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ANTINUTRITIONAL EFFECTS AND ECOLOGICAL SIGNIFICANCE OF DIETARY CONDENSED TANNINS MAY NOT BE DUE TO BINDING AND INHIBITING DIGESTIVE ENZYMES

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Abstract—The digestive enzymes alkaline phosphatase and 5′-nucleotide phosphodiesterase, solubilized from bovine intestinal mucosa and purified to homogeneity, were found to be strongly inhibited in vitro by condensed tannins (proanthocyanidins) purified from sorghum seeds and from quebracho. Tannin inhibition was prevented and reversed by the detergent Triton X-100 (protein-binding agent), by soluble polyvinylpyrrolidone (tannin-binding agent), or by phosphatidylcholine (membrane component). When tested as a crude particulate membrane fraction more characteristic of their in vivo condition, both enzymes were inhibited much less than either purified enzyme at the same tannin concentration. Because the enzymes appear to be relatively insensitive to inhibition by tannin in conditions which mimic in vivo conditions, and because the proportion of the dietary tannin which is available to interact with these enzymes in the digestive tract is likely to be rather small, we suggest that the antinutritional effects and ecological significance of dietary tannins are not due to tannin inhibition of these or other digestive enzymes by direct binding to them.

Key Words—Dietary tannins, digestive enzymes, phosphatase, 5′-nucleotide phosphodiesterase, enzyme inhibition.

INTRODUCTION

Tannins are plant polyphenols capable of forming strong complexes with proteins. Tannins are generally beneficial to the plants that produce them, func-

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tioning as part of the plants' chemical defense system against herbivores and/or microorganisms (Fellows, 1980). Tannins are effective antifeedants, but the mechanism of this effect has not been established (Mole and Waterman, 1987a). In animal diets tannins generally show antinutritional effects such as diminished weight gains and poor feed efficiency (Blair and Mitaru, 1983).

The growth-depressing effect of dietary tannins has been attributed, in part, to a decrease in protein digestibility (Vohra et al., 1966), possibly due to formation of a complex between the tannin and endogenous proteins such as digestive enzymes (Glick and Joslyn, 1970). Digestive enzymes such as trypsin and α-amylase have been reported to be inhibited in vitro by tannins (Griffith and Moseley, 1980). The digestive enzyme enterokinase, which plays a crucial role in the activation of digestive proteases (Maroux et al., 1971), has also been reported to be inhibited in vitro by (dietary) tannins (Oh and Hoff, 1986). These investigators also reported that tannin may either inhibit or stimulate in vitro digestion by intestinal proteases, depending upon the reaction conditions. Mole and Waterman (1985, 1987b) have also questioned the assumption that the effects of dietary tannins are due to their inhibition of digestive enzymes, and several observations inconsistent with inhibition of digestion as the sole or primary mechanism of the antinutritional effects of dietary tannin in mammals have been summarized (Butler et al., 1986). It has recently been concluded that dietary tannins do not reduce the nutritional value of insect foods by inhibiting digestion (Martin et al., 1987).

Condensed tannins (proanthocyanidins) have been shown to be highly selective in their binding; large, conformationally loose, proline-rich proteins are bound several orders of magnitude more strongly than small, compact proteins (Hagerman and Butler, 1981). Dietary tannin has opportunities for complexing with a wide variety of dietary proteins and other proteins of the digestive tract which have a very high affinity for tannin (Mehansho et al., 1983) before it is exposed to the major digestive enzymes. Tannin may therefore not be free to bind digestive enzymes when it encounters them. Moreover, some digestive enzymes occur in membrane-bound forms, which may be less susceptible to inhibition by tannin than the soluble forms purified and tested in vitro. In an attempt to assess the nutritional significance of the inhibition of digestive enzymes in vitro, we have investigated the inhibition, by condensed tannin purified from sorghum seeds and from quebracho, of two digestive enzymes as they occur in a particulate, membrane-bound form as well as the soluble form which has been purified and characterized in vitro. Because the effects of tannin on proteolytic enzymes are complicated by tannin binding to both the protease and the protein substrate, we have chosen to study phosphohydrolases, for which tannin binds only to the enzymes and not to the substrates. These enzymes, alkaline phosphatase (APase) and 5′-nucleotide phosphodiesterase (PDase), are from mucosal cells of the bovine intestinal brush border.

METHODS AND MATERIALS

Materials. The soluble forms of APase (Landt et al., 1978) and PDase (Landt and Butler, 1978) were purified from bovine intestine as previously described. Purified soluble enzymes were used in all experiments in which particulate enzyme is not specified. To prepare particulate enzymes (all steps carried out at 0–5°C), 2 g frozen bovine intestinal mucosa was suspended in 18 ml of buffer A (100 mM Tris HCl, 12 μM $ZnSO_4$, pH 8.0) using a ground glass homogenizer. The homogenate was centrifuged 20 min at 35,000*g* and the supernatant discarded. The pellet was suspended in 18 ml of buffer, and centrifuged again. The pellet from the second centrifugation was resuspended in buffer A and homogenized on a Brinkmann Polytron equipped with a PT-10 head, at full power for five bursts of 12 sec each, with 2-min intervals for chilling to 0°C. In order to show that Polytron treatment did not convert the enzyme to a soluble form, a sample of the homogenate was centrifuged 90 min at 200,000*g*. Most of the enzyme activity sedimented; only 3.5% of the APase activity and 4.5% of the PDase activity remained in the supernatant.

Substrates (4-nitrophenyl phosphate and 4-nitrophenyl phenylphosphonate), bovine liver phosphatidylcholine, soluble polyvinylpyrrolidone (PVP), average mol wt 10,000, and Triton X-100, average mol wt 625, were from Sigma (St. Louis, Missouri). Sorghum tannin was purified from the seed of the bird-resistant sorghum hybrid, BR 64, by the method of Hagerman and Butler (1980a). Quebracho tannin was purified from quebracho extract (Trask Chemical Corp., Marietta, Georgia) as previously described (Asquith and Butler, 1985).

Enzyme Assays. APase and PDase activities were determined as previously described (Landt and Butler, 1978) using 1 mM 4-nitrophenyl phosphate and 4-nitrophenyl phenylphosphonate, respectively, as substrates at pH 8.0.

RESULTS

Soluble highly purified PDase is strongly inhibited by tannin (Figure 1). Under the conditions used in these assays, no precipitation of the enzymes by tannin is observed; both PDase and APase are glycoproteins, which may account for the solubility of their complexes with tannin (Asquith et al., 1987). Inhibition is not instantaneous, but it is essentially complete in 15 min. Tannins purified from sorghum seeds or quebracho are equally effective as inhibitors, with 50% inhibition at about 20 μg/ml in the subsequent assay. The experiment shown in Figure 1 was carried out at pH 8.0. Similar effects were observed when the enzyme was incubated with tannin at pH 6.0 and assayed at pH 8.0; physiological pH in the small intestine should be between pH 6 and pH 8.

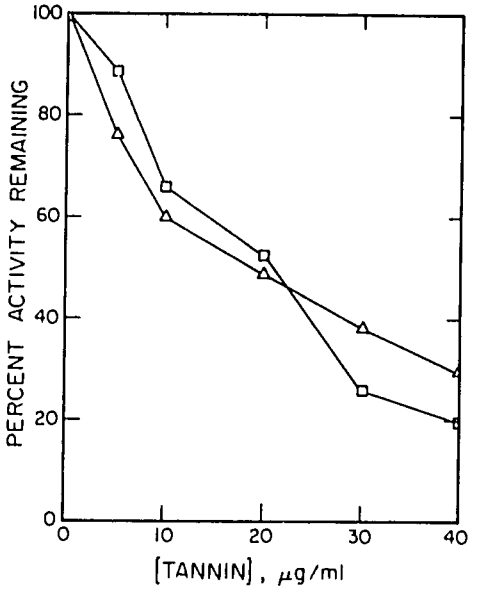

FIG. 1. PDase inhibition by sorghum and quebracho tannin. PDase was incubated for 15 min at 30°C in 0.1 M Tris (HCl), 10 μM $ZnSO_4$, pH 8.0 (buffer A) containing the indicated concentration of sorghum (squares) or quebracho (triangles) tannin. The mixture was then diluted 3.3-fold into assay mix and the activity measured as described in Methods and Materials.

Purified APase is somewhat less sensitive to inhibition by tannin (Figure 2). In contrast to PDase, the two sources of tannin are not equivalent for APase; up to eight times more quebracho tannin is required to obtain the same degree of inhibition observed with sorghum tannin. Sorghum tannin is likewise more effective than quebracho tannin in protein binding and precipitation assays (Asquith and Butler, 1985).

Effect of Triton X-100, Phospholipid, and PVP. Figure 3 shows the pronounced effect of 10 mM Triton X-100 on inhibition of PDase by tannin. Tannin concentrations, which otherwise produce 80% inhibition of PDase, are not inhibitory in the presence of Triton X-100. Tannin even slightly stimulates PDase when 10 mM Triton X-100 is present. Tannin likewise does not inhibit APase in the presence of 10 mM Triton X-100 (Butler et al., 1984). Similarly, in the presence of phosphatidylethanolamine, a prominent membrane component, tannin inhibition of PDase (Figure 4) and APase (not shown) is greatly reduced.

When Triton X-100 is added to tannin-inhibited APase, inhibition is completely reversed (Figure 5). The inhibitory effect of tannin, therefore, does not

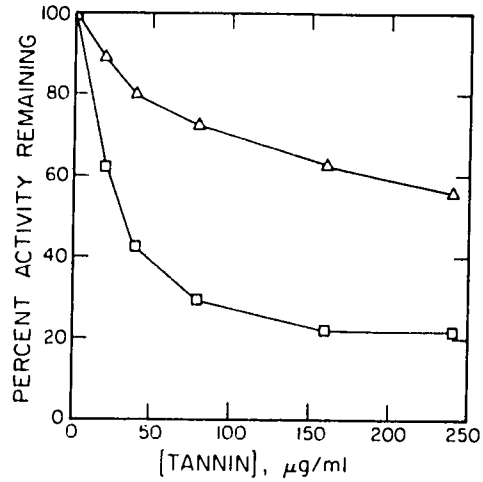

FIG. 2. APase inhibition by sorghum and quebracho tannin. APase was incubated for 15 min at 30°C in Buffer A containing the indicated concentration of sorghum (squares) or quebracho (triangles) tannin and assayed as in Figure 1.

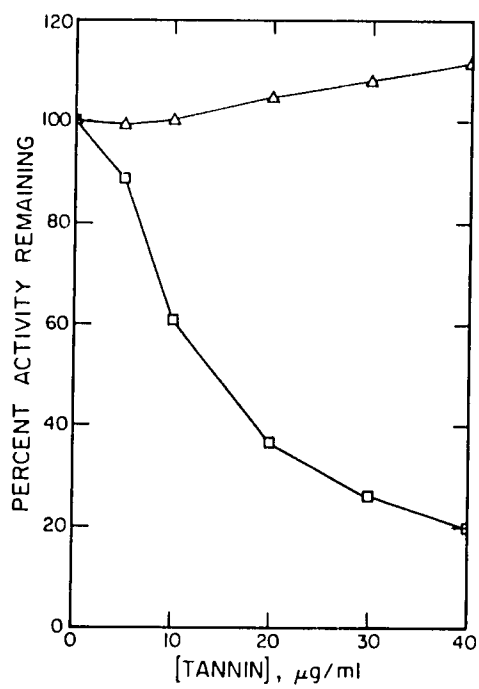

FIG. 3. Effect of Triton X-100 on tannin inhibition of PDase. PDase was incubated for 15 min in buffer A (squares) or in buffer A containing 10 mM Triton X-100 (triangles) in the presence of the indicated concentrations of sorghum tannin.

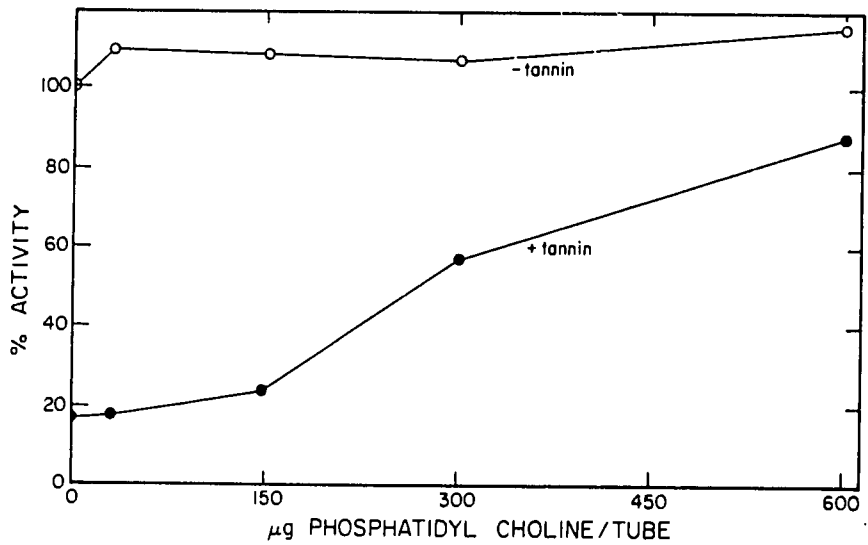

FIG. 4. Effect of phosphatidylcholine on tannin inhibition of PDase. PDase, 1.1 μg protein, was incubated for 30 min at 30°C with the indicated amount of phosphatidylcholine in buffer A after which 30 μg of sorghum tannin was added for a final volume of 0.5 ml another 30 min before assay.

depend on irreversible denaturation or modification of the enzyme. Triton X-100 also reactivated PDase after tannin inhibition (data not shown).

Addition of soluble PVP to tannin-inhibited PDase leads to complete reactivation (Figure 6). Tannin-inhibited APase was also reactivated by PVP, but the recovery of activity after PVP addition was not quite as complete as with PDase. Thus, the inhibition of these enzymes by tannin can be reversed by addition of agents that bind either protein (Triton X-100) or tannin (PVP). On the other hand, 10% (v/v) methanol, which enhances protein precipitation by tannin (Hagerman and Butler, 1980b), did not have a significant effect on tannin inhibition of PDase (data not shown).

When the enzyme sample is a particulate fraction instead of the purified soluble enzyme, the effect of tannin is much smaller. As shown in Figure 7, 50 μg/ml tannin only inhibits the particulate PDase by 11%, whereas the soluble purified PDase is inhibited 45% at this tannin concentration. The particulate enzyme preparation contained about the same level of enzyme activity as the soluble purified PDase, but it had approximately 200 times as much protein. In a previous study (Butler et al., 1984) a higher concentration of the crude particulate enzyme was used, and 50 μg/ml tannin caused only 3% inhibition of the PDase activity. APase activity of the particulate fraction is likewise less

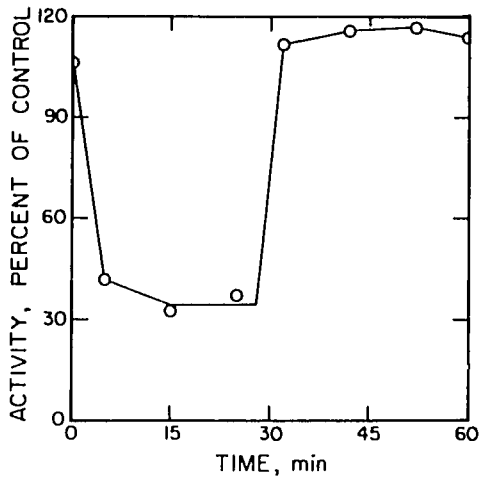

FIG. 5. Reversal of tannin inhibition of APase by Triton X-100. APase was incubated for the time shown at 30°C in buffer A containing 200 μg/ml sorghum tannin and 8 mg/ml methanol. A control sample contained methanol without tannin. At 28 min, both samples were made 12 mM in Triton X-100.

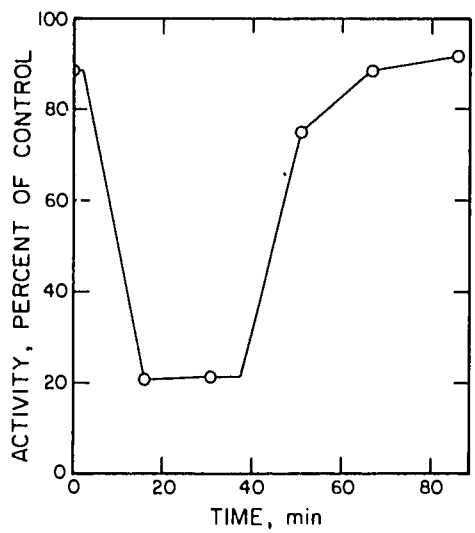

FIG. 6. Reversal of tannin inhibition of PDase by PVP. PDase was incubated for the time shown at 30°C in buffer A. At 2 min, the experimental sample, but not the control, was made 31.2 μg/ml in sorghum tannin. At 38 min, both samples were made 3.85 mg/ml in soluble PVP.

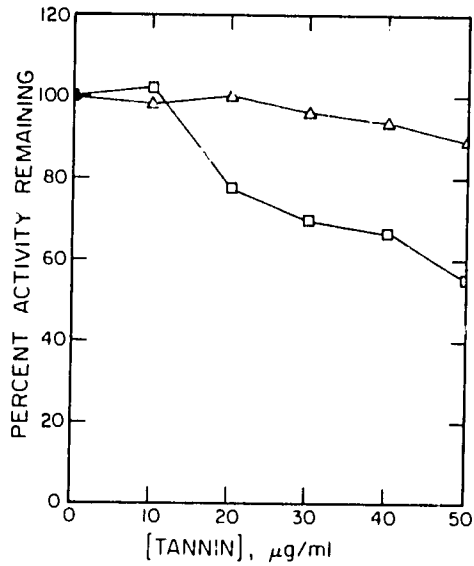

FIG. 7. Comparison of the tannin inhibition of soluble versus particulate PDase. Soluble purified PDase (squares) or the washed particulate fraction from bovine intestinal mucosa (triangles) was incubated for 30 min at 30°C in buffer A containing the indicated concentrations of sorghum tannin.

inhibited by 250 μg/ml tannin (44% inhibition) than is the purified soluble enzyme (79% inhibition).

DISCUSSION

Our results demonstrate, as expected, that in their purified soluble form these digestive enzymes are strongly inhibited by purified condensed tannins. This in vitro inhibition of digestive enzymes may not, however, account for the reported lower digestibility in ruminants (McBrayer et al., 1983; Kumar and Singh, 1984) and nonruminants (Featherston and Rogler, 1975; Martin-Tanguy et al., 1976) of diets containing tannin.

Both APase and PDase occur predominantly in a particulate, membrane-bound form is freshly prepared homogenates of intestinal mucosa; in the rat intestine, brush border membrane the binding of APase has been shown to be hydrophobic in nature (Seetharam et al., 1985). These enzymes in the washed particulate fraction proved to be less susceptible than the soluble form to inhibition by tannins at comparable tannin concentrations. This diminished susceptibility is likely due to the presence in the particulate form of other proteins

which compete for binding the tannin; the specific activity (units of activity per milligram of protein) of the particulate enzymes was approximately 0.5% that of the corresponding soluble purified enzymes. The membrane-bound enzymes may also be protected by bound phospholipids which, like Triton X-100, diminish tannin inhibition of the soluble form of the enzymes. In our early attempts to solubilize and purify PDase (Kelly et al., 1975), phospholipids were found to be strongly associated with this enzyme.

In addition to the diminished susceptibility to tannin of the enzymes in their predominant in vivo form, under physiological conditions dietary tannin is probably not accessible to digestive enzymes. Dietary proteins (Butler et al., 1984) or specialized tannin-binding proteins of the saliva (Mehansho et al., 1983) are available to form complexes with dietary tannin before it is exposed to digestive enzymes. Even if digestive enzymes are subject to inhibition by dietary tannins, the effect is likely to be reversed by detergents (Figure 5) (Oh and Hoff, 1986) such as bile acids or by various tannin-binding materials (Figure 6) encountered in the digestive tract. Our observations are consistent with those of Armstrong (1983), who found other digestive enzymes to exhibit full activity in the intestines of rats fed diets containing tannins from sorghum and other sources.

We conclude that the antinutritional effects (and ecological significance) of dietary tannin are probably not due to binding and inhibition of digestive enzymes by tannin, especially those which predominantly occur in a membrane-bound form. The relative insensitivity to tannin of membrane-bound enzymes suggests that other membrane-associated processes such as absorption may be little affected by dietary tannin. The antinutritional effects of dietary tannin that result in reduced protein digestibility may be due to formation of less-digestible complexes of dietary proteins with tannins (Butler et al., 1984). Other antinutritional effects are unrelated to digestibility (Rogler et al., 1985; Mehansho et al., 1985).

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