EFFECT OF SAPONIN AS DEFAUNATING AGENT ON *IN VITRO* RUMINAL FERMENTATION OF FORAGE AND CONCENTRATE

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ABSTRACT

This research was arranged with a 4x3 factorial design of treatments to investigate the effect of saponin level in fermentation medium (0, 0.1, 0.2, or 0.3 mg/ml), and many kinds of feed (king grass, rice bran, and king grass:rice bran, 60:40 w/w) on protozoa numbers, ammonia concentration, microbial protein, pH and cellullase activity. Each treatment was consisted of three replicates. Fermentation was done in syringe and used *in vitro* gas production medium. The data obtained were analyzed by variance analysis using factorial design (4x3). The differences between mean values were analyzed by *Duncan's new multiple range test* (DMRT). The result showed that protozoa numbers decreased 17.22, 42.73 and 49.57% ($P<0.01$) for 0.1, 0.2, and 0.3 mg/ml saponin, respectively from 8.19×10^3/ml in the control. The addition of 0.1 mg/l saponin increased ammonia concentration from 33.04 mg/100 ml (without saponin) to 37.12 mg/100 ml ($P<0,01$), whereas the addition of 0.2 and 0.3 mg/ml saponin decreased ammonia concentrations by 1.69 and 16.50% ($P<0.01$) compared to the control. Microbial protein, cellullase activity and pH were not affected neither by saponin nor kind of feed. Protozoal numbers and ammonia concentration in the rumen were lower ($P<0.01$) with king grass as substrat than that with rice bran, or king grass: rice bran. In general, no interactions between saponin and kind of feed were observed, except for ammonia concentration. It can be concluded that level of 0.2 mg/ml saponin have antimicrobial properties, particularly in suppressing protozoa, which may prove beneficial to ruminal fermentation and may lead to lower ruminal ammonia concentration, but it did not have negative effect on pH, microbial protein and cellullase activity. King grass as a substrate decreases protozoa numbers and ammonia concentration.

Keywords: defaunation, in vitro rumen fermentation, protozoa, saponin

INTRODUCTION

Saponins are glycosidic compounds composed of a steroid (C27) or triterpenoid (C30) sapogenin nucleus with one or more carbohydrate branches (Klita *et al*., 1996). Sarsaponin is a group of steroidal glycosides extracted from the *Yucca schidigera* plant. Steroidal saponins are present in a wide variety of plants, including the desert plant, *Yucca schidigera*. Saponins have antimicrobial properties, particularly in suppressing ciliate protozoa, peptidase-producing bacteria (Wallace *et al.,* 1994; Wang *et al.*, 2000), and cellulolytic bacteria (Wang *et al.*, 2000). Methanogenic bacteria were metabolically correlated with ciliate protozoa (Newbold *et al.*, 1995 *cit.* Pen *et al*., 2006). Because methane production has a negative correlation with energy utilization in ruminants (Ørskov *et al*., 1968), there have been many efforts to inhibit its production and to rechannel hydrogen to produce more VFA and microbial mass.

In tropical ruminant feeding systems, forages usually have a low digestibility and are deficient in N, which restricts the efficiency of feed utilization. Abreu *et al*. (2004) explained that feed utilization could be improved by manipulation of ruminal fermentation either through changes in diet composition (e.g., legume supplementation) or through manipulation of key ruminal microbial groups. Removing ruminal ciliate protozoa has been proposed as one of promising approach because their presence decreases bacterial count and the total amount of microbial protein leaving the rumen. A range of techniques for defaunation has been tested, but suitable methods for defaunation under normal farm conditions are still lacking.

Recently, plant secondary metabolites with an inherent capability to partially defaunate the

rumen have been described. The incorporation of pure saponins or saponin-rich feeds, such as *Sapindus saponaria* fruits, into the diet decreased the ruminal ciliate population, whereas bacterial and fungal biomass increased (Dıaz *et al*., 1993). Supplementing grass-alone and grass-legume diets with *S.saponaria* fruits resulted in favorable ruminal changes *in vitro*, but effects occasionally depended on the basal diet (Hess *et al*., 2003). Many compounds have been tested *in vitro* as methane inhibitors. However, ruminal microbial populations *in vivo* adapt or *in vitro* degrade many of these compounds, and favorable effects on animal performance have rarely been observed.

The objective of the present study was to investigate the effects of addition of saponin as defaunating agent in ruminal fermentation characteristics of forage and concentrate on protozoal numbers, pH, ammonia concentration, microbial protein and cellullase activity.

MATERIALS AND METHODS

Experimental Design

Treatments were arranged in a 4x3 factorial, with the main factors being level of saponin (containing 0, 0.1, 0.2, or 0.3 mg/ml saponins in fermentation medium) and kind of feed (king grass [*Pennisetum hybrid*], rice bran, and king grass-rice bran [60:40, w/w]). *In vitro* fermentation experiments were separately conducted for each treatment with three replicates.

In Vitro Batch Fermentation

Short-term *in vitro* incubation were carried out with rumen fluid from a fistulated Ongole Crossbred. The rumen fluid was withdrawn before the morning feeding and was squeezed through four layers of surgical gauze into an Erlenmeyer flask under continuous flushing with CO_2, and efforts were made to maintain the temperature at 38 to 39°C. The fluid was then mixed by a bicarbonic buffer for *in vitro* gas production pH 6.9 in a ratio of 1:2 (v/v) as described by Menke and Steingass (1988). The substrate (feed) was milled to pass through 1mm sieve and 300 mg was weighed in 100-ml glass syringes. After mixing, 30 ml of diluted rumen fluid was anaerobically transferred to glass syringe containing 300 mg of each substrate. Weighed amounts of saponin were added to achieve final concentrations of 0, 0.1, 0.2, or 0.3 mg/ml of fermentation medium. Each glass syringe was sealed by syringe cap and was incubated in a water bath at 39°C for 72 h.

At the end of the incubation period, protozoal numbers and pH was determined in fermentation fluid. Bacteria were isolated from fermentation fluid using differential centrifugation. In the first step, fermentation fluid was centrifuged at 3000 rpm for 15 min to separate bacteria from protozoa and feed particles. The resulting supernatant was analyzed for ammonia concentration. A part of supernatants were again centrifuged at 10.000 rpm for 15 min (5°C), the cell-free supernatant was analyzed for cellullase activity (CMC-ase) and the pellets were analyzed for microbial protein.

Measurement of *In Vitro* Fermentation Parameters

Protozoal Counts. Ruminal protozoa were counted by a 0.2-mm depth counting chamber (Dıaz *et al*., 1993). Before being counted, samples were fixed by the addition of 0.8 ml/ml of formaldehyde-saline solution (37% [v/v] formaldehyde and 0.9% [w/v] NaCl). Then, the samples were shaken to ensure homogenity and were transferred via a pipet to the edge of the cover slip, allowing the 0.2-mm deep chamber to fill by capillary action and thereby ensuring that there was no bubble formation under the cover glass. The primary square of the counting chamber was visualized under a microscope at 40 x magnification.

Fermentation fluid pH. Rumen fluid pH was immediately recorded using a pH meter.

Ammonia concentration. After centrifuging at 3.000 rpm for 15 min, supernatant was assayed for ammonia, which was analyzed by the phenol-hypochlorite method (Weatherburn, 1967). This method was based on iodophenol reaction resulting stable blue colour, and was measured at 630 nm.

Microbial protein. The pellets were resuspended using a 1 N NaCl solution and were analyzed for microbial protein by the Lowry method with bovine serum albumin (BSA) as the standard (Plummer, 1978). Five milliliter of 'alkaline solution' were added to 1 ml of the test solution, and were allowed to stand at room temperature for 10 min. Then it was added by 0.5 ml of diluted Folin-Ciocalteau reagent. After 30 min the result was read the extinction against the appropriate blank at 750 nm. The values obtained were converted to milligrams of bacterial mass protein per milliliter of broth.

Cellullase activity. To determine ruminal cellullase activity, the cell-free supernatant was analyzed for cellullase as described by Halliwell *et al*. (1985). Cellullase activity was determined by measuring reducing sugar released using ferricyanide reaction. One milliliter of supernatant was incubated by 1 ml of 1% CM-cellulose in 0.1 M sodium acetate buffer (pH 5.5) at 38°C for 45 min. The reducing sugar thus released was estimated by ferricyanide reaction. The optical density was calibrated by glucose solutions of known concentration. Appropriate blanks were used. Enzyme activity was expressed as unit per g of enzyme protein (U/g). One unit of each enzyme activity was defined as the amount of enzyme which released 1 μmol of reducing sugar per ml of sample per min under the condition indicated. The enzyme protein concentration was determined by the Lowry method with BSA as the standard (Plummer, 1978).

Statistical Analysis

The data in the main study were analyzed as a 4 x 3 factorial arrangement. The differences of mean value were analyzed by Duncan's new multiple range test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Results

Addition of saponin significantly (P<0.01) inhibited the protozoa growth *in vitro* fermentation fluid and decreased ammonia concentration, but other parameters (pH, microbial protein, and cellullase activity) were unaffected. The protozoa numbers were reduced by 17.22, 42.73, and 49.57% for 0.1, 0.2, and 0.3 mg/ml saponin, respectively from 8.19×10^3/ml in the control. The addition of 0.2 and 0.3 mg/ml saponin decreased ammonia concentrations by 1.69 and 16.50%, respectively compared to the control, with the lowest concentration in 0.3 mg/ml saponin (27.59 mg/100ml). The addition of 0.1 mg/ml saponin increased ammonia concentration compared to control. Cellullase activity was 30.13% lower in addition of 0.3 mg/ml saponin than in the controls, but this difference was not statistically significant (P>0.05).

Kind of feed also influenced significantly (P<0.01) on protozoal numbers and ammonia concentration, but it didn't affect on pH, microbial protein, as well as cellullase activity. Protozoa numbers in fermentation with king grass and king grass-rice bran as substrate were lower than that of rice bran, which were 21.34 and 24.83% lower than the control (7.45×10^3 /ml). The ammonia concentration with rice bran and king grass-rice bran were higher than that with king grass, and the highest concentration was in rice bran (37.65 mg/100ml).

In general, no interactions between saponin and kind of feed were observed. Except for ammonia concentration, which were reached the lowest concentration (23.53 mg/100ml) by the addition of 0.3 mg/ml saponin and king grass as a substrat.

Table 1. *In vitro* Fermentation Parameters at Different Level of Saponin and Kind of Feeds

Parameters	King grass				Rice bran				King grass-rice bran				Significance		
	0	0.1	0.2	0.3	0	0.1	0.2	0.3	0	0.1	0.2	0.3	KF	SPN	INT
Protozoal numbers ($x10^3$/ml)	8.62	6.00	4.87	3.94	7.69	6.00	4.69	4.03	8.25	8.34	6.75	6.47	**	**	ns
pH	7.02	6.97	6.99	7.00	7.06	6.99	7.05	7.03	7.06	7.05	7.05	7.03	ns	ns	ns
Ammonia concentration (mg/100 ml)	24.12	28.03	26.53	23.53	37	34.89	35.9	30.07	37.99	48.44	34.99	29.19	**	**	**
Cellullase activity (U/g)	1.53	1.37	1.04	0.73	0.84	1.17	1.07	2.52	0.92	0.69	0.61	1.41	ns	ns	ns
Microbial protein (mg/ml)	0.25	0.31	0.32	0.29	0.27	0.23	0.32	0.25	0.25	0.29	0.21	0.26	ns	ns	ns

** P<0.01
KF kind of feeds
SPN level of saponin (%)
INT interaction

Discussion

Microbial proteins were apparently unaltered by saponin, but protozoal numbers have shown that saponin affects the protozoa growth, and tended to increase cellullase activity. Included at 1%, *Yucca schidigera* extract stimulated *Prevotella ruminicola*, suppressed *Streptococcus bovis* and *Butyrivibrio fibrisolvens* and did not affect growth of *Selenomonas ruminantium* (Wallace *et al*., 1994). Decreasing protozoal counts with supplementation of saponins rich extract (Hristov *et al*., 1999) or pod and seed (Patra *et al.,* 2006) or fruits (saponin-rich fruits including *S. saponaria* (Diaz *et al*., 1993; Hess *et al*., 2003) have been reported. Saponins possibly bind with sterol of cell membrane of protozoa and change the permeability of cell membrane (Patra *et al.,* 2006). The most widely recognized biological effect of saponins is the haemolysis of red blood cells in vitro. It is generally accepted that saponins cause haemolysis by increasing the permeability of the plasma membrane (Price *et al*., 1987). Saponins from various sources have also been found to permeabilize the small intestinal mucosal cells of mammals (Johnson *et al.,* 1986), but the effect of saponins on the permeability of microbial cell walls has not been assessed. Klita *et al*. (1996) explained the susceptibility of rumen protozoa and lack of susceptibility of rumen bacteria to saponins by the presence of cholesterol in eukaryotic membrane (including protozoa), but not in prokaryotic bacteria cells.

The cellullase activities were not affected by saponin. Patra *et al.* (2006) demonstrated the specific activities of CMCase were not affected by any of the extracts tested, whereas specific activity of acetylesterase reduced significantly ($P<0.05$) by all the extracts. The numerically lower activities in the presence of different extracts on CMCase and xylanase might be due to its antiprotozoal activity, as it has been reported that about 38% of cellulase activity is associated with protozoa fraction of rumen liquor (Agarwal *et al*., 1991 *cit.* Patra *et al.,* 2006). A decrease in CMCase and xylanase activity by addition of yucca and quillaja saponins have been observed by Hristov *et al.* (2003).

The addition of 0.3 mg/ml saponin resulted in the lowest ammonia-N concentration. Hussain and Cheeke (1995) considered that the reduction in ammonia-N by saponins was due to the reduced urease activity, but Headon *et al*. (1991) *cit*. Hu (2005) explained it by ammonia binding properties of saponins. In the result study, Pen *et al*. (2006) demonstrated that NH3–N concentrations were reduced ($P<0.001$) by up to 48% with *Yucca schidigera* extract and tended to decrease by up to 21% by *Quillaja saponaria* extract. *Yucca* extract has two fractions, the glycofractions and saponins, and reduced NH3–N concentrations may be due to the ammonia binding ability of the glycofractions, while the saponin fraction may affect ammonia concentrations indirectly via toxicity to rumen ciliate protozoa (Wallace et al., 1994). Reduced ammonia concentrations in the rumen are typical when protozoal growth is inhibited, presumably as a result of depressed bacterial lysis (Pen *et al*., 2006).

CONCLUSION

In conclusion, supplying at 0.2 mg/ml saponin *in vitro* gas production buffer has antimicrobial properties, particularly in suppressing protozoa and reduce ammonia concentration, which may prove beneficial to ruminal fermentation and may lead to lower ruminal ammonia concentration, but it did not have negative effect on pH, microbial protein and cellullase activity. Using king grass as a substrat decreases protozoal numbers and ammonia concentration..

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