Mechanisms of tannin resistance and detoxification in the rumen

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ABSTRACT

Tannins are naturally occurring protein-binding polyphenols that occur in many forage plants and which reduce nutritive value through inhibitory effects on ruminal and intestinal functions. Some animals have adapted to tannins through the synthesis of tannin-binding salivary proteins, the presence of tannin-resistant or tannin-degrading ruminal/intestinal microorganisms, or other potential adaptations in the lower intestinal tract. *Streptococcus caprinus/gallolyticus* is found ubiquitously in the rumen of many animals browsing tannin-rich forage legumes. Biochemical studies have shown that this bacterium metabolises gallic acid to pyrogallol, although it does not metabolise pyrogallol, and produces extracellular polysaccharide (EPS) in response to tannins in the growth medium. Induction of EPS appears to be a bacterial defence mechanism that permits the bacterium to maintain its population when related species are dying. *Selenomonas ruminantium* K2 grows in the presence of hydrolysable or condensed tannins as a sole carbon source and secretes a tannin-inducible tannin acylhydrolase (TAH). This enzyme has been isolated and characterised, and the putative gene has been cloned and sequenced. Current studies focus on identifying the tannin-responsive element that control the gene. A number of other tannin-resistant bacterial species have been isolated and identified. In addition to effects of tannins on microbial function, intestinal function studies have revealed that tannins inhibit nutrient metabolism and uptake in the abomasum and small intestine of ruminants. Alkaline phosphatase and aminopeptidase-N activities are inhibited, intestinal microvilli structure is disturbed and signs of tissue fragility are evident. These studies indicate that the protein-complexing action of tannins may have a more profound effect on intestinal rather than rumen function and that microbial interactions in the rumen may reduce but not eliminate tannin toxicity.

Introduction

Phenolic secondary plant products, mainly comprising tannins, are ubiquitous in plants ranging in concentrations from <2% to more than 20% of the dry weight and pose a worldwide problem for grazing livestock because they often prevent effective utilisation of forage (1). Tannins characteristically bind with proteins, carbohydrates and minerals and dramatically inhibit digestive and absorptive processes in the rumen of grazing ruminants (2). Studies on intestinal structure suggest that post-fermentative changes to digestive functions may also occur in ruminant and monogastric animals (unpublished). Livestock consuming tannin-rich diets (>5% w/v tannin) usually develop a negative nitrogen balance and lose weight and body condition unless supplemented with non-

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protein nitrogen, carbohydrate and minerals. Phenolic compounds also interact with salivary and mucosa-associated proteins producing astringency which is reflected in reduced feed intakes in grazing animals.

Studies of feral ruminants (goats, camels) in Australia have demonstrated that these animals exhibit resistance to tannins, possibly mediated through rumen microbial populations that may modify or degrade these compounds (3,4). However, it is not clear how this occurs, nor what role these tannin-resistant organisms play in the overall ecology of the rumen. The studies described here report on possible mechanisms of tannin tolerance in 2 ruminal isolates, *Streptococcus caprinus* (*gallolyticus*) *and Selenomonas ruminantium* K2, and the cloning and partial sequencing of the tannin acylhydrolase gene from *S. ruminantium* K2. We also report on changes in intestinal function as a result of tannin interactions.

Materials and methods

Bacterial isolation

All bacteria used in this study were obtained from crude rumen fluid of feral goats or camels browsing *Acacia aneura* (Mulga), and were isolated after selection on Brain Heart Infusion (BHI) medium containing varying concentrations (up to 5% w/v) of tannic acid or condensed tannin (3). Isolates were colony purified and identified by metabolic, biochemical characteristics and 16 S rDNA mapping.

Condensed tannin isolation

Condensed tannin (CT) was isolated from Acacia leaves by 70 % acetone extraction followed by fractionation on Sephadex LH-20. The CT fraction was freeze dried and stored in the dark under anaerobic conditions. Tannin content was analysed by the butanol-HCL method (5)

Tannin acylhydrolase

Methods for assay and purification of tannin acylhydrolase (TAH) were as described (4).

Genomic library screening

K2 DNA was digested with Sau IIIA and ligated with BamHI-digested and phosphatased pBluescript (Stratagene). Recombinant colonies were selected on LB plates containing X-Gal and IPTG, and analysed by colony hybridisation using a PCR-generated 350 bp TAH probe. Inserts were sized by colony-PCR using M13 primers, and confirmed as TAH clones by PCR using TAH-specific primers. Clones containing TAH inserts were sequenced by automated sequencing.

Animal feeding experiments

Five experimental diets comprised oaten hay chaff (OHC) (*ad libitum*), oaten hay chaff (800 g/day), Mulga, oaten hay chaff plus PEG-4000, and Mulga plus PEG-4000. Twenty animals on OHC or OHC + PEG diets were pair fed with corresponding sheep fed Mulga or Mulga + PEG respectively.

Histochemistry of intestinal brush border enzymes

To determine whether tannins had effects downstream from the rumen, we examined enzyme profiles from regions of the intestine of sheep fed *Acacia aneura* (Mulga). Enzyme activity was measured in the duodenum, jejunum and ileum of sheep in 5 different groups.

Alkaline phosphatase

Sections were prefixed in formal calcium (1%w/v $CaCl_2$ 8.75 % v/v formalin in water) for 10 min, washed in 125 mM Tris-HCl (pH 9.2) at 39^{o}C, and incubated in AP substrate (3.1 mg naphthol AS-BI phosphate (Sigma), 10 mg Fast Red (BDH Chemicals, UK), 60 ul dimethylformamide in 10 ml 125 mM Tris-HCl pH 9.2) for 21 minutes. The reaction was stopped by immersing sections in ice-cold 125 mM Tris-HCl pH 7.5, fixed in 4% v/v formaldehyde at room temperature and mounted in warm glycerin jelly (ref). Samples were stored in the dark at 4^{o}C until analysis.

Aminopeptidase-N

Samples were fixed in formal calcium (as above) at 4^{o}C for 10 min, rinsed in 0.85 % w/v saline solution and incubated in 0.1 M $CuSO_4$ for 2 min. Treated samples were incubated at 39^{o}C in a substrate solution comprising 2 mg of L-alanine 4-methoxy-β-napthylamide dissolved in 0.05 ml ethanol, 0.45 ml distilled water, 5 ml of 0.1M sodium acetate buffer pH 6.5, 4 ml of 0.5% w/v saline , 0.5 ml of 13% w/v KCN and 5 mg of Fast Blue B (BDH Chemicals, UK). Assayed samples were stored at 4^{o}C until analysis. For each enzyme assay, precipitated reaction product was measured on a calibrated (density, brightness, perimeter) image analysis program. Measurements were made along the crypt-villous axis, recorded at 4 pixel intervals using a 492 nm polarised filter on an Olympus BH-2 microscope

Statistical analyses

Measurements were analysed in a repeated-measures ANOVA, including 'within sheep' factors of replication and region.

Results

Streptococcus caprinus

This organism grew in the presence of up to 5% w/v tannic acid or condensed tannins isolated from *Acacia*, but it did not utilise the tannins as a carbon source. Significant differences in the pre-exponential phase lag period were observed as the concentration of tannic acid or condensed tannin in the medium was increased. For *S. caprinus*, lag periods were 3, 5, 7, 16 and 23 h for concentrations of tannic acid in the medium of 0.5, 1.0, 2.0, 3.0, and 5.0% w/v (Fig 1). The presence of 0.5, 1.0 or 2.0% w/v condensed tannin caused a lag time for *S. caprinus* of 5, 8 and 11 h respectively.

GLC analysis of spent medium revealed the presence of produced in a time dependent manner when *S. caprinus* was incubated in the presence of tannic acid or gallic acid (Fig 2). The bacterium could not utilise pyrogallol for growth. Pyrogallol was not detected following anaerobic incubation of cell-free extracts prepared from *S. caprinus* with either tannic acid or gallic acid. However, incubation of gallic acid and tannic acid with washed whole *S. caprinus* cells resulted in the production of pyrogallol.

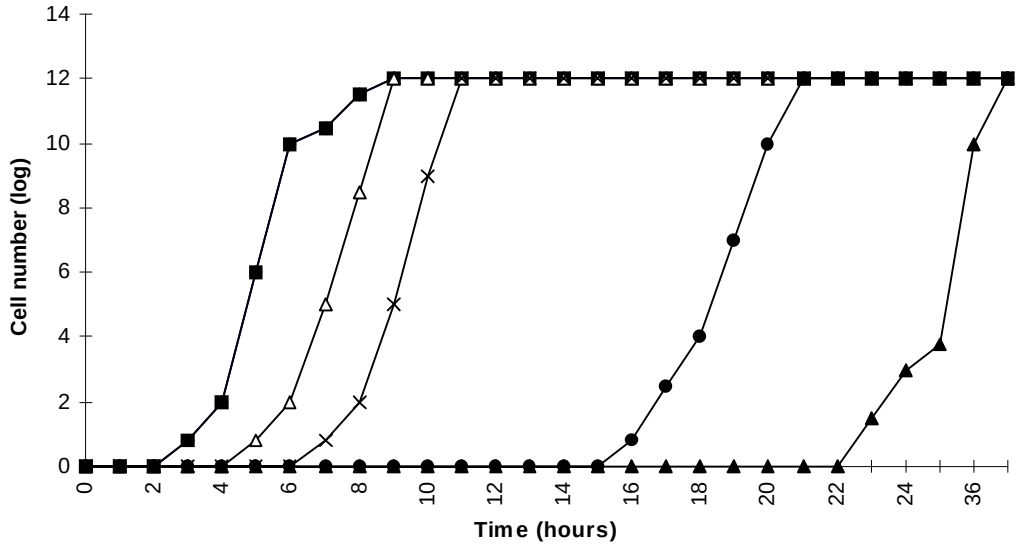

Fig. 1. The effect of tannic acid on the growth lag period of *S. caprinus*. Cultures containing increasing amounts of tannic acid were inoculated with *S. caprinus* and growth was measured in samples by serial dilution, plating on nutrient agar (without tannic acid) and viable cell count. ■,0; □, 0.5; Δ, 1.0; X, 2.0; ●, 3.0; ▲, 5.0 % (w/v) tannic acid. Counts are expressed as the log of cell number.

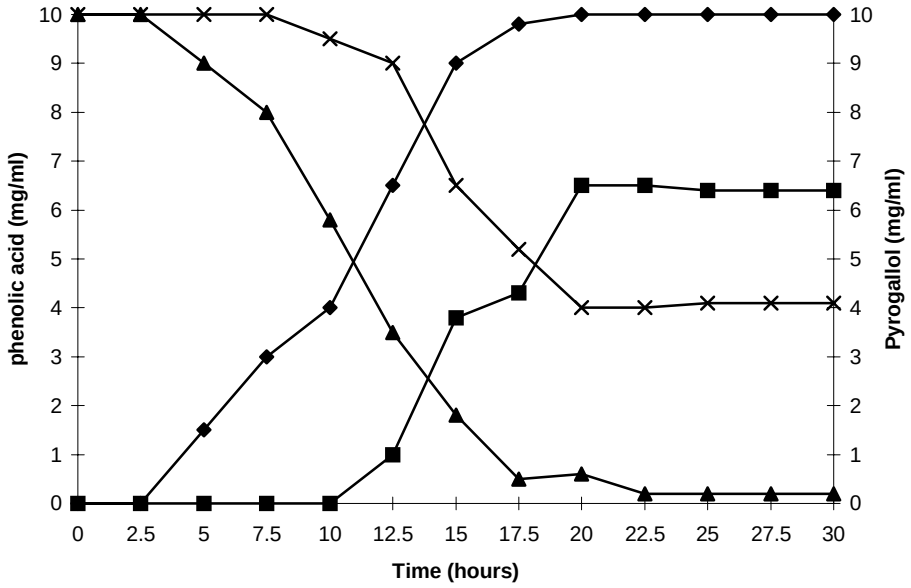

Fig. 2. Time-dependent production of pyrogallol by *S. caprinus* incubated with tannic acid or gallic acid. Cultures were incubated with 1% (w/v) tannic acid or gallic acid and samples of medium were extracted with ethyl acetate, derivatised with TMS and analysed by GLC for the production of pyrogallol. Data points represent the mean of triplicate assays.
Growth in; ▲, gallic acid; X, tannic acid.
Pyrogallol production from; ◆ gallic acid; ■, tannic acid.

The specific activity of gallate decarboxylase increased 4-fold when the bacteria were grown in gallic acid compared with cells grown in mBHI medium containing no phenolic acid. After growth in the presence of tannic acid, the specific activity of gallate decarboxylase was increased 2.5 fold. However, gallate decarboxylase activity did not appear to be significantly up-regulated after growth in the presence of condensed tannin or other phenolic acids including protocatechuic, vanillic, syringic or hydroxybenzoic acids.

When *S. caprinus*, grown in the absence of tannin, was observed under field emission scanning electron microscopy (FESEM), extracellular material surrounding the cells appeared globular in structure and was present in patches on the surface of the bacterium. Under the same growth conditions, no extracellular material was evident on *S. bovis*. With the addition of 0.2-0.5% w/v tannic acid to the growth medium, the amount of extracellular material surrounding both *S. caprinus* and *S. bovis* increased. However, at concentrations of tannic acid greater than 2% w/v, the extracellular material completely encased *S. caprinus* whereas growth of *S. bovis* ceased. A time course of EPS synthesis showed that little EPS accumulated during early logarithmic growth of *S. caprinus*, but increasing amounts were produced as the culture moved from late log into stationary phase (Fig 3).

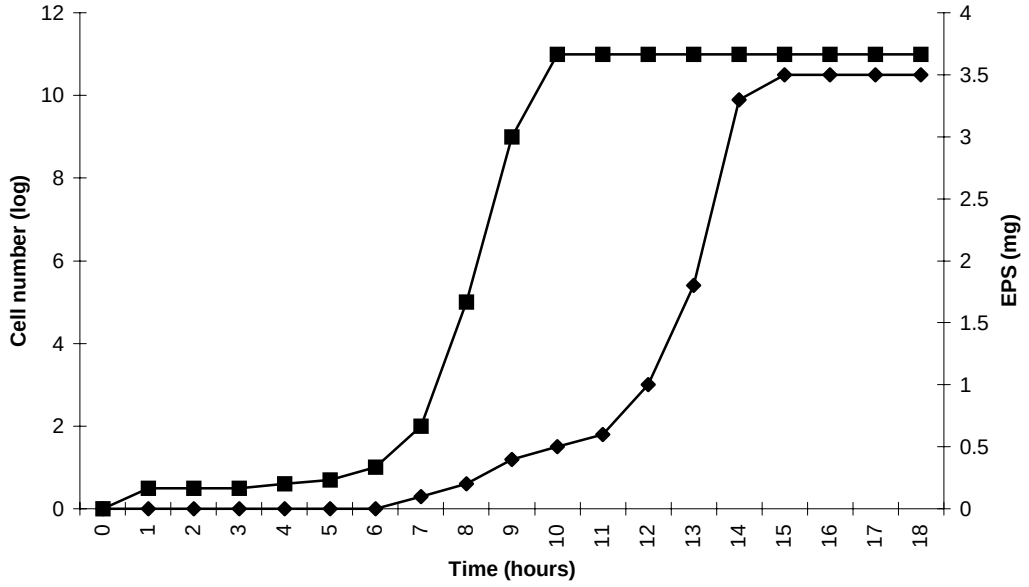

Fig. 3. Production of extracellular polysaccharide by *S. caprinus*. Cells were incubated in medium containing 1% (w/v) tannic acid and samples were removed at various times for viable cell counts. EPS was determined by colorimetric assay and expressed as glucose equivalents. Values represent the average of triplicate assays. ■, log viable cell count; ◆, EPS (mg).

Characterisation of extracellular material

The average yield of crude extracellular material isolated from *S. caprinus* was approximately 0.95±0.12 mg/mg cells (dry weight), the material had a molecular weight equal to or greater than blue dextran (2×10^6) and was principally associated with the bacterial cell surface. Analysis of the alditol acetate derivatives of hydrolysates by GLC and

GLC-MS indicated that the neutral sugar composition was primarily glucose with trace amounts of mannose (glucose:mannose = 1:0.2). Acyl and N-acyl residues were also detected. No uronic acids or hexosamines were present when *S. caprinus* was grown in mBHI media in the absence of tannic acid, but increased amounts of uronic acids were detected after growth in tannic acid-containing medium. Extracellular material of a similar molecular weight was isolated from cultures of *S. bovis* (0.8±0.06 mg/mg cells dry weight) grown in the absence of tannic acid. However, its composition differed, comprising mannose, glucose and galactose in the ratio of 1: 0.7: 0.2, as well as a larger amount of acyl and N-acyl groups, and some uronic acids. *S. bovis* did not grow (and therefore did not produce extracellular material) in the presence of tannic acid.

Selenomonas ruminantium K2

Isolated on BHI plates containing up to 5% w/v tannic acid, this organism was able to grow on either tannic acid or condensed tannin as a sole carbon source and was shown to produce gallic acid from tannic acid. Tannin acylhydrolase activity was demonstrated using gallic acid methyl ester (GAME) as an artificial substrate and activity was shown to increase by up to 35 fold when K2 was grown in the presence of tannic acid or GAME (Table 1), but not monomeric phenols. However, maximum specific activity of TAH was not observed until cells reached stationary phase at an A_{600} of greater than 1.0. The enzyme was demonstrated by zymogram to have a molecular weight of 59 kilodaltons and was purified by 2 dimensional gel electrophoresis and isoelectric focussing. Purified enzyme was sequenced at the N terminus by automated Edman degradation, and at an internal site following endo-lysC degradation.

Table 1. Effect of phenolic compounds on specific activity of TAH *in vivo*[a]

Growth medium	TAH activity
MBHI	0.13 ± 0.02
mBHI + 0.2 % tannic acid	4.52 ± 1.10
mBHI + 0.2% ferulic acid	0.12 ± 0.03
mBHI + 0.2% catechin	0.18 ± 0.04
mBHI + 0.1% GAME	2.10 ± 0.20
mBHI + 0.2% gallic acid	0.42 ± 0.09

[a]*Sel. ruminantium* K2 cells were grown in the presence or absence of phenolic compounds and cell-free extracts were assayed for TAH activity

PCR primers were synthesised from the amino acid sequence of each fragment with a 48 fold maximum level of degeneracy and were used to isolate a 350 bp fragment of the TAH gene by PCR. This fragment was cloned in *E.coli*, sequenced and used to probe Southern and Northern blots of K2 DNA and RNA respectively. The results (fig 4a) demonstrated specific hybridisation of the probe to 2 bands of EcoRI and HindIII digested K2 DNA and not DNA from a non-TAH *Selenomonas* control strain. We have established that the 350 bp fragment used as a probe does not contain internal EcoR1 or HindIII sites. This suggests that there may be at least 2 genes related to this probe. The 350 bp probe gave a positive reaction to RNA isolated from K2 cells grown in the presence, but not the absence of tannin (Fig. 4b).

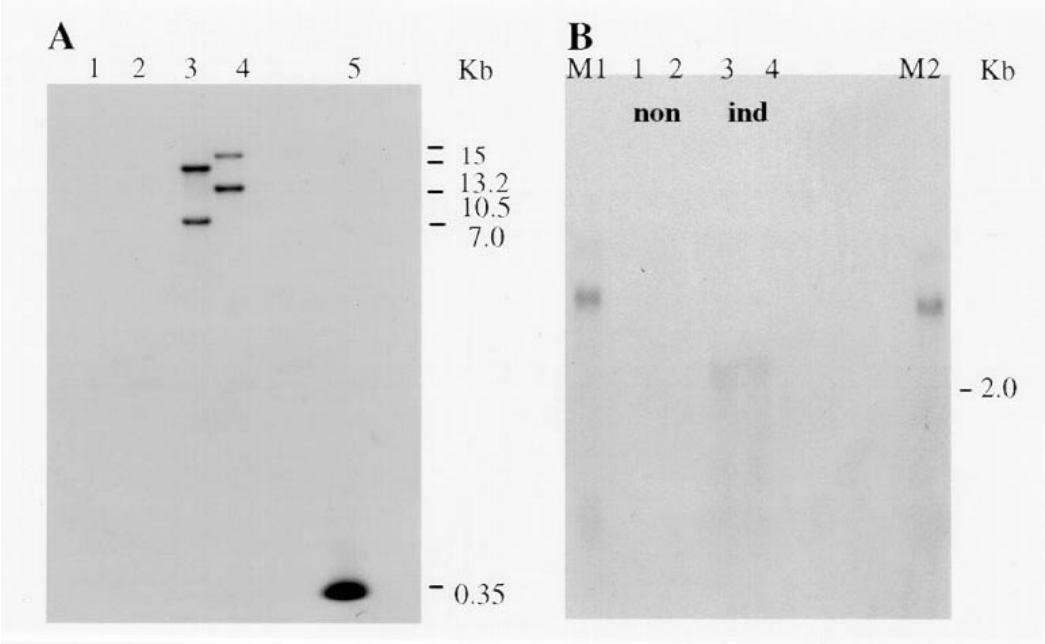

Fig. 4. Southern (A) and Northern (B) blots of DNA and RNA from *Sel. Ruminantium* K2 using the 350 bp PCR product as a probe. Panel A; DNA was cut with HindIII(lanes A1 & A3) or EcoRI (lanes A2 & A4). Lanes A1 & A2; non-TAH *Sel. Ruminantium* DNA, lanes A3 & A4; *Sel. Ruminantium* K2 DNA, lane A5; self hybridisation of the probe sequence. Panel B; RNA was from non-induced (non, lanes B1 &B2) or tannic acid-induced (ind, lanes B3 & B4) cells. Lanes B1 and B3 contain 25 μg of RNA, lanes B2 and B4 contain 50 μg of RNA. The marker lanes M1 and M2 contain a BRL molecular size ladder.

A genomic library of K2 DNA was prepared in pBluescript, and cloned in *E.coli*. The library was screened using the 350 bp PCR product. Two positive clones (TAH1 and TAH2) were identified and purified. In both, the insert was approximately 700 bp. Both clones were sequenced and shown to be identical and to contain the complete 350 bp sequence. Analysis of clone TAH1 demonstrated one major open reading frame (ORF) and several partial ORFs. At this stage, it is not clear which ORF is correct since screening of the Genbank database shows no extensive homology with any other DNA or amino acid sequence. To confirm the clone identity we will therefore prepare an expression library of K2 DNA and screen for expression of TAH activity *in vitro*, and for homology to clone TAH1.

Histochemistry of intestinal brush border enzymes

There were no significant differences in AP activity across the various regions of the intestine within treatment groups but between groups, activity in the Mulga-fed sheep was 50-60% lower than oaten hay chaff (OHC), OHC + polyethylene glycol (PEG) or Mulga + PEG fed sheep (Table 2).

Aminopeptidase-N (AP-N) specific activity was approximately 3 fold greater than AP activity, but the regional distribution was similar (Table 2). OHC, OHC + PEG and Mulga + PEG expressed similar AP-N activity, whereas activity in the duodenum, jejunum and ileum of Mulga-fed sheep was approximately 25% of the other treatment groups. The addition of PEG to the Mulga diet restored AP-N activity and regional distribution was demonstrated in the epithelial cells of the duodenum, and the ileum.

Table 2. Effect of diet on AP and AP-N activities in the intestinal tract of sheep fed *Acacia aneura*.

Enzyme and location	**Enzyme activities versus diet**				
	ad lib	OHC	Mulga	OHC+PEG	Mulga+PEG
Alkaline phosphatase[a]					
Duodenum	0.25±0.04	0.28±0.05	0.17±0.02	0.25±0.02	0.22±0.02
Jejunum	0.33±0.02	0.26±0.02	0.16±0.03	0.31±0.02	0.24±0.03
Ileum	0.26±0.03	0.21±0.03	0.17±0.04	0.32±0.03	0.31±0.03
Aminopeptidase N[a]					
Duodenum	0.79±0.02	0.81±0.02	0.15±0.01	0.51±0.11	0.59±0.02
Jejunum	0.55±0.08	0.80±0.02	0.10±0.01	0.77±0.03	0.88±0.03
Ileum	0.85±0.03	0.81±0.02	0.20±0.02	0.65±0.06	0.75±0.02

[a] Activities are expressed as mean absorbance/μm^2 of microvillus membrane

Discussion

The results with *S. caprinus* demonstrate that this bacterium reacts with several adaptive responses to the presence of tannins. The pre-exponential growth lag period increases, the activity of gallate decarboxylase is elevated and the synthesis of EPS is induced. However, the extent to which each of these contributes to the overall tolerance of *S. caprinus* to high concentrations of tannic acid or condensed tannins in the growth medium is unclear. The increased lag period suggests that some prior adaptation such as the synthesis of an enzyme or production of a glycocalyx may be a necessary requirement for continued growth. The fact that at low concentrations of tannin, a similar lag period occurs with *S. bovis* (result not shown), but at higher tannin concentrations the bacterium does not continue to grow, suggests that this bacterium is not able to develop the necessary protective strategy. The production of pyrogallol from gallate is another possible mechanism of resistance, but this is only relevant where tannic acid or catachin-gallates are present. Where condensed tannins are predominant, it is possible that the production of a protective extracellular coat is the best strategy for *S. caprinus*.

For *Sel. ruminantium K2*, the only strategy appears to be the synthesis of tannin acylhydrolase. A 35 fold induction of the enzyme results in significant cell-associated activity that cleaves the glucose moiety from hydrolysable tannins, and presumably allows the bacterium to ferment the glucose. We do not know how this organism develops tolerance to condensed tannins since it does not appear to secrete large quantities of EPS as does *S. caprinus*. The tannin acylhydrolase enzyme is not structurally identical to other acylhydrolases isolated from fungi and the partial gene sequence from TAH1 does not reveal any striking homologies with other genes. This is perhaps not surprising since there are very few genes sequenced from any *Selenomonas* and so direct comparisons are not possible. However, confirmation that TAH1 does indeed contain the correct gene awaits expression studies. The Southern data suggests that there are at least 2 similar genes but it is possible that only one of those is expressed.

The results of histochemical studies clearly demonstrate inhibitory effects on abomasal and intestinal function which are separate from effects on bacterial populations. Reduced activity of AP and AP-N were evident, and this was restored by the inclusion of PEG in the diet. The simplest explanation of this effect is that enzyme activity was inhibited by the protein-binding action of tannins and this was alleviated by pre-binding the tannins with PEG. However, an alternate explanation is that the tannins inhibited enzyme secretion by forming a lining on the intestinal mucosa, thus preventing the export of proteins from the intestinal epithelial cells. This second explanation is supported by additional data (not shown) which demonstrates that tannins induce histological changes in the intestinal mucosa including abnormal villous structure and disruption of cellular networks of communication.

Therefore these results indicate that tannins may inhibit several different processes including microbial and digestive tract functions, and that these effects may have an impact upon animal production over a range of grazing and browse feeds. However, recognition of the effects may be more problematic since the extent and duration of inhibition will depend upon a number of factors including diversity of forage available, intake, age of plant and other environmental influences. Resistance to tannins may therefore occur at several levels; microbial tolerance, tannin degradation and intestinal tract adaptation.

Acknowledgements

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