

## Effects of Sorghum (*Sorghum bicolor* (L.) Moench) Tannins on $\alpha$ -Amylase Activity and in Vitro Digestibility of Starch in Raw and Processed Flours

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**ABSTRACT:** The purpose of this study was to investigate the effects of tannins on starch digestion in tannin-containing sorghum extracts and wholegrain flours from 12 sorghum varieties. Extracts reduced amylase activity in a tannin concentration-dependent manner when the extract was mixed with the enzyme before substrate (amylopectin) addition, with higher molecular weight tannins showing greater reduction. Conversely, when the extract and substrate were combined before enzyme addition an enhancement in amylase activity was experienced. In uncooked, cooked, and cooked and stored wholegrain sorghum flours, rapidly digestible, slowly digestible, and resistant starches were not correlated with tannin content or molecular weight distribution. Resistant starch increased from 6.5% to 22–26% when tannins were added to starch up to 50% (starch weight). Tannin extracts both reduced and enhanced amylase activity depending on conditions, and, while these trends were clear in extracts, the effects on starch digestion in wholegrain flours was more complex.

**KEYWORDS:** digestion, Englyst test, polyphenols, resistant starch, slowly digestible starch, processing

### ■ INTRODUCTION

Grain sorghum (*Sorghum bicolor* (L.) Moench) can be divided into tannin-free and tannin-containing varieties based on the absence or presence of a pigmented testa containing polyphenolic compounds.<sup>1</sup> The major polyphenolic compounds in sorghum are condensed tannins, which are oligomers and polymers of catechins and epicatechins and belong to a broader class of polyphenolic compounds called procyanidins or proanthocyanidins.<sup>1</sup>

Tannin-containing extracts from many sources, including sorghum, can decrease  $\alpha$ -amylase activity,<sup>2–8</sup> which suggests that tannins may decrease starch digestion and contribute to reducing glycemic index and increasing resistant starch (RS).<sup>9–11</sup>

In tannin-containing extracts, reduction in amylase activity has been shown to be realized through interactions with the enzyme<sup>3,8</sup> and with the starch.<sup>10</sup> Because tannins can interact with both amylase and starch, it is plausible that the magnitude of amylase reduction would depend on whether enzyme was mixed with the tannin-containing extract and then the substrate was added, or the extract was mixed with the substrate and then the enzyme was added. However, the influence of order of addition of these components has not been explored.

Additionally, few studies have determined if diminished amylase activity is maintained when the whole food is used, rather than an extract. Regarding sorghum, only one such study exists, in which the authors mixed tannin-containing or nontannin sorghum brans with endosperm and determined in vitro starch digestion properties.<sup>9</sup> Two tannin-containing sorghums were used in this study, and, while

they both reduced starch digestion rate (estimated glycemic index), only one increased RS content. This disparity could be due to differences in tannin molecular weight<sup>3,8</sup> and merits further exploration. Furthermore, starch digestion properties are known to be affected by cooking and by cooking followed by storage (which induces retrogradation).<sup>12</sup> Because tannins interact with starch during cooking and may interfere with retrogradation,<sup>10,13</sup> tannins could affect starch digestion differently depending on processing conditions.

Thus, the objectives of this study were to investigate the effects of tannin extracts from sorghum on amylase activity and to determine differences in starch digestion properties of wholegrain sorghum flours. First to be examined was how sorghum extracts may affect amylase activity depending on whether the extract was mixed with the enzyme or substrate prior to the reaction. Then, the influence of sorghum extracts differing in molecular weight distribution on amylase activity was studied. Next, the in vitro starch digestion properties of uncooked, cooked, and cooked and stored sorghum flours that varied widely in tannin content and molecular weight distribution were determined. Finally, a determination of how purified tannins may affect starch digestion without the effects of other interfering compounds was made.

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Table 1. Selected Chemical Components of Non-Tannin and Tannin-Containing Sorghums<sup>a</sup>

variety	tannins (mg CE/g)						starch (mg/g)	free glucose (mg/g)	sucrose (mg/g)
	total	DP >22	DP 10–22	DP 3–9	DP 1–2	OP:MD			
Macia	ND	NA	NA	NA	NA	NA	685 ± 20	0.783 ± 0.017	6.23 ± 0.15
Ajabsido Y1	1.8	0.1	0.4	0.6	0.8	1.4	717 ± 14	0.338 ± 0.001	1.92 ± 0.01
Koro kollo Y2	4.6	0.2	1.0	1.7	1.7	1.8	699 ± 29	0.262 ± 0.005	4.00 ± 0.32
SC-103-12E Y1	6.3	0.7	1.7	2.1	1.7	2.6	688 ± 5	0.205 ± 0.010	7.40 ± 0.24
SC 599 Y1	6.5	0.3	1.6	2.2	2.4	1.7	715 ± 22	0.185 ± 0.001	5.14 ± 0.003
SC-103-12E Y2	12.9	1.0	3.0	4.0	4.9	1.7	682 ± 21	0.296 ± 0.018	5.20 ± 0.05
Shanqui red Y1	20.3	1.7	5.2	7.7	5.6	2.6	681 ± 29	0.311 ± 0.002	3.53 ± 0.12
Shanqui red Y2	29.7	1.0	5.9	10.2	12.6	1.4	680 ± 22	0.233 ± 0.013	3.93 ± 0.16
IS 8525 Y1	23.6	1.1	5.9	8.1	8.6	1.8	702 ± 6	0.225 ± 0.052	4.46 ± 0.07
IS 8525 Y2	27.6	1.7	5.8	9.1	11.0	1.5	688 ± 36	0.308 ± 0.002	3.64 ± 0.14
Sumac Y1	43.4	2.5	7.4	15.2	18.3	1.4	680 ± 40	0.431 ± 0.002	3.42 ± 0.15
Sumac Y2	50.6	2.0	9.5	16.7	22.4	1.3	678 ± 6	0.467 ± 0.022	3.46 ± 0.10

<sup>a</sup>Tannin data from Kaufman et al.;<sup>17</sup> carbohydrate data expressed as mean ± standard deviation (dry basis); *n* = 3 for starch; *n* = 2 for free glucose and sucrose; CE = catechin equivalents; ND = not detected; NA = not analyzed; Y1 and Y2 designate growing years.

## MATERIALS AND METHODS

**Samples.** Eleven sorghum varieties grown in Kansas, USA, were used in this study.<sup>14</sup> For comparison, a nontannin sorghum variety, Macia, was obtained from Ismail Dweikat (University of Nebraska-Lincoln, Lincoln, NE). The kernels were cleaned and sorted by hand, and then ground in a cyclone mill equipped with a 0.5-mm sieve (UDY Corporation, Fort Collins, CO, USA). Milled samples were stored at -20 °C until analysis.

**Grain Analysis.** Grain properties (color, hardness, diameter, kernel weight), protein content, and tannin content and molecular weight distributions of the tannin-containing sorghum varieties were reported previously.<sup>14</sup> Total starch was determined using an assay kit (K-TSTA, Megazyme, Wicklow, Ireland) following the dimethylsulfoxide format. Free glucose and sucrose contents were determined on 200 mg of sorghum flour using a sucrose/D-glucose kit (K-SUCGL, Megazyme, Wicklow, Ireland). All analyses were expressed on a dry weight basis.

**Effect of Order of Tannin Extract Addition on  $\alpha$ -Amylase Activity.** Tannin extracts from all 12 sorghum varieties were prepared by combining 0.15 g of milled sorghum with 8 mL of 1% v/v hydrochloric acid in methanol, incubating at 30 °C for 20 min with intermittent mixing, and centrifuging at 2000g for 20 min. Although there was some risk of tannin hydrolysis with the methanolic-HCl,<sup>15</sup> we opted to use this extraction solvent because acid results in higher levels of tannin extracted<sup>16</sup> and is essential for extracting some types of tannins.<sup>1</sup> Furthermore, size exclusion chromatographic analysis of tannins extracted with methanolic-HCl showed no difference in molecular weight distribution compared to purified tannins that were initially extracted with methanol containing 10 mM ascorbic acid. Polymers of >22 degrees of polymerization have been detected in such extracts.<sup>17</sup>

Substrate was prepared by boiling 0.5 g of waxy maize starch (Amioca; National Starch Food Innovation, Bridgewater, NJ) in about 60 mL of distilled water on a stirring hot plate, cooling to room temperature, adding 20 mL of 0.5 M sodium maleate buffer (pH 6.0), and then diluting to 100 mL. Waxy maize starch was used to minimize the effects of retrogradation on enzyme activity.

Porcine pancreatic  $\alpha$ -amylase (A3176; 21.6 U/mg solid, Sigma-Aldrich) was prepared by dissolving 46 mg of solid in 50 mL of 100 mM sodium maleate buffer (pH 6.0, containing 5 mM calcium chloride and 0.02% w/v sodium azide). One-half mL was then diluted to 5 mL with buffer such that the final concentration was 0.2 U/mL. Tannin extracts, enzymes, and substrate were prepared fresh prior to each experiment.

Enzyme activity in the presence of tannin extracts was assayed by two strategies: (1) when the substrate and tannin extracts were allowed to interact before adding the enzyme (amylopectin + tannins) and (2) when the enzyme and tannin extracts were allowed to interact before the substrate was added (amylase + tannins).

In the assay of amylopectin + tannins, 0.5 mL of waxy starch solution was pipetted into a glass tube and placed in a water bath at 37 °C.

Fifty  $\mu$ L of freshly prepared tannin extract was added to the starch solution and vortex mixed. The starch + tannin mixture was allowed to incubate for 30 min with shaking at 140 rpm, and then 0.5 mL of amylase solution was added. The reaction proceeded for 5 min and then was stopped by adding 1.5 mL of dinitrosalicylic (DNS) reagent (containing (per L): 10 g of DNS, 16 g of sodium hydroxide, and 300 g of Rochelle salt (sodium potassium tartrate)).<sup>18</sup> The tubes were then placed in a boiling water bath for 5 min, cooled under running water, and the absorbance was recorded at 540 nm. Reducing sugar content was determined using external calibration with glucose as the standard.

To correct for the effect of the methanolic HCl extraction solution itself, the control was assayed by adding this extraction solution in place of the sorghum extract. Thus, all reaction tubes, controls, and blank tubes contained the same volume of methanolic HCl (<5% v/v).

It was necessary to determine reducing sugar content at time zero to quantify the reducing sugars released by the enzyme. To determine this, blank tubes were assayed by adding solutions in the following order: 0.5 mL of starch solution, 0.05 mL of condensed tannin extract, 1.5 mL of DNS reagent, and then 0.5 mL of the enzyme preparation. This value was subtracted from the value obtained after the 5-min incubation. Results were expressed as a percent of amylase activity when extraction solvent only was used.

The amylase + tannin assay was similar to the starch + tannin approach, except 50  $\mu$ L of freshly prepared tannin extract was mixed with 0.5 mL of enzyme preparation in the first step and 0.5 mL of starch solution was added after 30 min.

**Effect of Condensed Tannin Molecular Weight on  $\alpha$ -Amylase Activity.** Kaufman et al.<sup>14</sup> previously reported the molecular weight profiles of tannins in the sorghum samples used in this study. Size exclusion chromatograms from these samples were divided into two regions, corresponding to oligo- and polymers (degrees of polymerization  $\geq 3$ ; OP) and monomers and dimers (MD). The ratio of relative peak areas for these two regions (OP:MD) ranged from 1.36 to 2.62 in all samples (Table 1). One sorghum variety (Shanqui red) contained quite different proportions of these two classifications depending on the year grown. These two samples were thus selected to determine the effect of tannin molecular weight on the activity of  $\alpha$ -amylase.

Tannins from the selected sorghum samples were extracted from 1 g of wholegrain flour using methanolic HCl and diluted to contain 0.3, 0.5, 0.8, 1.1, and 1.3 mg tannins/mL. Extraction solvent containing 0 mg tannins/mL was also included. Then,  $\alpha$ -amylase aliquots were added, incubated, and then mixed with starch substrate at timed intervals as previously described in the amylase + tannin scheme (see Effect of Order of Tannin Extract Addition on  $\alpha$ -Amylase Activity).

**In Vitro Starch Digestion of Processed Sorghum Flours.** Wholegrain sorghum flours that had been subjected to three treatments: uncooked, cooked (boiling water bath, 20 min), and cooked and stored to induce retrogradation (4 °C, 7 d),<sup>19</sup> were analyzed for in vitro starch digestion according to Englyst et al.<sup>20</sup> with some modifications.

Modifications were performed to decrease sample size and accommodate different sources of enzymes than those reported in the original method. In short, flour equivalent to 120 mg of starch was dispersed in 2 mL of water in a 15-mL tube. A positive reference consisting of normal maize starch (Argo, Oakbrook, IL, USA) and a blank with no starch were included. If samples were cooked, they were capped and placed in a boiling water bath for 20 min with vortex mixing several times during the first 5 min of cooking, and then cooled by placing the tubes in a water bath at 37 °C; samples that were not cooked were simply dispersed in the water and placed in the 37 °C water bath. Four mL of 3.6% w/v pepsin (3802 U/mg protein; Sigma, St. Louis, MO, USA) containing 0.5% w/v guar gum (TIC Gums, Belcamp, MD, USA) in 50 mM hydrochloric acid was added to the each tube, vortex mixed, and incubated for 30 min at 37 °C. Subsequently, six glass beads (6-mm diameter) were added to the tubes followed by 2 mL of sodium acetate buffer (0.5 M, pH 5.2, containing 5 mM calcium chloride). Starch digestion was initiated by adding 2.05 mL of an enzyme mixture prepared by dispersing pancreatin (P-7545; Sigma-Aldrich) in water (15% w/v) on a magnetic stirrer for 10 min and then centrifuging for 10 min at 4000g, whereupon 20  $\mu$ L of amyloglucosidase (3260 U/mL; Megazyme) and 13 mg of invertase (10600 U/g; S-75136; Fisher Science Education, Hanover, IL, USA) were added per mL of recovered pancreatin supernatant. Starch was digested over 2 h at 37 °C with horizontal shaking at 160 rpm. After exactly 20 and 120 min of digestion, 50  $\mu$ L of slurry was removed from the tube and mixed with 0.95 mL of 90% aqueous ethanol. The sampled mixtures were kept refrigerated overnight and then centrifuged at 8161g for 5 min. The glucose content was measured in the supernatant using the glucose oxidase-peroxidase method (Megazyme) and converted to starch by multiplying by a factor of 0.9. Results were expressed as rapidly digestible starch (RDS), defined as the weight of starch converted to glucose in the first 20 min of in vitro starch digestion; slowly digestible starch (SDS), which was the weight of starch converted to glucose between 20 and 120 min; and resistant starch (RS), the weight of starch that was not converted to glucose after 120 min, which was determined as the difference between total starch and the starch hydrolyzed at 120 min.<sup>23</sup> Results were adjusted for the content of free glucose and glucose from sucrose,<sup>20</sup> and expressed as a percentage of total starch.

**In Vitro Digestion of Starch in the Presence of Purified Tannins, Catechins, or Cellulose.** Tannins were extracted from 200 g of a high-tannin sorghum variety (Sumac Y1; Table 1) as described.<sup>21</sup> This resulted in a freeze-dried powder containing 85.7% tannins as determined by the modified vanillin-HCl method,<sup>22</sup> except decreasing the sample size to 15 mg. This was designated as "purified tannins".

Sixty mg of normal maize starch (Argo) and varying levels of purified tannins (0, 12.5, 25, 37.5, and 50% as a percentage of the starch used) were dispersed in 1 mL of water, and then cooked for 20 min. In vitro digestion then proceeded as described previously (see In Vitro Starch Digestion of Processed Sorghum Flours), except the volumes of reagents used were decreased by half. Catechin hydrate, the building block of tannins (monomer), was also assayed in the same manner for comparison. Because all tubes contained equivalent weight of starch with increasing solids added to the tubes (i.e., tannins or catechins), cellulose (Fisher Scientific, New York USA) was included to determine the effects of nonspecific interactions between enzymes or starch and the added solids.

**Data Analysis.** All data were analyzed using SAS software (version 9.2, SAS Institute, Cary, NC). Reduction in  $\alpha$ -amylase activity by tannin extracts was analyzed by least-squares regression. When comparing the effects of tannin extracts on  $\alpha$ -amylase activity with the treatment containing only starch, ANOVA was used followed by Dunnett's test.

Data for in vitro starch digestion were analyzed using two-way ANOVA. In the first model, sorghum variety and treatment (cooked, uncooked, and cooked and stored) varied as main effects; in the second model, components (purified tannins, catechin, or cellulose) and level (0, 12.5, 25, 37.5, and 50%) were the main effects. Fisher's least significant difference test was used to determine significant differences among main effects and interactions with significant F-tests. Only predetermined comparisons were evaluated: treatment effects

and variety effects within treatment in the first model, and component effects and level effects within component in the second model.

Because RDS, SDS, and RS were not independent because they summed to 100%, the effects of tannin content and contents of different molecular weight fractions on RDS, SDS, and RS were analyzed by two-stage least-squares methodology to simultaneously fit tannin content (continuous variable) to RDS, SDS, and RS. SDS and RS were modeled and coefficients for RDS were determined by difference.

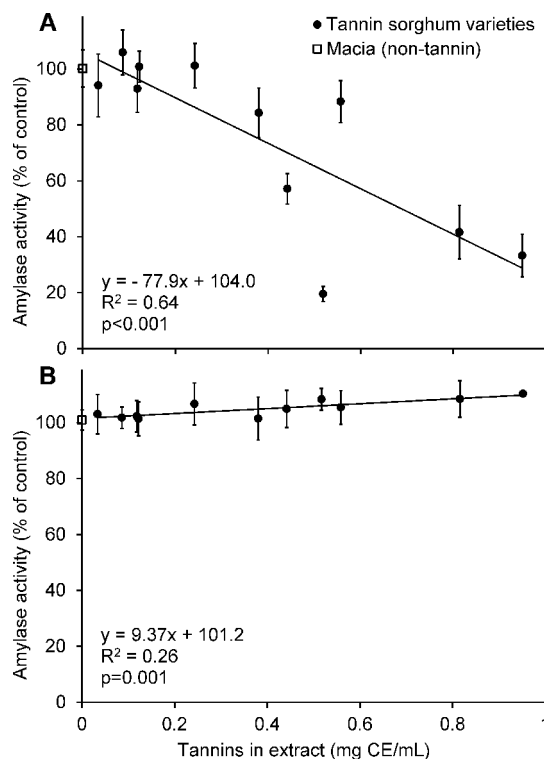
In all cases, at least three replicates of each analysis were run. Exceptions included free glucose and sucrose, the cooked and stored Sumac Y1 sample, and the 0% tannin sample for the purified tannin + amylopectin experiment (where  $n = 2$ ).

## RESULTS AND DISCUSSION

**Sorghum Composition.** The sorghum samples used in this study were selected for their wide range in tannin content and molecular weight distribution (Table 1). Interestingly, in every case where the same sorghum line was grown in consecutive years, the sample from the second year was higher in tannins and this was due to a higher proportion of monomers and dimers relative to oligomers and polymers. Thus, these data suggest that environment has a significant (and directionally consistent) effect on sorghum tannin content.

Total starch ranged from 68.0% to 71.7% (Table 1). This was similar to previous reports in nontannin and tannin-containing sorghums.

**Effect of Order of Tannin Extract Addition on  $\alpha$ -Amylase Activity.** Tannin extracts from the 11 tannin-containing sorghum lines proportionally reduced the activity of  $\alpha$ -amylases as a function of tannin concentration when the enzymes were allowed to interact with the tannin extracts before adding the substrate (amylase + tannin scheme; Figure 1A). The nontannin sorghum,

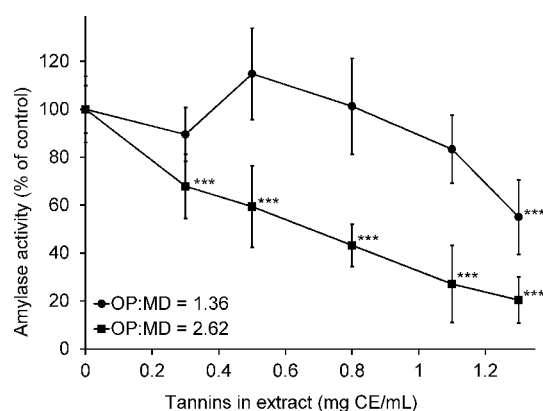


**Figure 1.** Effect of condensed tannin extracts from sorghum on porcine pancreatic  $\alpha$ -amylase activity when amylase was incubated with tannin extract before adding cooked, waxy starch (A) or when the starch was incubated with the tannin extract before adding amylase (B). Error bars show standard deviation;  $n \geq 3$ ; CE = catechin equivalents.



Macia, had no effect on amylase activity. These results are supported by previous studies in which tannins from various sources have been found to significantly reduce the activity of amylolytic enzymes.<sup>2–8</sup> In most of these studies the tannin extracts were put in direct contact with the enzyme before adding the substrate (this is not clear in all cases).

In contrast, a slight but significant increase in the activity of  $\alpha$ -amylase was observed when the tannin extracts were allowed to interact with the substrate before adding the enzyme (amylopectin + tannin scheme; Figure 1B). Barros et al.<sup>10</sup> reported that tannins interact with both amylose and amylopectin; however, unlike tannin interactions with amylose, those with amylopectin did not result in an increase in RS content. Indeed, they reported <1% RS content when amylopectin was cooked with tannin extracts compared to about 7% for normal maize starch. Barros et al.<sup>10</sup> suggested that tannin interactions with amylopectin were due to physical entanglement in the branched amylopectin structure.



**Figure 2.** Effect of condensed tannin extracts from Shanqui Red Y1 (ratio of oligo- and polymeric polyphenols to monomers and dimers (OP:MD) = 2.62) and Shanqui Red Y2 (OP:MD = 1.36) on porcine pancreatic  $\alpha$ -amylase activity. Error bars show standard deviation;  $n = 3$ ; \*\*\*significantly different from no tannins in extract ( $p < 0.001$ ); CE = catechin equivalents.

This entanglement could keep the branches in a more open and random configuration, improving enzyme access to the amylopectin chains and leading to the increase in amylopectin digestibility.

**Effect of Condensed Tannin Molecular Weight on  $\alpha$ -Amylase Activity.** To evaluate how differences in tannin molecular weight impact  $\alpha$ -amylase activity, tannin extracts from one sorghum variety grown in two consecutive years and containing tannins with very different molecular weight profiles were prepared and tested for the ability to reduce  $\alpha$ -amylase activity. While extracts from both Shanqui Red Y1 (with a high proportion of oligomers and polymers) and Shanqui Red Y2 (with a high proportion of monomers and dimers) displayed decreases in amylase activity at the higher concentrations tested, the extract from Shanqui Red Y1 reduced activity to a greater extent and displayed a linear decrease with concentration (Figure 2). No decrease in  $\alpha$ -amylase activity was experienced with the Shanqui Red Y2 extract at the lower concentrations tested; as much as 1.3 mg tannins/mL was required to induce a significant decrease.

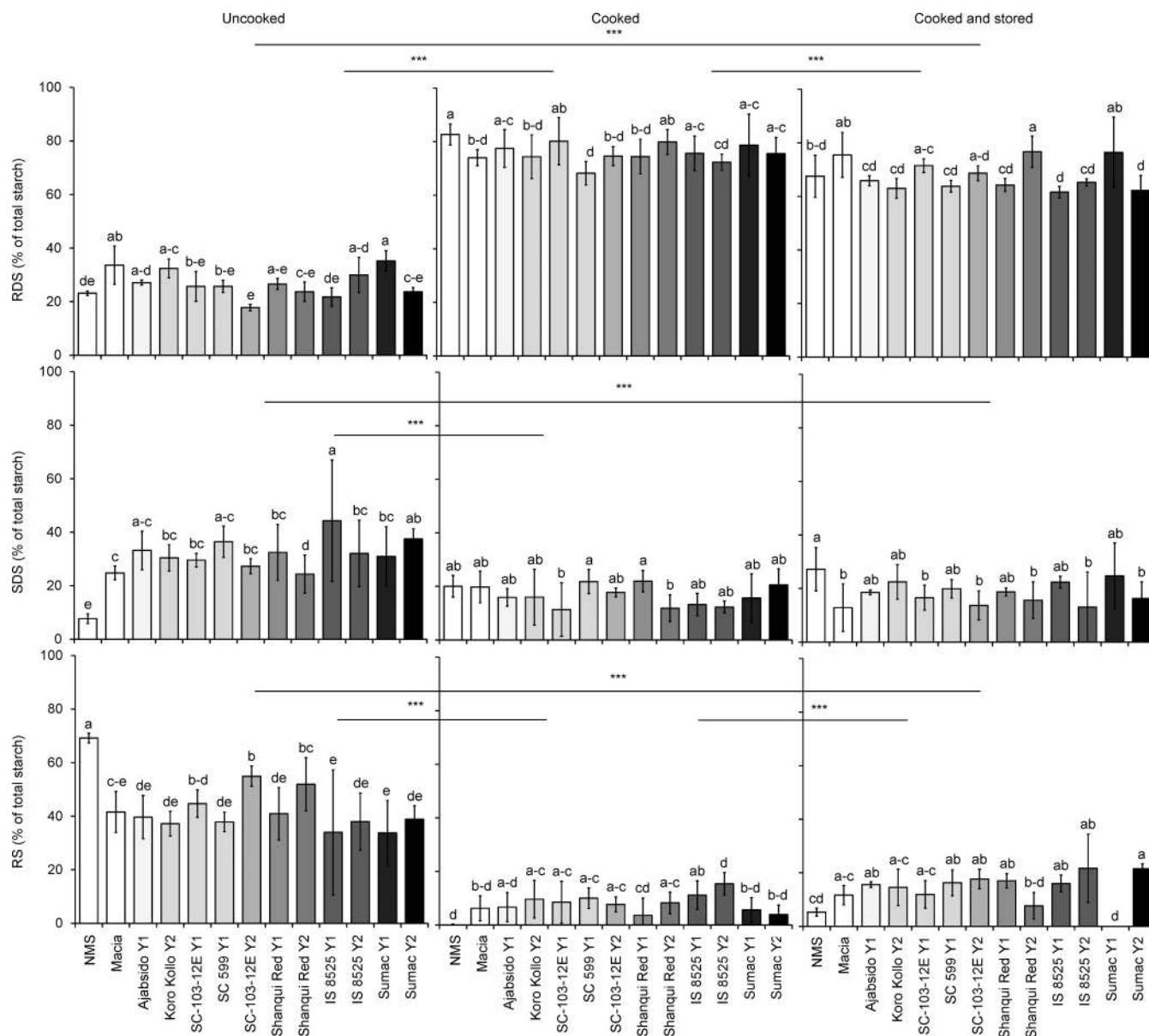
These results confirm reports that higher molecular weight<sup>3</sup> or more complex tannin structures<sup>8</sup> cause a greater reduction in  $\alpha$ -amylase activity than lower molecular weight. Thus, tannin chemistry is important in understanding how tannins may impact starch digestibility, not simply tannin content. These data also illustrate the impact the environment can have on tannin content and activity in sorghum, since these extracts came from the same sorghum line but were grown in different years. Therefore, the effect of the environment on tannin chemistry should be considered when attempting to select or breed tannin containing sorghum lines for attributes that may positively impact human health.

**In Vitro Starch Digestion of Processed Sorghum Flours.** Sorghum flours were categorized into uncooked, cooked (freshly), and cooked and stored based on treatments prior to digestion (Figure 3). On average, uncooked samples contained 26.6% RDS, 30.0% SDS, and 43.2% RS. The

**Table 2. Estimated Coefficients for Regression Model of Tannin Content or Degrees of Polymerization (DP) on Percent Rapidly Digestible Starch (RDS), Percent Slowly Digestible Starch (SDS), and Percent Resistant Starch (RS)<sup>a</sup>**

source	%RDS		%SDS		%RS		
	estimate	estimate	SE	P-value	estimate	SE	P-value
Uncooked							
tannin content	0.33	0.82	1.11	0.47	-1.15	1.22	0.35
DP 1–2	0.85	2.01	2.48	0.42	-2.86	2.73	0.30
DP 3–9	1.35	2.25	3.27	0.50	-3.59	3.59	0.32
DP 10–22	0.03	3.83	6.11	0.54	-3.86	6.77	0.57
DP >22	4.47	14.12	22.84	0.54	-18.59	25.22	0.47
Cooked							
tannin content	0.44	0.09	0.06	0.88	-0.53	0.50	0.29
DP 1–2	0.86	0.20	1.35	0.88	-1.06	1.12	0.35
DP 3–9	1.34	0.33	1.78	0.85	-1.67	1.46	0.26
DP 10–22	2.60	0.30	3.33	0.93	-2.91	2.75	0.29
DP >22	14.21	1.63	12.36	0.90	-15.84	10.06	0.12
Cooked and Stored							
tannin content	0.12	-0.10	0.76	0.89	-0.02	0.87	0.98
DP 1–2	0.05	-0.24	1.70	0.89	0.20	1.94	0.92
DP 3–9	0.49	-0.17	2.24	0.94	-0.32	2.56	0.90
DP 10–22	1.06	-1.39	4.11	0.74	0.34	4.69	0.94
DP >22	9.68	5.61	15.93	0.73	15.29	18.00	0.40

<sup>a</sup>Estimates for SDS and RS calculated simultaneously and RDS calculated by difference; SE = standard error.



**Figure 3.** Rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) contents in cooked, uncooked, and cooked and stored (4 °C, 7 d) wholegrain sorghum flours. Shading is proportional to the tannin content, with darker indicating higher tannin content; error bars show standard deviation;  $n \geq 3$  except for the cooked and stored Sumac Y1 sample where  $n = 2$ . Significant differences among processing conditions for each starch digestible fraction are denoted by lines where \*\*\* $p < 0.001$ ; significant differences within each processing category and starch digestion fraction are denoted by different letters ( $p < 0.05$ ). NMS = normal maize starch.

presence of the high amounts of RS and SDS in uncooked sorghum flours is consistent with previous research on maize starch,<sup>23</sup> though reports of RS levels in raw sorghum have varied widely.<sup>24</sup>

Cooking greatly increased the digestibility of sorghum flours (Figure 3), as evidenced by an increase in RDS (26.6% to 76.0%) and a decrease in SDS (30.0% to 16.8%) and RS (43.2% to 7.21%). The RS content of the cooked samples ranged from 3.60% to 15.4%. Others have reported RS contents of 2–10% in whole grain products,<sup>25,26</sup> while Austin et al.<sup>9</sup> reported RS contents of 3–14% when decorticated sorghum endosperm was cooked with added sorghum bran. Most of the samples fell within these ranges, with significant variation among samples.

Sorghum flours were also cooked and stored at 4 °C for 7 d (Figure 3). Matalanis et al.<sup>19</sup> demonstrated that this is the ideal

storage regimen to induce starch retrogradation in sorghum and maize starch. This could also allow for starch–tannin interactions to fully develop and stabilize. RDS contents in cooked and stored sorghum flours were less than those observed for cooked sorghum flours (67.8% versus 76.0%). Though there was no difference in SDS content, cooked and stored flours exhibited higher RS compared to freshly cooked sorghum flours (13.5% versus 7.21%). The presence of the reduced RDS accompanied by increased RS in comparison with freshly cooked sorghum flours suggested that retrogradation had occurred to some extent, which reduced the susceptibility of starch to enzymatic hydrolysis.<sup>12</sup> The decrease in RDS and increase in RS content in these samples would be expected to beneficially impact health.<sup>26</sup>

While there were significant differences in the RDS, SDS, and RS contents among the sorghum varieties (Figure 3), these

differences could not be explained by the tannin content or molecular weight (Table 2). In our studies above, tannin extracts did not reduce amylopectin hydrolysis when the extracts were mixed with the amylopectin before introduction of the amylase (Figure 1B). In the present digestion procedure, the tannins from the sorghum flours were first exposed to the starch during the cooking and pepsin digestion stages prior to adding the starch-degrading enzymes. This could explain the lack of an association between tannin content and starch digestible fractions.

As mentioned, Austin et al.<sup>9</sup> showed a reduction in estimated glycemic index (which would be similar to a decrease in RDS and an increase in SDS in the present study) when high-tannin sorghum bran was mixed with sorghum endosperm at a 15:85 ratio compared to an endosperm only sample. Notably, however, the magnitude of reduction was relatively minor: from 100 to 89–95 depending on variety of sorghum used. Furthermore, of the two tannin-containing sorghum brans, only one resulted in an increase in RS content. Our study is consistent with these results in that some tannin-containing sorghum samples showed reduction in RDS and/or increase in SDS or RS compared with the nontannin sorghum, Macia, while others did not (Figure 3).

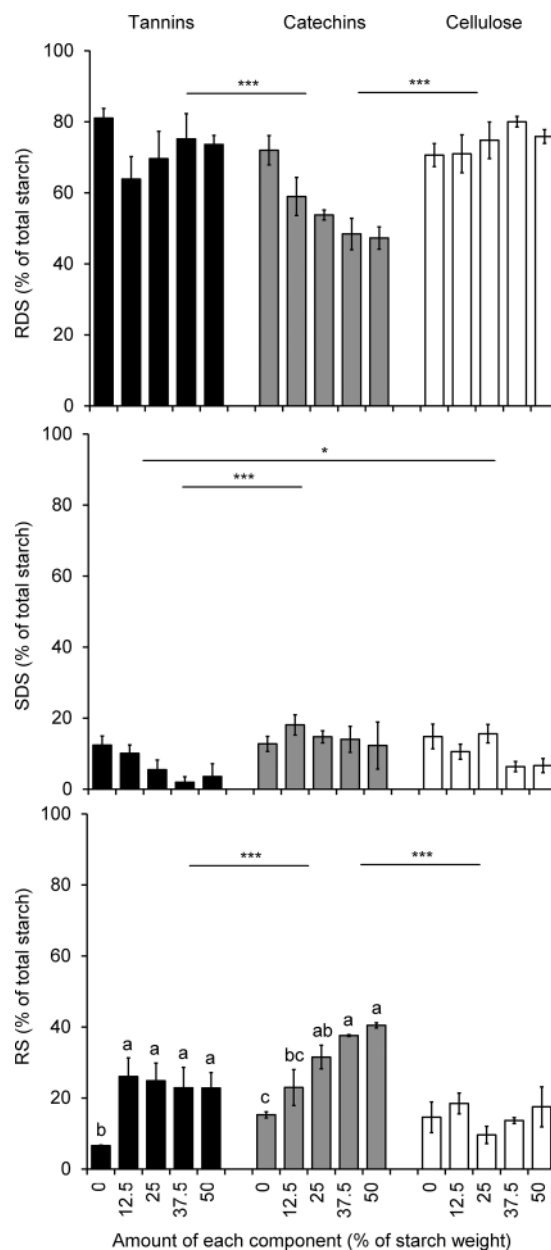
**In Vitro Digestion of Starch in the Presence of Purified Tannins, Catechins, or Cellulose.** It is possible that the concentrations of tannins in sorghum flours may not have been high enough to demonstrate an effect on starch digestion using the Englyst method for starch digestion. Alternatively, the high concentration of enzymes used may have overwhelmed the effects of tannins on starch digestibility. Indeed, others have indicated that reduced enzyme concentrations are needed to determine how structural changes in starch affect digestion rate.<sup>27</sup> Sorghum flour also contains other components that may affect starch digestion. Therefore, tannins were purified from one tannin-containing sorghum variety (Sumac grown in 2003) and mixed with normal maize starch at levels up to 50% of the starch weight. This allowed for the determination the effects of much higher concentrations of tannins on starch digestibility while minimizing the effects of interfering compounds.

Components (tannins, catechin, and cellulose) were added to normal maize starch at increasing levels to determine the effects on starch digestible fractions. Increasing cellulose levels from 0 to 50% of the starch weight had no effect on RDS, SDS, or RS content of the samples ( $p = 0.48$ ,  $p = 0.12$ , and  $p = 0.32$ , respectively). The observation that the cellulose did not affect starch digestion suggested that any changes induced by catechins or tannins were brought about through specific interactions with the starch or enzymes.

Component (tannin, catechin, and cellulose)\*level (0, 12.5, 25, 37.5, and 50%) interactions were not significant for RDS and SDS ( $p = 0.10$  and  $p = 0.45$ , respectively); therefore, mean separations within component were not calculated. However, catechin resulted in a significantly lower RDS values compared with cellulose and tannin (Figure 4). Additionally, SDS was suppressed in samples containing tannin compared with cellulose and catechin. These data suggest that catechins reduce RDS and tannins reduce SDS.

The component\*level interaction was significant for RS ( $p = 0.03$ ). Catechins resulted in a concentration-dependent increase in RS content (Figure 4). The RS content was increased by tannins compared with only starch, but was not affected by concentration.

Depressed SDS and enhanced RS when tannins were added to starch can be explained by the possible interactions between



**Figure 4.** Starch digestibility profiles upon inclusion of different components (purified tannins, catechins, or cellulose). Error bars show standard deviation;  $n \geq 3$  except for the 0% tannin sample where  $n = 2$ . Significant differences among components for each starch digestible fraction are denoted by lines where \* $p < 0.05$  and \*\*\* $p < 0.001$ ; significant differences within component for RS are denoted by different letters ( $p < 0.05$ ); significant differences within component for RDS and SDS were not calculated because the interaction, component\*level, was not significant ( $p = 0.095$ ).

tannins and amylopectin and amylose. The decrease in SDS is consistent with our amylase activity studies above indicating that tannins increase amylopectin digestion when allowed to interact before enzyme addition, while the increase in RS agrees with Barros et al.<sup>10</sup> who showed that tannins bind amylose, possibly through hydrophobic interactions, and increase RS.

Tannin extracts were found to reduce amylopectin hydrolysis by amylase, but only when the tannins were allowed to interact with the enzyme prior to introduction of amylopectin. Indeed, when the extract was mixed with the amylopectin prior to enzyme addition, the converse was true. The magnitude of

amylase activity reduction depended on tannin molecular weight, with high molecular weight being more effective. Significant variability was seen in the amount of RDS, SDS, and RS in cooked sorghum samples from a diverse set of tannin-containing sorghum varieties; however, tannin content and molecular weight distribution was not correlated with RDS, SDS, or RS content. When purified tannins were mixed with starch at up to 50% of the starch weight, a decrease in SDS and an increase in RS were found. Thus, tannins may decrease SDS content, possibly by entanglement in amylopectin branches and keeping the structure of amylopectin more open and accessible to the enzyme. Tannins may increase RS content by binding to amylose and reducing enzyme hydrolysis. However, additional work is needed to investigate how other factors in the grain (hardness, starch structure, and ash content, etc.) may influence starch digestibility.

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### Notes

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## ABBREVIATIONS

RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch

## REFERENCES

- (1) Dykes, L.; Rooney, L. W. Sorghum and millet phenols and antioxidants. *J. Cereal Sci.* **2006**, *44*, 236–251.
- (2) McDougall, G. J.; Shpiro, F.; Dobson, P.; Smith, P.; Blake, A.; Stewart, D. Different polyphenolic components of soft fruits inhibit  $\alpha$ -amylase and  $\alpha$ -glucosidase. *J. Agric. Food Chem.* **2005**, *53*, 2760–2766.
- (3) Gonçalves, R.; Mateus, N.; de Freitas, V. Inhibition of  $\alpha$ -amylase activity by condensed tannins. *Food Chem.* **2011**, *125*, 665–672.
- (4) Gu, Y.; Hurst, W. J.; Stuart, D. A.; Lambert, J. D. Inhibition of key digestive enzymes by cocoa extracts and procyanidins. *J. Agric. Food Chem.* **2011**, *59*, 5305–5311.
- (5) Hargrove, J. L.; Greenspan, P.; Hartle, D. K.; Dowd, C. Inhibition of aromatase and  $\alpha$ -amylase by flavonoids and proanthocyanidins from *Sorghum bicolor* bran extracts. *J. Med. Food* **2011**, *14*, 799–807.
- (6) Kunyanga, C. N.; Imungi, J. K.; Okoth, M.; Momanyi, C.; Biesalski, H. K.; Vadivel, V. Antioxidant and antidiabetic properties of condensed tannins in acetonitrile extract of selected raw and processed indigenous food ingredients from Kenya. *J. Food Sci.* **2011**, *76*, C560–C567.
- (7) Sudha, P.; Zinjarde, S. S.; Bhargava, S. Y.; Kumar, A. R. Potent  $\alpha$ -amylase inhibitory activity of Indian Ayurvedic medicinal plants. *BMC Complementary Altern. Med.* **2011**, *11*, 5.
- (8) Barrett, A.; Ndou, T.; Hughey, C. A.; Straut, C.; Howell, A.; Dai, Z.; Kaletunc, G. Inhibition of  $\alpha$ -amylase and glucoamylase by tannins extracted from cocoa, pomegranates, cranberries, and grapes. *J. Agric. Food Chem.* **2013**, *61*, 1477–1486.
- (9) Austin, D. L.; Turner, N. D.; McDonough, C. M.; Rooney, L. W. Effects of brans from specialty sorghum varieties on *in vitro* starch digestibility of soft and hard sorghum endosperm porridges. *Cereal Chem.* **2012**, *89*, 190–197.
- (10) Barros, F.; Awika, J. M.; Rooney, L. W. Interaction of tannins and other sorghum phenolic compounds with starch and effects of *in vitro* starch digestibility. *J. Agric. Food Chem.* **2012**, *60*, 11609–11617.
- (11) Lemlioglu-Austin, D.; Turner, N. D.; McDonough, C. M.; Rooney, L. W. Effects of sorghum [*Sorghum bicolor* (L.) Moench] crude extracts on starch digestibility, estimated glycemic index (EGI), and resistant starch (RS) contents of porridges. *Molecules* **2012**, *17*, 11126–11138.
- (12) Zhou, X.; Lim, S. T. Pasting viscosity and *in vitro* digestibility of retrograded waxy and normal corn starch powders. *Carbohydr. Polym.* **2012**, *87*, 235–239.
- (13) 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.
- (14) Kaufman, R. C.; Herald, T. J.; Bean, S. R.; Wilson, J. D.; Tuinstra, M. R. Variability in tannin content, chemistry and activity in a diverse group of tannin containing sorghum cultivars. *J. Sci. Food Agric.* **2013**, *93*, 1233–1241.
- (15) Esatbeyoglu, T.; Winterhalter, P. Preparation of dimeric procyanidins B1, B2, B5, and B7 from a polymeric procyanidin fraction of black chokecherry (*Aronia melanocarpa*). *J. Agric. Food Chem.* **2010**, *58*, 5147–5153.
- (16) Maxson, E. D.; Rooney, L. W. Evaluation of methods for tannin analysis in sorghum grain. *Cereal Chem.* **1972**, *49*, 719–729.
- (17) Kaufman, R. C.; Tilley, M.; Bean, S. R.; Tuinstra, M. R. Improved characterization of sorghum tannins using size-exclusion chromatography. *Cereal Chem.* **2009**, *86*, 369–371.
- (18) Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428.
- (19) Matalanis, A. M.; Campanella, O. H.; Hamaker, B. R. Storage retrogradation behavior of sorghum, maize and rice starch pastes related to amylopectin fine structure. *J. Cereal Sci.* **2009**, *50*, 74–81.
- (20) Englyst, K. N.; Englyst, H. N.; Hudson, G. J.; Cole, T. J.; Cummings, J. H. Rapidly available glucose in foods: An *in vitro* measurement that reflects the glycemic response. *Am. J. Clin. Nutr.* **1999**, *69*, 448–454.
- (21) Hagerman, A. E. *The Tannin Handbook*; <http://www.users.muohio.edu/hagermae/> (accessed 07/31/2012).
- (22) Dykes, L.; Rooney, L. W.; Waniska, R. D.; Rooney, W. L. Phenolic compounds and antioxidant activity of sorghum grains of varying genotypes. *J. Agric. Food Chem.* **2005**, *53*, 6813–6818.
- (23) Zhang, G.; Venkatachalam, M.; Hamaker, B. R. Structural basis for the slow digestion property of native cereal starches. *Biomacromolecules* **2006**, *7*, 3259–3266.
- (24) Taylor, J. R. N.; Emmambux, M. N. Developments in our understanding of sorghum polysaccharides and their health benefits. *Cereal Chem.* **2010**, *87*, 263–271.
- (25) Englyst, K. N.; Englyst, H. N. Carbohydrate bioavailability. *Br. J. Nutr.* **2005**, *94*, 1–11.
- (26) Englyst, K. N.; Liu, S.; Englyst, H. N. Nutritional characterization and measurement of dietary carbohydrates. *Eur. J. Clin. Nutr.* **2007**, *61*, S19–S39.
- (27) Zhang, G.; Sofyan, M.; Hamaker, B. R. Slowly digestible state of starch: Mechanism of slow digestion property of gelatinized maize starch. *J. Agric. Food Chem.* **2008**, *56*, 4695–4702.