NUTRITIONAL IMPLICATIONS OF BOUND PROANTHOCYANIDINS

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ABSTRACT
The correlation between protein binding capacity of proanthocyanidin-rich neutral detergent fiber (NDF) and their proanthocyanidin (PAs) content was very low (r = -0.21; n = 6). These PAs-rich NDF did not affect growth of a tannin-sensitive strain of Clostridium perfringens. A substantial amount of bound PAs (75 to 92 %) disappeared from the NDF in 24 h of fermentation in an in vitro incubation medium containing rumen microbes. Addition of a tannin-binding agent, polyethylene glycol to the incubation medium containing rumen microbes and browses made free of extractable tannins or NDF rich in bound PAs increased the gas production up to 100 %. The results suggest that bound PAs seem to be inert and do not affect microbial fementation. However, these bound PAs get released into the medium as a result of microbial action and then affect microbial fermentation.

KEYWORDS
tannins, polyphenols, condensed tannins, proanthocyanidins

INTRODUCTION
Tannins are polyphenolic compounds which form complexes with proteins, carbohydrates, alkaloids, vitamins and minerals. Tannins are present in extractable (free) and unextractable forms (bound to macromolecules). The free or extractable tannins have been extensively studied and have been shown to have both adverse and beneficial effects depending on their content and nature and physiological state of the animal. The bound proanthocyanidins form a significant portion of the total proanthocyanidins (Makkar and Singh, 1991; Terrill et al., 1992). Not much is know on the nutritional implications of bound proanthocynidins. In the present study we report protein binding capacity and antibacterial activity of proanthocyanidin-rich neutral detergent fibers (NDF) and fate of proanthocyandins bound to NDF in an in vitro rumen fermentation system and their effects on fermentation.

EXPERIMENTAL
Air dried tannin-rich leaves were used for preparation of NDF according to the standard procedure as described by Goering and Van Soest (1970) without the addition of decaline and sodium sulfite. The NDF was dried using a lyophilizer after wasing it thoroughly with distilled water till it is free of sodium dodecylsulfate (SDS). The proanthocyanidins (PAs) bound to NDF were measured by the butanol-HCl-iron reagent as described in Makkar et al. (1995a). The SDS-free NDF was used for all the studies.

Protein binding capacity (PBC) of NDF. To the NDF (650 mg) in a beaker was added 15 ml of bovine serum albumin (BSA) solution (5 mg/ml acetate buffer [pH 5.0. 0.2 M contining 0.17 M NaCl]). The contents were stirred for 15 min at room temperature and then placed in a refrigerator (ca 4°C) for 30 min. The contents were poured in crucibles and washed throughly in the acetate buffer (ca 300 ml) to remove BSA losely bound to the NDF. The NDF residue was again lyophilized. To different quantities of this residue (25, 50, 75 and 100 mg) was added 2 ml of 1 % SDS to remove the BSA bound to the NDF. Contents were vortexed and centrifuged to collect supernatant containing BSA desorbed from the NDF. The BSA in the supernatant was determined by the method of Lowry et al. (1951). Proper blanks were run in a manner similar to that mentioned above except that BSA was omitted from the acetate buffer. The values of proteins obtained in the blanks were subtracted from the corresponding test samples. The release of proteins from the NDF in blanks could be due to pH of the SDS solution (1 %; pH 4.0) which is different from the neutral pH of the neutral detergent solution which also has SDS. The PBC of the NDF were obtained from the regression coefficients (slopes) of linear regressions fitted to measurements performed at four different concentrations.

Activity of NDF against Clostridium perfringens. Both leaf and NDF samples (50 mg and 200 mg respectively) were added to a suspension of C. perfringens type A strain in sterile physiological saline and the mixture was kept in a 50 ml screw cap bottle in a shaking water bath (80 rpm) adjusted at 37°C. After 20 min, aliquots of 2 x 0.1 ml from the mixture were spread evenly with a sterile spatula on two plates of Tryptose sulfute cycloserine (TSC) agar. The plates were then transferred into anaerobic glove box (39°C) containing an atmosphere of 88 % N_2, 7 % H_2 and 5 % CO_2. Blackstained colonies as Colony Forming Units (cfu) were counted after 48h. The cfu did not decrease in tannin-free controls in 20 min of incubation in a shaking water bath.

Fate of NDF bound PAs. Samples of NDF (500 mg) were incubated in 100 ml calibrated glass syringes with 40 ml of the rumen-fluid medium as described earlier (Makkar et al., 1995). After 24 h of incubation at 39°C, the contents of syringes were emptied in the refluxing beaker and the syringes were washed with two 20 ml portions of double strength neutral detergent solution (NDS) to quantitatively recover all the material of the syringes. The contents were refluxed by the standard procedure as described above.

Effects of released PAs . Leaf samples were made free of soluble tannins by treating with 70 % aq. acetone (3 times) and 50 % aq. methanol treatment (3 times) together with ultrasonic treatment and removal of supernatant each time following centrifugation. The residues were lyophilized. These samples free of soluble tannins (0.5 g) and the NDF (0.5 g) were incubated without and with (1 g) polyethylene glycol 6000 (PEG) in presence of a rumen liquor containing medium (Makkar et al., 1995a) to study the effect of bound proanthocyanidins released in the medium during fermentation.

RESULTS AND DISCUSSION
Protein binding capacity of NDF. The PBC of NDF from six tannin-rich leaves varied from 0.30 to 1.48 µBSA/mg NDF and the bound PAs levels in these NDF were from 0.58 to 4.0 absorbance units (550 nm)/10 mg NDF. The correlation between PBC and bound PAs was insignificant (r = -0.21, n = 6). It shows that bound PAs do not play a role in binding to proteins and is also not likely to affect the microbial fermentation. The NDF from wheat straw and white clover (free of bound PAs) have also been found to bind BSA (1.16 and 1.0 µg/mg NDF respectively). These observations also suggested that NDF has non-specific sites (Allen et al., 1985) where protein can bind to the fiber fraction.

Activity of NDF against C. perfringens. In a previous study we found that an exposure (10 min) of C. perfringens type A strain to tannin-containing extracts from various browses did not form any colony on the TSC agar, and this antibacterial effect was not observed on removing tannins from the extracts with a tannin-binding agent, insoluble polyvinyl pyrrolidone. Furthermore, purified tannins also

completely inhibited the growth of C. perfringens type A strain, suggesting this strain to be highly sensitive to tannins (unpublished results). In the present study, cfu were also not observed when incubations were carried out with 50 mg of the tannin-rich whole samples containing both soluble and insoluble tannins. On the other hand, NDF samples (200 mg) rich in bound PAs did not decrease the cfu. These observations suggested that bound PAs do not have antibacterial activity.

Fate of NDF bound PAs. A substantial amount of bound PAs (75 to 92 %) disappeared from the NDF fraction in 24 h of fermentation (Table 2). Our previous study (Makkar et al., 1995b) showed that PAs are not hydrolysed by rumen microbes. Therefore, PAs which emanated from the NDF would be present in the solution and might influence microbial activity. This was examined in the next study. Bravo et al. (1993) have demonstrated that non-extractable PAs from carob pods were not digested in the rat intestinal tract.

Effect of bound PAs. Addition of PEG in the rumen liquor containing incubation medium and the residual sample after removal of free tannins by aq. acetone and methanol treatments increased gas production (% increase: Bauhinia spp. 2.1; Acacia saligna 25.1; Erica spp. 79.6; Azadirachta indica 19.3). The PEG binds to tannins and make them inert. The increase in gas on addition of the PEG suggested that tannins which are tightly bound to feed matrix and are released in the process of digestion affect the microbial digestion. It could be that the present approach of removal of free tannins did not completely remove all soluble tannins from the leaf samples. Therefore, we also investigated the effect of PEG on increase in the gas from NDF of tannin-rich leaves. An increase in gas of up to 100 % was observed, confirming that bound PAs could affect the microbial fermentation.

Conclusions. Condensed tannins (PAs) in the bound form seem to be inert and do not affect microbial fementation. However, these bound PAs get released into the medium as a result of microbial action and affect microbial fermentation.

REFERENCES

Allen, M.S., I.M. McBurney and P.J. Van Soest. 1985. Cation-exchange capacity of plant cell walls at neutral pH. J. Sci. Food Agric. **36**: 1065-1072.

Bravo, L., E. Manas and F. Saura-Calixto. 1993. Dietary non-extractable condensed tannins as indigestible compounds: effects on fecal weight, and protein and fat excretion. J. Sci. Food Agric. **63**: 63-68.

Goering, H.K. and P.J. Van Soest. 1970. Forage Fiber Analyses. ARS, USDA Washington, DC, Agricultural Handbook, no. 379, 20 pp.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265-275.

Makkar, H.P.S. and B. Singh. 1991. Distribution of condensed tannins (Proanthocyanidins) in various fibre fractions in young and mature leaves of some oak species. Anim. Feed Sci. Technol. **32**: 253-260.

Makkar, H.P.S., M. Blümmel, M. and K. Becker. 1995a. Formation of complexes between polyvinyl pyrrolidones and polyethylene glycols with tannins and their implications in gas production and true digestibility in in vitro techniques. Br. J. Nutr. **73**: 897-913.

Makkar, H.P.S., M. Blümmel, M. and K. Becker. 1995b. In vitro effects and interactions between tannins, saponins and fate of tannins in the rumen. J. Sci. Food Agric. **69**: 481-493.

Terrill, T.H., A.M. Rowan, G.B. Douglas, T.N. Barry. 1992. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. J. Sci. Food Agric. **58**: 321-329.

Table 1

Effect of incubation of tannin-rich and tannin-free samples and their neutral detergent fiber (NDF) fractions with a suspension of *Clostridium perfringens* type A strain for 20 min on growth (cfu, colony forming units) of the microbe

	cfu					
			Tannin-rich			Tannin-free
	Prosopis cineraria	*Eucalyptus punctata*	*Eugenia jambolana*	*Robinia pseudoacacia*	*Quercus incana*	*Wheat straw*
Control	5×10^6	5×10^6	6×10^6	2.8×10^6	3.6×10^6	4.0×10^6
50 mg whole leaf sample	Negative	Negative	Negative	7.5×10^1	3.5×10^3	4.2×10^6
200 mg NDF	1×10^6	5×10^6	6×10^6	2.6×10^6	3.5×10^6	4.0×10^6

Table 2

The contents of neutral detergent fiber (NDF) and proanthocyanidins (PAs) in NDF fractions of leaf samples before and after 24 h fermentation

	Leaf sample incubated			NDF following incubation	
Tannin-rich leaves	Dry wt. (mg)	NDF[a] (mg)	PAs in NDF[b] (A550nm units)	Dry wt. of NDF (mg)	PAs in NDF (A550nm units)
Dichostachys cinerea	470	305.1	23.64	238.8 + 16.7	4.86 + 0.29
Cassia sieberiana	470	279.5	57.69	93.2 + 18.4	4.70 + 0.80
Robinia pseudoacacia	465	210.9	44.37	121.1 + 6.3	4.76 + 0.24
Acioa barteri	467	331.1	27.54	260.5 + 2.9	6.91 + 0.55

* Mean + SD, [a] average of two values; the deviation of each value from the mean was not more than 5 %, [b] average of at least four values; the deviation of each value from the mean was not more than 8%.