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The Specificity of Proanthocyanidin-Protein Interactions*

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The proanthocyanidins or condensed tannins, phenolic polymers which are synthesized by many plants, characteristically bind and precipitate proteins. The specificity of the interaction was investigated using a competitive binding assay to compare directly the affinities of various proteins and synthetic polymers for the tannin obtained from *Sorghum bicolor* (Linn.) Moench. At pH 4.9, the relative affinities range over more than 4 orders of magnitude, indicating that this proanthocyanidin interacts quite selectively with protein and protein-like polymers. The affinity for tannins is an inverse function of the size of the polymer, and peptides with less than six residues interact very weakly with tannin. Proteins are precipitated by proanthocyanidins most efficiently at pH values near their isoelectric points. Proline-rich proteins and polymers have very high affinities for tannin. Tightly coiled globular proteins have much lower affinities for tannin than conformationally loose proteins.

The proanthocyanidins, or condensed tannins, of plants are phenolic polymers which precipitate protein and which form colored anthocyanidins when heated with acid (1). The polymers are composed of catechin (M_r = 390) and its steroisomer epicatechin linked by carbon-carbon bonds between C-4 of one flavan-3-ol monomer and C-6 or C-8 of the adjacent monomer (2, 3). Molecular weights ranging from 800 to 6000 have been reported for condensed tannins isolated from various plant tissues (3). Organized tertiary structures such as helices have been proposed for these proanthocyanidins (4).

Little is known at the molecular level about the interactions of proanthocyanidins with globular proteins. Direct determination of the composition of tannin-protein complexes is not possible because tannin interferes with conventional assays for protein (5). Separation of unbound proanthocyanidin from complexes cannot be accomplished by dialysis, because of the polymeric nature of tannin, or by gel filtration, because of the affinity of aromatic compounds for normally inert chromatographic supports (6).

Proanthocyanidin-protein interactions are similar to antigen-antibody interactions in that a binding agent and ligand of comparable sizes associate multivalently to form soluble and insoluble complexes. Methods developed for the study of antigen-antibody interactions might therefore be applied to investigations of tannin-protein interactions. With the competitive immunoassay, the relative affinities of various antigens for an antibody can be determined (7). We describe here a competitive binding assay for tannin in which tannin is the binding agent, iodine 125-labeled protein is the labeled ligand, and unlabeled proteins, polymers, or small molecules are the competing ligands. With this assay we have obtained definitive information on the specificity and mechanism of proanthocyanidin-protein interactions.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were reagent grade or the best grade available. Sorghum (*Sorghum bicolor* (Linn.) Moench) condensed tannin was purified as previously described (8). Calfskin gelatin was from Eastman (No. 1099) and basic proline-rich protein from rat parotid glands (9) was a gift from D. M. Carlson (Purdue University). Soluble polyvinylpyrrolidone was from Sigma (PVP-360) and poly(α-amino acids) were from Sigma or Miles Yeda. The *N*-acetyl proline methyl ester was from Bachem (Torrance, CA 90505).

The specific rotation of the poly(L-proline) in aqueous solution was negative, indicating that the polymer was in the Form II (*trans*) conformation (10). Polyproline with an average molecular weight of 13,000 was fractionated in water on a Sephadex G-50 column and the eluant was monitored at 226 nm. The molecular weight of each polyproline fraction was calculated from its elution volume using commercially prepared tetraproline, bacitracin, and insulin A chain as standards. Upon lyophilization the peptides larger than tetraproline formed powders; tetraproline and smaller peptides formed oils (11). For competition assays approximately 10 μM solutions of the lyophilized products were prepared and the concentrations were calculated from the absorbance at 226 nm (12).

The concentrations of protein solutions were determined spectrophotometrically at 280 nm or with the biuret assay (13) with bovine serum albumin as the standard. For the determination at 280 nm the following values of $E^{1\%}_{1cm}$ were used: bovine serum albumin 6.6; histone F1, 1.8; lysozyme, 26.4; ovalbumin, 7.5. The concentrations of poly(α-amino acid) solutions were determined by nitrogen analysis. A hydroxamate assay for esters (14) was used to determine the concentrations of *N*-acetyl proline methyl ester solutions, with phenylalanine methyl ester as the standard.

Bovine serum albumin and lysozyme were labeled with iodine 125 as previously described (15) and diluted with unlabeled protein after removal of the unbound iodine by gel filtration. The diluted serum albumin, in buffer A (0.20 M acetic acid, 0.17 M NaCl, adjusted to pH 4.9 with NaOH), and lysozyme, in 0.2 M phosphate buffer, pH 6.0, were stored frozen. The final specific radioactivity of the labeled protein was about 10^5 to 10^6 cpm/mg of protein, and trichloroacetic acid precipitated 98% of the radioactivity (15).

Competitive Binding Assay—The procedure for determining protein in a precipitated tannin-protein complex (15) was modified slightly for competitive tannin binding assays. The competitor was mixed with the labeled tracer protein in a total of 2 ml of buffer, and a 0.25-ml aliquot of a methanol solution of tannin (0.05 mg) was added. Thus, the proanthocyanidin was exposed simultaneously to the tracer protein and to the unlabeled competitor. After 15 min, insoluble complexes were removed by centrifugation (5000 × *g*, 15 min) and a 1.0-ml aliquot of the supernatant was counted (Beckman Gamma 300 gamma counter). The amount of tracer protein precipitated in the presence of competitor was expressed as a percentage of the amount precipitated in the absence of competitor, and the per cent inhibition was the difference between that value and 100.

Titration of tannin with either tracer protein gave curves similar to those previously obtained (15). The amount of protein precipitated is proportional to the amount of protein added until an equivalence

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point is reached; beyond that point protein is present in excess and a constant amount of protein is precipitated. At pH 4.9 (buffer A) 1.0 mg of iodine 125-labeled bovine serum albumin was used as the tracer protein. The background precipitation of 0.05 mg of bovine serum albumin in control samples containing no tannin was subtracted from the apparent amount precipitated in the presence of tannin. In the absence of competitor 0.50 to 0.75 mg of bovine serum albumin was precipitated. At pH 7.8 (buffer B; 0.1 M phosphate, pH 7.8) iodine-125-labeled lysozyme was used as the tracer protein. This protein is not precipitated by proanthocyanidin as efficiently as is bovine serum albumin, so each sample contained 5.5 mg of lysozyme; 0.20 mg was precipitated in the tannin-free controls. From 0.65 to 0.80 mg was precipitated in the absence of competitor after accounting for the background precipitation. Although different tracer proteins were used at the two pH values, the results can be directly compared because the same amount of tannin was used at both pH values.

When approximately equal amounts of labeled bovine serum albumin and unlabeled bovine serum albumin, or of labeled lysozyme and unlabeled lysozyme, were used in the competition assay described below, the precipitation of tracer was inhibited by 50% (Table I). This indicates that interaction of proanthocyanidin with labeled protein is indistinguishable from that with unlabeled protein (7).

RESULTS

The addition of another ligand bound by tannin to an excess of tracer protein diminishes the amount of tracer subsequently precipitated by proanthocyanidin. The amount of tracer precipitated, expressed as a percentage of the amount precipitated in the absence of competitor, is plotted as a function of the logarithm of the amount of competitor added (Fig. 1). The plots are sigmoidal as expected from analogous competitive immunoassays (7). The nonparallel curves may be due to heterogeneity of sites or to cooperative binding between tannin and protein (16). Because the proanthocyanidin-protein interaction includes an irreversible precipitation step, true affinity constants for the proanthocyanidin-protein interaction cannot be calculated from this data. Instead, we have defined the amount of competitor required to cause 50% inhibition of the precipitation of tracer protein as the relative affinity of the competitor for the proanthocyanidin. The observed differences in the relative affinities of proteins for tannin (Table I) are due to differences in their strengths of interaction with tannin rather than to differences in their rates of reaction with tannin; for example, ovalbumin forms complexes with proanthocyanidin twice as fast as bovine serum albumin (17) but an 80-fold excess of ovalbumin is required to inhibit the precipitation of bovine serum albumin by 50% (Table I).

The relative affinities of various proteins, polyamino acids, and polymers for tannin vary over more than 4 orders of magnitude at pH 4.9 and over more than 3 orders of magnitude at pH 7.8 (Fig. 1; Table I). The competitors were arranged in the same order and over a similar range of relative affinities when considered on a weight basis rather than a molar basis.

The relative affinity of polyproline for proanthocyanidin increases in a nonlinear fashion with the molecular weight of the polyamino acid (Table II). Like the smallest polyproline

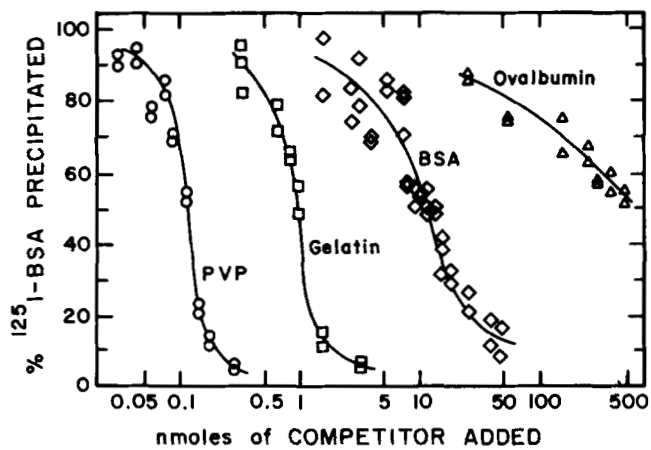


FIG. 1. **Inhibition of tannin bovine serum albumin precipitation by proteins and polymers at pH 4.9.** The standard competitive binding assay with 1 mg (15 nmol) of iodine 125-labeled bovine serum albumin and 0.05 mg of tannin was performed as described in the text. The amount precipitated in the absence of competitor was set at 100%. PVP, polyvinylpyrrolidone; BSA, bovine serum albumin.

TABLE I

Inhibition of the precipitation of tracer protein by proanthocyanidin at pH 4.9 and pH 7.8

The standard competitive binding assay with 1.0 mg (15 nmol) of iodine 125-labeled bovine serum albumin (pH 4.9) or 5.5 mg (380 nmol) of iodine 125-labeled lysozyme (pH 7.8) and 0.05 mg of tannin was performed as described in the text.

Competitor	$M_r \times 10^{-3}$	pI	Amount required for 50% inhibition	
			pH 4.9	pH 7.8
			nmol	
Polyvinylpyrrolidone	360		0.11	0.17
Calfskin gelatin	65	4.7	0.93	1.5
Rat parotid proline-rich protein	16	>10	1.9	2.6
Polyproline	13		1.9	3.1
Porcine pepsin	35	<1	12	
Bovine serum albumin	65	4.9	13	40
Calf thymus histone F1	21.5	>10	14	10
Porcine pancreatic lipase	48	5.2	~100[a]	
Sperm whale myoglobin	17	7.0	140	
Bovine hemoglobin	32.5	7.0	190	
Bovine milk α-lactalbumin	14.4	5.2	400[b]	
Horse heart cytochrome *c*	12.4	10	450	
Jack bean concanavalin A	50	7.1	>400[c]	
Bovine pancreatic α-chymotrypsinogen A	25	9.1	>700[c]	
Hen ovalbumin	45	4.6	800	>800
Oxidized bovine pancreatic ribonuclease A	13.7	~6	~800[a]	
Bovine pancreatic ribonuclease A	13.7	9.5	>1400[c]	
Bovine milk β-lactoglobulin	36	5	~1500[a]	
Hen egg white lysozyme	14.4	11	2700	250

[a] Value graphically extrapolated from amount of inhibition caused by smaller amounts of competitor.
[b] Assay performed with suspended protein due to low solubility.
[c] No inhibition by the amount of competitor indicated.

TABLE II

Inhibition by polyproline of the precipitation of tracer protein by proanthocyanidin at pH 4.9

The standard competitive binding assay with 1.0 mg (15 nmol) of iodine 125-labeled bovine serum albumin and 0.05 mg of tannin was performed as described in the text.

Molecular weight	Amount of competitor added	Inhibition[a]	Inhibition/nmol
	nmol	%	%
13,000	1.9	50	26
9,000	4.9	50	10
2,100	330	37	0.11
1,500	600	49	0.082
1,300	900	45	0.050
1,000	950	40	0.042
800	1150	40	0.035
600	950	26	0.027
500	1400	5	<0.01
≤400	3400	2	<0.01

[a] The per cent inhibition obtained with the indicated amount of competitor.

peptides, glycylproline, *N*-acetyl proline methyl ester, and pentaglycine do not inhibit the precipitation of bovine serum albumin when used in the competition assay in amounts varying from 1 to 30 μmol. Similarly, the affinities of several low molecular weight compounds for proanthocyanidin are at least a thousand-fold lower than those of proteins and large polymers (Tables I and III).

Polyproline and polysarcosine (poly(*N*-methyl glycine)) are similar in their affinities for proanthocyanidin (Table IV). However, other structurally similar polyamino acids such as polyhydroxyproline have far lower affinities for tannin (Table IV).

TABLE III

Inhibition by low molecular weight compounds of the precipitation of tracer protein by proanthocyanidin at pH 4.9

The standard competitive binding assay with 1.0 mg (15 nmol) of iodine 125-labeled bovine serum albumin and 0.05 mg of tannin was performed as described in the text.

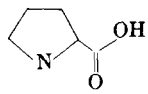
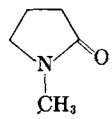
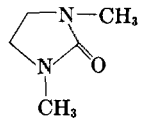
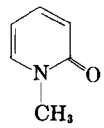
Competitor	Structure	Amount required for 50% inhibition
		nmol
L-Alanine		2.1×10^6
Glycine		2.3×10^6
L-Proline		3.0×10^6
1-Methyl-2-pyrrolidinone		3.0×10^6
1,3-Dimethyl-2-imidazolidinone		2.2×10^6
1-Methyl-2-pyridone		1.5×10^6
N,N-Dimethyl formamide		8.8×10^6
N-Methyl formamide		1.5×10^7
Formamide		2.3×10^7

TABLE IV

Inhibition by polyamino acids of the precipitation of tracer protein by proanthocyanidin at pH 4.9

The standard competitive binding assay with 1 mg (15 nmol) of iodine 125-labeled bovine serum albumin and 0.05 mg of tannin was performed as described in the text.

Competitor	Molecular weight	Amount required for 50% inhibition
		nmol
Polyhydroxyproline	10,000	~700[a]
Polyproline	9,000	4.9
Polysarcosine	4,800	10
Poly(prolyl-glycyl-proline)	2,450	600
Polyproline	1,500	~700[a]

[a] Value graphically extrapolated from amount of inhibition caused by smaller amounts of competitor.

DISCUSSION

Large differences in the relative affinities of proteins for proanthocyanidin were not expected since there are few previous reports of specificity in the interaction between tannin and protein (18). The variation over several orders of magnitude in the affinities of proteins for tannin at two pH values suggests that proanthocyanidin interacts with protein in a specific and selective manner.

The relative affinities of proteins and polypeptides for proanthocyanidin is influenced by the size of the polymer. With the exception of proline-rich protein (9), all of the proteins examined which have molecular weights less than 20,000 have rather low affinities for tannin. The extremely low affinities of small peptides and nonpolymeric compounds for tannin, and the nonlinearity of the increase in affinity with increase in polyproline size implies that the proanthocyanidin-polypeptide interaction involves multiple binding sites.

Previous studies (19) suggested that the proanthocyanidin-protein interaction is strongest near the isoelectric pH where protein-protein electrostatic repulsion is minimized. In accordance with those results, globular proteins with acidic isoelectric points like bovine serum albumin have greater affinities for tannin at pH 4.9 than at pH 7.8, and basic proteins like lysozyme have higher affinities at the higher pH.

A common characteristic of proteins and polypeptides with high affinity for tannin is their high proline content. Gelatin contains 18 mol % proline plus hydroxyproline (17) and rat parotid proline-rich protein contains 40 mol % proline (9). Moreover, the heterocyclic vinyl pyrrolidone subunits of polyvinylpyrrolidone bear some structural resemblance to the pyrrolidine ring of proline. Such heterocyclic ring structures do not have a high intrinsic affinity for tannin, as is shown by the low affinities of proline, its *N*-acetyl methyl ester, and several other heterocyclic compounds for tannin. Low molecular weight heterocyclic compounds have affinities similar to the affinities of the noncyclic amino acids alanine and glycine (Table III).

Hydrogen bonding between phenolic hydroxyl and peptide carbonyl is a major force stabilizing proanthocyanidin-protein complexes (15, 17). Such bonding is strengthened by alkyl substitution on the amide nitrogen adjacent to the carbonyl (20). Thus, the substituted derivatives *N*-methyl formamide and *N,N*-dimethyl formamide are stronger hydrogen bond acceptors than the parent compound formamide (20), and the substituted derivatives have higher affinities for tannin than the parent compound (Table III). The *X*-proline peptide bond contains a substituted nitrogen adjacent to the carbonyl, so polypeptides with high proline content are strong hydrogen bond acceptors. Their high affinities for tannin are due in part to the enhanced strength of the hydrogen bonding.

In addition, the tertiary structure of these polypeptides is either a collagen-like helix (21) or a random coil (9, 22). The carbonyl oxygens of the peptide bonds in either structure are more exposed and available for hydrogen bonding than those of a compactly folded protein. It is therefore reasonable to believe that proteins such as gelatin (22) and proline-rich protein (9) and polyamino acids like polyproline and polysarcosine (21) which contain large regions of random coil or collagen-like helices have high affinities for proanthocyanidin. Interchain hydrogen bonding which diminishes the accessibility of the carbonyl oxygens of polyhydroxyproline and poly(prolyl-glycyl-proline) (21) may prevent these helical polyamino acids from interacting with tannin as strongly as polyproline, which has no hydrogen bond donor and cannot form interchain bonds.

Proteins which have compact globular structures, such as ribonuclease A, cytochrome *c*, lysozyme, and myoglobin (23),

have lower affinities for tannin than do the loosely structured globular proteins like bovine serum albumin and histone F1 (23), probably because of the increased accessibility of the peptide backbone of the less compact proteins. Performic acid oxidized ribonuclease A, which has a random coil structure (24), has an affinity for tannin at least twice as large as that of the compact native protein, and α-lactalbumin has an affinity eight times as large as that of lysozyme, which has a similar amino acid sequence but more compact structure (23). In both of these pairs of proteins not only does the protein with greater affinity have a more open structure, but also it has a more acidic pI value (25, 26), further enhancing its affinity for tannin at pH 4.9.

Although tannins are generally considered to be nonspecific protein binding agents, our results show that proanthocyanidin may efficiently precipitate one protein in the presence of a large excess of another protein. The specificity of interaction is a function of the size, conformation and charge of the protein molecule. The high affinities of proline-rich polypeptides for tannin are at least partially due to their open conformations and their capacities to form strong hydrogen bonds with proanthocyanidins.

The reduced growth (27) and impaired proline utilization (28) of animals fed sorghum or other tannin-containing feeds may be due to diminished digestibility of proline-rich proteins which interact specifically with the dietary proanthocyanidin. Sorghum grain (8) and saliva (9) contain proline-rich proteins that interact very strongly with tannin and may to some extent protect other dietary and digestive proteins from interaction with tannin. Selective, high affinity interactions between proanthocyanidin and foreign proteins may protect the plant from pathogens or predators.

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