Protein-tannin complexes are susceptible to proteolytic degradation

A. DIAZ-HERNANDEZ, M.J. NIXON, F.M. BALL, R.A. LENG AND J.B. ROWE

Department of Animal Science, University of New England, Armidale, NSW 2350, Australia.

ABSTRACT

In vitro studies were carried out to determine the factors that affect formation and dissociation of the protein-tannin complex (PTC) under conditions simulating those found in the digestive tract of the ruminant. For this study purified condensed tannins (CT) from the following tropical plants were used: *Acacia harpophylla, Acacia aneura, Acacia saligna, Acacia holosericea, Tipuana tipu, Albizia chinensis, Flemingia macrophylla, Grevilea robusta, Casuarina cristata, Casuarina cunninghamiana, Azadarachta, Leucaena leucocephala, Leucaena pallida and Leucaena diversifolia.* English spinach was selected as the source of soluble protein and during its growing phase the plants were exposed to a ^{14}C-CO_2 atmosphere for 48 h. Following exposure, the foliar protein ribulose 1,5 biphosphate carboxylase/oxygenase, (rubisco) was isolated. The radioactive labeled protein was incubated with the isolated samples of CT for 2 h at 39°C. The mixtures were then centrifuged and the percentages of bound protein (precipitate) and unbound protein (fluid phase) were determined. Formation of the protein-tannin complex (PTC) occurred over a range of pH 3-7. Above pH 7 no PTC was formed. To determine the effects of pH on the dissociation of the PTC, the precipitate was incubated with buffers over a range of pH 3-9. It was found that acidic or alkaline pH was responsible for only 8 and 14% of the dissociation of the PTC, respectively. However, addition of abomasal and intestinal fluid from both sheep and cattle increased dissociation of PTC to more than 60%. Further analysis showed that pepsin and trypsin are largely responsible for the dissociation which occurs with the abomasal and intestinal fluids, respectively.

Keywords: Condensed tannins; protein-tannin complex; tropical plants; pepsin; trypsin.

INTRODUCTION

Condensed tannins (CT) are polyphenolic compounds known to bind to proteins and form insoluble complexes (Haslam, 1979; Swain, 1979). They appear to have detrimental nutritional effects such as reducing feed intake, reducing feed digestibility and increasing faecal nitrogen excretion (Barry and Duncan, 1984; Pritchard *et al.*, 1988). On the other hand, they also have beneficial effects, which include the prevention of bloat (Clarke and Reid, 1972) and the protection of protein against degradation in the rumen (Waghorn *et al.*, 1987). According to Jones and Mangan (1977) the protein-tannin complexes (PTC) are stable and insoluble at rumen pH 5.5-7.0, but dissociate and release protein under acidic conditions (pH 2-3) in the abomasum, rendering the protein available for digestion and absorption in the small intestine.

There is little known about the interactions of purified condensed tannins from tropical plants with the major leaf proteins (Perez-Maldonado, *et al.*, 1995). Our study aimed firstly, to determine the capacities of purified CT isolated from tropical plants to bind to the foliar protein ribulose 1,5 biphosphate carboxylase/oxygenase, (rubisco), and secondly, to determine whether the protein-tannin complex (PTC) can be dissociated by changing pH, by extracts of digesta or by purified proteolytic enzymes.

MATERIALS AND METHODS

Plant material and isolation of tannins: Leaf material was taken from the following range of trees and shrubs growing at Brian Pastures Research Station (Gayndah, Queensland): *Acacia harpophylla, Acacia aneura, Acacia saligna, Acacia holosericea, Tipuana tipu, Albizia chinensis, Flemingia macrophylla, Grevilea robusta, Casuarina cristata, Casuarina cunninghamiana, Azadarachta, Leucaena leucocephala, Leucaena pallida and Leucaena diversifolia.* All samples collected were oven dried in a forced-draught dehydrator for 48 h at 70°C and then ground to pass through a 1 mm sieve.

Preparation of condensed tannins (CT): Leaf material was extracted three times with 70% acetone, followed by extractions (3 times each) with diethyl ether and ethyl acetate. The resulting extract was purified by passage through chromatography on Sephadex LH-20 (Pharmacia, Sweden) according to the procedure of Strumayer and Malin (1975). Tannic acid was obtained from Merck Chemical Co. (Germany).

^{14}C Ribulose 1,5 biphosphate carboxylase/oxygenase: Actively growing english spinach plants were placed in an air-tight chamber under continuous illumination. The label was introduced by injecting as ^{14}C-$NaCO_3$ (2mCi/mmol) into a continuously mixing solution of 5M H_2SO_4. After 48 h, the extraction of the ^{14}C-rubisco protein was performed as described previously by Jones and Lyttleton (1972). The final specific radioactivity was about 7000 dpm/150 ug protein/0.1 ml. Trichloroacetic acid precipitated around 90% of the label, indicating that the label was covalently attached to the protein.

Formation of PTC: 0.2 ml of CT (100 ug/100 ul) dissolved in 10% methanol was added to 0.1 ml solution of ^{14}C- rubisco (100 ug/0.1 ml) prepared in 0.05 M phosphate buffer, pH 7.0 containing 0.1 % (w/v) ascorbic acid and 1mM mercaptoethanol. The solutions were mixed,

incubated for 2 h at 39°C and then centrifuged at 13,000 x g for 10 minutes. Samples (0.1 ml) from the supernatant were taken and their radioactivity determined using a toluene/Triton-X solution (9/4 v/v) containing 0.4% 1, 4-bis-[2-(5-Phenyoxazole)]-Benzene (PPO) and 0,02% 2, 5-Diphenyoxazole (POPOP), and a scintillation spectrophotometer (Packard Instrument Company, Inc.). The amount of protein precipitated as PTC was determined on the basis of the volume of supernatant and amount of radioactivity remaining in solution. Controls showed that protein was not precipitated in the absence of tannin.

To determine the effect of pH on the formation of the PTC, an aliquot from the CT was transferred into buffer solutions (0.05M) with different pH values. Phosphate buffers were used for pH 6-7, Tris-HCl was used for pH 8-10, and for pH 3-5, Citrate-HCl buffer was used. The final concentrations of tannin in the buffer solutions was 0.1 mg/ml.

Dissociation of the PTC: After removing all the supernatant and washing the pellet with phosphate buffer solution (pH 7.0), a number of test solutions (described below) were added to the pellet, mixed thoroughly and re-incubated for 2 h at 39°C. After centrifugation (13,000 x g, 10 min), the radioactivity from an aliquot of supernatant was determined. The amount of radioactivity in the supernatant was used to determine the amount of protein dissociated from the pellet.

The test solutions used to study the dissociation of the protein from the PTC were a) buffers, b) physiological fluids, and c) commercial enzymes. The buffer solutions were the same as described in the experiment on the formation of the PTC. The physiological fluids from the rumen, abomasum, small intestine and gall bladder were obtained from animals killed in a commercial abattoir. After collection, all physiological fluids were immediately stored in ice and then centrifuged at 22,000 x g for 2 h. They were kept frozen at -20°C until used. The effects of pepsin and trypsin on the digestion of rubisco-tannin complexes were determined by adding 0.1 ml of either pepsin or trypsin (2 mg/ml of 0.001 N HCl) (Sigma Chemical Co., USA) and 0.1 ml of Tris-HCl buffer (pH 8.0) or 0.1 ml of citrate-HCl buffer (pH 3.0) to tubes containing the protein-tannin complex. These tubes were then incubated at 39°C for 2 h with periodic shaking. After centrifugation (13,000 x 10 min), aliquots were taken from the supernatant to determine the amount of ^{14}C-protein released by digestion.

RESULTS AND DISCUSSION

Binding capacities

For all tannins studied, the amount of protein precipitated by adding increasing amounts of tannins to a constant amount of protein, increased curvilinearly until a saturation point was achieved. Further addition of tannin did not affect the amount of protein precipitated (Fig. 1). In order to calculate the ratio of bound protein/tannin (as ug of protein precipitated/100 ug of CT present in the PTC) the data were plotted as tannin concentration/% protein precipitated on the ordinate vs. tannin concentration on the horizontal axis. The slope of this relationship was used to calculate the protein/tannin ratio.

FIGURE 1: Amount of protein precipitated (%) with increasing concentrations of tannins.

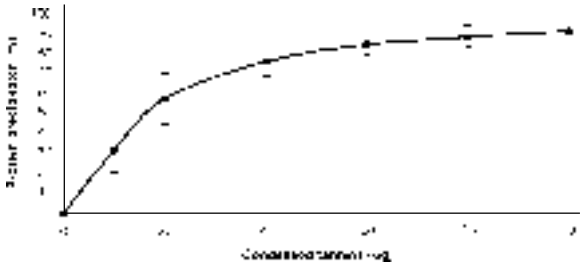


Table 1 shows the ratios of bound protein/tannin and it is clear that values were similar for most of the tannins studied. The slightly higher protein/tannin ratio found for tannic acid might be explained by the presence of other polyphenolic compounds usually found in commercial batches of tannic acid.

TABLE 1: Bound protein/tannin ratios for a range of tropical fodder trees and shrubs.

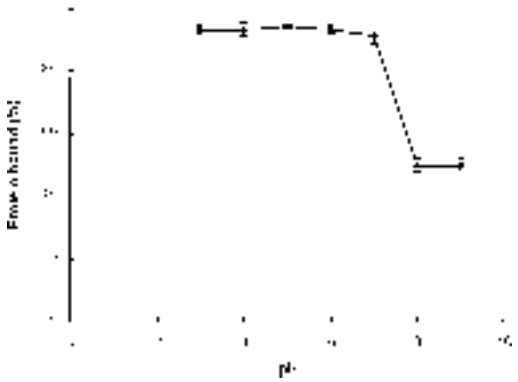
Plant species.	Bound protein / tannin (ug protein/100 ug CT)
L. food leucocephala	68 ± 2.4
L. pallida	72 ± 0.1
L. diversifolia	70 ± 1.0
A. aneura	76 ± 1.0
A. harpophylla	72 ± 0.1
A. holosericea	68 ± 2.4
A. saligna	71 ± 2.6
Azadarachta	73 ± 2.8
G. robusta	79 ± 1.9
A. chinensis	76 ± 0.1
T. tipu	69 ± 1.0
F. macrophylla	74 ± 1.7
C. cristata	72 ± 1.1
C. cunninghamiana	69 ± 0.5
Tannic acid	78 ± 4.4

Effect of pH on the formation and dissociation of the protein-tannin complex

The effect of pH on the formation of the protein-tannin complex is shown in Fig. 1. It can be seen that the binding of the protein to the tannins occurred only in the pH range 3-7, and that similar proportions of the protein were bound by all the tannins tested. Above pH 7, almost no precipitation occurred. This finding is consistent with the results of Martin *et al.*, (1985) and Mole and Waterman (1987), who found that condensed tannins do not form strong complexes with proteins at alkaline pH.

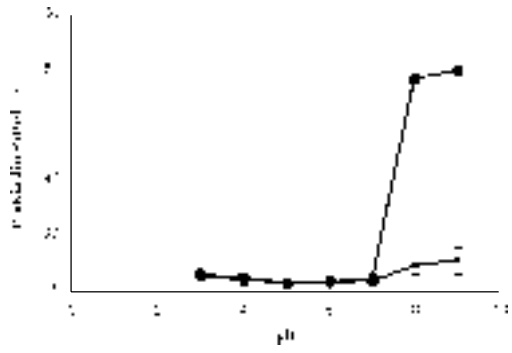
Fig. 2 shows the dissociation of the PTC over a range of pH and shows that once the PTC was formed, the addition of buffer solutions in the pH range 3-9 had little effect, with the exception of tannic acid, on the dissocia-

FIGURE 2: The effect of pH on the formation of the protein-tannin complex for all of the plants studied.



tion of the PTC . This implies that forces other than hydrogen bonding must be involved in the protein-tannin interactions. Oh *et al.*, (1980), Osawa and Walsh (1993), and Hagerman and Butler (1980) have suggested that hydrophobic interactions could be the most important forces in the formation of the PTC. Tannic acid, on the other hand, is a hydrolyzable tannin and the forces involved in the binding of the PTC are susceptible to changes in pH (Schultz *et al.*, 1981).

FIGURE 3: Effects of pH on the dissociation of protein from the protein-tannin complex for: (■) Tannic acid and (●) tropical plants studied.



Our results show that both fomation and dissociation of the PTC are largely unaffected by a wide range of pH. Therefore, only a minor dissociation of the PTC could be expected in the alkaline environment of the small intestine, unless some other agent causes dissociation of the complex. If the dissociation is purely dependent on pH most of the PTC would remain undigested in the small intestine and be excreted in the faeces. This is consistent with the observations with ruminants in which CT increased the nitrogen loss in the faeces and reduced nitrogen retention.

Effects of abomasal and intestinal fluids on the dissociation of the protein-tannin complex.

When the PTC pellets were incubated with either abomasal fluid (adjusted to pH 2.5) or intestinal fluid (adjusted to pH 8.0) from sheep there was partial dissociation of the protein from the complex (Table 2). Similar results were obtained using abomasal and intestinal fluids from cattle. These results suggested that proteolytic enzymes present in abomasal and intestinal digesta were responsible for dissociation of the PTC

TABLE 2: Effects of abomasal and intestinal fluids from sheep on the percentage of protein dissociated from the protein-tannin complex.

Condensed tannin	Protein dissociated (%)	
	Abomasal fluid	Intestinal fluid
L. leucocephala	49 ± 2.5	53 ± 0.4
L. pallida	41 ± 2.7	48 ± 4.4
L. diversifolia	48 ± 1.9	56 ± 1.8
A. aneura	50 ± 0.9	49 ± 0.8
A. harpophylla	56 ± 2.4	63 ± 2.0
A. holosericea	49 ± 5.4	94 ± 9.4
A. saligna	41 ± 5.1	41 ± 4.7
Azadarachta	42 ± 0.2	58 ± 2.0
G. robusta	40 ± 4.5	56 ± 0.6
A. chinensis	38 ± 0.2	53 ± 2.9
T. tipu	48 ± 4.1	65 ± 3.3
F. macrophylla	51 ± 0.4	61 ± 3.2
C. cristata	46 ± 1.7	58 ± 9.3
C. cunninghamiana	45 ± 1.2	74 ± 6.3
Tannic acid	62 ± 0.7	94 ± 1.1

This was confirmed by the results obtained from incubations of the PTC with commercial enzymes pepsin (pH 3) and trypsin (pH 8) and found that both caused partial dissociation of the protein from the complex (Table 3). However, sequential digestion of the protein-tannin pellets with pepsin and trypsin showed that their dissociative effects are only partially additive (Table 3).

The results of this study with respect to the dissocia-

TABLE 3: Dissociation of protein from the protein-tannin complex by digestion for 2h with pepsin (pH 3) or trypsin (pH 8).

Condensed tannin	Protein dissociated (%)		
	Pepsin	Trypsin	Sequential pepsin and trypsin
L. leucocephala	53 ± 8.5	46 ± 1.0	67 ± 2.7
L. pallida	61 ± 4.4	43 ± 1.3	62 ± 1.9
L. diversifolia	43 ± 2.9	52 ± 0.5	64 ± 0.4
A. aneura	63 ± 7.4	50 ± 0.2	69 ± 1.5
A. harpophylla	80 ± 3.2	70 ± 1.0	73 ± 3.0
A. holosericea	65 ± 5.8	70 ± 0.7	80 ± 3.6
A. saligna	33 ± 0.2	46 ± 1.1	41 ± 0.8
Azadarachta	66 ± 0.6	66 ± 0.6	62 ± 4.0
G. robusta	56 ± 4.4	66 ± 0.5	70 ± 5.8
A. chinensis	74 ± 1.3	54 ± 1.2	77 ± 0.5
T. tipu	44 ± 2.0	70 ± 1.0	71 ± 0.4
F. macrophylla	67 ± 11.1	62 ± 0.1	75 ±7.9
C. cristata	57 ± 3.5	51 ± 6.2	67 ± 1.9
C. cunninghamiana	70 ± 1.5	89 ± 4.9	73 ± 0.1
Tannic acid	51 ± 3.4	78 ± 7.9	61 ± 5.1

tion of the PTC differ from those reported in the literature. Most other studies used incubation times of 10-15 minutes, whereas we incubated our mixtures at 39° for 2h, in an attempt to simulate more closely conditions in the rumen. We had found in earlier work (Díaz *et al.*, unpublished) that at least 90 minutes of incubation is required to maximize the formation of the PTC. This suggests that longer incubation is necessary to form a strong complex between the protein and the tannin. Shorter incubations, may therefore result in greater dissociation of the PTC. This would have the effect of attributing too much importance to the effect of pH and the possibility that free tannins may react and inhibit the proteolytic enzymes and in this way mask their important effect on the PTC.

CONCLUSIONS

The factors influencing the formation and dissociation of insoluble complexes between rubisco, the major protein found in leaf material, and condensed tannins isolated from tropical browse plants include pH and proteolytic digestive enzymes. Although protein-tannin complexes form only at pH < 7, pH itself has little effect on the dissociation of the complex once it has formed. The incubation of the protein-tannin complex with abomasal and intestinal fluids released about half the protein in the complex and we believe that it is the proteolytic activity in the components of the digestive tract, rather than change in pH, which is responsible for the release of protein from the PTC.

ACKNOWLEDGEMENTS

A. Diaz-Hernandez is grateful to the Universidad Central de Venezuela for the support provided by her postgraduate scholarship. The research was funded by an Australian Research Council grant.

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