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Effects of Mixtures of Tween80 and Cellulolytic Enzymes on Nutrient Digestion and Cellulolytic Bacterial Adhesion*

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ABSTRACT : A series of *in vitro* and *in vivo* experiments were conducted to investigate the effects of the mixture of Tween 80 and cellulolytic enzymes (xylanase and cellulase) on total tract nutrient digestibility and rumen cellulolytic bacterial adhesion rates in Holstein steers. Ground timothy hay sprayed with various levels of Tween 80 and cellulolytic enzymes was used as substrates in an *in vitro* experiment to find out the best combinations for DM degradation. The application level of 2.5% (v/w) Tween 80 and the combination of 5 U xylanase and 2.5 U cellulase per gram of ground timothy hay (DM basis) resulted in the highest *in vitro* dry matter degradation rate ($p<0.05$). Feeding the same timothy hay to Holstein steers also improved *in vivo* nutrient (DM, CP, CF, NDF and ADF) digesibilities compared to non-treated hay ($p<0.05$). Moreover, Tween 80 and enzyme combination treatment increased total ruminal VFA and concentrations of propionic acid and isovaleric acid with decreased acetate to propionate ratio ($p<0.001$). However, adhesion rates of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* determined by Real Time PCR were not influenced by the treatment while that of *Ruminococcus albus* was decreased ($p<0.05$). The present results indicate that a mixture of Tween 80 and cellulolytic enzymes can improve rumen environment and feed digestibility with variable influence on cellulolytic bacterial adhesion on feed. (**Key Words :** Tween 80, Cellulolytic Enzymes, Cellulolytic Bacteria, Total Track Digestibility, Adhesion)

INTRODUCTION

Non ionic surfactant, Tween 80, has been used as a feed additive to improve rumen environment and animal productivity. Previous studies indicate that Tween 80 may provide some positive effects on exo- and endogenous enzyme activity of rumen bacteria, total bacterial growth rate, VFA, gas production, DM intake and milk production in cattle (Kamande, 1994; McAllister et al., 2000; Lee et al., 2003; Wang et al., 2003; Kim et al., 2004; Baah et al., 2005). Various modes of action for these beneficial effects of Tween 80 have been proposed and some of them are: increased microbial growth (Lee et al., 2003; Goto et al., 2003a), cellulolytic enzyme activity (Lee and Ha, 2003), and enzyme binding on substrates (Goto et al., 2003b). Although adhesion of fibrolytic bacteria is regarded as an obligated step in fiber digestion (Cheng et al., 1991; Flint and Forsberg, 1995), we could show that Tween 80 actually reduced adhesion of major fibrolytic bacteria to rice straw (Lee et al., 2007). Therefore, Tween 80 may improve fiber digestion by some other mechanism. For instance Eriksson et al. (2002) have suggested that non-ionic surfactant, Tween 80, surrounds lignin parts of substrates through strong hydrophobic interaction, which makes bacterial or enzymatic degradation of cellulose or hemicellulose easier. Moreover, non-ionic surfactants decrease the absorption of enzymes to substrates, which may be helpful to maintain enzymatic reaction (Kim et al., 2006).

Responses by ruminant animals to exogenous enzymes have been variable in the literature. However, there are

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Table 1. Application levels of the combination between Tween 80 and cellulolytic enzymes in *in vitro* experiment

	Control	T+E	0.5T+E	T+0.5E	0.5T+0.5E
Tween 80	-	2.5%	1.25%	2.5%	1.25%
Xylanase	-	5 U	5 U	2.5 U	2.5 U
Cellulase	-	1.25 U	1.25 U	0.625 U	0.625 U

Control: water treated timothy; T+E: 2.5% (v/w) Tween 80 per timothy g DM+5 U xylanase and 1.25 U cellulase per timothy g DM; 0.5 T+E: 1.25% (v/w) Tween 80 per timothy g DM+5 U xylanase and 1.25 U cellulase per timothy g DM; T+0.5E: 2.5% (v/w) Tween 80 per timothy g DM+2.5 U xylanase and 0.625 U cellulase per timothy g DM; 0.5T+0.5E: 1.25% (v/w) Tween 80 per timothy g DM+2.5 U xylanase and 0.625 U cellulase per timothy g DM.

studies which show enhanced fiber digestion by exogenous enzymes under *in vitro, in situ* and *in vivo* conditions (Feng et al., 1996; Lewis et al., 1996; Yang et al., 1999). Beauchemin and Rode (1996) suggested that exogenous enzymes could enhance fibrolytic enzyme activity by less than 15%, and DM disappearance and bacterial colonization of lucerne hay treated with exogenous enzymes prior to feeding (Yang et al., 1999). Wang et al. (2001) reported that addition of exogenous enzymes improved bacterial adhesion rates to feed particles. In addition, Hristov et al. (1998b) suggested that when enzymes were treated to feed or administrated into the abomasum, intestinal viscosity was reduced, which resulted in 1.2-1.5% increase in total tract DM digestibility.

Recently, there have been some attempts to determine effects of the mixtures of Tween 80 and cellulolytic enzymes on ruminal fermentation and feed digestion with inconsistent results. Kim et al. (2005) reported that there were no significant effects of the mixture of Tween 80 and exogenous enzymes on VFA production, DM degradation rates, methane production, except for enzyme activity and pH values. However, Baah et al. (2005) suggested that the mixture of Tween 80 and cellulolytic enzymes increased propionate production, slowly disappearing fraction of orchardgrass and degradation rate of barley grain.

Present study was conducted to investigate effects of combinations of Tween 80 and cellulolytic enzymes on nutrient digestion and to see if the combination has any effects on cellulolytic bacterial adhesion.

MATERIALS AND METHODS

Treatment of timothy hay with Tween 80 and cellulolytic enzymes

Tween 80 (P1754), xylanase (X2753) and celluase (C8546) were obtained from SIGMA-ALDRICH KOREA (Kyunggi-do, Korea). Details of treatment of hay have been published elsewhere (Lee et al., 2007). Briefly, treatment solutions were prepared by dissolving the mixture of Tween 80 and cellulolytic enzymes (xylanase and cellulose) in distilled water, and sprayed to timothy in an amount of 2 ml per gram timothy hay (DM basis). The optimal concentration of Tween 80 and enzymes was determined in previous *in situ* experiments (data not shown), where concentration of 2.5% Tween 80 (v/w), 5 U xylanase and 1.25 U cellulase per gram rice straw DM gave the best result in dry matter digestion. In both *in vitro* and *in vivo* experiments, timothy hay was used as a substrate. Timothy was ground to have mean particle size of 1 mm-1.5 mm or 15 cm-20 cm for *in vitro* and *in vivo* experiments, respectively and the treatment solutions were sprayed onto timothy, mixed vigorously by hand and then left at room temperature for 24 h.

In vitro study

To determine the optimal level of Tween 80 and cellulolytic enzymes (xylanase and cellulase), an *in vitro* culture study was conducted. The treatment levels of Tween 80 and enzymes are presented in Table 1. Rumen fluid was collected before morning feeding, squeezed through eight layers of cheesecloth, and then mixed with McDogall buffer (McDogall, 1948) in the ratio of 1:4 (v/v). Serum bottles (60 ml) that contained 0.7 g ground timothy treated with different mixture levels of Tween 80 and cellulolytic enzymes (Table 1) were flushed with O_2-free CO_2. The rumen fluid-buffer mixture (30 ml) then was added to the serum bottles (Wheaton Scientific) and incubated at 39°C. The digestibility of dry matter was calculated from the amount reduced after incubation and original sample weight.

In vivo study

Four rumen cannulated Holstein steers (average 708 kg of BW) were used in a digestion trial in a 2×2 change over design. The basal diet fed to the steers contained 60% timothy hay and 40% commercial concentrate at the level of 2% BW. The analyzed compositions of non-treated and treated timothy hay were 90.34 and 96.46% of DM, 7.87 and 7.86% of CP, 3.24 and 3.91% of EE, 75.82 and 74.72% of NDF and 50.67 and 50.51% of ADF, respectively. Timothy hay which was treated with water (control) or the mixture of Tween 80 and cellulolytic enzymes (treatment) were fed twice a day at 0800 and 2000 together with a concentrate, which contained 12.5% CP and 71% TDN. Amount of feed consumed and refused were recorded and orts were collected for analysis. The experiment was conducted for 16 days with 10 days of adaptation period and 6 days of sampling period. From 11 d to 13 d, before morning feeding, entire feces of each cow were collected,

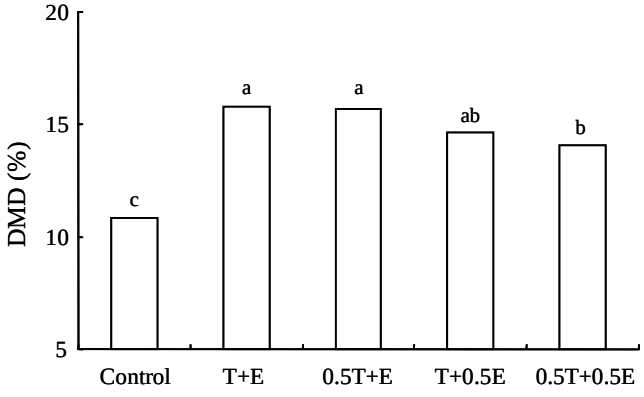

Figure 1. DM degradation of timothy as influenced by different application levels of Tween 80 and cellulolytic enzymes. [a, b] Means with different letters differ significantly ($p<0.05$).

measured for total weight, mixed well and then subsamples (1/10 of whole feces) were taken. Subsamples were dried at 60°C immediately and ground to pass through 1 mm screen before analysis. On day 14, rumen fluid was taken through rumen cannula at 0 (before morning feeding; 0800), 2, 4, and 8 h after feeding and then pH values were measured immediately. The collected rumen fluid samples were frozen at -20°C until VFA analysis. On day 15 and 16, nylon bags (5×10 cm; pore size, 53 μm) which contained ground timothy hay treated with water or the mixture of Tween 80 and cellulolytic enzymes were introduced into the rumen before morning feeding to measure cellulolytic bacterial adhesion rate. The nylon bags were retrieved from each steers after 0, 15 min, 30 min, 2 h, 8 h and 24 h incubation, and washed with 39°C tap water until it ran clear and then squeezed by hand to remove excess water (Lee et al., 2007). The washed samples were frozen at -20°C before quantitative analysis of fiber-attached bacterial populations by real time PCR. All treatments were replicated three times.

Quantification for cellulolytic bacteria by RT-PCR

DNA extraction, primers for *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* and real time PCR conditions were same as previously reported (Lee et al., 2007).

Chemical analyses

Feed and fecal samples were ground to pass a 1-mm screen before DM, CP and ether extract determination (AOAC, 1990). Concentrations of NDF and ADF were determined according to Van Soest et al. (1991) except that sodium sulfite and heat-stable amylase were used for NDF analysis. Ruminal VFA (mM) was measured by Erwin et al. (1961). For VFA analysis 2 ml subsample of rumen fluid was centrifuged at 12,000 rpm for 10 min and 1 ml of supernatant was treated with 25% (w/v) metaphosphoric acid and VFA concentration was measured by using Gas Chromatography (HP 6890).

Statistical analysis

Statistical analysis was conducted using the ANOVA procedure of the Statistical Analysis System Institute, Inc. (SAS, 1996). Variables in the model were treatment, time and treatment×Time. Differences among treatments were declared as significant at $p<0.05$.

RESULTS AND DISCUSSION

***In vitro* experiments**

Effects of different combinations of Tween80 and cellulolytic enzymes on i*n vitro* ruminal dry matter degradation of ground timothy hay are presented in Figure 1. Dry matter degradation (DMD) rate of all treated hay was greater ($p<0.05$) than control. Of the concentrations tested in this study, combination of 2.5% (v/w) Tween 80 plus 5 U xylanase and 1.25 U cellulase and 1.25% Tween 80 plus 5 U xylanase and 1.25 U cellulose gave the best results in terms of DMD with no differences between two treatments. Since data on effects of combinations of Tween 80 and enzymes on DMD are limited, it is not possible to validate present results with published values. Although a previous report (Kim et al., 2005) indicated that there were no effects of combination of Tween 80 and enzymes on *in vitro* DMD at 1% Tween 80 and commercial enzyme mixture, it is certain that effects of the mixture of Tween 80 and enzymes can be different when types and amount of enzymes and substrates are different (Beauchemin et al., 1995; Feng et al., 1996; Lewis et al., 1999).

***In vivo* experiment**

The mixture of 2.5% (v/w) Tween 80 and 5 U xylanase and 1.25 U cellulase was selected as the best combination and their effects on nutrient digestion was assessed in a subsequent digestion trial. Pretreatment of timothy hay with the mixture for 24 h prior to feeding improved ($p<0.05$) total tract digestibility of dry matter, crude protein, crude fiber, NDF and ADF of a diet containing 40% commercial concentrate and 60% timothy hay in steers (Figure 2). The same treatment also increased ($p<0.05$) total VFA concentration compared to non-treated control (Figure 3). The treatment also increased ($p<0.05$) propionic acid and decreased acetate to propionate ratio (A/P) ($p<0.05$). The results of present experiments are similar to those of Baah et al. (2005) who reported that the application of combination of Tween 80 and fibrolytic enzymes increased propionic acid significantly. The same authors also observed that treatment of the mixture of Tween 80 and fibrolytic enzymes increased slowly disappearing NDF

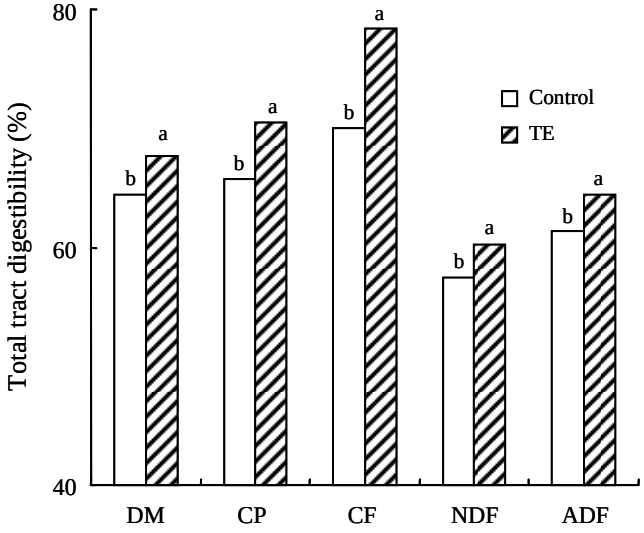

Figure 2. Total track digestibility as influenced by the mixture of Tween 80 and cellulolytic enzymes (TE). [a, b] Means with different letters differ significantly ($p<0.05$).

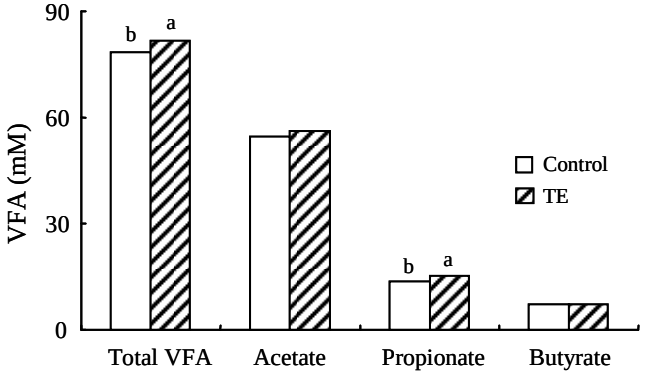

Figure 3. VFA production as influenced by the mixture of Tween 80 and cellulolytic enzymes (TE). [a, b] Means with different letters differ significantly ($p<0.05$).

fraction (b) of orchardgrass hay and barley grain. Based on these results, they suggested that there were positive interactions between Tween 80 and fibrolytic enzymes, even though individual treatment (Tween 80, fibrolytic enzymes and the mixture of Tween 80 and fibrolytic enzymes) did not significantly affect DM, N, NDF and ADF total track digestibility. Contrary to observations by Kim et al (2005) who did not see any effects of the mixture of Tween 80 and fibrolytic enzymes on total VFA and acetate to propionate ratio, application of Tween 80 and fibrolytic enzymes mixture resulted in increased total VFA and propionate concentration in present experiment. Increased total VFA is considered due to improved DMD, however, effects on propionate by the treatment is not clearly explained from present data. It is widely accepted that increased fiber digestion would increase acetate rather than propionate concentration. Supplementation of Tween 80 and enzyme mixture may have resulted in microbial population change leading to increased propionate. Effect of Tween 80 and enzyme mixture on rumen microbial population is not clearly documented in the literature.

Cellulolytic bacterial adhesion

Adhesion of cellulolytic bacteria on timothy hay as influenced by pretreatment of the mixture of Tween 80 and cellulolytic enzymes for 24 h prior to incubation in the rumen of cattle is shown in Table 2. Pretreatment of Tween 80 and cellulolytic enzymes tended ($p = 0.05$) to decrease the adhesion rate of *Ruminococcus albus* at 30 min and 2 h after incubation in the rumen. However, adhesions of *Fibrobacter succinogenes* and *R. flavefacience* to timothy hay were not affected by the treatment. Bacterial attachment is one of the most important factors governing feed digestion in the rumen (Cheng et al., 1991; Flint and Forsberg, 1995). Previous studies suggested that Tween 80 might enhance rumen bacterial adhesion rate when measured by indirect method such as microbial growth rate and Scanning Electron Microscopy (Lee et al., 2003; Lee et al., 2007). However, when bacterial adhesion rate was detected by direct method using Real Time PCR, it was observed that Tween 80 actually decreased cellulolytic bacterial adhesion rate (Lee et al., 2007). On the contrary, treatment of cellulolytic enzymes (cellulase and xylanase) improved cellulolytic bacterial colonization onto feed

Table 2. Cellulolytic bacterial adhesion rate (Log copy number/g rice straw DM) as influenced by the mixture of Tween 80 and cellulolytic enzymes (TE)

	Incubation time, hr					Mean	p value		
	0.25	0.5	2	8	24		Trt	Time	Trt×time
Fibrobacter succinogenes									
Control	8.24	8.39	10.20	11.03	11.64	9.90	0.723	<0.001	0.509
TE	8.36	8.87	9.30	10.73	11.77	9.80			
Ruminococcus albus									
Control	7.65	8.23	9.35	10.02	9.90	9.03	0.05	<0.001	0.259
TE	7.85	7.35	8.64	9.37	10.03	8.65			
Ruminococcus flavefaciens									
Control	7.80	7.90	9.07	9.96	10.06	8.96	0.718	<0.001	0.707
TE	7.94	7.79	9.21	10.06	9.95	8.99			

particles (Yang et al., 1999). Present results may indicate that the positive effects of enzymes on bacterial adhesion was masked by Tween 80. However exact mechanism can not be provided from results of current study. It would be interesting if the effects of order of application of Tween 80 and enzymes on bacterial adhesion be investigated.

In summary the mixture of Tween 80 and the combination of 5 U xylanase and 2.5 U cellulase per gram of ground timothy hay (DM basis) improved nutrient digestion through improved fermentation in the rumen, but that was not directly related to adhesion capability of major fibrolytic bacteria. More research with different type and level of surfactants and enzymes are warranted to make clear conclusion on the relationship between surfactants and cellulolytic enzymes in feed digestion and bacterial adhesion.

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