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Interaction of Proteins with Sorghum Tannin: Mechanism, Specificity and Significance

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ABSTRACT

The grain of some varieties of sorghum contains 2% or more condensed tannin; many other varieties contain no tannin at all. Agronomic advantages, e.g., resistance to bird depredation, are associated with high-tannin sorghums, which have relatively low nutritional value for nonruminants. The biological effects of tannin are a result of its propensity for binding proteins; both hydrogen bonding and hydrophobic interactions are involved. Sorghum tannins can bind dietary proteins and reduce their digestibility. Purified digestive enzymes are inhibited by tannin, but significant inhibition in vivo is unlikely. Proteins differ greatly in their affinity for tannin. Those with highest affinity are large, have an open structure, contain no bound carbohydrate and are rich in proline. Sorghum proteins of the alcohol-soluble prolamine fraction associate strongly with tannin, are difficult to remove during tannin purification and are found combined with tannin in the indigestible residue after in vitro digestion with pepsin. On germination, the seed may sacrifice a portion of these proteins to bind the tannin that might otherwise interfere with metabolism by inhibiting seed enzymes. During seed development, tannin molecules are relatively short and do not effectively precipitate proteins; as the seed dries, tannins undergo polymerization to an average of ca. 6 flavan-3-ol units/molecule. The antinutritional effects of sorghum tannins can be eliminated by soaking the grain in dilute aqueous alkali, but not by cooking. When rats are put on high-tannin sorghum diets, their parotid glands undergo hypertrophy and produce a group of unique salivary proteins with extremely high affinity for tannin. These proteins contain over 40% proline and are devoid of sulfur-containing and aromatic amino acids. This metabolic adaption may protect rats against tannin by binding and inactivating it immediately when it enters the digestive tract.

INTRODUCTION

Tannins are plant secondary substances (not in metabolic pathways providing energy for growth and reproduction) that are characteristically rich in phenolic hydroxyl groups (1). Tannins exhibit a wide variety of biological effects thought to be caused by their capacity to bind and coagulate proteinaceous tissue (astringency) (2). Indeed, the name "tannin" is from their historically important use in tanning hides into leather by binding proteins such as collagen in animal skins.

Both major structural classes of tannins are widely distributed in plants (1). Hydrolyzable tannins are phenolic carboxylic acids, e.g., gallic acid, esterified to sugars such as glucose. Condensed (nonhydrolzable) tannins, chemically known as proanthocyanidins, are polymers of flavin-3-ols linked by carbon-carbon bonds.

Mature grain of the important cereal *Sorghum bicolor* (L.) Moench may contain up to 2% or more condensed tannin, although many lines contain no tannin at all; immature grain of high-tannin sorghums shows even higher levels of tannin in chemical assays (3). Hydrolyzable tannins have not been reported from sorghum. Under optimal conditions, sorghum tannin is capable of binding and precipitating at least 12 times its own weight of protein (4). Because sorghum grain contains ca. 10% protein (5), the grain of high-tannin cultivars contains more than enough tannin to bind all the seed protein, thus profoundly affecting the properties of the protein. Other tannin-containing crops, e.g., barley, rye and common beans, contain lower amounts of tannin and higher levels of protein (6); the protein of these crops is less affected by the presence of tannin.

The purpose of this article is to review the current knowledge of the mechanism, specificity and significance of the interaction of sorghum tannins with proteins.

MECHANISM

Proteins have been shown to interact with tannins by means of hydrogen bonding (12), hydrophobic interaction (13), electrostatic attraction (14) and covalent bonding associated with oxidation (15).

Electrostatic attraction does not apply to the sorghum tannin-protein system. The only ionizing groups of condensed tannins are phenolic hydroxyls; in order to ionize these groups the pH would be so high that most proteins would have similar negative charges, so any electrostatic interaction would be repulsion rather than attraction. The precipitation of soluble proteins by tannin is maximal near the isoelectric point of the protein, when the net charge on the protein is zero (16). Proteins with high isoelectric points, e.g., egg white lysozyme, not only precipitate over a broad pH range (16), but also enhance coprecipitation of more acidic proteins at intermediate pH values, probably by electrostatic interaction between proteins of opposite net charge (17).

No evidence exists that sorghum tannins bind protein covalently, with the possible exception that purified sorghum tannin always contains ca. 2% (by weight) protein contaminant (18). Precipitates of protein with sorghum tannin (17), as well as protein complexes with other tannins

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(13), are readily and completely dissolved in alkaline detergent solutions, suggesting no covalent bonds are formed. Also, all the protein of a tannin-protein complex is extractable by phenol, which is not a solvent for condensed tannin (17).

The effects on tannin-protein interactions of solutes such as formamide and its mono- and dimethyl derivatives, with varying capabilities for competing for hydrogen bonds, strongly affirm the importance of hydrogen bonding in interactions between sorghum tannin and proteins (19). The role of, and specificity for, peptide-bound proline also implicates hydrogen bonding in the interaction.

On the other hand, the effects of organic solvents on protein precipitation by sorghum tannin are consistent with a nonpolar, hydrophobic contribution to the protein-tannin interaction (4). Moreover, our previously unpublished data shown in Table I shows a reversible inhibition by moisture of the extraction of tannin from seed, which is consistent with hydrophobic interaction of tannin with adjacent materials in the seed. In the absence of water, the tendency for nonpolar materials to associate to decrease their contact with water is minimal; thus tannin readily extracts from dry seed but not from seed that has taken up moisture.

We conclude that interactions between sorghum tannin and proteins involve both hydrogen bonding and nonpolar hydrophobic association. The binding process is highly cooperative, involving multiple interactions; low MW models of protein or of tannin do not have similar interactions to the macromolecular forms (17).

SPECIFICITY

Although tannins were originally defined as binding agents for proteins such as collagen in animal skins, the binding of other proteins has been widely noted (1). Tannins have come to be regarded as nonspecific protein binding agents (20), and have been used in such diverse applications as clarifying beer and treating wounds. Previous investigations of the specificity of tannin-protein interactions focused on the interaction of a single protein with a variety of condensed tannins from grapes (21) or with hydrolyzable tannins from several sources (2,22). By using an immobilized form of condensed tannin (23) and hydrolyzable tannin (24), proteins were found to bind, but sugars, amino acids, peptides and nucleotides do not; proteins could be separated into 3 classes depending on whether or not they absorb to the material, and whether salt concentration affects the absorption (25).

We have systematically investigated the specificity of the binding of proteins by purified sorghum tannin, using a competitive binding assay (19). In this assay, a standard radioisotope-labeled protein is mixed with another protein of unknown affinity for tannin, and a limiting amount of tannin is added. The amount of standard protein precipitated by the tannin is diminished to a degree that depends on the amount of unknown protein added, as well as its affinity for tannin. The assay detects all interactions of the unknown protein with tannin, whether or not they result in precipitation of the unknown. In this way, relative affinities of proteins for tannins are readily established, and the effects of other tannin-binding materials can also be assessed.

In contrast to the assumed nonspecificity of protein binding, we found that the relative affinity of different proteins for sorghum tannin varies by over 4 orders of magnitude (19). A protein such as gelatin, with a high affinity for tannin, can be selectively precipitated by tannin out of a hundred-fold excess of a low-affinity protein such as lysozyme. By testing proteins with a wide variety of properties, the characteristics that determine the proteins' affinity for tannin became apparent. In general, proteins that bind sorghum tannin strongly are relatively large, have a loose, open structure, and are rich in proline. All of these characteristics maximize the opportunity for forming multiple hydrogen bonds between tannin molecules and the peptide backbone of the protein and for nonpolar interactions. Proline residues disrupt the α-helix, in which the peptide carbonyl oxygens and amide hydrogens are all internally hydrogen bonded. Proline-rich peptides tend to form open structures with carbonyl and amide groups extending into the solvent (26). Free proline and polyproline peptides containing six or less proline residues do not significantly bind tannin. Polyproline of higher MW binds tannin with increasing strength as the MW increases, but peptides containing 100% proline apparently bind no more strongly than those of the same size containing only ca. 30% proline dispersed throughout the molecule (17).

Glycoproteins are reported to be comparatively resistant to denaturation by tannins; the carbohydrate material may somehow protect the protein material from interacting with tannin (27).

Protein contaminants persist through several extraction and chromatography steps during purification of sorghum tannin. In accord with the above observations on specificity of protein binding, these tannin-associated proteins are not a random mixture of all the proteins of the sorghum seed, but largely consist of 3 discrete components with an apparent high relative affinity for tannin (18). Two of these proteins, from the alcohol-soluble (prolamine) fraction, are quite hydrophobic, and one of them contains over 20% proline (18).

The presence of tannin in high-tannin sorghum alters the fractionation of the seed proteins into solubility classes. Tannin causes a decrease in the saline-soluble fraction (albumins and globulins) and an increase in the relatively insoluble glutelin fraction (28,29).

TABLE I

Effects of Water and Time on Extraction of Tannin from Sorghum Seed[a]

Treatment	Extractable tannins as protein precipitable phenols (ΔA_{510}/g original dry seed)
Time seed was moistened before grinding and extraction	
1 min	1.68
6 hr	0.55
1 day	0.22
2 days	0.18
4 days	0.12
5 days	0.10
5 days, then 2 days drying before grinding and extraction	1.64
Controls	
Grind and extract seed	1.65
Add 15% water to above extract	1.60
Grind dry seed, add 15% water, extract	1.36

[a]The sorghum grain was the high-tannin hybrid, DeKalb BR 64.

SIGNIFICANCE OF SORGHUM TANNIN-PROTEIN INTERACTIONS IN THE DIET

The presence of tannin significantly diminishes the nutritional value of high-tannin sorghums, as determined by feeding trials (30-33). Also, in vitro assays show the protein of high-tannin sorghums is less digestible (34-36). In experiments not previously reported, we carried out in vitro

digestion by pepsin under conditions in which low-tannin sorghums are completely digested but high-tannin sorghums leave a pepsin-indigestible residue. In order to identify the undigested protein, the residue was extracted with phenol and the extracted protein was subjected to SDS gel electrophoresis (Fig. 1). The indigestible residue of the high-tannin sorghums, IS 4225 and IS 6881, consisted mainly of prolamines (alcohol-soluble storage proteins). The low-tannin sorghum, RS 610, produced no indigestible protein residue. Treatment of the whole grain with ammonia before grinding and digestion made the protein of high-tannin grain completely digestible (Fig. 1).

In addition to decreasing the digestibility of dietary protein, dietary tannin could interfere with digestion by inhibiting digestive enzymes. Protein-digesting enzyme activities in the intestine of rats on diets of high-tannin sorghum were reported to be equal to, or greater than, those on corresponding diets of low-tannin sorghum (37, 38). This observation could be accounted for by a lack of inhibition by tannin, or by inhibition accompanied by a compensatory increase in enzyme secretion.

Phenols inhibit many enzymes in vitro (39,40), including digestive enzymes, e.g., trypsin and α-amylase (37). In previously unreported experiments, we have examined the inhibition of 2 digestive enzymes from bovine intestinal mucose, alkaline phosphatase and 5′-nucleotide phosphodiesterase, for inhibition by sorghum tannin in vitro. These enzymes are glycoproteins (41) and might be relatively insensitive to inhibition by tannin (27). Sorghum tannin strongly inhibits these purified enzymes in vitro (Figs. 2 and 3). When tested in the presence of the nonionic detergent, Triton S-100, which apparently provides an environment similar to their original membrane-bound state, phosphodiesterase is insensitive to inhibition by tannin (Fig. 3). Furthermore, amounts of tannin that inhibit the membrane-free purified enzyme show little or no inhibition of the membrane-bound form of the enzyme (Fig. 4).

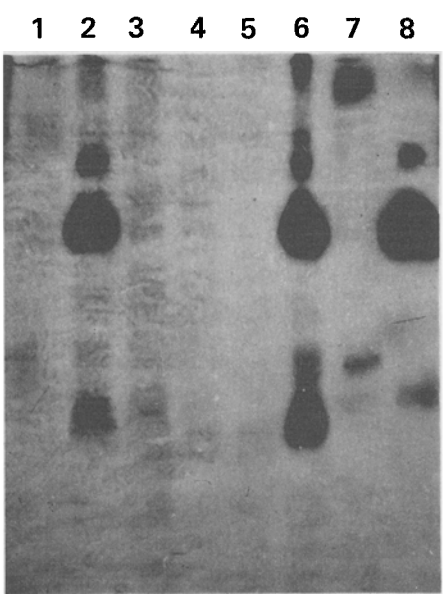

FIG. 1. Pepsin-indigestible proteins of high-tannin sorghum and their ammoniation to overcome the effect of tannin. Lane #1, ammoniated IS 4225 (high tannin); #2, untreated IS 4225; #3, ammoniated RS 610 (low tannin); #4, untreated RS 610; #5, ammoniated IS 6881 (high tannin); #6, untreated IS 6881; #7, pepsin (no sorghum); #8, prolamine fraction of sorghum protein (not treated with pepsin). Complete experimental details for the results presented in the figures and tables can be obtained on request from the senior author.

Taken as a whole, the evidence suggests that sorghum tannins reduce the digestibility of dietary protein by forming less digestible complexes with dietary protein, not by forming complexes with digestive enzymes and inhibiting them. The grinding, cooking or other processing of

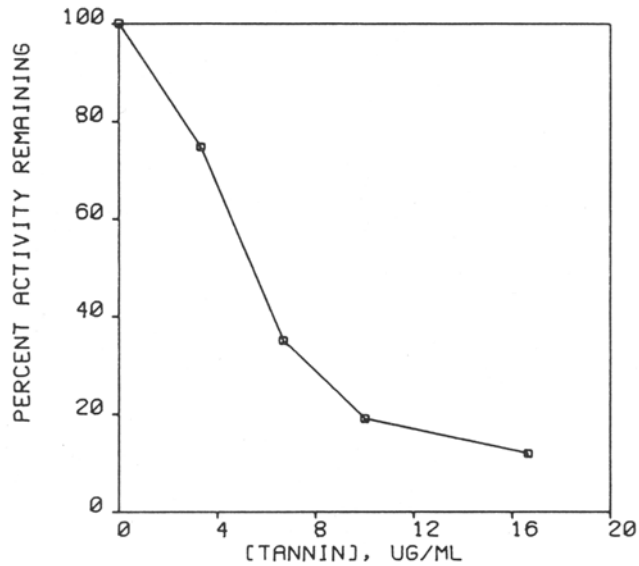

FIG. 2. Inhibition of purified bovine intestinal alkaline phosphatase by sorghum tannin. Assays were run 1 hr after mixing tannin with the enzyme.

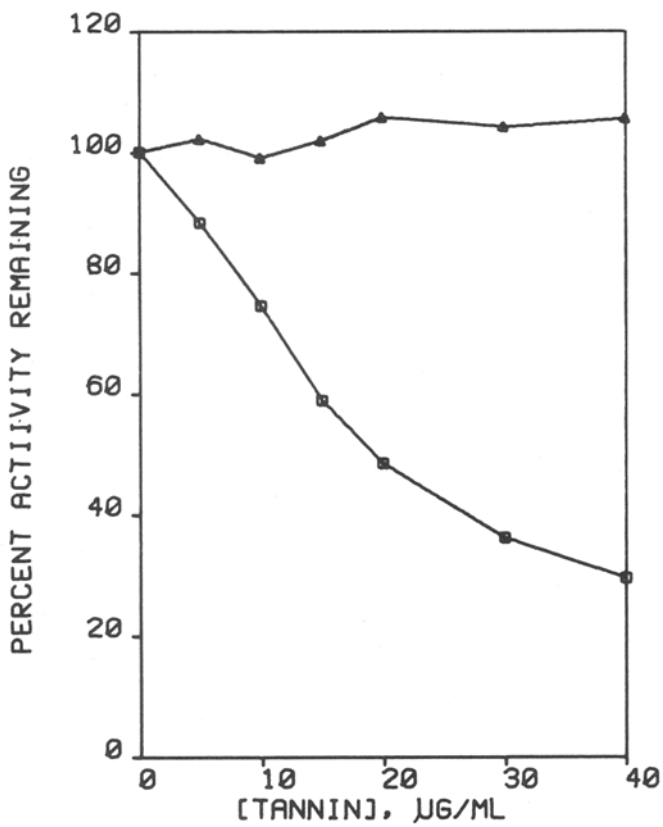

FIG. 3. Triton X-100 prevents tannin from inhibiting purfied bovine intestinal 5′-nucleotide phosphodiesterase. Assays were run 30 min after mixing tannin with the enzyme. Samples in the upper curve contained 10 mM Triton X-100; samples in the lower curve contained no Triton X-100 but were otherwise identical.

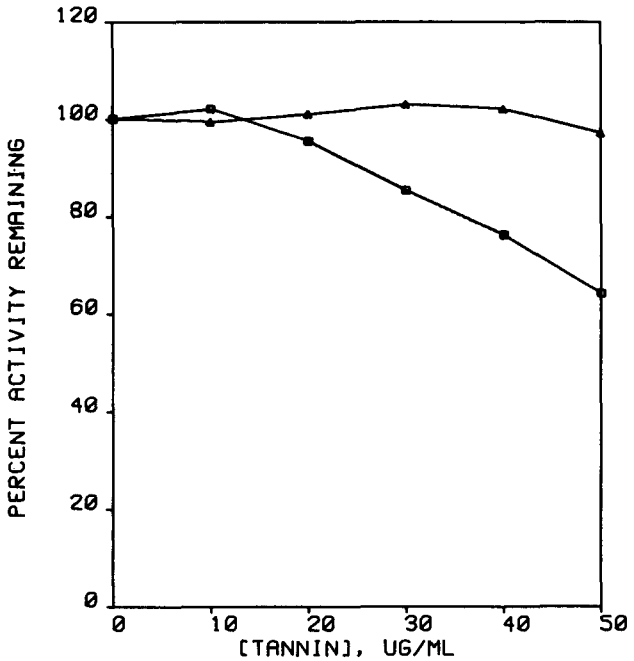

FIG. 4. Differential tannin inhibition of purified soluble 5'-nucleotide phosphodiesterase and its crude membrane-bound form. Assays were run 10 min after mixing tannin with the enzyme. Squares, 0.34 μg of purified soluble enzyme; triangles, washed particulate fraction of bovine intestinal mucosa.

high-tannin sorghum enhances the opportunity for the interaction of tannin with dietary protein before it encounters digestive enzymes. In experiments not reported previously, we found that the more thoroughly the grain is disrupted while wet, the less tannin can subsequently be extracted from the grain after drying (Table II). After grinding and cooking in water, tannin can no longer be extracted from high-tannin sorghum, but its antinutritional effects are undiminished (42), which agrees with the above suggestion.

Our colleagues have recently found another effect of dietary sorghum tannin on proteins of the digestive tract. When placed on diets largely composed of high-tannin sorghum, rats, mice and presumably other monogastric animals rapidly respond by hypertrophy of the parotid gland, with production of an unusual class of proteins that bind tannin very strongly (43). These proteins contain ca. 45% proline but no aromatic or sulfur-containing amino acids. These proteins apparently constitute a system whereby the organism protects itself against dietary tannin by forming a complex with specific proteins as soon as it enters the digestive tract. Blockage of the synthesis of these proteins apparently makes the rat unusually sensitive to dietary tannin; enhancement of the synthesis of these proteins might be expected to diminish the antinutritional effects of dietary tannin (42).

TABLE II

Effect on Extraction of Tannin of Disruption of Moist Seed Before Drying and Extraction

	Tannins as procyanidins (ΔA_{550}/mL)
Control (not moistened)	2.05
Whole grain (moistened but not disrupted)	1.67
Cracked	1.04
Coarsely ground	0.32
Finely ground	0.09

Each sample consisted of 25 seeds of sorghum BR 64. Intact seeds were kept moist for 24 hr, disrupted, dried for 48 hr, ground, extracted and assayed for procyanidins (12). Values are averages of duplicates.

SIGNIFICANCE OF TANNIN TO THE PLANT

The biological effects associated with sorghum tannins are generally beneficial in the field and harmful in the diet. In parts of the semiarid African tropics (7) and in certain other areas of the world, including the southeastern US, low-tannin sorghum cultivars cannot be reliably produced because of severe damage by birds. High-tannin cultivars are usually less heavily damaged and may be the only types produced where bird problems are acute (8). High-tannin sorghums are also reported to be less vulnerable than low-tannin sorghums to preharvest seed deterioration by fungi (9) and to vivipary (10). When used as feed, however, high-tannin sorghum grain results in lower weight gains and feed efficiency and, for poultry, lower egg production than corresponding diets of low-tannin sorghum (11).

Beyond its agronomic benefits, e.g., bird resistance, tannin represents a potential threat to the plant. Crucial metabolic reactions of the tannin-producing plant could be seriously disrupted if tannin bound and inhibited endogenous enzymes. How this is prevented is worth noting.

Tannin apparently occurs only in reproductive tissue of sorghum, the seed and glumes (44). In seed, tannin and associated polyphenol pigments, when present, occur in the testa layer just external to the aleurone layer of cells (45). During seed development, tannins first appear in small vesicles and usually accumulate until the cellular structure of the testa layer is disrupted by an almost continuous layer of tannin (46), sometimes with 2 overlapping layers (45). Similar vesicles are seen in cells growing in tissue culture (47). These vesicles are apparently bounded by a membrane that limits accessibility of the tannin to cellular proteins. Moreover, during seed development, the tannin molecules are relatively short and do not bind proteins as well as the longer (hexameric) molecules characteristic of mature dry grain (48).

When high-tannin sorghum is germinated and then milled, the activity (49) and the solubility (50) of enzymes involved in malting are diminished compared with low-tannin lines. However, little inhibition is seen if the grain is left intact (49). On germination, tannin apparently binds to some component of the seed, possibly prolamine proteins in the outer portion of the endosperm, and thereby becomes unextractable (Table I). This bound form of the tannin probably is much less able than free tannin to inhibit enzyme activity of the seed.

POSSIBLE MEANS OF LIMITING INTERACTION OF DIETARY PROTEINS WITH TANNINS

The antinutritional effects of sorghum tannin may be alleviated by treating the grain with dilute aqueous ammonia (51), strong alkalies (52-54) and formaldehyde (55) or by dehulling (35). Whether any of these measures are suitable and simple enough for widespread adoption is not yet clear. In the long view of the problem, providing a genetic solution in terms of agronomically superior (e.g., bird-resistant) lines with good nutritional quality (e.g., low-tannin) is desirable. Two approaches being explored are the development of lines in which tannins never polymerize to the long antinutritional forms, and lines rich in a component that stimulates the secretion of salivary proline-rich tannin-binding proteins in increased amounts so that more complete protection against dietary tannin is obtained.

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