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The impact of condensed tannins from dock (*Rumex obtusifolius*) on the growth of rumen proteolytic bacteria *in vitro*

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ABSTRACT

Dock (*Rumex obtusifolius*) is commonly regarded as a weed, but it prevents bloat in cattle and the condensed tannins (CT) in dock are able to reduce the viability of gastrointestinal parasite eggs and larvae under *in vitro* conditions. These benefits, with other attributes of CT from a variety of forages promoted this study to examine effects of CT extracted from dock on the growth of five strains of proteolytic rumen bacteria *in vitro*. *Streptococcus bovis* NCFB 2476, *Eubacterium* sp. C124b, *Prevotella bryantii* B14, *Butyrivibrio fibrisolvens* H17c and *Clostridium proteoclasticum* B316^T were tested against 200, 400 and 600 µg CT/ml. In the absence of CT, all bacterial strains showed typical growth and reached maximum optical density (OD 600 nm) after 6 - 8 h of incubation at 39^{0}C. All strains continued to grow in the presence of 200 µg of the CT from dock per ml but attained significantly lower ($P<0.001$) OD 600 nm values than their counterparts in the control incubation. The addition of 400 and 600 µg CT/ml reduced ($P<0.001$) the growth of all bacterial strains tested compared to controls. All strains except *P. bryantii* did not initiate growth after the addition of 600 µg/ml relative to the growth before addition of CT. *C. proteoclasticum* was the most sensitive to the action of CT followed by *B. fibrisolvens*, *S. bovis*, *Eubacterium* sp. and *P. bryantii*. These results suggest that the dock CT have the ability to modify the growth of rumen proteolytic bacteria either directly or indirectly by preventing access to the protein.

KEY WORDS: dock, condensed tannins, rumen bacteria

INTRODUCTION

Dock is a common weed in dairy pastures, but it may have significant value for grazing ruminants. Dock is readily accepted by cattle when not fouled by faeces,

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reduces soluble protein concentrations in the rumen and prevents bloat in cattle (Waghorn and Jones, 1989). Dock contains high concentrations of protein and moderate amounts of condensed tannins (CT) in the leaves (Waghorn and Jones, 1989). These authors demonstrated the binding between dock CT and lucerne (*Medicago sativa* L.) protein and suggested this may account for a lower incidence of bloat in cattle consuming dock with lash pasture. Binding between CT and plant material in the rumen is associated with medium proteolysis in diets containing CT such as *Lotus* species, sulla (*Hedysarum coronarum*) and an increased flow of non-ammonia nitrogen to the small intestine (Wang et al., 1996). Other benefits attributable to dietary CT have included reduction in gastrointestinal parasites numbers, improved growth rates, especially in parasitised animals, increased wool growth and resistance to fly strike (Niezen et al., 1995). A considerable nematicidal activity was demonstrated for CT extracted from dock and other forages (Molan et al., 2000).

The objective of the present study was to investigate the effect of condensed tannins extracted from dock (*Rumex obtusifolius*) on the growth of a range of rumen proteolytic bacteria.

MATERIAL AND METHODS

Rumen bacterial strains

The bacterial strains used in this study were obtained from the Rumen Microbiology Culture Collection, AgResearch Ltd., Palmerston North (New Zealand). The bacterial strains were *Streptococcus bovis* NCFB 2476, *Eubacterium* sp. C124b, *Prevotella bryantii* B14, *Butyrivibrio fibrisolvens* H17c and *Clostridium proteoclasticum* B316^T. The bacterial strains were grown as described previously (Molan et al., 2001; Sivakumaran et al., 2004).

Preparation of condensed tannin extracts

Condensed tannins were extracted from dock. Frozen whole plants were homogenized with 70% acetone containing 1 g/l ascorbic acid in a Waring Blender. Acetone extracts were reduced to the aqueous phase by evaporation under reduced pressure at 50°C. The resulting aqueous phase was washed 3 times with methylene chloride in a separating flask and the upper layer, containing the CT was collected. Traces of methylene chloride were removed by rotary evaporation. The resulting fraction was freeze-dried, redissolved in 1:1 methanol/H_2O (v/v) and then purified by using a column containing Sephadex LH-20 (Pharmacia, Sweden). The Sephadex LH-20 extracts were freeze-dried and stored at -20°C until required.

The effect of CT on the growth of rumen bacteria

A series of in vitro experiments were undertaken to determine the effect of CT on the growth of five strains of rumen proteolytic bacteria. Condensed tannins were dissolved in CO_2-saturated artificial saliva and filter sterilized. The CT were added to culture media in Hungate tubes to final concentrations of 200, 400, and 600 µg/ml 2 h after the addition of the bacterial inocula (except for *S. bovis* where CT were added after 1 h due to its rapid growth), and all cultures were incubated at 39°C. Growth of the cultures was monitored spectrophotometrically by measuring optical density at 600 nm against blanks of uninoculated media containing 0, 200, 400 and 600 µg CT/ml. Initial absorbance values after inoculation (0 h) were subtracted from these readings to give adjusted optical density readings.

Statistical analysis

The effects of concentration and time were tested using analysis of variance (ANOVA; proc GLM, SAS ver. 8). T-tests were used to compare pairs of treatments of particular interest.

RESULTS

In the control treatments (i.e. in the absence of CT), all bacterial strains showed typical growth, reaching maximum OD600 values after 6-8 h of incubation and then declining slowly (Figure 1). Although the dock CT decreased the *in vitro* growth of all strains to a significant extent (Figure 1), the bacterial strains responded differently to the action of CT. At 200 µg/ml of CT, all strains continued to grow but overall growth was lower ($P<0.001$) in all strains except *S. bovis* compared to incubations without CT (Figure 1). At 400 µg/ml CT, *S. bovis* and *B. fibrisolvens* continued to grow while the remaining strains did not initiate growth after the addition of CT and in all strains the overall growth was significantly lower ($P<0.001$) than the growth in the control incubations (Figure 1). *Streptococcus bovis, Eubacterium* sp., *P. bryantii and C. proteoclasticum* did not show any sign of growth after the addition of 600 µg/ml (Figures 1A, B, C and E). However, *B. fibrisolvens* grew slowly during the first 6 h after the addition of 600 µg/ml (Figure 1D) and then declined sharply.

Figure 2 shows the relationship between the growth of the 5 strains of rumen bacteria and the concentration of dock CT after 8 h of incubation. In all strains the growth declined with increasing the CT concentration.

Exposure of the rumen bacterial strains to high concentrations of CT (400 and 600 µg/ml) from dock resulted in inhibition of separation of cells after their division and consequently appearance of long chains (Figure 3) of cells.

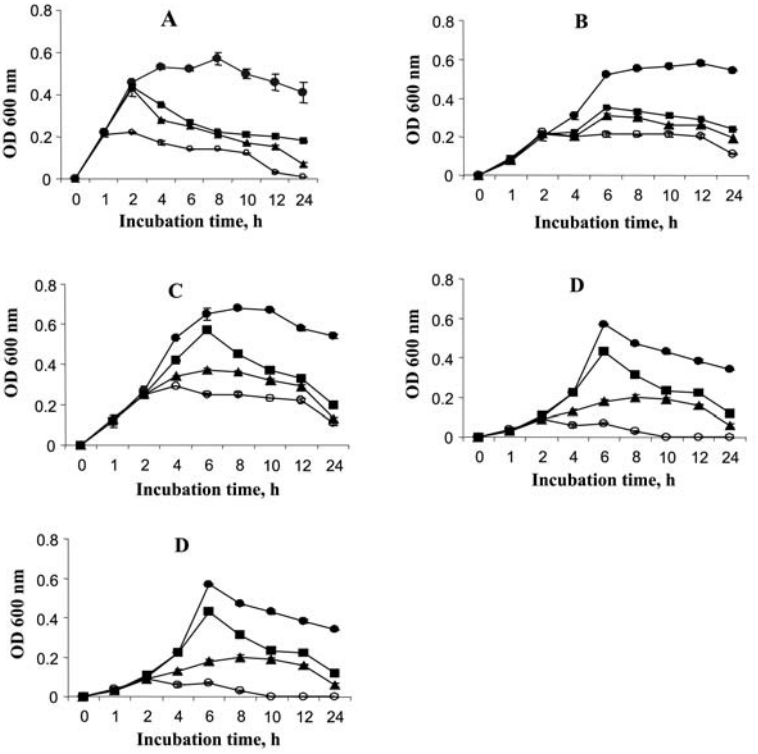

Figure 1. Growth of proteolytic strains in the presence of condensed tannins (CT) extracted from dock (*Rumex obtusifolius*). Growth of *Streptococcus bovis* NCFB 2476 (A)**,** *Eubacterium* sp. C124b (B)**,** *Prevotella bryantii* B14 (C)**,** *Butyrivibrio fibrisolvens* H17c (D) and *Clostridium proteoclasticum* B316^T (E) in the absence (●) and presence of 200 (■), 400 (▲), and 600 (O) µg CT per ml. Values are the means of duplicate cultures from two identical experiments. Vertical bars represent standard errors

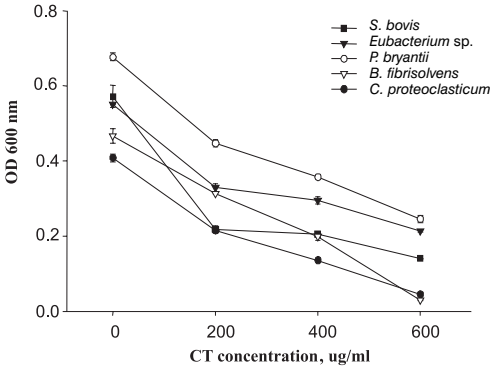

Figure 2. Growth of *Streptococcus bovis* NCFB 2476**,** *Eubacterium* sp. C124b**,** *Prevotella bryantii* B14**,** *Butyrivibrio fibrisolvens* H17c and *Clostridium proteoclasticum* B316^T after 8 h in the presence or absence of CT extracted from dock (*Rumex obtusifolius*). Values are the means of duplicate cultures from two identical experiments. Vertical bars represent standard errors

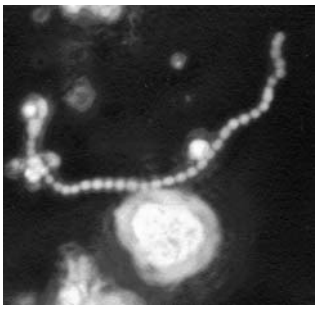

Figure 3. Light micrograph of *Streptococcus bovis* NCFB 2476 incubated with CT from dock. *S. bovis* cells were incubated for 24 h at 39 °C in the presence of 400 μg/ml of dock CT. Micrograph of bacteria at the end of the incubation showing long chain of cells that failed to separate after division

DISCUSSION

The present results demonstrate that the CT from dock are very effective at reducing the growth of rumen proteolytic bacteria under *in vitro* conditions. Five strains of proteolytic rumen bacteria were exposed to different concentrations of CT extracted from dock. The CT had direct effects on the growth of the rumen bacteria as evidenced by their ability to reduce or inhibit their growth *in vitro*. Jones et al. (1994) found that sainfoin CT had profound effects on the growth of five strains of proteolytic rumen bacteria. Molan et al. (2001) studied the effects of CT extracted from *Lotus corniculatus* (LC) and *L. pedinculatus* (LP) on the same bacterial strains tested in this study and found that CT from *L. pedinculatus* were more effective at reducing the growth of these bacteria and they responded differently to the action of CT from LC and LP. Recently, Sivakumaran et al. (2004) studied the antibacterial activity of CT extracted from *Dorycnium rectum* against pure cultures of microbes selected from the ruminal population to represent fibre degrading, proteolytic and hyper ammonia producing bacteria and found that the CT fractions were effective at reducing the growth of these bacteria. The activity of CT was significantly dependent on their structure and the bacterial strains responded differently to the action of CT. Although the mode of action of CT is not fully known, this ability may be attributed to the capacity of these substances to bind strongly to protein and polysaccharides and to reduce the activity of microbial enzymes (Scalbert, 1991).

Condensed tannins have been shown to inhibit endogenous proteinases activity in the small intestine (Waghorn, 1996). At higher tannin/protein ratio the inhibition of proteolysis is assumed to be due to polyphenolic compounds covering the protein surface (McManus et al., 1981) leading to interference with the interaction of enzyme and substrate. Molan et al. (2001) studied the mechanism of CT action by following the degradation of large subunits (LSU) of ribulose-1,5-bisphosphate carboxylase/

oxygenase (Rubisco) after bacterial cells or Rubisco were preincubated with CT extracted from *Lotus corniculatus* and *L. pedunculatus*. Preincubations of forage protein and bacterial cells decreased LSU degradation but they differed in their response to polyethylene glycol (PEG) addition. Addition of PEG to CT-Rubisco preincubations negated the effects of CT while PEG addition to CT-bacteria preincubations did not. It has been suggested that the CT-bacterial interaction is stronger than the CT-Rubisco interaction or the interaction is of a different type and not reversible by PEG.

In this study, we found that CT from dock targeted the cell wall of rumen bacteria as evidenced by the inhibition of separation of bacteria cells after their division and consequently leads to formation of long chains of cells (Figure 3). Similarly, Jones et al. (1994) found that CT from sainfoin caused the normally single rods of *B. fibrisolvens* A38 to become filamentous and inhibited the separation of these cells after division. Similarly cell separation was inhibited in *S. bovis* 45 SI, leading to the appearance of chains of 50 or more cells.

It was concluded that the cell wall of proteolytic bacteria was the target of dock CT toxicity and that more research is needed to support this finding.

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