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Effects of Tannic Acid on the Fibrolytic Enzyme Activity and Survival of some Ruminal Bacteria

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ABSTRACT

Plant biomass is the main diet of the herbivores and their biodegradation in the gastrointestinal tract of the animals is mainly depends on the fibrolytic activities of the gut microbes. Some plant sources, however, contain various anti-nutritional factors, such as tannins, which retards the digestion of the ingested plant particles by host animals. The current study explored the possible effects of the tannic acid on the growth rate and fibrolytic enzyme activities of five rumen originated anaerobic bacterial strains, *Ruminococcus albus* SY3, *Fibrobacter succinogenes* S85, *Streptococcus bovis* ES1, *Prevotella bryantii* B14, *Butyrivibrio fibrisolvens* JW11. Parallel with an increase in tannic acid concentration (0.1-0.6%) used in the culture medium, growth rates of all bacterial strains were notably reduced. Similar results were obtained for the CMCase and xylanase activities of those strains that various concentration levels (0.05-0.30%) of tannic acid hampered the activity of these fibrolytic enzymes significantly. © 2011 Friends Science Publishers

Key Words: Cellulase; Xylanase; Rumen bacteria; Tanin; Tannic acid

INTRODUCTION

Grazing animals supply their energy requirements mainly via the degradation of plant material in their digestive system, which principally occur through the activity of microbial polysaccharidases. Therefore, rumen microbial enzymes have been studied in detail for all main fibrolytic microbes of the rumen such as protozoa, fungi and bacteria (Akin *et al*., 1989; Coleman, 1992; Comlekcioglu *et al*., 2010), because of their importance in animal nutrition (Tauqir *et al*., 2009). As one of the main microbial population of the rumen, cellulolytic rumen bacteria play a remarkable role for plant biomass degradation in the gastrointestinal tract of most herbivores and possess a variety of fibrolytic enzymes (Ekinci *et al*., 1997; Ozkose *et al*., 2004; Jun *et al*., 2007). Polyphenolic compounds (such as tannin) and anti-nutritional factors in plants can influence the activity of polysaccharidases in the rumen and hamper the biodegradation of plant biomass ingested by the host herbivores (Jones *et al*., 1994; Gamble *et al*., 1996). On the other hand, some microorganisms seem tolerate tannin (Krause *et al*., 2005) and degrade that phenolic polymer by producing tannase enzyme (Sabu *et al*., 2006).

Tannins are water-soluble polyphenolic compounds that can be found in significant quantities in plant tissue. The chemical structure of tannins is highly variable. The hydrolysable tannins are polyesters of gallic acid, pyrogallol, resorcinol and simple sugars, while condensed tannins are group of polyhydroxy flavan-3-ol oligomers and polymers of carbon-carbon bound flavonoids (Schofield *et al*., 2001). Both these classes of tannins are rich in highly reactive hydroxyl groups, which emanate from each of the benzene rings and form complexes with proteins, including enzymes (Mangan, 1988; Khanbabaee & Van Ree, 2001), resulting in a remarkable reduction in the biodegradation of the fibrolytic polymers such as cellulose and hemicellulose in the rumen (Priolo *et al*., 2000). Both hydrolysable and condensed tannins are generally regarded as anti-nutritional factors for ruminants, because of depression of feed intake and dry matter digestibility (McSweeney *et al*., 2001), but their capacity to precipitate proteins reversibly at rumen pH may be nutritionally beneficial since tannin-protein complexes are stable at the pH range of 3.5 to 7.0 (Mangan, 1988).

Tannic acid (TA), typical hydrolysable tannin, is even toxic to both ruminant and monogastric animals particularly when it is available in the diets of those animals in excess amounts (Zhu *et al*., 1992). Although tannic acid is toxic to most farm animals, some of them (particularly ruminants) have developed a defense mechanism against that type of anti-nutritional factors via microbial ecosystem inhabiting in their gastrointestinal tract. The main pathways of the decorboxylation of that type of phenolics occur in the rumen and some ruminal bacteria, such as *Selenomonas* sp., are

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able to detoxify tannic acids besides some other anti-nutritional factors of ruminant diets (Bhat *et al*., 1998; Singh *et al*., 2001).

The effects of tannin on the growth of rumen bacteria and on microbial proteolysis have been described for a few bacterial species (Bae *et al*., 1993; Jones *et al*., 1994) and our knowledge about the effects of tannins upon the fibrolytic activities of pure culture rumen microorganisms is quite limited. This work, therefore, aimed to study the survival rate of some rumen bacteria at the different level of TA in laboratory conditions. Moreover, the effect of TA on the activity of different rumen anaerobic bacterial polysaccharidases (cellulases & xylanases) and determination of inhibition level of TA on these lignocellulolytic enzymes were also examined.

MATERIALS AND METHODS

Microorganisms and culture maintenance: The anaerobic rumen bacteria, *Ruminococcus albus* SY3, *Fibrobacter succinogenes* S85, *Streptococcus bovis* ES1, *Prevotella bryantii* B14, *Butyrivibrio fibrisolvens* JW11, were courtesy of Rowett Research Institute, Aberdeen, UK. Cultures were maintained at 37°C without shaking in complex liquid medium (originally designed for rumen fungi) containing 30% (v/v) clarified rumen fluid and 0.3% (w/v) glucose, 0.2% (w/v) cellobiose as energy sources (pH 6.7). The medium was prepared by adding 10 mL of medium into Hungate tubes (16 x 125 mm, Bellco Glass Inc., Vineland, NJ, USA) under anaerobic conditions then sterilized by means of autoclaving at 121°C for 15 min. TA (Merck, Germany) was prepared as concentrated stock solution using anaerobic distilled water and filter sterilized under anaerobic conditions by passing through a 0.22 µm universal filters.

Enzyme sources: Bacterial cell and culture supernatants from experimental cultures were harvested after 1-2 days of incubation. Culture supernatants were centrifuged for 10 min (11,000 *g* at room temperature) and the clarified enzyme solutions and cell extracts kept at −70°C until needed for enzyme assays, which were conducted in the presence and absence of TA concentrations. The enzyme source for all enzyme reactions was a solution of the appropriate enzyme diluted with sodium phosphate buffer (pH: 6.5) at the ratio of 1:1 (v/v).

Enzyme assays: The protein content of samples was determined with the aid of the method described by Lowry *et al*. (1951). Xylanase and carboxymethylcellulase (CMCase) activities were determined by measuring the reducing sugar released from xylan and carboxymethylcellulose (CMC) as described by Lever (1977) at pH 6.5 and 37°C. A 10 mg mL^{-1} (in final concentration) soluble xylan, obtained from oat spelt xylan according to Ghangas *et al*. (1989), or 10 mg mL^{-1} CMC suspended in 0.05 M sodium phosphate buffer (pH 6.5) was used as substrate. Substrate (0.9 mL) was mixed with 0.1 mL of extract and incubated at 37°C for 60 min and 0.1 mL from this mixture was withdrawn and added to test tubes containing 5 mL of PAHBAH (parahydroxybenzoic acid hydrozide) solution as described by Lever (1977). This mixture was heated at 70°C for 10 min and after cooling the absorbance was read at 410 nm wavelength (Spectramax Plus, UK). A solution of 0.1 mg mL^{-1} glucose in 0.05 M sodium phosphate buffer (pH 6.0) was used as standard. Units (IU) of enzyme activity are defined as mM of product released per minute under assay conditions. In assay reaction mixture, different concentrations of TA [in final concentration 0, 0.10, 0.15, 0.20, 0.25 & 0.30% (w/v) TA] were added. In both, treatment and control tubes, the reaction mixtures were incubated for 60 min at 37°C for reducing sugar determination.

Statistical analysis: The significant differences among treatment means were calculated using general linear model procedures of statistical analysis system (SAS, 1985) package with the variables fitted being different levels of TA treatments.

RESULTS AND DISCUSSION

Survival of anaerobic rumen bacteria in the presence of tannic acid: The complete medium containing different concentration of TA (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6) were inoculated in triplicate with a standardized cell suspension (Jones & Pickard, 1980) of anaerobic rumen bacteria, *R. albus* SY3, *F. succinogenes* S85, *S. bovis* ES1, *P. bryantii* B14, *B. fibrisolvens* JW11 and incubated under anaerobic conditions at 37°C. In the presence of TA growth of bacteria were quantified using same medium containing 1.5% agar in Hungate roll tubes by serial dilutions and viable colony counts.

The growth of 5 rumen anaerobic bacteria in the absence or presence of different concentrations of TA was tested (Table I). In the absence of TA (control group), all bacterial strains showed typical growth. In general, the growth of the bacterial strains hampered in parallel to the increase of final TA concentration in the medium (Table I). Addition of TA (0.2-0.6% TA) resulted a remarkable ($P<0.05$) reduction in the growth rate of most bacterial strains tested compared to control group (0.0% TA). However, *R. albus* SY3 cultured in the medium containing 0.1% TA showed transient increases in its growth, but not at higher (>0.2%) concentrations of TA. This enhancement of bacterial growth may be caused by structural changes in the substrate protein by its interaction with TA as also suggested by earlier reports (Mole & Waterman, 1985), allowing easier access of extracellular enzymes to their specific substrates (Min *et al*., 2005).

TA had relatively little effect on the growth ratio of *F. succinogenes* S85 and *P. bryanti* B14, which was able to grow in the presence of the highest TA concentration (0.6% TA) used in current study. Exposure of *F. succinogenes* S85 to 0.5% TA inhibited the growth of *F. succinogenes* S85

(Table I). At concentrations below 0.5% TA growth of *F. succinogenes* S85 and *P. bryanti* B14.was not seriously inhibited. In contrast, the cultures of *F. succinogenes* S85 and *P. bryanti* B14 supplied with 0.6% TA in the growth medium, the numbers of viable bacteria were notably decreased. Although growth of cells at these higher concentrations was severely inhibited, as relatively lower number of the cells remained viable at the end of the incubation period. However, approximately 50% of the viable cells of *B. fibrisolvens* JW11 and *S. bovis* ES1 reduced in the presence of 0.1% TA and in the higher concentrations of TA, growth of these strains drastically inhibited.

Inhibition of the growth of bacterial cells by TA may involved in the action of tannins on *R. albus* SY3, *B. fibrisolvens* JW11 and *S. bovis* ES1 cell wall structures (Jones *et al*., 1994; O'Donovan & Brooker, 2001). Tannins bound to cell coat polymers of bacterial strains and caused morphological changes in *B. fibrisolvens* and *S. bovis*, which prevent cell division and failure of daughter cells to separate (Jones *et al*., 1994). The cell wall composition in *R. albus, S. bovis* and probably in *B. fibrisolvens* too (Hespell *et al*., 1993) is characteristic of gram-positive bacteria, which is a target of tannin toxicity (Jones *et al*., 1994). It could be suggested that, despite complex formation with cell coat polymers in these strains, tannin penetrated to the cell wall in sufficient concentration to react with one or more ultrastructural components and to selectively inhibit cell wall synthesis. Although, growth of *F. succinogenes* (Bae *et al*., 1993) and *P. ruminicola* (Jones *et al*., 1994) in the medium containing various level of tannins resulted in production of large amounts of surface material, although bacterial growth was not completely inhibited, which is supported by the findings of current study. The bacteriostatic properties of tannins and differences in tannins sensitivity among the rumen bacteria reported earlier (Bae *et al*., 1993). Although tannins were undeniably seems to be bacteriostatic to rumen bacteria used in this experiment, the recovery of living cells of *F. succinogenes* S85 and *P. bryanti* B14 from cultures containing 0.6% of TA demonstrates that these compounds are not total bactericidal particularly for that species.

Effect of different concentrations of tannic acid on the enzyme activities: Total culture supernatant and cell-associated CMCase and xylanase activities of ruminal bacteria were tested in the absence or presence of different concentrations of TA (Fig. 1 & 2). All observations are reported alongside the enzyme activity observed for the corresponding, with zero ratio of TA as control assays. CMCase and Xylanase activities of all bacterial strains were mainly associated with the culture supernatant. In the absence of TA (control) *R. albus* SY3 and *F. succinogenes* S85 appeared to be highly active CMC degraders, while the other bacterial strains had moderate to lower rate of CMC degradation. In general, in the presence of TA, supernatant and cell-associated CMCase activities from different bacterial sources showed a dose response in relation to TA concentration ($P < 0.05$; Fig. 1). As TA concentration increased, a reduction in CMCase activities of bacterial strains were observed, apart from the CMCase activities for two group of microbes, *F. succinogenes* S85 and *P. bryantii* B14. Exposure to TA caused a continuous decrease in extracellular CMCase activities up to 0.3% for all strains. These reductions in extracellular CMCase activities were apparent at concentrations of TA even as low as 0.05% (Fig. 1).

The total CMCase activity in cultures of *P. bryantii* B14 was 36% higher ($P < 0.05$) in the presence of 0.1% TA than in the control. TA inhibited the CMCase activity of *B. fibrisolvens* JW11. Total CMCase activity of *B. fibrisolvens* JW11 was reduced approximately 60% in the presence of 0.1% TA (Fig. 1). Several reports have also shown that low levels of CT and TA increase bacterial growth and enzyme activity and moreover, cause a remarkable decrease of bacterial survival (O'Donovan & Brooker, 2001; Krause *et al*., 2005).

At all concentration of tannic acid the activity of xylanase was significantly ($P < 0.01$) lower (Fig. 2) than that observed for the strains cultured in the medium containing zero level of tannin. For all xylanases from different organisms, as TA concentration increased, a remarkable reduction in xylanase activity was observed (Fig. 2). The inhibition of xylanase activities from all bacterial sources initiated from low concentrations of TA was almost half that inhibit the CMCase activities. This result suggests that xylanases of these strains are more susceptible against the existence of TA and may have more affinity compare to CMCases.

In the absence of TA *S. bovis* ES1 appeared to be highly active against xylan, while the other bacterial strains had showed moderate to lower rate of xylan degradation. Total cell-associated and culture supernatant xylanase activities of *F. succinogenes* S85, *P. bryantii* B14 and *S. bovis* ES1, were reduced about 50%, ($P < 0.01$), in the presence of 0.05% TA and the reduction of xylanase activities continued in the presence of 0.1% TA (Fig. 2). Although the reduction of xylanase activity in culture of *R. albus* SY3 was observed, this reduction was not as fast as the other bacterial xylanases in the present of same concentration of TA (Fig. 2).

The inhibitory effects of tannins on cellulose and xylan digestion may not be solely related to their inactivation of fibrolytic enzymes through the formation of tannin-enzyme complexes. Adhesion is thought to be essential for the digestion of cellulose and xylan (Morris & Cole, 1987; Gong & Forsberg, 1989). Differences observed in the inhibition of CMCases and xylanases from different bacterial sources could be due to differences in secondary and tertiary structure, amino acid composition and degree of glycosylation characteristics inherent in the enzymes themselves (Barahona *et al*., 2006). It has been observed that the proteins, which are rich in proline content and are

Table I: Effect of tannic acid on growth of five anaerobic ruminal bacteria

Bacterial species	Concentration of tannic acid (%)						
	0	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**
R. albus SY3	$2.0\pm0.0010\times10^{8}$	$7.0\pm0.0035\times10^{8}$	$4.6\pm0.0023\times10^{7}$	$2.0\pm0.0009\times10^{6}$	$0.8\pm0.0004\times10^{5}$	$2.6\pm0.0012\times10^{4}$	$1.2\pm0.0007\times10^{3}$
F. succinogenes S85	$3.2\pm0.0016\times10^{8}$	$2.6\pm0.0013\times10^{8}$	$8.2\pm0.0041\times10^{7}$	$2.8\pm0.0014\times10^{8}$	$6.4\pm0.0032\times10^{7}$	$3.6\pm0.0018\times10^{6}$	$1.8\pm0.0009\times10^{4}$
P. bryantii B_{14}	$1.2\pm0.0006\times10^{8}$	$1.4\pm0.0007\times10^{8}$	$8.4\pm0.0042\times10^{7}$	$4.8\pm0.0024\times10^{7}$	$2.6\pm0.0013\times10^{7}$	$7.2\pm0.0035\times10^{6}$	$1.8\pm0.0008\times10^{7}$
B. fibrisolvens JW11	$4.3\pm0.0021\times10^{7}$	$2.4\pm0.0012\times10^{7}$	$0.6\times\pm0.000310^{6}$	$4.3\pm0.0021\times10^{4}$	$1.2\pm0.0005\times10^{5}$	$3.2\pm0.0014\times10^{3}$	$4.7\pm0.0019\times10^{2}$
S. bovis ES1	$2.7\pm0.0013\times10^{8}$	$1.4\pm0.00068\times10^{8}$	$1.6\pm0.0008\times10^{7}$	$1.3\pm0.0006\times10^{5}$	$2.2\pm0.0010\times10^{4}$	$1.2\pm0.0005\times10^{3}$	$0.7\pm0.0003\times10^{3}$

Fig. 1: Total culture supernatant (A) and cell-associated (B) CMCase activities of bacterial strains in the presence of different concentration of TA. Since CMCase activity of strain ES1 was found negligible, the data representing effect of TA on this enzyme was not presented. Bars represent the standard deviation of means

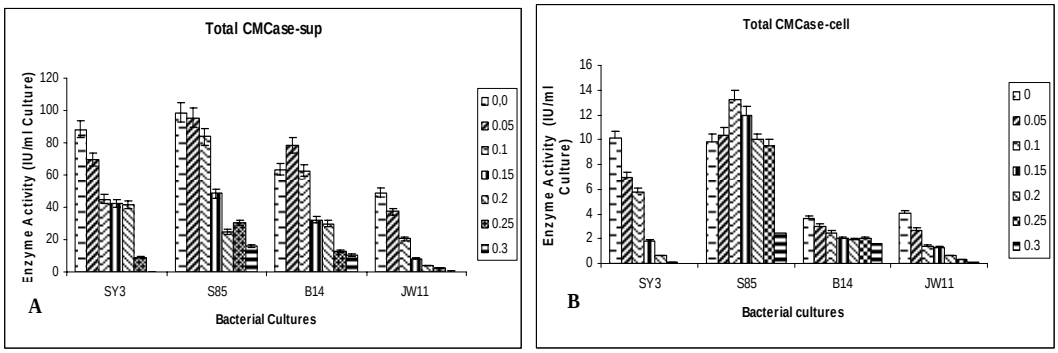
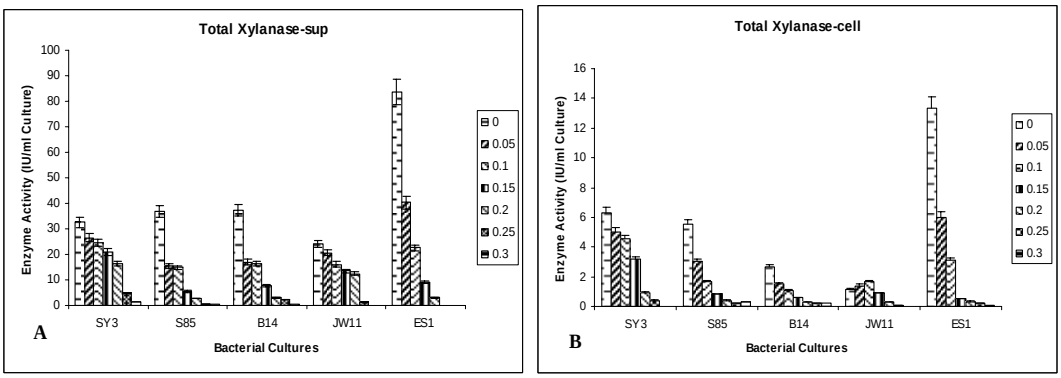


Fig. 2: Total culture supernatant (A) and cell-associated (B) xylanase activities of bacterial strains in the presence of different concentration of TA. Bars represent the standard deviation of means



highly hydrophobic (Hagerman & Butler, 1981) might enhance the binding of tannins to proteins and protecting tannin-fed animals against tannins deleterious effects (Siebert *et al*., 1996). As observed for many bacterial CMCases and xylanases (Gilkes *et al*., 1991), it is possible that the CMCases and xylanases of anaerobic rumen bacteria used here differ in proline-rich sequences and in secondary and tertiary structures, to an extent that could account for their different susceptibilities to the inhibitory actions of tannis. Besides, proteins with loose, open conformations and their isoelectric point and conformational flexibility can also influence their binding affinities for tannins. Tannins also bind to other macro-molecules besides protein, variation in the inhibition of bacterial and fungal enzymes by tannins could be related to factors inherent in the substrate hydrolyzed by these enzymes (Barahona *et al*., 2006). Some studies reported that TA deprived substrate rather than acting directly on the enzyme (Hagerman & Butler, 1981). Most substrates used in the assays reported herein were in soluble form. On the other hand, CMC and xylan are substrates of much higher molecular weights, with polymeric chains that vary in their degree of substitution. These factors could affect the binding affinity of CMC and xylan to tannins. The residues attached to CMC and xylan could lead to stronger tannin–polysaccharide interactions. Possible effects of the pH level of the assay conditions (after adding TA) on the enzyme activities of the strains were also investigated. However, there was no significant differences for the pH level of the medium containing different level of tannic acid (data not shown) and therefore the changes in enzyme activities for different level of TA was not related to the pH level.

CONCLUSION

Tannin acid had inhibitory effects on the survival some ruminal bacteria. Furthermore, TA hampered the cellulolytic and hemicellulolytic enzymes of these bacteria although their enzymes showed different level of responses to various level of TA. Further *in vivo* investigations on the possible roles of tannin in tannin-fed ruminants in relation to their structure–activity relations are imperative.

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