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Effect of 2-Bromoethanesulfonic Acid on *In vitro* Fermentation Characteristics and Methanogen Population*

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ABSTRACT : An *in vitro* incubation study was conducted to investigate effects of 2-bromoethanesulfonic acid (BES) on ruminal fermentation characteristics and methanogen population. BES at the final concentration of 0, 1 and 5 mM with two different substrates having a different ratio of timothy and concentrate (100% timothy vs. 40% timothy-60% concentrate) was incubated for 0, 24, 48 and 72 h in a 39°C incubator. Total DNA extracted from culture fluid was used as a template for real-time PCR to measure the population of methanogens. Four different primer sets were used for amplification of total bacteria, total methanogens, the order *Methanobacteriales* and the order *Methanomicrobiales*. BES reduced ($p<0.01$) total gas and methane production in a dose-dependent manner. BES at 5 mM inhibited methane production by more than 95% compared to the control. An interaction between substrate and level of BES in total gas and methane was detected ($p<0.01$). The decrease of methane production with increasing BES level was more pronounced on mixed substrate than on timothy alone. However, hydrogen production was increased by BES treatment ($p<0.01$). Total VFA concentration was not affected, but molar percentage of propionate and butyrate was increased and acetate to propionate ratio was reduced by BES treatment ($p<0.01$). BES did not affect the population density of total bacteria but reduced ($p<0.01$) the population of total methanogens, the order *Methanobacteriales* and the order *Methanomicrobiales* in a dose-dependent manner. The type of substrate did not influence the trend, although the magnitude of response was different between all-roughage and 40% roughage substrate. (**Key Words** : Methane, 2-Bromoethanesulfonic Acid, Fermentation, Methanogen)

INTRODUCTION

Methane production in the rumen is a nutritionally wasteful process which represents 2 to 15% feed energy loss (Moss, 1993). In addition, methane production by animals, principally ruminants, is estimated to account 15 to 20% of the global production of methane (Crutzen et al., 1986). Therefore, extensive research effort has been made to find ways to reduce methane production in the rumen. Various compounds have been used to decrease ruminal methane production by various methods (Bhatta et al., 2007), and some of examples are: ionophores (Van Nevel and Demeyer, 1988), organic acids (Martin, 1998), fatty acids (Czerkawski et al., 1966; Dohme et al., 2001), plant extracts (Busquet et al., 2005) and halogenated compounds (Martin and Macy, 1985).

Halogenated compounds such as bromoethanesulfonic acid (BES) are known to be the most effective inhibitors due to their direct inhibitory effects to methanogenic bacteria. BES is known to inhibit the action of methyl coenzyme M reductase in the last step of methanogenesis (Balch and Wolfe, 1979). Martin and Macy (1985) observed that 30 μM BES reduced methane production by 76% in mixed cultures of rumen fluid. However, Immig et al. (1996) observed that methane production was recovered after 4 days of BES infusion into the rumen of sheep. Ungerfeld et al. (2004) observed different sensitivity to methane inhibitors including BES by pure culture of ruminal methanogens. *Methanobrevibacter ruminantium* was the most sensitive to BES, *Methanosarcina mazei* was the least sensitive and *Methanomicrobium* was intermediate.

Traditional culture-based techniques have allowed for the isolation and identification of only a limited number of

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Table 1. The primers used for real-time PCR assay

Target group	Forward primer	Reverse primer	Amplicon size (bp)	Annealing temp. (°C)
Bacteria[1]	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC	130	60
Methanogens[2]	TTCGGTGGATCDCARAGRGC	GBARGTCGWAWCCGTAGAATCC	140	60
Methanobacteriales[3]	CGWAGGGAAGCTGTTAGT	TACCGTCGTCCACTCCTT	343	60
Methanomicrobiales[3]	ATCGRTACGGGTTGTGGG	CACCTAACGCRCATHGTTTAC	506	63
Methanosarcinales[3]	GTAAACGATRYTCGCTAGGT	GGTCCCCACAGWGTACC	354	60
Methanococcales[3]	TAAGG GCTGG GCAAG T	CACCT AGTYC GCARA GTTTA	337	60

[1] Adapted from Denman and McSweeney (2006). [2] Adapted from Denman et al. (2007). [3] Adapted from Yu et al. (2005).

species of ruminal methanogens due to their fastidious growth requirement (Joblin, 2005). However, non-cultured techniques developed in recent years made identification and enumeration of methanogens easier. Some of molecular techniques used for this purpose include DNA hybridization, the development of clone libraries of 16S rRNA gene sequences, quantitative real-time PCR, denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) (Lin et al., 1997; Tajima et al., 2001; Skillman et al., 2006; McSweeney et al., 2007; Nicholson et al., 2007). Yu et al. (2005) developed group-specific primer to detect four orders of methanogens (*Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales*, and *Methanococcales*) using quantitative real-time polymerase chain reaction (qPCR).

Although previous experiments clearly show that BES reduces methane production, which can be related to the number and composition of methanogens (Sparling and Daniels, 1987; Van Nevel and Demeyer, 1995), direct evidence on the relationship between BES and methanogen population has not been clearly presented in the literature. Therefore, current *in vitro* study was conducted to find relationship between methane production and the population of methanogen and to determine effects of BES on ruminal fermentation characteristics with two different substrates.

MATERIALS AND METHODS

In vitro incubation

Rumen fluid was obtained from rumen-cannulated Holstein steers before morning feeding. Steers were fed twice a day with a ration consisting of 60% timothy and 40% commercial concentrate mixture (12% crude protein and 75% TDN). The collected rumen content was filtered through four layers of cheesecloth and then mixed with two volume of buffer (Menke and Steingass, 1988) under O_2-free CO_2 gas. The 50 ml of rumen fluid-buffer mixture was dispensed anaerobically into serum bottles containing 0.5 g of substrates which had two different ratios of timothy and concentrate (100% timothy vs. 40% timothy-60% concentrate). The solution of BES sodium salt (Aldrich, WI, USA) was added to have the final concentration of 0, 1 and 5 mM in the bottle, which then was filled with O_2-free CO_2 gas and capped with a rubber stopper. The bottles were incubated for 0, 24, 48 and 72 h in a 39°C incubator.

Analyses

Total gas production was measured after 24, 48 and 72 h incubation by the method of Theodorou et al. (1994) and then headspace gas in the bottle was collected for the analysis of methane and hydrogen. Methane and hydrogen were measured with a gas chromatography (Varian 3800, USA) equipped with Carbosieve S 8100 mesh column (Supelco, USA). The culture fluid was subsampled for the determination of pH, volatile fatty acid (VFA) concentration and total DNA extraction. VFA analysis was performed with a gas chromatography (HP 6890) as described by Erwin et al. (1961).

DNA extraction and real-time PCR

DNA extraction : Total DNA was extracted according to Lee et al. (2007). The culture fluid was mixed with TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0), Tris-buffered phenol, sterilized glass beads (0.5 mm) and 10% sodium lauryl sulfate solution in the 2 ml tube. The mixture was shaken for 2 min two times using Mini-beadbeater (BioSpec. Product Inc., USA). The mixture was cooled down on ice for 2 min after each bead-beating. The tubes were centrifuged at 14,000×g for 5 min and supernatant was collected. The RNA was removed by incubation with DNase-free RNase (iNtRON Biotechnology, Korea). The extracted DNA solution was filtrated using MicroSpin S-200 HR Columns (GE Healthcare, UK) and then DNA concentration was determined using spectrophotometer (UV-1601PC, Shimadzu, Japan).

PCR primers : The PCR primer sets used in this study for amplification of total bacteria, total methanogens and different groups of methanogen (the order *Methanobacterials*, the order *Methanomicrobiales*, the order *Methanosarcinales* and the order *Methanococcales*) were the same as developed by Denman and McSweeney (2006), Denman et al. (2007) and Yu et al. (2005), respectively as shown in Table 1.

Real-time PCR : The extracted DNA from culture fluid was amplified and quantified with an iCycler iQ real-time

Table 2. Effects of 2-bromoethanesulfonic acid (BES) on *in vitro* total gas, methane and hydrogen production

Item	Treatment[1]						SEM[2]	Significance		
	Timothy (mM)			Mixed (mM)						
	0	1	5	0	1	5		Sub	BES	Sub×BES
Total gas (ml)										
24 h	77.7	70.4	65.4	94.0	84.1	77.3	2.41	**	**	NS
48 h	93.2	84.1	75.5	110.6	92.7	88.1	2.71	**	**	*
72 h	103.5	90.8	75.2	110.0	92.5	88.1	2.66	**	**	**
Methane (ml)										
24 h	5.7	2.1	0.3	8.7	2.4	0.2	0.74	**	**	**
48 h	7.5	3.8	0.3	11.2	4.3	0.4	0.94	**	**	**
72 h	9.3	5.3	0.4	11.5	4.8	0.2	1.01	**	**	**
Hydrogen (ml)										
24 h	0.01	0.52	1.16	0.04	1.15	2.41	0.202	**	**	**
48 h	0.01	0.01	0.44	0.01	0.02	0.92	0.085	**	**	**
72 h	0.01	0.01	0.10	0.01	0.05	0.10	0.012	NS	**	NS

[1] Within timothy and mixed substrate, BES was treated at the final concentration of 0, 1 and 5 mM. [2] SEM = Standard error of means.
*, ** Significant at $p<0.05$ and $p<0.01$, respectively; NS = Not significant ($p>0.05$).

PCR system (Bio-Rad Inc., USA). The iQ SYBR Green Supermix (Bio-Rad Inc., USA) was used for PCR amplification according to the manufacturer's protocol. PCR conditions were as follows: one cycle at 50°C for 2 min and at 95°C for 3 min for initial denaturation, 40 cycles at 95°C for 15 sec and at each annealing temperature (Table 1) for 1 min for primer annealing and product extension. The melting curve was obtained by increasing the temperature at a rate of 1°C/10 sec from 60 to 95°C. The values of cycle threshold (Ct) after real-time PCR were used to determine changes of different microbial population relative to 0 h control without BES treatment as described by Denman and McSweeney (2006).

Statistical analysis

Obtained data were analyzed using the ANOVA procedure of Statistical Analysis System (SAS, 2002) and main effect of substrate (n = 2) and BES (n = 3), and their interactions were analyzed and differences among main effects were compared with the method of least significant differences (LSD) (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Total gas, methane and hydrogen production

Total gas production at 0% BES was higher when the substrate was concentrate and timothy mixture than timothy alone regardless of incubation time ($p<0.01$) (Table 2). Increased gas production with mixed substrate in present study is expected result considering close relationship between rumen fermentation and gas production. It is well accepted that gas production in the rumen is the result of fermentation of substrates, mainly carbohydrates (Wolin, 1960). Therefore, the mixture which contained more easily fermentable carbohydrates produced more gas than slowly fermentable timothy (Menke and Steingass, 1988).

Methane production at 0% BES was higher with mixed substrate ($p<0.01$) (Table 2). It is well known that not only type of substrates, but also level of substrates can influence methane production. According to Van Nevel and Demeyer (1995), methane production increased with increasing apparent digestibility of the ration at maintenance level, but the opposite was true when feeding level was three times maintenance. According to Bonhomme (1990) and Krumholz et al. (1983), readily fermentable carbohydrates at maintenance level increased the population of the ciliate protozoa, so that methane production increased due to an increase in hydrogen transfer between these microorganisms. The level of concentrate in the mixture substrate of present study was 40%, which is regarded as the level for maximum protozoal growth, and therefore methane production was higher possibly due to higher hydrogen transfer to methanogens. Excess amount of concentrate over the level used in present study may have decreased protozoal growth and reduced methane production in the rumen compared to roughage alone as reported in previous studies (Moe and Tyrrell, 1979; Moss et al., 1993). Lower methane production at high concentrate level may also be attributed to shift in VFA proportions in favor of propionate, and inhibition of methanogenic bacteria and rumen ciliates (Van Nevel and Demeyer, 1988; Van Kessel and Russell, 1996). In addition, McAllister et al. (1996) reported properties of the forage that lower digestibility and longer residency within the rumen generally increased methane production. Results of an *in vitro* study by Lee et al. (2003) also indicated that methane production at 24 h incubation was highest with grains, followed by brans and hulls, oil seed meals and roughages.

BES reduced total gas and methane production in dose-dependent manner regardless of the type of substrate ($p<0.01$). BES at 5 mM inhibited methane production by 94.5-97.2% compared to control at 24 h incubation and

Table 3. Effects of 2-bromoethanesulfonic acid (BES) on pH and VFA production at 48 h incubation

Item	Treatment[1]						SEM[2]	Significance		
	Timothy (mM)			Mixed (mM)						
	0	1	5	0	1	5		Sub	BES	Sub×BES
pH	6.43	6.42	6.37	6.41	6.39	6.36	0.006	**	**	NS
Total VFA (mM)	67.62	65.32	66.56	81.49	84.03	82.97	1.606	**	NS	NS
Acetate (mol %)	62.5	55.5	53.8	53.0	49.9	48.5	0.917	**	**	**
Propionate (mol %)	19.7	22.7	22.1	22.4	22.8	23.6	0.410	**	**	**
Butyrate (mol %)	12.4	14.7	15.6	18.8	20.9	21.6	0.429	**	**	NS
A/P	3.18	2.45	2.43	2.37	2.20	2.06	0.088	**	**	**

[1] Within timothy and mixed substrate, BES was treated at the final concentration of 0, 1 and 5 mM. [2] SEM = Standard error of means.
** Significant at $p<0.01$; NS = Not significant ($p>0.05$).

maintained methane production below 0.5% of total gas at 72 h incubation. BES is known as a specific inhibitor of methanogenesis and its effects on methane production is well documented (Martin and Macy, 1985; Sauer and Teather, 1987; Choi et al., 2004). Moreover, an interaction between substrate and level of BES was detected at 48 and 72 h for total gas production, and at all incubation times for methane production (Table 2). The degree of reduction in total gas and methane production with increasing BES level was more pronounced with mixed substrate than with timothy alone.

Unlike methane, more hydrogen was accumulated by BES treatment in dose-dependent manner ($p<0.01$) (Table 2). Hydrogen production was low (below 0.1% of total gas) in the control during 72 h incubation. However, BES treatment increased the accumulation of hydrogen gas, which accounted for 3% of total gas in the treatment of BES 5 mM with mixed substrate at 24 h incubation. Low hydrogen concentration is normally observed in the rumen because most of hydrogen produced from fermentation is used for the reduction of CO_2 to CH_4 by methanogenic bacteria (Hungate et al., 1970). However, hydrogen accumulation was noticed when methanogenesis was inhibited by BES treatment (Sauer and Teather, 1987; Nollet et al., 1997). Hydrogen accumulation tended to be gradually reduced with increasing incubation time, and the concentration of hydrogen in the BES 5 mM treatment reached lower than 0.1% of total gas at 72 h incubation. Accumulated hydrogen due to inhibition of methane production may stimulate hydrogen-consuming processes and increase more reduced fermentation products (i.e., propionate) (Nollet et al., 1997). There was an interaction between substrate and level of BES for hydrogen production at 24 and 48 h incubation (Table 2). The increase of hydrogen production with increasing BES level was higher with mixed substrate than with timothy alone.

pH and VFA production

Incubating mixed substrate resulted in lower pH compared to timothy only ($p<0.01$) (Table 3). BES treatment decreased pH in dose-dependent manner ($p<0.01$). Total VFA concentration was higher with mixed substrate than with timothy only ($p<0.01$) (Table 3). However, total VFA production was not influenced by BES treatment. Mixed substrate also decreased molar proportion of acetate ($p<0.01$) but increased that of propionate and butyrate ($p<0.05$). Present results are in agreement with previous observations in that substrates containing more readily fermentable cell contents showed higher total VFA and propionate production and lower A/P ratio (Demeyer and Van Nevel, 1975; Zicarelli et al., 2008).

BES treatment decreased molar proportion of acetate ($p<0.01$) and increased that of propionate and butyrate ($p<0.01$), so that the ratio of acetate and propionate (A/P) was decreased ($p<0.01$). Many previous studies showed that depressed methanogenesis resulted in higher molar proportion of propionate and lower A/P ratio (Trei et al., 1971; Nollet et al., 1997) and that methane production had the inverse relationship with propionate production (Mitsumori and Sun, 2008). Therefore, it can be expected that any intervention causing shifts in VFA proportions in favor of propionate will result in lower methane production (Van Nevel and Demeyer, 1995) and vice versa as was seen in present study. There was an interaction between substrate and level of BES in molar proportion of acetate and propionate, and A/P ratio ($p<1.01$). The decrease of molar proportion of acetate and A/P ration or the increase of molar proportion of propionate with increasing BES level was more noticed with timothy substrate than on mixed one.

Microbial profile

The population of total bacteria was increased by more than 2 times compared to 0 h control in all treatments during the first 24 h incubation, but gradually decreased thereafter (Table 4). The decrease in total bacteria after 24 h incubation might be due to lack of energy for maintenance and growth. Total bacteria tended to be higher with mixed substrate than with timothy only until 48 h incubation ($p>0.05$), while reverse trend was observed at 72 h incubation ($p<0.01$). BES treatment did not influence total bacteria population and an interaction between substrate and BES was not detected in the population of total bacteria

Table 4. Effects of 2-bromoethanesulfonic acid (BES) on relative quantification of different microbial groups

Item	Treatment[1]						SEM[2]	Significance		
	Timothy (mM)			Mixed (mM)						
	0	1	5	0	1	5		Sub	BES	Sub×BES
Total bacteria										
24 h	2.60	2.22	2.10	3.14	2.31	2.27	0.137	NS	NS	NS
48 h	1.45	0.98	1.05	1.51	1.17	1.05	0.089	NS	NS	NS
72 h	0.68	0.60	0.70	0.39	0.39	0.45	0.037	**	NS	NS
Total methanogen										
24 h	1.38	1.37	1.04	1.47	1.49	0.98	0.074	NS	*	NS
48 h	1.57	0.92	0.42	1.92	0.92	0.46	0.142	NS	**	NS
72 h	1.11	0.60	0.27	1.17	0.52	0.31	0.098	NS	**	NS
Methanobacteriales										
24 h	1.17	1.10	0.52	1.39	1.14	0.49	0.099	NS	**	NS
48 h	0.98	0.71	0.22	1.15	0.93	0.37	0.085	*	**	NS
72 h	0.68	0.37	0.17	0.58	0.45	0.24	0.050	NS	**	NS
Methanomicrobiales										
24 h	1.19	0.18	0.17	1.57	0.20	0.13	0.152	NS	**	NS
48 h	1.90	0.13	0.09	2.25	0.21	0.14	0.418	NS	**	NS
72 h	0.91	0.18	0.06	1.41	0.03	0.02	0.368	*	**	*

[1] Within timothy and mixed substrate, BES was treated at the final concentration of 0, 1 and 5 mM. [2] SEM = Standard error of means.
*, ** Significant at $p<0.05$ and $p<0.01$, respectively; NS = Not significant ($p>0.05$).

during 72 h incubation.

The population of total methanogen was not influenced by substrate, although there was tendency of increased methanogen population with mixed substrate (Table 4). However, BES treatment reduced the population of total methanogen in dose-dependent manner after 24 h incubation ($p<0.01$) with more pronounced trend at later incubation times. BES 5 mM treatment reduced the population of total methanogen by more than 50% at 48 h incubation, and by about 70% at 72 h incubation. There are a few previous studies which determiined effects of BES on change of methanogens with different techniques. Agarwal et al. (2008) reported that BES suppressed methanogen population by 7 fold on lucerne hay and by 8.5 fold on maize hay at 24 h incubation. Dong et al. (1999) also observed that BES inhibited growth of methanogenic bacteria estimated by the MPN method with no effect on either the total or cellulolytic bacteria population. Since BES specifically inhibits methanogenesis, the population of methanogens also must be reduced by BES treatment. Similar trend was obtained in present study in that both methane production and the methanogen population was reduced by BES, however, the degree of reduction at a given concentration of BES was different between methane gas production and metananogen population.

Effect of substrate on the population of *Methanobacteriales* was detected only at 48 h incubation ($p<0.05$) (Table 4) with more decrease with timothy substrate than with mixed one at 48 h incubation. Similar to the population of total methanogen, BES treatment reduced the population of *Methanobacteriales* in dose-dependent manner at all incubation times ($p<0.01$). BES 5 mM treatment reduced the population of the order *Methanobaceriales* by 63-78% at 48 h incubation and by 76-83% at 72 h incubation.

Significant effect of substrate on the population of the order *Methanomicrobiales* was seen only at 72 h incubation ($p<0.05$) (Table 4). However, BES treatment reduced *Methanomicrobiales* population compared to control at all incubation times ($p<0.01$), but there was no difference in the population of *Methanomicrobiales* between BES 1 mM and 5 mM treatment. BES treatment reduced the population of the order *Methanomicrobiales* by 79-91% at 48 h incubation and by 82-98% at 72 h incubation.

Many investigators have reported that the most prevalent methanogenic species in the rumen is the order *Methanobacteriales* (Skillman et al., 2006; Nicholson et al., 2007; Wright et al., 2007). Others have reported that species of the order *Methanomicrobiales* is the most (Tajima et al., 2001) or second most (Wright et al., 2007) prevalent one in the rumen. Some studies also reported the existence of the orders *Methanosarcinales* and *Methanococcales* in the rumen (Lin et al., 1997), although the abundance of these methanogens was much less than that the orders *Methanobacteriales* and *Methanomicrobiales*. There was no detection of the order *Methanosarcinales* and the order *Methanococcales* at any samples in this study. Behlke (2007) also showed no detection of these two orders and concluded that either i) methanogens of these two orders were not present in the rumen or ii) their presence was less than the detection limit.

Ungerfeld et al. (2004) studies BES effects on methane production with different species of methanogens and reported that *Methanobrevibacter ruminantium* was the

most sensitive to BES, *Methanosarcina mazei* was the least sensitive, and *Methanomicrobium mobile* was intermediate. According to Ungerfeld et al. (2004), methanogens that can synthesize coenzyme M (CoM) exhibit lower rates of transport of external CoM and also its analogue BES into the cell, so that they are likely to be more resistant to BES. *Methanobrevibacter ruminantium* can not synthesize CoM intracellularly, but *Methanosarcina mazei*, *Methanomicrobium mobile* and *Methanbrevibacter smithii* can synthesize CoM (Balch and Wolfe, 1979). However, all species of methanogens reduced methane production at the level of 250 μM of BES.

Although BES treatment inhibited methane production more than 63% and 5 mM BES reduced the number of methanogens compared to control (no BES) at 24 h incubation, total methanogens was not decreased compared to 0 h. Significant difference in population of total methanogens between control and BES treatment was seen from 48 h incubation in present study.

Denman et al. (2007) also reported this discordance between methane production and methanogen population using bromochloromethane (BCM) as a methane inhibitor. They reported that methane production was rapidly reduced to 59% within two hours of BCM dosing compared to control, but methanogen population was decreased from 8 hr after dosing. According to Denman et al. (2007), the observed delay for a reduction in methanogen numbers would suggest that inhibition of methanogenesis affected the growth of organisms which was reflected in a decline in numbers several hours after initial exposure. Similar to BCM, BES used in current study also inhibited methanogenesis but did not reduce methanogens population simultaneously.

CONCLUSION

BES reduced total gas, methane and molar percentage of acetate but increased hydrogen production, molar percentage of propionate and butyrate. In addition, BES did not affect total bacteria but reduced the population of total methanogens, the order *Methanobacteriales* and the order *Methanomicrobiales* in dose-dependent manner. Present study also confirmed that reduction of methane production and methanogens by BES did not coincide. Further research is guaranteed on the detection of other methanogens with samples from animals or with more sensitive detection methods to clarify relationship between methane production and methanogens.

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