JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Interaction of Tannins and Other Sorghum Phenolic Compounds with Starch and Effects on in Vitro Starch Digestibility

Frederico Barros,* Joseph M. Awika, and Lloyd W. Rooney

Cereal Quality Lab, Department of Soil & Crop Sciences, Texas A&M University, College Station, Texas 77843-2474, United States

ABSTRACT: This study investigated interactions of sorghum proanthocyanidins (PAs) with starch molecules and the effect on in vitro starch digestibility. High tannin (predominant in PA), black (monomeric polyphenols), and white (low in polyphenols) sorghum phenolic extracts were mixed and cooked with starches varying in amylose content. Starch pasting properties, polyphenol profile, and resistant starch (RS) were determined. PAs decreased setback of normal starch and were least extractable after cooking with all starches. Pure amylose interacted more strongly with oligomeric and polymeric PA compared to amylopectin. The PA extract increased the net RS in normal starch by about 2 times more than the monomeric polyphenol extract; debranching amylopectin increased the difference by 4.3 times. Only treatments with PA increased RS in high amylose starch (52% higher than the control). Sorghum PAs interact strongly with starch, decreasing starch digestibility. The interactions appear to be specific to amylose and linear fragments of amylopectin, suggesting hydrophobic interactions are involved.

KEYWORDS: *sorghum, condensed tannins, resistant starch*

INTRODUCTION

Sorghum is an important food crop in semiarid parts of Africa and Asia. It is also finding increased use as an "ancient grain" and gluten free food ingredient in the United States.[1] This growth in popularity is mainly due to agronomic advantages such as high drought tolerance, high yields, low cost, and potential health benefits including slow starch digestibility, cardiovascular disease reduction, antioxidant activity, anti-inflammatory and anticarcinogenic properties.[2,3]

Special sorghum varieties are good sources of phenolic compounds such as condensed tannins (proanthocyanidins), 3-deoxyanthocyanins, and other flavonoids concentrated in the sorghum bran.[4] Condensed tannins, specifically the high molecular weight ones, have more powerful antioxidant activity in vitro and in vivo than do simple phenols and other natural antioxidants.[5,6] Other than their high antioxidant activity, tannins reduce nutrient digestibility by interacting with proteins [7] and digestive enzymes.[8,9]

Starch is the major component of cereals and the main source of calories in cereal products. Amylose is the main starch component responsible in decreasing starch digestibility (i.e., forming resistant starch).[10] Decreasing starch digestibility is important because it helps lower caloric intake, providing benefits against obesity and type 2 diabetes. Sorghum has the lowest raw starch digestibility among cereals due to strong association between the starch granules and endosperm proteins (kafirins), which restrict accessibility to starch by α-amylase.[11] Even after cooking, sorghum flour has lower starch digestibility compared to corn, due to interaction between starch and cross-linked kafirins.[12]

Other components such as polyphenols may decrease in vitro starch digestibility by inhibiting digestive enzymes[8,13] and interacting with starch. There are limited studies showing interactions between starch and phenolic compounds. Condensed tannins are bound/adsorbed by raw starch.[9,14] Small phenolic compounds including gallic acid, ferulic acid, and catechins were reported to change functional properties of starch[15−17] by interacting with starch molecules.

Poor nutrient digestibility of sorghum has been seen as a negative aspect for animal feeding.[18] Sorghum polyphenols, especially high molecular weight condensed tannins, are known to bind with proteins, severely limiting their digestibility. However, interactions with starch and effects on starch digestibility have not been demonstrated. Lowering caloric density is an advantage for human health to prevent obesity and diabetes. Thus, this study aimed to investigate the interactions of condensed tannins and other sorghum phenolic compounds with starch, specifically with amylose and amylopectin, and the effects on in vitro starch digestibility.

MATERIALS AND METHODS

Sorghum Samples and Preparation of Phenolic Extracts. Three sorghum varieties grown in College Station, TX were chosen based on their different polyphenol concentration and profiles. High tannin sorghum (high in polymeric proanthocyanidins) and two other varieties without tannins: a white food-type sorghum (low in polyphenols) and black sorghum (TX430 black, high in monomeric polyphenols) were used. Sorghum brans were obtained by decorticating 1 kg batches in a PRL mini-dehuller (Nutama Machine Company, Saskatoon, Canada) and were separated with a KICE grain cleaner (model 6DT4-1, KICE Industries Inc., Wichita, KS). The brans (approximately 10% of the original grain weight) were milled to pass through a 0.5 mm screen using a UDY cyclone mill (model 3010−030, UDY Corporation, Fort Collins, CO). They were kept at −20 °C until used.

Phenolic extracts from white, black, and high tannin sorghums were obtained by extraction of the ground bran (15 g) with 70% (v/v) aqueous acetone (900 mL) with stirring for 2 h. Extracts were then centrifuged (3100g) for 10 min, and the acetone was immediately

Received: August 6, 2012
Revised: October 27, 2012
Accepted: November 5, 2012
Published: November 5, 2012

dx.doi.org/10.1021/jf3034539 | J. Agric. Food Chem. 2012, 60, 11609−11617

removed from the supernatant under vacuum at 40 °C; the aqueous extracts were freeze-dried and stored at 4 °C until used.

Starch, Amylose, and Amylopectin. Normal corn (amylose content = 23.9% ± 1.3), waxy (amylose content = 0.36% ± 0.04), and high amylose (amylose content = 66.5% ± 2.5) starches were obtained from National Starch Food Innovation (Bridgewater, NJ). Amylose from potato and amylopectin from corn were obtained from Sigma (St. Louis, MO).

The total starch was determined using the total starch kit (AACC method 76-13), and the amylose content was determined using the amylose/amylopectin ratio kit, both from Megazyme.

Reagents. All solvents were HPLC or analytical grade. Gallic acid, catechin hydrate, Folin-Ciocalteu reagent, and ethanolamine were obtained from Sigma (St. Louis, MO).

Material Characterization. Total phenol content was measured using the modified Folin-Ciocalteu method of Kaluza et al.[19] Crude protein percentage (% nitrogen multiplied by 6.25) was determined based on a combustion method.[20] SDS−PAGE [21] was used to identify different molecular weight proteins in the sorghum phenolic extracts.

An Agilent 1100/1200 HPLC system with a diode array (wavelengths of 280, 330, 360, and 480 nm) and fluorescence detectors (excitation wavelength of 230 nm and emission at 321 nm) (Agilent Technologies, Santa Clara, CA) was used to identify and quantify polyphenols. The method described by Awika et al. [2] was used with modifications to identify and quantify phenolic acids and anthocyanins in the samples. A reversed phase 150 × 2.00 mm, 5 μm, C-18 column (Phenomenex, Torrance, CA) was used. The freeze-dried phenolic extracts were dissolved in methanol, filtered, and then injected in the column. HPLC conditions were as follows: injection volume, 10.0 μL; flow rate, 1.0 mL/min. The mobile phase consisted of (A) 2% formic acid in water and (B) 2% formic acid in acetonitrile. The 43 min elution gradient for (B) was as follows: 0−3 min, 10% isocratic; 3−4 min, 10−12%; 4−5 min, 12% isocratic; 5−8 min, 12−18%; 8−10 min, 18% isocratic; 10−12 min, 18−19%; 12−14 min, 19% isocratic; 14−18 min, 19−21%; 18−22 min, 21−26%; 22−28 min, 26−28%; 28−32 min, 28−40%; 32−34 min, 40−60%; 34−36 min, 60% isocratic; 36−38 min, 60−10%; 38−43 min, 10% isocratic.

A normal-phase HPLC method described by Langer et al.[22] was used to separate proanthocyanidins based on the degree of polymerization (DP) in the tannin sorghum phenolic extract. The column was a Develosil Diol (250 mm × 4.6 mm, 5 μm particle size; Phenomenex, U.K.).

Starch Pasting Properties. A Rapid Visco Analyzer (RVA) was used in order to investigate the effects of sorghum phenolic extracts on starch pasting properties. Distilled water was added to normal corn starch (3.0 g, dry basis) and freeze-dried sorghum phenolic extracts at four levels (0%, 5%, 10%, and 20% starch basis) in the RVA canister to obtain a total constant sample weight of 28 g. The slurry was manually homogenized to prevent lump formation and the pH was recorded with a portable pH meter (model Russel RL060P, Thermo Scientific, Beverly, MA) before the RVA run. Pasting properties of corn starch and mixtures with freeze-dried sorghum phenolic extracts were determined using a Rapid Visco-Analyzer (RVA model 4, Newport Scientific PTY Ltd., Warriewood, Australia).

The temperature profile used was the RVA Standard 2 provided by the instrument manufacturer. There was a sample equilibration at 50 °C for 1 min followed by a linear temperature increase from 50−95 °C in 7.5 min, and then a holding step at 95 °C for 5 min, cooling to 50 °C within 7.5 min, and another holding step at 50 °C for 2 min, for a total of 23 min. The viscosities were reported in rapid visco units (RVU). Peak time (min), peak viscosity (RVU), final viscosity (RVU), breakdown (RVU), and setback (RVU) were determined using Thermocline version 2.2 (Newport Scientific PTY Ltd., Warriewood, Australia). Pastes obtained from the RVA were immediately frozen in liquid nitrogen, kept at −50 °C, and then freeze-dried. The freeze-dried material was stored at 4 °C. The control (freeze-dried sorghum phenolic extracts in water without starch) was included to determine heat sensitivity of the sorghum phenolic extracts to RVA cooking.

Interactions of Sorghum Polyphenols with Amylose/Amylopectin. In order to demonstrate the interactions between starch molecules and sorghum polyphenols, changes in the phenol content and concentration of different molecular weight proanthocyanidins before/after cooking were evaluated. Pure amylose, pure amylopectin, waxy, normal, and high amylose starches (10% w/v in distilled water) were mixed with freeze-dried sorghum phenolic extracts (10% starch basis) in a shaker for 1 h. The mixtures were frozen in liquid nitrogen and freeze-dried. The freeze-dried material was mixed with 1% HCl in methanol and analyzed for total phenol content (phenol concentration in the supernatant before cooking). In addition, the freeze-dried material containing tannin sorghum phenolic extracts (0.2 g) was mixed with methanol (10 mL), and the supernatant was filtered through a 0.45 μm membrane and injected (20 μL) in the HPLC to determine the concentration of proanthocyanidins and their molecular weight profile (DP).

Waxy and normal starches (10% w/v in distilled water) were mixed with freeze-dried sorghum phenolic extracts (10% starch basis) in a shaker for 1 h. Then, samples were cooked at 95 °C for 20 min to gelatinize the starches, frozen and freeze-dried. The same procedure was followed using high amylose starch (10% w/v in distilled water) mixed with sorghum phenolic extracts (10% starch basis). In order to gelatinize the high amylose starch, the mixture was cooked in an autoclave (121 °C for 30 min). The control (freeze-dried sorghum phenolic extracts in water, 3 mg/mL, without starch) was included for both cooking treatments. The total phenol assay and HPLC analysis for tannins were done in the freeze-dried material as described above (before cooking). For the control, 5 μL was injected in the HPLC.

In Vitro Starch Digestibility. The resistant starch (RS) content of freeze-dried samples from RVA was directly measured using the assay kit from Megazyme (AACC method 32-40).

In order to better understand the effects of the polyphenol−starch interactions on in vitro starch digestibility, normal, waxy and high-amylose starches (25% w/v in distilled water) were cooked with sorghum phenolic extracts (10% starch basis) in an autoclave at 121 °C for 30 min, cooled at room temperature and then stored at 4 °C overnight. This was repeated 2 more times (3 heating/cooling cycles) and the samples were freeze-dried and RS content determined. Compared to RVA, this method will produce more RS because of the drastic heating conditions (more amylose and amylopectin in solution) and cooling at 4 °C [the optimum temperature for starch (mainly amylose) retrogradation].

Furthermore, normal starch was pretreated with isoamylase (Catalogue No. E-ISAMY, 1000 units; Megazyme), and the hydrolyzed material was subjected to 3 heating/cooling cycles as described above. Debranching of amylopectin by the action of isoamylase will produce more linear molecules and will help to understand possible interactions between linear molecules and sorghum tannins and their effects on RS formation. The pH of a slurry of normal starch at a concentration of 5% (w/v) in distilled water was adjusted to 4.5, instantly heated to 70 °C for gelatinization, and quickly reduced to 45 °C within 1 min. Isoamylase at 1% (based on starch weight) was added and hydrolysis took place for 24 h. Then, the enzyme was deactivated (boiling temperature), and the sorghum phenolic extracts were added (10% starch basis). Samples were subjected to 3 heating/cooling cycles, as described above, in an autoclave (121 °C for 30 min) and then freeze-dried. The RS content was determined in the freeze-dried material.

Statistical Analysis. Data were analyzed using a one-way analysis of variance (ANOVA) to determine significant differences among them. Fisher's least significant difference (LSD) ($P \leq 0.05$) was used to compare multiple means. The software used was SPSS version 16.0 for windows (SPSS Inc., Chicago, IL). All tests were done in three replications.

RESULTS AND DISCUSSION

Properties of Sorghum Phenolic Extracts. White, black, and high-tannin sorghum freeze-dried phenolic extracts had yields of 4, 12, and 11% respectively based on bran weight. Phenol content (mg GAE/g) of the sorghum phenolic extracts

Table 1. Proanthocyanidin Content[a] of Tannin Sorghum Phenolic Extract (TSPE)[b]

DP[c]	TSPE	TSPE after cooking (95 °C for 20 min)	TSPE after cooking (121 °C for 30 min)	TSPE + NS before cooking	TSPE + NS after cooking	TSPE + amylopectin	TSPE + amylose
1	0.25 ± 0.01	0.78 ± 0.06	3.37 ± 0.18	nd	0.05 ± 0.0	nd	nd
2	0.58 ± 0.03	1.74 ± 0.09	3.43 ± 0.29	0.031 ± 0.0	nd	nd	nd
3	1.43 ± 0.10	1.88 ± 0.11	nd[e]	0.12 ± 0.01	nd	0.07 ± 0.01	0.045 ± 0.0
4	2.21 ± 0.09	3.67 ± 0.26	nd	0.17 ± 0.02	nd	0.094 ± 0.01	0.069 ± 0.0
5	2.64 ± 0.11	3.96 ± 0.11	nd	0.24 ± 0.08	nd	0.12 ± 0.0	0.076 ± 0.0
6	4.67 ± 0.08	5.57 ± 0.47	nd	0.50 ± 0.03	nd	0.20 ± 0.01	0.12 ± 0.01
7	4.74 ± 0.35	5.16 ± 0.33	nd	0.54 ± 0.05	nd	0.22 ± 0.0	0.12 ± 0.01
8	5.13 ± 0.24	4.73 ± 0.37	nd	0.53 ± 0.02	nd	0.27 ± 0.02	nd
9	4.62 ± 0.32	4.18 ± 0.39	nd	0.46 ± 0.04	nd	0.20 ± 0.01	nd
10	3.96 ± 0.18	nd	nd	0.38 ± 0.04	nd	0.19 ± 0.01	nd
P[d]	98.90 ± 4.30	91.10 ± 6.80	35.62 ± 1.60	10.60 ± 0.94	0.52 ± 0.03	3.63 ± 0.23	1.32 ± 0.10
Total	129.10 ± 5.81	122.80 ± 9.0	42.40 ± 2.07	13.60 ± 1.20	0.57 ± 0.03	5.0 ± 0.30	1.75 ± 0.13

[a]The values are expressed as mg/g, expressed in the catechin equivalent (corrected by molecular weight). [b]These values were obtained: before and after cooking at 95 °C for 20 min and at 121 °C for 30 min; mixed with normal starch (NS) before and after cooking (95 °C for 20 min); and after mixing with pure amylose and amylopectin. Values are means ± standard deviation. [c]Given as the degree of polymerization. [d]A mixture of polymers with DP > 10. [e]Not detected.

were 438.0 ± 25.4 (tannin), 366.0 ± 16.1 (black), and 48.1 ± 3.5 (white).

Starch was not detected in the sorghum freeze-dried phenolic extracts. About 50 mg/g crude protein was detected in the sorghum freeze-dried phenolic extracts. Since acetone was used in the phenolic extractions, these proteins were not expected to interact with tannins. Acetone inhibits formation of tannin−protein complexes [23] by precipitating high molecular weight proteins, which are the ones that have high affinities for tannins.[24] Proteins with molecular weights less than 20 000 have low affinities for tannins.[24] SDS−PAGE showed that there were only small molecular weight (below 10 000) proteins present in the phenolic extracts (data not shown).

Phenolic acids such as caffeic and ferulic acid were previously identified in sorghum.[25,26] In this work, phenolic acids were identified (data not shown) by HPLC in the white, black, and high tannin sorghum phenolic extracts. The major 3-deoxyanthocyanins (luteolinidin and apigeninidin) were not detected in white and tannin sorghum phenolic extracts but were the major polyphenols in black sorghum phenolic extracts (data not shown), which agrees with previous findings.[2,26] The tannin sorghum phenolic extract contained mostly proanthocyanidins (129 mg/g), with a high ratio (77%) of polymeric (DP >10) proanthocyanidins (Table 1 and Figure 1A) as previously reported.[27]

Effect of Sorghum Phenolic Extracts on Starch Pasting Properties. Black and tannin sorghum phenolic extracts significantly ($P \leq 0.05$) affected normal starch pasting properties, and the effect was dependent on phenolic extract concentrations (Table 2). Peak viscosity was higher ($P \leq 0.05$) for black and tannin treatments (376.3−393.4 RVU) at all levels compared to the control (353.5 RVU) (Table 2). Viscosity values increased as phenolic extract concentration increased. The same trend was observed for peak time which ranged from 8.3 to 8.8 min compared to 8.1 min for the control. Above 10%, black sorghum phenolic extract had a slightly higher peak time than that of the other treatments. There were no significant ($P > 0.05$) differences in pH among the treatments (Table 2).

As the concentration of sorghum phenolic extracts increased, the starch−phenolic extract mixture had higher solids content which may have affected the RVA parameters mentioned before. However, white sorghum phenolic extracts mixed at all levels with starch did not differ ($P > 0.05$) from the control in the peak viscosity and peak time (Table 2), implying that the solid content was not the major contributor. As previously mentioned, black and tannin sorghum phenolic extracts had the highest concentration of phenols (about 10 times more than the white sorghum phenolic extract). Thus, changes observed in starch pasting properties when black and tannin sorghum freeze-dried phenolic extracts were mixed with starch and cooked could be due either to the presence of more phenols in solution which compete for water with starch for hydration[16] or to possible interactions of black and tannin sorghum polyphenols with starch.

During cooling, the final viscosity increased as black and white sorghum phenolic extract concentrations increased (Table 2). The same trend was not observed for tannin sorghum phenolic extracts which had a similar final viscosity to control. Setback increased as white and black sorghum phenolic extract concentrations increased; however, it tended to decrease as the concentration of tannin sorghum phenolic extract increased (Table 2). This suggests some interaction of tannins with leached amylose, which may help retard starch retrogradation. The evidence indicates that low molecular weight polyphenols (in white and black sorghum) and the proanthocyanidins (in tannin sorghum) interact with starch via different mechanisms.

There are a few reports on the effect of polyphenols on starch properties. Tea catechins were shown to interact with rice starch and retard its retrogradation.[15] Zhu et al.[16] demonstrated that a diverse set of phenolic compounds changed wheat starch functional properties; they suggested that the changes were due to possible alteration of solution pH by the polyphenols as well as hydrogen bonding. In this study, the sorghum phenolic extracts did not affect solution pH, thus the observed differences are mostly attributed to their phenolic composition.

Interactions between Sorghum Polyphenols and Amylose/Amylopectin. *Changes in Phenol Content of Starch−Phenolic Extract Mixtures.* The phenolic content of freeze-dried extracts before RVA cooking, as previously mentioned, was higher for tannin sorghum phenolic extracts (438 mg GAE/g) than for either black (366 mg GAE/g) or

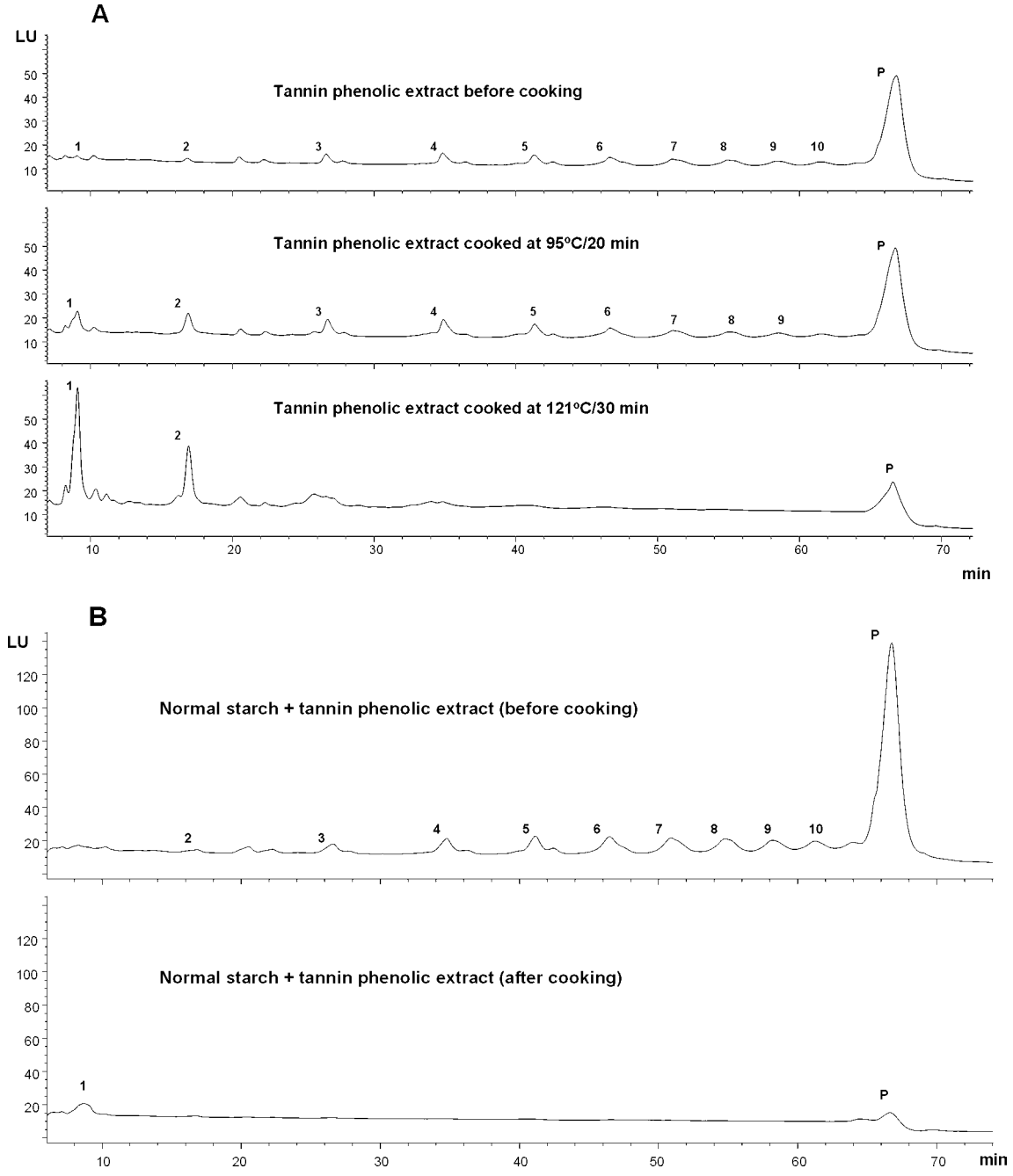

Figure 1. Normal phase HPLC procyanidin profiles before and after cooking (95 °C for 20 min and 121 °C for 30 min) of (A) tannin sorghum phenolic extract without starch (control), and the profiles before and after cooking (95 °C/20 min) of (B) tannin sorghum phenolic extracts mixed with normal starch. Numbers on peaks denote degree of polymerization. *P* = polymers with DP >10.

white (48.1 mg GAE/g). However, after RVA cooking, treatments with the black sorghum phenolic extract had significantly ($P \leq 0.05$) higher phenol content at all levels (Table 2). The evidence indicates that sorghum proanthocyanidins may be interacting with the starch molecules (forming insoluble complexes) to a greater extent than the simple phenolics in the black sorghum phenolic extracts. This may partly explain the observed differences in RVA pasting properties of the tannin sorghum phenolic extract compared to the black sorghum phenolic extract. There were no significant ($P > 0.05$) differences in the phenol content of the control (freeze-dried phenolic extracts cooked without starch) before and after RVA cooking (data not shown).

In order to completely gelatinize starch and investigate specific interactions of amylose and amylopectin with sorghum polyphenols, mixtures of waxy and normal starch with phenolic extracts were cooked at 95 °C for 20 min, and the mixture with high amylose starch was cooked at 121 °C for 30 min. Before cooking, adsorption of sorghum polyphenols to raw starch, the difference between added (10% of the phenol content of the sorghum freeze-dried phenolic extracts) and extractable polyphenols (Table 3), was significantly higher for the tannin sorghum phenolic extract (20.5−36.4%) than either the black sorghum phenolic extract (10.8−16.2%) or the white sorghum phenolic extract (4.1−10.4%) (Table 3). Corn starch (and other cereal starches) contains large surface pores (up to 1 μm

dx.doi.org/10.1021/jf3034539 | J. Agric. Food Chem. 2012, 60, 11609−11617

Table 2. Effect of Sorghum Phenolic Extracts on Normal Starch Pasting Properties[a]

		pasting properties				
treatments	phenol content[b]	pH	peak viscosity (RVU)	peak time (min)	final viscosity (RVU)	breakdown (RVU)	setback (RVU)
control (corn starch)	–	5.6 a	353.5 ab	8.1 ab	347.5 ab	168 a	162 bc
white (5%)	0.87 a	5.7 a	338.4 a	8.1 ab	334.0 a	160.2 a	155.8 b
white (10%)	2.5 bc	5.5 a	337.2 a	8.0 a	348.0 ab	168.4 a	179.2 d
white (20%)	4.55 d	5.6 a	346.9 ab	8.2 a	429.0 d	157.3 a	239.4 e
black (5%)	2.61 c	5.6 a	376.7 bc	8.5 c	368.2 b	175.3 a	166.9 b
black (10%)	5.82 e	5.5 a	378.5 c	8.7 d	382.4 c	169.7 a	173.6 cd
black (20%)	14.2 g	5.5 a	392.3 d	8.8 d	424.8 d	158.2 a	190.6 d
high tannin (5%)	2.1 b	5.5 a	376.3 bc	8.3 bc	347.7 ab	180.2 a	151.6 ab
high tannin (10%)	4.7 d	5.6 a	379.4 c	8.3 bc	345.4 ab	178.3 a	144.2 ab
high tannin (20%)	8.9 f	5.5 a	393.4 d	8.6 c	369.0 b	160.8 a	136.4 a

[a]Means followed by the same letter in a column are not significantly different ($P \leq 0.05$). [b]Measured after cooking (mg GAE/g).

Table 3. Total Phenol Content (mg GAE/g) Before and After Cooking of Sorghum Phenolic Extracts (10% Starch Basis) Mixed with Waxy, Normal, and High Amylose Starches[a]

	sorghum phenolic extracts					
	white		black		high tannin	
	before cooking	after cooking	before cooking	after cooking	before cooking	after cooking
controls						
control 1 (95 °C for 20 min)	48.0 a	48.1 a	369.0 b	364.9 b	442.3 c	423.1 c
control 2 (121 °C for 30 min)	48.0 a	47.4 a	369.0 c	351.8 b	442.3 e	381.9 d
cooking 1 (95 °C for 20 min)						
waxy starch	4.6 b	3.4 a	32.8 d	8.9 c	27.7 d	4.1 ab
normal starch	4.3 b	2.9 a	30.7 d	9.4 c	35.0 d	4.2 b
cooking 2 (121 °C for 30 min)						
high amylose starch	4.6 b	3.1 a	31.4 d	8.7 c	33.9 d	4.5 b

[a]Means followed by the same letter in a row are not significantly different ($P \leq 0.05$).

diameter),[28] which are likely sites for poyphenol adsorption into the intact granule. The larger tannin molecules are more likely to be physically trapped within the pores and thus become 'unextractable' compared to the smaller polyphenols. Additionally, hydrogen bonding is likely to increase the stability of the polyphenols within the starch granule.

Previous research has demonstrated that 40−60% condensed tannins are adsorbed on raw starches, and this adsorption was dependent on the starch surface area with higher surface area having the highest condensed tannins adsorbed.[9] Bourvellec at al.[14] suggested that due to presence of pores containing amylose chains on raw starch granules, condensed tannins would not only be adsorbed on the starch surface but could interact with amylose forming inclusion complexes.

There was a large decrease in extractable phenols after cooking for all treatments (Table 3). This difference was the highest for tannin sorghum treatments which had a further average decrease in extractable phenols of 87% after cooking. The drop in extractable phenol content was around 70% for black sorghum treatments and 30% for white sorghum treatments (Table 3). The result agrees with the RVA data, where lower extractable phenols were present in the tannin sorghum phenolic extract compared to black sorghum phenolic extract treatments after cooking. There was no significant ($P > 0.05$) difference in the phenol content of the control (freeze-dried phenolic extracts cooked without starch) before and after cooking at 95 °C for 20 min (Table 3). However, there was a slight but significant ($P \leq 0.05$) decrease in the phenol content of control after cooking at 121 °C for 30 min (Table 3).

The large changes in the extractable phenols after cooking indicate that condensed tannins and the simple phenolic compounds in sorghum chemically interact with gelatinized amylose and amylopectin molecules. The increased swelling and opening of amylose and amylopectin chains likely enabled the polyphenols to bind to specific sites on the molecules via hydrogen bonds and, likely, hydrophobic interactions. The hydrophobic interactions are likely for 3-deoxyanthocyanins and sorghum proanthocyanidins, which tend to be less polar than their analogs from fruits and vegetables. The chemical interactions were apparently strongest for the proanthocyanidin-containing phenolic extracts. The larger molecular weight proanthocyanidins provide more hydroxyl groups for hydrogen bonding and also contain more hydrophobic domains that would promote stronger interactions with gelatinized starch.

Changes in Molecular Weight Profile Proanthocyanidins Cooked with Starch. To better understand the possible interactions of condensed tannins with starch molecules, the treatments with the tannin sorghum phenolic extract were profiled using normal-phase HPLC. The controls (tannin sorghum phenolic extracts without starch) had a significant ($P \leq 0.05$) increase in catechins (monomers) and dimers after cooking at 95 °C for 20 min, and even more so at 121 °C for 30 min (Table 1 and Figure 1A). The concentration of monomers and dimers after cooking at 95 °C for 20 min increased from 0.25 to 0.78 mg/g and from 0.58 to 1.74 mg CE/g, respectively (Table 1). The concentration of monomers increased more than 10 times from 0.25 to 3.4 mg/g, and the dimers increased from 0.58 to 3.4 mg CE/g after cooking at 121 °C for 30 min (Table 1). In addition, the oligomeric proanthocyanidins up to

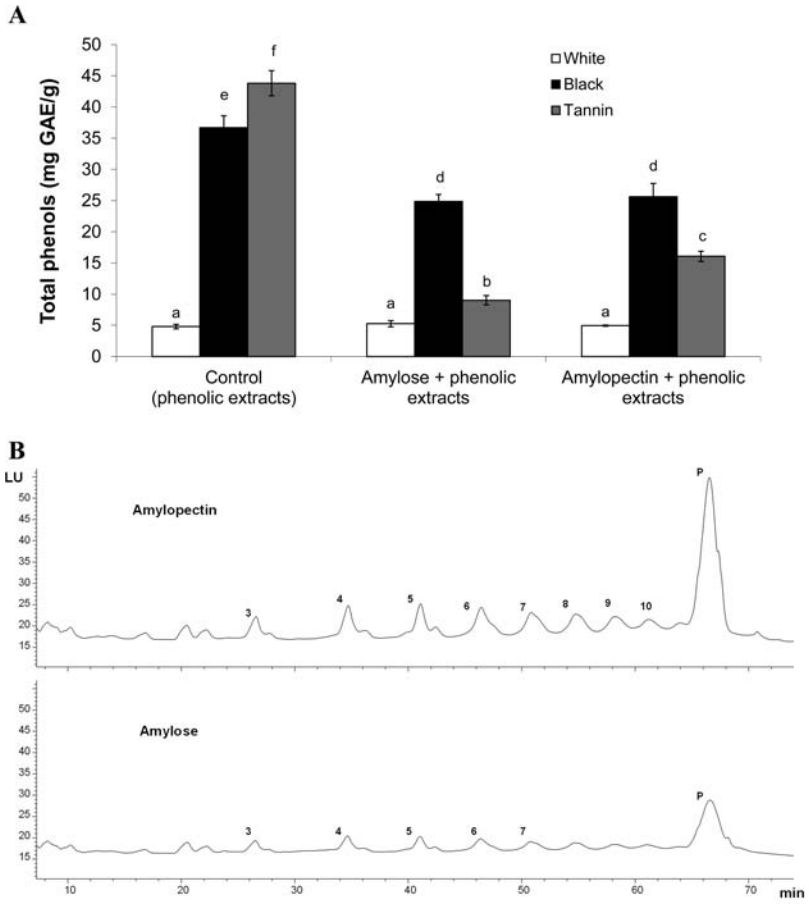

Figure 2. (A) Extractable phenol content (mg GAE/g) of sorghum phenolic extracts mixed with amylose and amylopectin. (B) Normal-phase HPLC procyanidin profiles of tannin sorghum phenolic extract mixed with amylose and amylopectin. Errors bars indicate ± standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$). Numbers on peaks denote degree of polymerization. *P* = polymers with DP >10.

DP 6 increased in the phenolic extract cooked at 95 °C for 20 min. This indicates that even relatively mild heat treatment induces significant depolymerization of condensed tannins. The concentration of polymeric tannins decreased upon cooking at 95 °C for 20 min (from 98.9 to 91.1 mg CE/g) and after autoclave cooking (from 98.9 to 35.6 mg CE/g) due to depolymerization and thermal degradation. Thermal-induced depolymerization of sorghum tannins after severe heat treatment was previously demonstrated.[27] The heat-induced depolymerization of tannins into monomers and dimers observed in this study may increase bioavailability of sorghum tannins.[29]

After cooking (95 °C for 20 min) normal starch with tannin sorghum phenolic extracts, there was a decrease in the oligomeric and polymeric tannins to mostly undetectable levels (Table 1 and Figure 1B) which indicated that almost all of the sorghum condensed tannins (oligomers and polymers) interacted with amylose/amylopectin in solution. Moreover, the appearance of the monomeric (catechin) peak was observed (Table 1 and Figure 1B). The same trend was observed for treatment with waxy starch (cooked at 95 °C for 20 min) and high amylose starch (cooked at 121 °C for 30 min). However, autoclaved treatment produced much higher levels of monomeric catechin (200 μg/g compared to cooking at 95 °C for 20 min, 50 μg/g). Thus, it is likely that during thermal treatment, depolymerization of 'free' proanthocyanidins proceeds simultaneously with their chemical interactions with gelatinized starch to form insoluble complexes. The fact that monomeric forms and very small amounts of polymeric proanthocyanidins were detectable in the cooked mixtures indicates that oligomers and polymers are mostly strongly involved in tannin−starch interactions. Thus, like proteins, starch may be interacting with the proanthocyanidins through hydrogen bonding,[24,30] as well as hydrophobic interactions, as previously mentioned.

Reaction of Pure Amylose and Amylopectin with Sorghum Phenolic Extracts. To further investigate the relative interactions of sorghum polyphenols with amylose and amylopectin, pure amylose and pure amylopectin were mixed with sorghum phenolic extracts at room temperature. There was no significant ($P > 0.05$) difference between extractable phenol content of white or black sorghum phenolic extracts mixed with amylopectin or amylose (Figure 2A). However, phenol concentration of tannin sorghum phenolic extracts mixed with amylose was significantly ($P \leq 0.05$) lower (9.0 mg GAE/g) than when mixed with amylopectin (16.1 mg GAE/g) (Figure 2A). In addition, compared to the starting material, the decrease in extractable phenol content was more dramatic for the tannin sorghum phenolic extract treatments than the black (or white) sorghum phenolic extract. Furthermore, the concentration of different molecular weight proanthocyanidins (oligomers and polymers) were significantly ($P \leq 0.05$) lower in the presence of amylose compared to amylopectin (Table 1 and Figure 2B).

The fact that only the sample with proanthocyanidins interacted more strongly with amylose compared to amylopectin suggests that the linear nature of amylose and the structure of sorghum proanthocyanidin polymers afford a more optimum configuration for stronger bond formation between starch and polyphenols in solution. This suggests that hydrophobic interactions are a major contributor to the tannin–starch interactions, as has been demonstrated for proteins.[31] The physical conformation of the polymeric proanthocyanidins provides more hydrophobic sites than is possible with the monomeric polyphenols, while the linear nature of amylose makes its hydrophobic core more accessible in solution compared to amylopectin. While the amylopectin side chains not involved in the double helix structure also provide limited hydrophobic sites, steric hindrance would likely interfere with its ability to efficiently interact with the polymeric tannins. Thus, a portion of unextractable polymeric proanthocyanidins might be physically trapped within the bulky amylopectin matrix without necessarily chemically interacting with the starch. Obviously steric hindrance would be less of an issue for the monomeric polyphenols, which explains why black and white sorghum phenolic extract polyphenols bound similarly to amylose and amylopectin. The hydrophobic interactions with amylose is likely to favor larger proanthocyanidin molecules; this was observed in this study (Figure 2B), which demonstrated that as proanthocyanidin DP increased, its apparent binding efficiency with amylose also increased (i.e., extractability decreased). Thus, this work demonstrates for the first time, albeit indirectly, specific DP-dependent proanthocyanidin–starch interactions.

In Vitro Starch Digestibility. *Effect of Cooking on Resistant Starch Content of Starch–Polyphenol Mixtures.* Sorghum tannin phenolic extracts significantly increased resistant starch content of normal starch cooked in RVA (Figure 3). There were no significant ($P > 0.05$) differences between the control and white sorghum phenolic extract treatment. At the 5% level, tannin sorghum phenolic extracts had almost double (5.5%) the RS content compared to the control (2.9%), whereas no significant ($P > 0.05$) difference was observed for the black sorghum phenolic extracts (Figure 3). At the 10% level, tannin sorghum phenolic extracts still had the highest RS content (8.3%), and black sorghum phenolic extracts had 5.6%. At the 20% level, both tannin and black sorghum phenolic extracts had the same effect on resistant starch formation; the RS content was about 8.5% for both treatments (Figure 3).

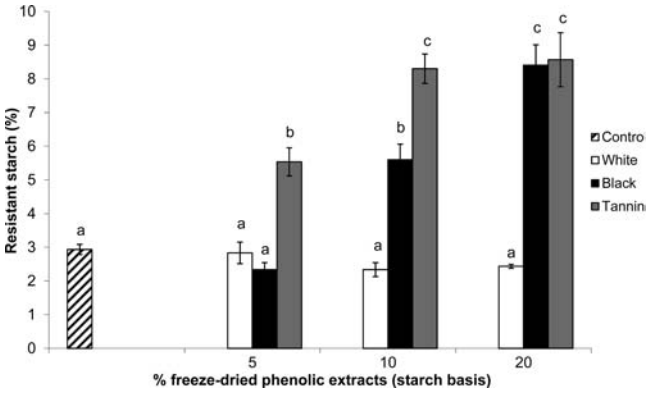

Figure 3. Effect of sorghum phenolic extracts on resistant starch content of normal starch cooked in a RVA. Errors bars indicate ± standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$).

As previously mentioned in this study, black sorghum phenolic extracts cooked with starch in RVA had significantly smaller reduction in extractable phenols compared to the tannin sorghum phenolic extract treatments and still presented less RS content up to the 10% level compared to the tannin sorghum phenolic extract treatment. This shows that the amount of extractable phenols in solution was not the most important cause of an increase in RS.

Davis and Hoseney[9] reported that condensed tannins can be adsorbed on raw starch and act as α-amylase inhibitors. Recently, Hargrove et al.[8] demonstrated that both tannin and black sorghum (without tannins) phenolic extracts inhibited α-amylase, and this inhibition increased as concentration of phenolic extracts increased. Tannin sorghum phenolic extracts inhibited the enzyme more strongly than the black sorghum phenolic extracts; however, as concentration increased, the inhibition of both phenolic extracts became similar. This may explain the higher increase in RS when high concentration of black sorghum phenolic extract was used (20% level).

Thus, it was observed that condensed tannins played a greater role in the formation of RS compared to 3-deoxyanthocyanins and other simple phenols in sorghum. This is partly explained by the stronger interactions of polymeric proanthocyanidins with starch molecules observed in this study.

Effect of Cooking–Cooling Cycles on Resistant Starch Content of Starch–Phenol Mixtures. Multiple heating/cooling treatments are known to increase RS content in foods.[32] The goal was to use this technique to enhance retrogradation of amylose and optimize RS formation and to investigate how amylose content affects interaction with sorghum polyphenols and formation of RS. This helped to better understand the effect of interactions between the tannin and amylose (linear molecule) on RS formation.

Tannin sorghum phenolic extract significantly increased resistant starch content of normal and high amylose starches (Figure 4). The control and treatment containing white sorghum phenolic extract did not differ ($P > 0.05$) in RS content when normal starch was used (Figure 4). Their RS

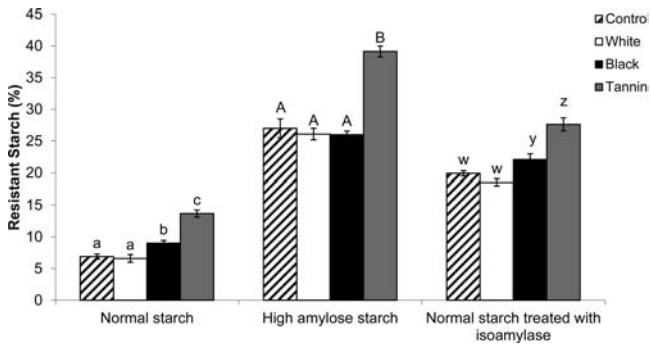

Figure 4. Effect of sorghum phenolic extracts (10% starch basis) on resistant starch content of normal starch (with and without treatment with isoamylase) and high amylose starch cooked in an autoclave (121 °C for 30 min) and cooled (4 °C) overnight (3 heating/cooling cycles). Errors bars indicate ± standard deviation. Means followed by the same letter within treatment are not significantly different ($P \leq 0.05$).

content was around 6.7%, which is more than twice higher than controlled cooking in the RVA (2.9%). Treatment containing black sorghum phenolic extracts had an RS content around 9% (Figure 4), compared to 5.6% when it was cooked in the RVA. The highest value of RS (13.7%) was obtained with tannin sorghum phenolic extract treatment (Figure 4); this value was around 8.4% when cooked in the RVA.

RS content reached over 40% when tannin sorghum phenolic extract was cooked with high amylose starch, whereas there were no significant ($P > 0.05$) differences among control and treatments containing white and black sorghum phenolic extracts (RS content around 26%) (Figure 4). This further supports the theory that hydrophobic interactions are dominant in explaining polyphenol−starch interactions. Both amylose and polymeric proanthocyanidins from sorghum have relatively strong hydrophobic regions which are more readily exposed during heat treatment, allowing for more efficient interactions. Repeated heating−cooling cycles allows for further alignment of these regions, and thus an increase in formation of RS over and beyond that observed from the amylose−amylose interaction.

RS content below 1% was observed (data not shown) when waxy starch was cooked with all sorghum phenolic extracts. This suggests that enzyme inhibition does not play a role in the RS formation observed in this study. Interaction between sorghum tannins and amylose during cooking was the main reason for the observed increase in RS.

Effect of Isoamylase Pretreatment of Normal Starch on Resistant Starch Formation. In order to understand the effect of amylopectin debranching on the polyphenol−starch interaction and RS formation, normal starch was treated with isoamylase and the autoclave heating−cooling cycle treatment as previously described. Isoamylase was to produce linear chains from amylopectin. The product of this debranching process is a starch solution with long (amylose) and short (from amylopectin branches) linear molecules. The highest RS content (28.6%) was obtained when tannin sorghum phenolic extract was cooked with the debranched starch (Figure 4). There was no significant ($P > 0.05$) difference between the control and treatment with white sorghum phenolic extract (RS around 20%) (Figure 4). Treatment with black sorghum phenolic extract had an RS content of 22% (Figure 4).

To reconcile all the RS data, it is important to consider 'net RS' formation in presence of the sorghum phenolic constituents, i.e., RS formation beyond those observed for corresponding controls (RS treatment − RS control expressed as mg/g). Interestingly, the net formation of RS attributable to the monomeric polyphenols in black sorghum versus polymeric polyphenol-containing tannin sorghum followed different trends. In black sorghum treatments (10% starch basis), the net RS was 27 mg/g in RVA-cooked normal starch versus 23 mg/g in the heating−cooling cycle-treated normal starch (Figures 3 and 4). When the isoamylase treatment was added to the heating−cooling cycle, the net RS formation declined modestly to 20 mg/g (Figure 4). By contrast, the tannin sorghum phenolic extract (10% starch basis) produced a net RS of 55 mg/g in RVA-cooked normal starch (Figure 3). In the heating−cooling cycle-treated normal starch, the net RS formation increased to 70 mg/g (Figure 4); debranching treatment further increased the RS formation to 86 mg/g (Figure 4).

As previously explained, repeated heating−cooling cycles of starch will favor increased RS formation, attributed largely to an increased amylose crystallinity due to double helical crystallite formation.[33] The polymeric condensed tannins may likely form complexes with the single helical amorphous regions of amylose, stabilized by hydrophobic and hydrogen bonding. Repeated heating−cooling would improve alignment of the starch−tannin hydrophobic regions and thus increase the formation of such complexes. The starch−tannin-complexed regions would obviously be resistant to enzyme attack. The fact that net RS formation decreased for monomeric polyphenol starch in RVA treatment compared to the autoclave−cooling cycle treatment suggests that the simple polyphenols probably complex with starch primarily via hydrogen bonds which can be disrupted by a high heat treatment.[31] Furthermore, the fact that debranching only increased the net RS in the tannin sorghum treatments further demonstrates the involvement of linear starch molecules in starch−tannin interactions.

Another interesting observation which confirms the different specific interaction of the polymeric sorghum tannins (as opposed to the monomeric ones) with amylose was a large increase in net RS formation observed for high amylose starch treated with a tannin sorghum phenolic extract (140 mg/g) (Figure 4). This is in sharp contrast to no net RS formation in the presence of monomeric polyphenol extracts (Figure 4). Increasing amylose content increased the available amorphous hydrophobic domains to which the polymeric tannins could complex. Thus, it is apparent from the data that the polymeric sorghum tannins are more likely to increase the RS content of starch than the monomeric polyphenols, probably due to the added advantage of strong hydrophobic interactions with starch, not possible for the monomeric molecules.

In conclusion, this is the first study that demonstrates specific interactions between condensed tannins and starch molecules (amylose and amylopectin). Sorghum condensed tannins are more effective in interacting with amylose possibly through hydrophobic and hydrogen bonding, significantly increasing the RS content of normal and high amylose starches compared to that of monomeric sorghum polyphenols, such as phenolic acids and 3-deoxyanthocyanins. Thus, high molecular weight polyphenols may provide new opportunities to produce functional food ingredients that reduce caloric density of starch-containing products while providing added health benefits.

AUTHOR INFORMATION

Corresponding Author

*Address: Cereal Quality Lab, Department of Soil & Crop Sciences, Texas A&M University, 370 Olsen Blvd., TAMU 2474, College Station, TX 77843-2474, USA. E-mail: fredbarros@tamu.edu. Tel: (979) 845-3838. Fax: (979) 845-0456.

Funding

We thank Texas AgriLife Research (a part of the Texas A&M System), USCP (United Sorghum Checkoff Program), and USAID INTSORMIL CRSP for partial financial support.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge the suppliers for raw materials.

ABBREVIATIONS USED

CE, catechin equivalent; DP, degree of polymerization; GAE, gallic acid equivalent; RS, resistant starch; RVA, Rapid Visco-Analyzer; RVU, Rapid Visco units

REFERENCES

(1) Asif, M.; Rooney, L. W.; Acosta-Sanchez, D.; Mack, C. A.; Riaz, M. N. Uses of sorghum grain in gluten-free products. *Cereal Foods World* **2010**, *55*, 285−291.

(2) Awika, J. M.; Yang, L.; Browning, J. D.; Faraj, A. Comparative antioxidant, antiproliferative and phase II enzyme inducing potential of sorghum (Sorghum bicolor) varieties. *LWT—Food Sci. Technol.* **2009**, *42*, 1041−1046.

(3) Awika, J. M.; Rooney, L. W. Sorghum phytochemicals and their potential impact on human health. *Phytochemistry* **2004**, *65*, 1199−1221.

(4) Awika, J. M.; McDonough, C. M.; Rooney, L. W. Decorticating sorghum to concentrate healthy phytochemicals. *J. Agric. Food Chem.* **2005**, *53*, 6230−6234.

(5) Hagerman, A. E.; Riedl, K. M.; Jones, G. A.; Sovik, K. N.; Ritchard, N. T.; Hartzfeld, P. W.; Riechel, T. L. High molecular weight plant polyphenolics (tannins) as biological antioxidants. *J. Agric. Food Chem.* **1998**, *46*, 1887−1892.

(6) Tian, Y.; Zou, B.; Li, C.; Yang, J.; Xu, F.; Hagerman, A. E. High molecular weight persimmon tannin is a potent antioxidant both ex vivo and in vivo. *Food Res. Int.* **2012**, *45*, 26−30.

(7) Hagerman, A. E.; Robbins, C. T.; Weerasuriya, Y.; Wilson, T. C.; McCarthur, C. Tannin chemistry in relation to digestion. *J. Range Manage.* **1992**, *45*, 57−62.

(8) Hargrove, J. L.; Greenspan, P.; Hartle, D. K.; Dowd, C. Inhibition of aromatase and α-Amylase by flavonoids and proanthocyanidins from sorghum bicolor bran extracts. *J. Med. Food* **2011**, *14*, 799−807.

(9) Davis, A. B.; Hoseney, R. C. Grain sorghum condensed tannins. I. Isolation, estimation, and selective adsorption by starch. *Cereal Chem.* **1979**, *56*, 310−314.

(10) Leeman, A. M.; Karlsson, M. E.; Eliasson, A.-C.; Bjorck, I. M. E. Resistant starch formation in temperature treated potato starches varying in amylose/amylopectin ratio. *Carbohydr. Polym.* **2006**, *65*, 306−313.

(11) Rooney, L. W.; Pflugfelder, R. L. Factors affecting starch digestibility with special emphasis on sorghum and corn. *J. Anim. Sci. (Champaign, IL, U.S.)* **1986**, *63*, 1607−1623.

(12) Zhang, G.; Hamaker, B. R. Low alpha amylase starch digestibility of cooked sorghum flours and the effect of protein. *Cereal Chem.* **1998**, *75*, 710−713.

(13) Thompson, L. U.; Yoon, J. H. Starch digestibility as affected by polyphenols and phytic acid. *J. Food. Sci.* **1984**, *49*, 1228−1229.

(14) Bourvellec, C. L.; Bouchet, B.; Renard, C. M. G. C. Non-covalent interaction between procyanidins and apple cell wall material. Part III: Study on model polysaccharides. *Biochim. Biophys. Acta* **2005**, *1725*, 10−18.

(15) Wu, Y.; Chen, Z.; Li, X.; Li, M. Effect of tea polyphenols on the retrogradation of rice starch. *Food Res. Int.* **2009**, *42*, 221−225.

(16) Zhu, F.; Cai, Y. Z.; Sun, M.; Corke, H. Effect of phenolic compounds on the pasting and textural properties of wheat starch. *Starch/Staerke* **2008**, *60*, 609−616.

(17) Beta, T.; Corke, H. Effect of ferulic acid and catechin on sorghum and maize starch pasting properties. *Cereal Chem.* **2004**, *81*, 418−422.

(18) Serna-Saldivar, S.; Rooney, L. W. Structure and Chemistry of Sorghum and Millets. In *Sorghum and Millets: Chemistry and Technology*; Dendy, D. A. V., Ed.; American Association of Cereal Chemists: St Paul, MN, 1995; pp 69−124.

(19) Kaluza, W. Z.; McGrath, R. M.; Roberts, T. C.; Schroder, H. H. Separation of phenolics of Sorghum bicolor (L.) Moench grain. *J. Agric. Food Chem.* **1980**, *28*, 1191−1196.

(20) Sweeney, R. A. Generic combustion method for determination of crude protein in feeds: Collaborative study. *J.−Assoc. Off. Anal. Chem.* **1989**, *72*, 770−774.

(21) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(22) Langer, S.; Marshall, L. J.; Day, A. J.; Morgan, M. R. A. Flavanols and methylxanthines in commercially available dark chocolate: A study of the correlation with nonfat cocoa solids. *J. Agric. Food Chem.* **2011**, *59*, 8435−8441.

(23) Hagerman, A.; Robbins, C. T. Implications of soluble tannin-protein complexes for tannin analysis and plant defense mechanisms. *J. Chem. Ecol.* **1987**, *13*, 1243−1259.

(24) Hagerman, A. E.; Butler, L. G. The specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.* **1981**, *256*, 4494−4497.

(25) Hahn, D. H.; Faubion, J. M.; Rooney, L. W. Sorghum phenolic acids, their high performance liquid chromatography separation and their relation to fungal resistance. *Cereal Chem.* **1983**, *60*, 255−259.

(26) Yang, L.; Allred, K. F.; Geera, B.; Allred, C. D.; Awika, J. M. Sorghum phenolics demonstrate estrogenic action and induce apoptosis in nonmalignant colonocytes. *Nutr. Cancer* **2012**, *64*, 419−427.

(27) Awika, J. M.; Dykes, L.; Gu, L.; Rooney, L. W.; Prior, R. L. Processing of sorghum (Sorghum bicolor) and sorghum products alters procyanidin oligomer and polymer distribution and content. *J. Agric. Food Chem.* **2003**, *51*, 5516−5521.

(28) Fannon, J. E.; Hauber, R. J.; Bemiller, J. N. Surface pores of starch granules. *Cereal Chem.* **1992**, *69*, 284−288.

(29) Deprez, S.; Mila, I.; Huneau, J. F.; Tome, D.; Scalbert, A. Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells. *Antioxid. Redox Signaling* **2001**, *3*, 957−967.

(30) Butler, L. G.; Riedl, D. J.; Lebryk, D. G.; Blytt, H. J. Interaction of proteins with sorghum tannin: Mechanism, specificity and significance. *J. Am. Oil Chem. Soc.* **1984**, *61*, 916−920.

(31) Siebert, K. J.; Troukhanova, N. V.; Lynn, P. Y. Nature of polyphenol-protein interactions. *J. Agric. Food Chem.* **1996**, *44*, 80−85.

(32) Yadav, B. S.; Sharma, A.; Yadav, R. B. Studies on effect of multiple heating/cooling cycles on the resistant starch formation in cereals, legumes and tubers. *Int. J. Food Sci. Nutr.* **2009**, *60*, 258−272.

(33) Zobel, H. F. Starch crystal transformations and their industrial importance. *Starch/Staerke* **1988**, *40*, 1−7.