



Effects of Tween 80 Pretreatment on Dry Matter Disappearance of Rice Straw and Cellulolytic Bacterial Adhesion

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ABSTRACT : An *in situ* experiment was conducted to find out whether Tween 80 improves rice straw digestion through increased adhesion of major fibrolytic bacteria. Rice straw was sprayed with various levels of Tween 80 non-ionic surfactant or SDS ionic surfactant 24 h before incubation in the rumen of Holstein steers. Dry matter (DM) disappearance and adhesion of *F. succinogenes*, *R. flavefaciens* and *R. albus* on rice straw after *in situ* incubation were measured by real-time PCR. Application of Tween 80 increased DM disappearance, which was more noticeable at an application level of 1% compared to lower application levels. Application of SDS resulted in an opposite response in DM disappearance with highest reduction in DM disappearance at 1% level. In a subsequent *in situ* experiment, higher Tween 80 was applied to rice straw in an attempt to find the optimum application level. Tween 80 at 2.5% gave better DM disappearance than 1% with a similar result at 5%. Therefore, an adhesion study was carried out using rice straw treated with 2.5% Tween 80. Our results indicated that Tween 80 reduced adhesion of all three major rumen fibrolytic bacteria to rice straw. Present data clearly show that improved DM disappearance by Tween 80 is not due to increased bacterial adhesion onto substrates. (**Key Words :** Tween 80, SDS, Cellulolytic Bacteria, Attachment, Rice Straw)

INTRODUCTION

Non-ionic surfactants (NIS) such as Tween 80 have been shown to improve production of ruminant animals (Kamande, 1994; McAllister et al., 2000; Lee et al., 2003; Wang et al., 2003; Kim et al., 2004). Also, Tween 80 has been shown to enhance ruminal fermentation and total tract digestibility (Kim et al., 2004) and to increase DM intake and milk production in cattle (Lee et al., 2003). In addition, Wang et al. (2003) observed that Tween 80 increased the rate of gas and volatile fatty acid production during *in vitro* fermentation of corn and orchardgrass silages. Baah et al. (2005) also indicated that Tween 80 with and without fibrolytic enzyme enhanced *in situ* disappearance of orchardgrass hay with no influence on total tract digestibility in Holstein cows.

Various modes of action for these possible beneficial

effects of NIS have been proposed. In previous *in vitro* studies, the nonionic surfactant Tween 80 increased numbers of viable bacteria (Akin, 1980), microbial growth rates (Lee et al., 2003), cellulase activity and stability (McAllister et al., 2000; Lee and Ha, 2003; Lee et al., 2003) and enzymatic accessibility and degradation of grain and forage substrates (Goto et al., 2003a, b). Kamande et al. (2000) reported that Tween 80 promoted the binding of enzymes to their substrates and increased microbial protease and cellulase activities *in vitro*, although Kim et al. (2006) indicated that Tween 80 decreased enzyme adsorption to substrate but enhanced enzyme activity.

Since the attachment of fibrolytic bacteria is regarded as an obligatory step in fiber degradation (Sung et al., 2007), we aimed to determine whether beneficial effects of Tween 80 on improvement of fiber digestion is due to enhanced microbial attachment on fibrous substrate.

MATERIALS AND METHODS

Preparation and treatment of surfactant solution

Tween 80 (P1754) and SDS (sodium dodecyl sulfate) (L4390) were obtained from SIGMA-ALDRICH KOREA (Kyunggi-do, Korea). Each surfactant solution for treatment was made by dissolving surfactant in water which was

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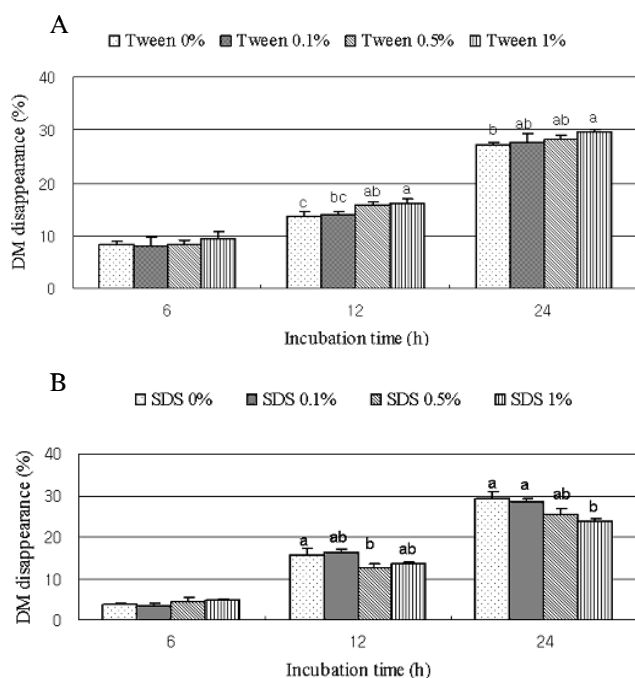


Figure 1. Effects of types and levels of surfactants on DM disappearance in *in situ* study. Rice straw was treated with 0, 0.1, 0.5, and 1% Tween 80 (v/w) (A) or SDS (w/w) (B) per unit rice straw DM. ^{a, b, c} Means with different letter differ significantly ($p < 0.05$).

applied to rice straw in a way that 2 ml solution resulted in final Tween 80 concentrations of 0, 0.1, 0.5 and 1% or 0, 1, 2.5 and 5% per unit rice straw DM. Final concentration of SDS was 0, 0.1, 0.5 and 1% per unit rice straw DM. Rice straw was ground to have a mean particle size of 1 mm-1.5 mm and Tween 80 or SDS solution was sprayed onto ground rice straw, mixed vigorously by hand and then left at room temperature for 24 h.

In situ study

Two Holstein steers with a permanent rumen cannula were maintained on 60% commercial concentrate mixture and 40% rice straw. Nylon bags (5×10 cm; pore size, 53 μm) containing 1.5 g of untreated (control)-, Tween 80 or SDS treated-ground rice straw were placed into the rumen immediately prior to feeding (08:00 h). Bags were removed from the rumen after 6, 12 and 24 h incubation for the determination of DM disappearance rate. Also, bags for the determination of attached fibrolytic bacteria on substrate were incubated for 1, 5, and 30 min, 2 h and 12 h. After removal from the rumen, bags were washed with 39°C distilled water until it ran clear and then squeezed by hand to remove excess water. All treatments were replicated three times. Samples were kept in the freezer at -20°C until quantitative analysis of fiber-attached bacterial populations by real-time PCR. Dry matter disappearance was determined by weight difference before and after incubation

in the rumen.

Quantification of cellulolytic bacteria by RT-PCR

DNA extraction : Total DNA was extracted according to Purdy et al. (1996). Dried rice straw samples treated with Tween 80 or distilled water at the 2.5% level from *in situ* incubation were mixed with TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0), Tris-buffered phenol, sterilized glass beads (0.5 mm, BioSpec. Product Inc. USA) and 10% sodium lauryl sulfate solution. Total DNA was extracted by shaking the mixture for 2 min and standing on ice for 2 min and the procedure was repeated three times; then the tubes were centrifuged at 13,000×g for 2 min and supernatant was collected. Total DNA was collected from pooled supernatant using a hydroxyapatite chromatography column (Hydroxyapatite Bio-Gel HTP Gel, Bio-Rad Laboratories, Inc. USA). The RNA was removed by DNase-free RNase and subsequent gel filtration (MicroSpin S-200 HR Columns, Amersham Biosciences, UK). The quality and quantity of total DNA were analyzed using a Biomate 5 spectrophotometer (Thermo Spectronic, USA).

PCR primer : Species-specific PCR primers for *F. succinogenes*, *R. flavefaciens* and *R. albus* reported by Koike and Kobayashi (2001) were used. Primers for *F. succinogenes*, *R. flavefaciens* and *R. albus* were: Fs219f (5'-GGT ATG GGA TGA GCT TGC-3') and Fs654r (5'-GCC TGC CCC TGA ACT ATC -3'); Rf154f (5'- TCT GGA AAC GGA TGG TA-3') and Rf425r (5'-CCT TTA AGA CAG GAG TTT ACA A-3'); Ra1281f (5'-CCC TAA AAG CAG TCT TAG TTC G-3') and Ra1439r (5'-CCT CCT TGC GGT TAG AAC A-3'), respectively. Amplification sizes from PCR reactions for the three bacterial species were 446, 259 and 175 bp and annealing temperatures were 62, 55 and 55°C, respectively.

Real-time PCR : Bacterial DNA was amplified and quantified with an iCycler iQ real-time PCR system (Bio-Rad Inc. USA). The iQ Syber Green Supermix (Bio-Rad INC. USA) was used for PCR amplification according to the manufacturer's protocol. PCR conditions were: one cycle of initial denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 30 s, followed by annealing at each temperature of strains for 30 s and then an extension at 72°C for 30 s. Thereafter, the melting point of PCR product was analyzed to detect specificity of application. The melting curve was obtained by a 0.1°C/s increase of heating temperature from 65 to 95°C with fluorescence detection at 0.1°C intervals.

Bacterial population was defined as log copy number of 16S rDNA which was calculated from a standard curve of control plasmid with an insert of specific fragment of 16S rDNA amplified with primers specific to each species. The plasmid was constructed using the pGEM-T and pGEM-T

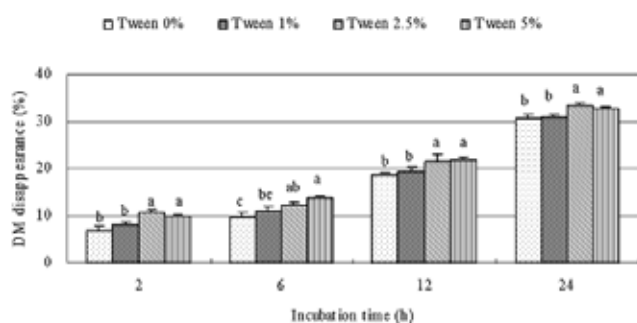


Figure 2. DM disappearance of rice straw as affected by levels of Tween 80. ^{a, b, c} Means with different letters differ significantly ($p < 0.05$).

Easy Vector System (Promega, USA) according to the manual procedure. The standard curves were made by plotting Ct values for serial dilutions of the control plasmid. *F. succinogenes* (19169), *R. albus* (27210) and *R. flavefaciens* (19208) used to obtain species-specific gene fragments were purchased from American Type Culture Collection (ATCC).

Statistical analysis

Data were analyzed using the ANOVA procedure of the Statistical Analysis System Institute, Inc. (SAS) (1996). Differences among means were tested using the least significant difference (LSD) procedure of SAS (1996).

RESULTS AND DISCUSSION

In situ DM disappearance

Effects of surfactants on rice straw DM disappearance are presented in Figure 1. Tween80, non-ionic surfactant, at a 1% level (Figure 1A) increased ($p < 0.05$) DM disappearance rate compared to the control after 24 h incubation. However, negatively charged surfactant, SDS (Figure 1B) decreased the DM disappearance of rice straw. Especially, the decrease in DM disappearance was significant when 1% SDS was applied to rice straw and incubated for 24 h ($p < 0.05$). The positive effects of Tween 80 on DM disappearance are similar to previously reported studies (Lee and Ha, 2003; Lee et al., 2003), although the degree of improvement was not high in the present study. In most studies which showed improved digestion, Tween 80 was either mixed with feed or directly added to incubation medium, while in the present study Tween 80 was applied to rice straw and dried before incubation in the rumen, which might have reduced potential beneficial effects of Tween 80. Our results clearly showed a distinctive difference between non-ionic and ionic surfactant in fiber digestion in the rumen. Regarding beneficial effects of Tween 80, Eriksson et al. (2002) have indicated that non-ionic surfactant surrounds lignin parts of substrates via

Table 1. Adhesion of *Fibrobacter succinogenes* (Log copy number/g rice straw DM) as influenced by Tween 80

| Incubation time | Control | Tween 80 | SEM |
|-----------------|-------------------|-------------------|------|
| 1 min | 8.16 ^a | 7.97 ^b | 0.04 |
| 5 min | 8.26 ^a | 8.12 ^b | 0.04 |
| 30 min | 8.68 | 8.51 | 0.05 |
| 2 h | 9.55 ^a | 9.08 ^b | 0.11 |
| 12 h | 10.40 | 10.34 | 0.03 |

^{a, b} Means with different letter differ significantly ($p < 0.05$).

SEM: Standard error of means.

Table 2. Adhesion of *Ruminococcus albus* (Log copy number/g rice straw DM) as influenced by Tween 80

| Incubation time | Control | Tween 80 | SEM |
|-----------------|-------------------|-------------------|------|
| 1 min | 5.29 ^a | 5.10 ^b | 0.05 |
| 5 min | 5.85 ^a | 5.65 ^b | 0.06 |
| 30 min | 6.10 | 5.96 | 0.05 |
| 2 h | 7.37 ^a | 6.79 ^b | 0.13 |
| 12 h | 8.28 | 8.40 | 0.05 |

^{a, b} Means with different letter differ significantly ($p < 0.05$).

SEM: Standard error of means.

Table 3. Adhesion of *Ruminococcus flavefaciens* (Log copy number/g rice straw DM) as influenced by Tween 80

| Incubation time | Control | Tween 80 | SEM |
|-----------------|-------------------|-------------------|------|
| 1 min | 5.97 ^a | 5.53 ^b | 0.10 |
| 5 min | 6.39 ^a | 5.87 ^b | 0.12 |
| 30 min | 6.80 ^a | 6.16 ^b | 0.15 |
| 2 h | 7.88 ^a | 7.34 ^b | 0.13 |
| 12 h | 8.63 | 8.44 | 0.05 |

^{a, b} Means with different letter differ significantly ($p < 0.05$).

SEM: Standard error of means.

strong hydrophobic interaction and then enables easier degradation of cellulose or hemicellulose for bacteria and cellulolytic enzymes. Moreover, non-ionic surfactants decrease the absorption of enzymes to substrates, therefore Tween 80 is helpful to maintain enzymatic reaction (Kim et al., 2006). On the other hand, SDS is negatively charged, and hence it is probable that SDS inhibited the accessibility of relatively negatively charged enzymes to rice straw.

Since the degree of beneficial effects of Tween 80 on DM digestion was low, we conducted an additional *in situ* experiment with higher Tween 80 levels (2.5 and 5%) to select an optimum level. As in Figure 2, 2.5% Tween 80 increased DM disappearance ($p < 0.05$) compared to the control and 1% Tween 80 treatments. However, further improvement in rice straw dry matter disappearance was not obtained by increasing Tween 80 to 5% after 24 h incubation, and 2.5% treatment resulted in the highest DM disappearance among all treatment levels. Therefore, the level of 2.5% Tween 80 treatment was selected for further work.

Cellulolytic bacterial adhesion

In the rumen, the dominant cellulolytic bacteria are *F.*

succinogenes, *R. albus* and *R. flavefaciens* (Miron et al., 2001). It has been reported that attachment of these cellulolytic bacteria to their substrates is an essential step for fiber digestion and most adhesion of cellulolytic bacteria is achieved within 10 min, although bacterial adhesion increased up to 6 h and then decreased slowly after 24 h (Koike et al., 2003). In the present study, the adhesion rate of cellulolytic bacteria rapidly increased up to 2 h and then increased at a slower rate to 12 h incubation. The initial adhesion of cellulolytic bacteria is the most important factor to determine effects of each treatment because, under *in situ* conditions, the Tween 80 would be washed out from substrates by continuous ruminal movement and then Tween 80 would be degraded by rumen bacteria as indicated by Goto et al. (2003a). As shown in Table 1-3, Tween 80 decreases adhesion of all three major fibrolytic bacteria to rice straw, contrary to generally accepted expectation. In most studies it has been assumed that Tween 80 treatment would increase cellulolytic bacterial adhesion, which would be one possible cause of improved DM digestibility, VFA production, enzyme activity and milk yield (Lee et al., 2003; Wang et al., 2003; Kim et al., 2006). Animals in the present study were maintained on a 60% concentrate diet and effects of Tween 80 on bacterial adhesion might have been different, which should be determined in future studies. Based on the existing literature there have been no experiments which examined effects of Tween 80 on the adhesion of rumen bacteria to rice straw during *in situ* incubation. However, there have been studies which showed that Tween 80 induced detachment of rumen bacteria from particles (Leedle et al., 1987; Whitehouse et al., 1994).

It is not apparent how Tween 80 improved DM degradation without increased bacterial attachment. No direct evidence is available, but this phenomena can be explained by the results of some previous studies. For instance, Dehority and Grubb (1980) indicated that surfactant increased total viable bacterial count in rumen contents stored for 0 or 6 h at 0°C and therefore, it is possible that Tween 80 increased total fibrolytic bacteria, but not attached bacterial numbers. Another possible mode of action is that Tween 80 modifies substrate characteristics and enzyme availability. There is relatively abundant information on effects of Tween 80 on enzyme availability. For instance, Tween 80 increases enzyme production and secretion, activity and binding to substrate (Eriksson et al., 2002; Goto et al., 2003a, b; Lee et al., 2003; Kim et al., 2006). Therefore overall improvement of enzyme availability could have been a major factor in improved DM degradation by Tween 80 observed in the present study. Also, it is possible that Tween 80 exerted substrate modification to some degree as reported in other studies.

Helle et al. (1993) reported that surfactants altered cellulose ultrastructure and increased vulnerability to enzymatic attack. Increased wettability of substrates by Tween 80 also has been proposed as a beneficial factor for improved enzyme attack (Goto et al., 2003b).

In conclusion, improved DM digestibility by Tween 80 is not dependent on increased adhesion of ruminal bacteria and other factors, such as improved enzyme availability and/or modification in substrate characteristics, may play a more important role. Further studies on effects of Tween 80 on the detachment of rumen bacteria and on populations of other rumen microorganisms under different dietary conditions may provide clues for the mode of action of Tween 80.

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