (F_{extract}) / (F_{control})] \times 100$. IC_{50} values were calculated from % inhibition values obtained at all tested concentrations.

To test the linearity of the system, glycation experiments were conducted over 3, 5 and 7 days and simultaneously evaluated for the formation of fluorescent AGEs. Relative fluorescence revealed a linear increase in AGE formation with longer incubation times (R = 0.998, data not shown).

Total phenolics. The concentration of total phenolics in the extracts was quantitated using the Folin-Ciocalteau colorimetric reaction. Aliquots of 250 μL of extract (1 mg/mL and 4 mg/mL) were added to test tubes containing 1.25 mL of Folin-Ciocalteau (FC) reagent (Sigma) and mixed by vortex. After 8 min, 1.0 mL of 7.5% sodium carbonate anhydrate reagent (Fisher, Ottawa, ON) was added. Samples were then incubated in darkness at room temperature for 2 h and transferred to clear 96-well plates for spectrophotometric analysis at 725 nm with a SpectraMax M5 microplate reader. For quantitative purposes, concurrently analysed standards of luteolin (25, 50, 100 and 200 μg/mL) were prepared and the absorbances converted to milligrams of luteolin equivalents/g extract.

Statistical analyses. All values are expressed as mean ± standard error (SE) for $n = 5$ unless otherwise indicated. The results were analysed by Student's paired *t*-test and 2-way ANOVA with Tukey's *post hoc* analysis. Statistics were performed using SigmaPlot and Systat v7.0 (Richmond, CA).

RESULTS AND DISCUSSION

Thirteen modern maize genotypes and one land race were grown in two replicate plots and their silk subsequently extracted for analysis of antiglycation activity. Based on the observed IC_{50} value for aminoguanidine in our system (44 μg/mL), each silk sample was initially tested at 40 μg/mL, revealing a wide range of results among genotypes, with four inhibiting glycation to a similar extent as aminoguanidine (CO354, CO431, CO433, B73) and one exhibiting greater activity (CO441) (Table 1). This confirms previous reports of antiglycation activity in corn silk components (Suzuki *et al.*, 2003) and extends the result over a much larger set of genotypes (Table 1). Autofluorescence of the silk extracts was less than 15% and, if present, usually resulted in a quenching effect, thereby underestimating the actual level of inhibition (data not shown).

Interestingly, the highest levels of inhibition were elicited by extracts from the newest resistant inbred lines (CO441, CO431 and CO433). These three inbreds were specifically bred for increased resistance

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Table 1. Antiglycation activity and disease resistance of 14 maize genotypes. Mean inhibition of non-enzymatic glycation is reported for standard concentrations of 40 μg/mL (silk extract or aminoguanidine). Data for disease severity were averaged over 3 years (2001–2003) with low numbers indicating greater resistance

Genotype (sample)	Percent inhibition ± SE (%) (plots 1 + 2)	Plot	Percent inhibition ± SE (%) (by plot)	Disease severity		
				Earrot-K[a]	Earrot-S[b]	Smut[c]
CO266	–10.2 ± 2.8	1	–5.9 ± 3.3	6.3	5.4	3.5
		2	–14.5 ± 3.8			
CO272	25.2 ± 3.4	1	17.3 ± 3.6	6.5	3.1	6.6
		2	33.2 ± 2.6			
CO325	31.3 ± 3.9	1	20.8 ± 3.0	6.5	3.1	6.6
		2	41.9 ± 1.9			
CO354	40.1 ± 3.2	1	48.9 ± 1.4	7	6.9	6.8
		2	31.3 ± 2.4			
CO359	32.5 ± 5.1	1	47.6 ± 1.5	6.3	6.6	4.3
		2	17.4 ± 1.7			
CO388	–0.7 ± 2.0	1	4.2 ± 1.2	5.8	5.4	4.3
		2	–5.7 ± 2.0			
CO389	–5.4 ± 4.3	1	6.2 ± 3.0	6.7	4.7	7
		2	–17.0 ± 2.5			
CO430	37.2 ± 3.0	1	28.6 ± 1.1	3.3	3.5	3.4
		2	45.2 ± 1.5			
CO431	51.5 ± 1.6	1	56.0 ± 1.1	4.8	4.8	4
		2	47.0 ± 0.2			
CO432	24.3 ± 7.1	1	45.2 ± 1.7	3.1	2.6	2.8
		2	3.5 ± 2.4			
CO433	41.1 ± 3.5	1	51.4 ± 1.2	4.1	3.4	2.9
		2	30.8 ± 1.2			
CO441	60.1 ± 5.4	1	76.2 ± 1.2	2.9	2.7	2.2
		2	44.0 ± 0.8			
B73	41.5 ± 1.2	1	42.7 ± 0.6	6.2	5.6	5.8
		2	40.3 ± 2.3			
Zapalote chico (ZC)	27.6 ± 1.2			N/A	N/A	N/A
Amino-guanidine	45.0 ± 3.1			N/A	N/A	N/A

[a] Earrot-K, resistance to *Fusarium graminearum* infection through kernel wounds.
[b] Earrot-S, resistance to *F. graminearum* infection through silk wounds.
[c] Smut, resistance to *Ustilago maydis* infection through silk wounds.

to gibberella ear rot and demonstrated reduced susceptibility to both this disease and common smut during a 3 year study (resistance levels for each genotype provided in Table 1).

A significant difference between plots (two-way ANOVA, $p < 0.0001$) was also observed with a trend toward stronger inhibition of glycation from the samples grown in the eastern plot (plot 1) compared with their congenetics grown to the west (plot 2). Moreover, two-way ANOVA analysis revealed a significant interaction between plot and genotype. This variation may be attributed to differences in soil conditions or exposure to pathogens, insects and weather. To explore environmental effects verses genotype more comprehensively, a full agronomic study should be considered with a randomized block design. Due to the variability between plots, samples from plots 1 and 2 were also considered independently (Table 1).

A selection of 15 active samples representing 10 genotypes, including ZC, was then assessed for IC_{50} values (Table 2). The IC_{50}s ranged from 9.5 to 82 μg/mL with seven samples yielding values similar to aminoguanidine and one, sample CO441-1, that was five times lower.

Because antiglycation activity has been ascribed to natural phenolic compounds such as flavones, the total phenolic content of each silk sample was measured and

Table 2. IC_{50} values for non-enzymatic glycation and total phenolic content of selected maize samples

Sample (Genotype-plot)	IC_{50} (μg/mL)[a]	Total phenolic content (mg/g extract)[b]
CO325-1	82.2 ± 21.1	17.8 ± 0.1
Zapalote chico	78.7 ± 15.7	27.9 ± 0.3
CO354-2	76.5 ± 35.5	17.9 ± 0.2
CO433-2	71.9 ± 29.6	28.5 ± 0.6
CO430-1	70.6 ± 17.1	34.9 ± 0.5
CO432-1	70.5 ± 49.1	14.4 ± 0.3
CO325-2	53.3 ± 19.9	33.5 ± 0.2
CO359-1	49.7 ± 19.0	49.2 ± 0.3
CO441-2	45.8 ± 6.6	44.8 ± 0.5
CO431-2	45.5 ± 12.1	47.5 ± 0.4
Aminoguanidine	44.4 ± 12.5	
CO354-1	41.2 ± 8.6	30.3 ± 0.2
CO430-2	40.4 ± 24.4	48.1 ± 0.7
CO433-1	38.5 ± 30.7	48.1[c]
CO431-1	36.2 ± 10.4	47.0 ± 1.6
CO441-1	9.5 ± 18.1	53.0 ± 1.1

[a] IC_{50} ± 95% confidence interval.
[b] Total phenolic content expressed as mg of luteolin equivalents per gram of silk extract ± SEM.
[c] Estimated based on regression between phenolics and IC_{50}, Fig. 1 (not included in analysis).

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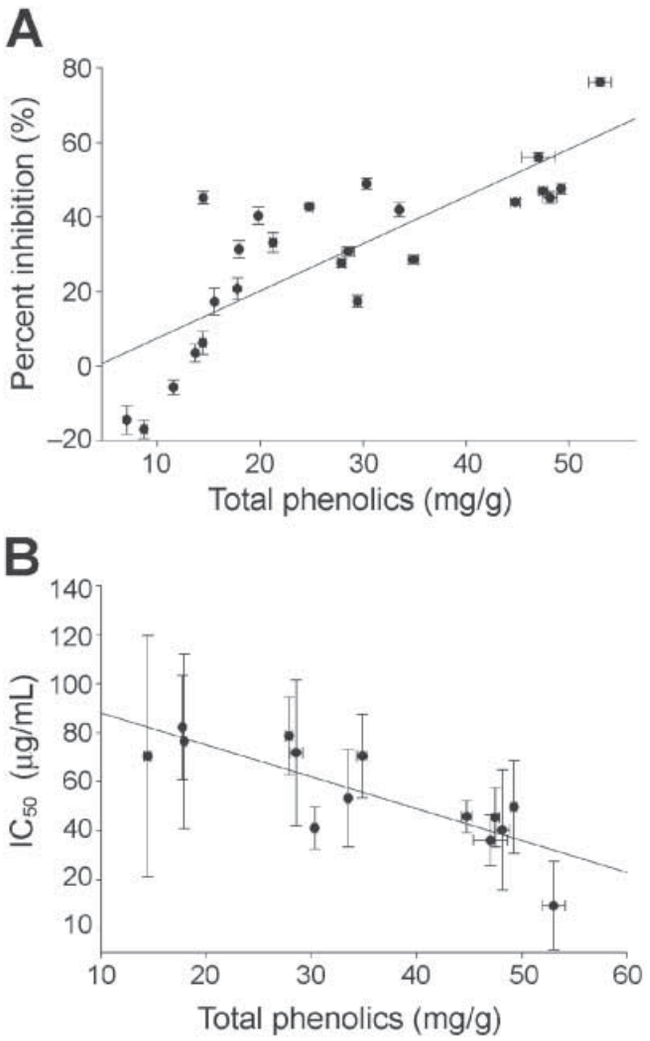

Figure 1. Antiglycation activity varies according to total phenolic content of silk extracts. (A) % inhibition versus total phenolics, $r^2 = 0.63$, $p = 0.0001$; and (B) IC_{50} versus total phenolics, $r^2 = 0.67$; $p = 0.0003$.

expressed as luteolin equivalents (Table 2). Samples from genotypes CO441, CO431, CO430, CO433, CO359, CO354, CO325 and ZC contained the highest relative concentrations of phenolics and, with the exception of ZC, also represent the nine most potent extracts, all with IC_{50}s below 55 µg/mL (Table 2). Accordingly, a clear correlation between increasing phenolic content and inhibition of glycation was observed among all samples for both inhibition at 40 µg/mL ($r^2 = 0.63$, $p = 0.0001$; Fig. 1A) and IC50 values ($r^2 = 0.67$, $p = 0.0003$; Fig. 1B).

The moderate antiglycation activity elicited by ZC was somewhat surprising considering its known abundance of maysin, a luteolin glycoside, and the high relative phenolic content (Snook *et al*., 1993). However, considering that the quantitation of total phenolics is a non-specific measurement, the identity and profile of phenolics in the inbred lines is likely distinct from those of the evolutionarily distant Mexican land race, whose phenolic compounds appear to be less potent inhibitors of glycation.

A proposed mechanism for flavonoid inhibition of glycation is free radical scavenging (Matsuda *et al*., 2003; Lou *et al*., 2001), a well-documented activity of plant phenolics. Although antioxidant activity was not evaluated in the current study, our results indirectly support this claim since total phenolics, which has repeatedly been proven a good predictor of antioxidant capacity (Prior *et al*., 1998; Wang and Liu, 2000; Moyer *et al*., 2002; Spoor *et al*., 2006), was highly correlated with antiglycation activity.

Though the modern resistant inbreds were the most effective inhibitors of glycation and the most phenolic-rich of the tested genotypes, no clear relationship between resistance to fungal infection and antiglycation activity or total phenolics was observed (Tables 1 and 2). Indeed, when all samples were included in analyses, only resistance to ear rot through kernel was significantly correlated with antiglycation activity ($r^2 = 0.159$, $p = 0.043$) (data not shown). Resistance to ear rot through silk wounds did not correlate with glycation inhibition or total phenolics. However, when analysing only those samples listed in Table 2 (the more active samples), inbreds with high resistance to common smut were generally more effective inhibitors of glycation than more susceptible inbreds ($r^2 = 0.280$, $p = 0.017$), as was the case with resistance to ear rot infection through the kernel ($r^2 = 0.240$, $p = 0.029$) (data not shown). A similar trend was observed between phenolics and susceptibility to smut ($r^2 = 0.194$, $p = 0.035$) and ear rot through kernel inoculation ($r^2 = 0.188$, $p = 0.039$), with higher phenolic content correlating with greater resistance (date not shown)

It is important to note that exposure to fungal pathogens (*F. graminearum* and/or *U. maydis*) enhances the production of active phenolic compounds in maize silk, particularly among more resistant inbreds (Reid *et al.*, 1992). Furthermore, Reid *et al.* (1992) demonstrated that phenolic levels in maize do not correlate with resistance to infection by *F. graminearum* when this fungus is not introduced. It is therefore possible that, if maize silk is not exposed to fungal spores, fewer inducible phenolics will be produced and correspondingly less antiglycation activity will be observed. The corollary of this argument is that infected maize silk will contain more total phenolics and possess greater antiglycation activity. Although plants were not inoculated in the current study, signs of infection were seen in some silk samples and may have contributed to the observed variation between plots.

While the resistance level of maize genotypes did not prove to be a reliable predictor of antiglycation activity, the modern disease resistant inbreds were consistently the best inhibitors of glycation, providing some practical advantages in terms of developing corn silk as a natural therapy for the treatment/prevention of diabetic complications. Disease resistant lines would both protect the primary product (corn) and also likely yield a more valuable silk. Further investigation is required to understand the interactions of genotype, secondary metabolism, fungal invasion and environment in corn silk.

The assay used depended solely on the formation of fluorescent AGEs such as pentosidine and argpyrimidine and therefore only measures a subset of AGEs. The fact that several maize samples displayed comparable activity to aminoguanidine, an AGE inhibitor tested in clinical trials for the treatment of diabetic complications, justifies further investigation. To gain more insight into the antiglycation capacity of corn silk extracts, inhibition of early glycation adduct formation,

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non-fluorescent AGE formation and AGE-derived crosslinking should be evaluated.

In conclusion, the majority of maize genotypes displayed some capacity to reduce non-enzymatic protein glycation, many to a similar degree as aminoguanidine. The phenolic content and pathogen resistance of corn silk samples accounted for nearly 70% and 30% of the observed variation in antiglycation activity, respectively (not additive), demonstrating that chemical ecology can have predictive power in medicinal applications. Our results also suggest that modern resistant and high phenolic maize inbreds are attractive candidates for development as a therapeutic for diabetic complications or the degenerative effects of aging. Interestingly, the land race with very high C-glycosyl flavonoid content had only moderate activity. This suggests that maysin, the active flavone in ZC, does not reduce AGE formation as efficiently as the other phenolics present in corn silk. Isolation and structural elucidation of the various active constituents is required to identify those phenolic compounds most efficacious at preventing AGE formation.

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