Change in Nitrogen Fractions and Ruminal Nitrogen Degradability of Orchardgrass and Alfalfa during the Ensiling Process and the Subsequent Effects on Nitrogen Utilization by Sheep

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ABSTRACT : In order to determine the extent of change in nitrogen fractions and *in vitro* ruminal degradability of forage protein during ensilage and the influence on nitrogen utilization by sheep, orchardgrass (*Dactylis glomerata L.*) and alfalfa (*Medicago sativa L.*) were ensiled in separate 120 L silos for 5, 21 and 56 days. With respect to nitrogen fractions, fraction 1 (buffer solution soluble nitrogen), fraction 2 (buffer solution insoluble nitrogen-neutral detergent insoluble nitrogen), fraction 3 (neutral detergent insoluble nitrogen-acid detergent insoluble nitrogen), and fraction 4 (acid detergent insoluble nitrogen) were determined. Fractions 1 and 2 accounted for more than 80% of total nitrogen in orchardgrass and 90% of that in alfalfa. The proportion of fraction 1 in orchardgrass increased from 33.0% at day 0 to 52.0% after day 56 of ensiling. In the case of alfalfa silage it was 41.7% and 62.9%, respectively. Seventy percent of this increase occurred within the first 5 days of ensiling. A similar change of *in vitro* ruminal degradability of total nitrogen was also observed in both forages. Nitrogen retention in sheep tended to decrease as the length of ensiling increased, with a significantly positive correlation between urinary nitrogen and fraction 1, and *in vitro* ruminal degradability of total nitrogen. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 11 : 1524-1528)***

Key Words : Orchardgrass, Alfalfa, Ensiling, Nitrogen Fractions, Ruminal Degradability, Nitrogen Utilization

INTRODUCTION

Qualitative changes in forage protein occur during the ensiling process to a variable extent. For example, more than half of the original forage protein may breakdown to non-protein nitrogen (NPN); such an extensive breakdown of proteins may result in inefficient nitrogen utilization by ruminants (McDonald et al., 1991; Chamberlain and Wilkinson, 1996). Normally, about three weeks are required before the silage enters stable stage. There are two main stages during this period: the first is plant cell respiratory-aerobic microbial active stage and the second is lactic acid bacterial active stage, which are completed within the first few days and 21 days, respectively (Barnett, 1954; Ensminger et al., 1990; McDonald et al., 1991).

The recent methods to evaluate protein quality of feedstuffs are based on the estimation of their ruminal degradability and the partition of them into several fractions that are assumed to be well correlated with ruminal degradability data (Krishanamoorthy et al., 1983; Sniffen et al., 1992; Calsamigia et al., 2000). However, the degree to which nitrogen fractions and degradability of forage protein changes as the ensiling process advances is still not clear.

In this study, orchardgrass and alfalfa were ensiled for 5, 21 and 56 days in order to determine the extent of change in nitrogen fractions and *in vitro* ruminal degradability of forage protein during each stage, i.e. the first, second and stable stages. In addition, the subsequent effects on nitrogen utilization by sheep were also investigated.

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MATERIALS AND METHODS

Forage preparation and ensilage

Orchardgrass (*Dactylis glomerata L.*) and Alfalfa (*Medicago sativa L.*) were the first cut, harvested in the early heading and flowering stage, respectively, from the experimental farm of Obihiro University of Agriculture and Veterinary Medicine (Hokkaido, Japan). The two forages were wilted for 8 h and picked up using a precision chop forage harvester (theoretical chop length 2 cm). The two forages were treated individually using the following procedures: about 1 ton of parental material was mixed and sampled. One fourth was then stored immediately at -15°C for later use as material grass in nitrogen balance trials. Chemical composition of the material grasses is shown in Table 1. The remaining three fourths was ensiled in nine 120 L capacity laboratory silos. Three silos from each forage type were opened after 5, 21, 56 days of ensiling. A representative sample from each silo was mixed and sub-sampled. The remaining content of the three silos was mixed and frozen at -15°C before being fed to the animals in the nitrogen balance trials.

Table 1. Chemical composition of material grasses

	Orchardgrass	Alfalfa
Dry matter (%)	31.4	28.9
Crude protein (% DM)	11.3	18.8
Neutral detergent fiber (% DM)	55.8	46.4
Acid detergent fiber (% DM)	32.1	31.6
Acid detergent lignin (% DM)	2.6	9.6
Water soluble carbohydrates (% DM)	9.5	6.2
Gross energy (MJ/kg DM)	18.28	16.90

Fractionation of forage nitrogen

The fractionation scheme applied was basically from the Cornell Net Carbohydrate and Protein System (Sniffen et al., 1992) where feed proteins are partitioned into five fractions (A, B_1, B_2, B_3 and C). However, in this study A and B_1 were not separated since the proportion of B_1 is a very small part of the total nitrogen of forages and it is also assumed to be totally degraded in the rumen as is the A fraction (Krishnamoorthy et al., 1982; Sniffen et al., 1992). Therefore, the following four fractions were studied: Fraction 1 (TN-BSIN); Fraction 2 (BSIN-NDIN); Fraction 3 (NDIN-ADIN); and Fraction 4 (ADIN). Buffer solution insoluble nitrogen (BSIN) was determined by the method of Krishnamoorthy et al. (1982). Neutral and acid detergent residues (Van Soest et al., 1991) were analyzed for N to determine the neutral detergent insoluble nitrogen (NDIN) and acid detergent insoluble nitrogen (ADIN), respectively.

Estimation of ruminal degradability of forage nitrogen

In vitro procedure of Coblentz et al. (1999) was applied to estimate the degradability of forage nitrogen in the rumen. The protease enzyme Actinase E from *Streptomyces griseus*, (Kaken Pharmaceutical Inc., Tokyo, Japan), which contained 10^6 activity units/g of solid, was used in this study. The fresh enzyme solution was prepared in a borate-phosphate buffer (pH 8.0). Samples containing15 mg of nitrogen were put into 80 ml capacity glass bottles with screw caps and incubated for 1 h at 39°C in a 40 ml borate-phosphate buffer. Following the 1 h buffer incubation, 10 ml of prepared protease solution, containing 33 activity units/ml, was added to each bottle yielding the final enzyme activity concentration of 6.6 units/ml in the incubation medium. The bottles were then continuously incubated for 4 h at 39°C. After incubation, samples were filtered immediately through Toyo No.5A filter paper (Toyo Roshi Kaisha, Ltd., Japan) and the N content of each residue was determined by Kjeldahl's method.

Nitrogen balance trials

Two trials were conducted separately for the two forages. For each forage trial, four treatments (i.e. material grass and the three silages obtained after 5 days, 21 days, and 56 days of ensiling) were assigned to four wethers in a 4×4 Latin square design. Animals were kept in metabolism cages with free access to water and mineral block (Nihonzenyaku Inc., Tokyo, Japan). The feeds were offered to the animal at the maintenance energy level (2% of body weight on a dry matter basis), available in two equal feedings at 08:00 and 17:00 hours daily. Each experimental period consisted of 7 days of adaptation to the respective experimental feed and 5 days of recording feed intake, fecal and urinal output. Fecal and urine samples were immediately stored in a freezer at -15°C after collection.

Table 2. Fermentation characteristics of silages*

	Silages			
	5d	21d	56d	SE
Orchardgrass				
pH	4.72[a]	4.34[b]	4.19[c]	0.00
Lactic acid (%DM)	2.5[c]	4.4[b]	5.3[a]	0.04
Acetic acid (%DM)	0.5[c]	0.7[b]	1.0[a]	0.01
Propionic acid (%DM)	-	-	-	-
Butyric acid (%DM)	0.1[c]	0.2[b]	0.4[a]	0.00
Ammonia-N (%TN)	4.8[c]	6.6[b]	8.0[a]	0.05
WSC (%DM)	4.4[a]	1.7[b]	1.2[c]	0.01
Alfalfa				
pH	5.03[a]	4.34[b]	4.19[c]	0.00
Lactic acid (%DM)	2.6[c]	5.4[b]	6.4[a]	0.02
Acetic acid (%DM)	1.2[a]	0.8[b]	0.4[c]	0.01
Propionic acid (%DM)	-	-	-	-
Butyric acid (%DM)	0.2	-	-	-
Ammonia-N (%TN)	4.4[b]	5.1[a]	5.4[a]	0.01
WSC (%DM)	1.6[a]	1.4[b]	1.3[c]	0.01

* Values are mean of three silos.
[a,b,c]Mean within rows with unlike superscripts are significantly different ($p<0.05$)

Chemical analyses

Material grasses and silages were dried for 24 h in a freeze-dryer to determine dry matter (DM). Feces was initially dried in a forced air oven at 60°C for 72 h, and the sub-samples were then dried in a drying oven at 135°C for 2 h to determine DM content. Samples of dried feed and feces were ground in a Wiley mill (1 mm screen) for subsequent analyses. Total nitrogen (TN) was determined by Kjeldahl's method using an automatic apparatus (UDK 130 D'VELP Scientifica, Italia) and crude protein (CP) was calculated as N×6.25. Neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were determined as described by Van Soest et al. (1991). Water soluble carbohydrate (WSC) was determined colorimetrically using the Anthrone method of Masaki (1971). The silage extract was prepared for measuring pH (pH meter/Horiba F-7AD), lactic acid (Barker and Summerson, 1961), volatile fatty acids (VFA; capillary column gas chromatography/ Shimadzu CG-14A), and ammonia nitrogen (Conway and O'Malley, 1942). Gross energy (GE) content in feed was

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Table 3. Nitrogen fractions (% of TN) of material grasses and silages*

	Material grass	Silages			
		5 d	21 d	56 d	SE
Orchardgrass					
Fraction 1	33.0	46.3[b]	51.1[a]	52.0[a]	0.64
Fraction 2	49.2	39.0[a]	35.5[b]	35.1[b]	0.76
Fraction 3	6.9	3.2[a]	1.3[b]	0.8[b]	0.36
Fraction 4	11.0	11.6[b]	12.3[a]	12.1[a]	0.16
Alfalfa					
Fraction 1	41.7	55.9[c]	59.2[b]	62.9[a]	0.57
Fraction 2	48.5	35.4[a]	32.2[b]	29.1[c]	0.65
Fraction 3	3.5	2.6	2.6	1.9	0.35
Fraction 4	6.3	6.2	6.0	6.1	0.18

Fraction 1=100 BISN, Fraction 2=BSIN-NDIN, Fraction 3=NDIN-ADIN, Fraction 4=ADIN.
* Values are mean of three silos except for material grass.
[a,b] : Mean within rows with unlike superscripts are significantly different ($p<0.05$).

determined by adiabatic bomb calorimeter (CA-4P, Shimadzu, Japan).

Statistical analysis

Differences between feeds were analyzed by analysis of variance (ANOVA) according to the General Linear Model (GLM) procedures of Statistical Analysis System (SAS) Institute Inc. (1997) with mean separation by Duncan's multi-range test ($p<0.05$). Pearson correlation coefficients between variables were also computed using procedures of SAS Institute Inc. (1997).

RESULTS AND DISCUSSION

The fermentation characteristics of silages are shown in Table 2. WSC plays a main role as a substrate for silage fermentation and WSC contents of both silages were significantly lower at day 5 of the fermentation process compared with fresh material (Table 1), and continuously decreased ($p<0.05$) as the length of ensiling increased. As a result, lactic acid concentration increased ($p<0.05$), thereby pH values decreased ($p<0.05$) in both forage silages. Yahaya et al. (2002) and Guan et al. (2002) also reported the similar observations on orchardgrass silage and sorghum silage, respectively. The silages obtained from day 56 of the fermentation process of both forages were judged to be as good as those specified by McDonald and Whittenbury (1973), Woolford (1984), McDonald et al. (1991), Chamberlain and Wilkinson (1996).

The nitrogen fractions of material grasses and subsequent silages are shown in Table 3. Fraction 1 consists of mostly NPN (NH_3, amines, amides, amino acids, peptides) and a small amount of true protein (globulins, some albumins) that are rapidly degraded in the rumen (Krishnamoorthy et al., 1982; Sniffen et al., 1992). Generally, the proportion of total nitrogen that is present as NPN is lower in grass than leguminous herbage (Oshima and McDonald, 1978; Sanderson and Wedin, 1989). Hergaty and Peterson (1973) reported that about 10-25% of the N in fresh herbages is present in NPN form. In this study, the proportion of fraction 1 in material grass was 33.0% and 41.7% for orchardgrass and alfalfa, respectively, being substantially higher than those values. This is mainly because proteolysis took place during the 8 h wilting period on the field before ensiling (McDonald et al., 1991; Nguyen et al., 2004). The proportion of fraction 1 was higher in silages than in material grass of either orchardgrass or alfalfa, and increased ($p<0.05$) as ensiling advanced. This increase could be the result of the degradation of true protein in fraction 2 and fraction 3 during the ensiling process (McDonald et al., 1991). After 56 days of ensiling, the proportion of fraction 1 reached 52.0% and 62.9% in orchardgrass and alfalfa silage, respectively of which, 70% of this increase occurred within the first 5 days of ensiling. Oshima and McDonald (1978) and Woolford (1984) pointed out that extensive protein breakdown occurs during the early stages of ensilage, and plant enzymes have a dominant role while microbial enzymes contribute to a lesser extent in this breakdown. Fraction 2 mainly consists of albumins and glutelins that are partially degraded in the rumen and partially degraded in the small intestine (Sniffen et al., 1992). Although the proportion of fraction 2 was largest among the four fractions in the material grasses, it became smaller than that of fraction 1 in the silages of both forages. After 56 days of ensiling, it decreased to 35.1 and 29.1% in orchardgrass and alfalfa silage, respectively, in contrast to fraction 1, which saw a 70% decrease within the first 5 days of ensiling. Fraction 3 is prolamines that are slowly degraded in the rumen, part of which is cell wall protein (Sniffen et al., 1992). In this study, the fraction 3 proportion was the smallest among the four fractions in material grass and silages of both forages, and was smaller in silages than material grass. Five days after ensiling it was 3.2% and 2.6% in silage of orchardgrass and alfalfa, respectively. Fraction 4 is bound or unavailable proteins associated with the ADF in feeds (Sniffen et al., 1992). In this study, it was 11.0% and 6.3% in material grass of orchardgrass and alfalfa, respectively. No considerable change in proportion was observed in either of the forages after ensiling.

Although all *in vitro* techniques currently available for protein quality evaluation have limitations (Stern et al., 1997), *in vitro* enzymatic methods may still be acceptable in many situations, particularly when the determination of relative differences between forages, rather than absolute accuracy, are the most important considerations (Coblentz et al., 1999). *In vitro* ruminal degradability of total N and insoluble N of material grasses and silages are shown in Table 4. Total N degradability was higher in silages than

Table 4. *In vitro* ruminal degradability (%) of total N and insoluble N of material grasses and silages*

	Material grass	Silages			SE
		5 d	21 d	56 d	
Orchardgrass					
Total N degradability	67.4	71.1[c]	72.5[b]	73.8[a]	0.35
Insoluble N degradability	51.4	46.1[a]	43.7[b]	45.3[ab]	0.68
Alfalfa					
Total N degradability	68.3	72.6[b]	73.6[b]	75.5[a]	0.52
Insoluble N degradability	45.7	38.0[a]	35.2[ab]	33.9[b]	1.03

Total N degradability=(TN-undegraded N)/TN×100

Insoluble N degradability=(BSIN-undegraded N)/BSIN×100.

* Values are mean of three silos except for material grass.

[a-c] Means within rows with unlike superscripts are significantly different ($p<0.05$).

that of material grass, and increased ($p<0.05$) as ensiling advanced in both forages. These increases could be the result of the increase of fraction 1 during ensiling which is subsequently totally degraded in the rumen (Krishnamoorthy et al., 1982; Sniffen et al., 1992; Chalupa and Sniffen, 1996). In contrast, the insoluble N degradability was lower in silages than that of material grass and tended to decrease as ensiling progressed in both forages. This change is probably due to the decrease of easily degradable proteins in fraction 2 and 3 caused by the proteolytic activities that occur during the ensiling process (Oshima and McDonald, 1978).

The nitrogen balance in sheep fed on material grasses and subsequent silages, and the correlation coefficient between the nitrogen balance parameters and nitrogen fractions and *in vitro* ruminal nitrogen degradability are shown in Table 5 and 6, respectively. In sheep fed on orchardgrass silages, N retention tended to decrease as ensiling advanced, and was significantly lower ($p<0.05$) for 56 day old silage than for the material grass. A similar trend was also observed in sheep fed on alfalfa silages, although there was no significant difference between that for 56 day old silage and for the material grass. With respect to the percentage of N excreted in feces and urine, which directly influences N retention, for fecal N there was no significant difference among treatments in either of the forages. However, urinary N for silage increased as ensiling advanced, with a significant difference ($p<0.05$) between for 56 day old silage and for the material grass in orchardgrass. A similar trend was also observed in sheep fed on alfalfa silages. This increase of urinary N is thought to be due to the increase of fraction 1 and total N degradability that resulted in the rapid release of a larger amount of ammonia-N in the rumen. The ammonia-N that exceeds the rumen microbes' capacity to assimilate will be absorbed into the blood, carried to the liver and converted into urea. Although some of this urea is recycled to the rumen, generally most of it is excreted in the urine and consequently, is a waste of nitrogen (MacDonald et al.,

Table 5. Nitrogen balance in sheep fed on material grasses and silages*

	Material grass	Silages			SE
		5 d	21 d	56 d	
Orchardgrass					
N intake (g/day)	20.1[a]	19.7[ab]	19.3[ab]	19.0[b]	0.21
Fecal N (% of N intake)	38.2	37.3	36.2	37.6	1.32
Urinary N (% of N intake)	45.8[b]	56.0[ab]	57.9[ab]	65.3[a]	3.62
Retained N (% of N intake)	16.0[a]	6.7[ab]	5.9[ab]	-2.9[b]	3.34
Alfalfa					
N intake (g/day)	36.9[a]	35.2[b]	34.1[d]	34.9[c]	0.07
Fecal N (% of N intake)	25.0[b]	26.2[a]	26.6[a]	26.0[a]	0.22
Urinary N (% of N intake)	46.9	53.8	54.4	56.0	2.91
Retained N (% of N intake)	28.1	20.0	19.0	18.0	2.93

* Values are mean of four sheep. [a-d] Means within rows with unlike superscripts are significantly different ($p<0.05$).

Table 6. The correlation coefficient between nitrogen fractions, *in vitro* ruminal nitrogen degradability and nitrogen balance parameters

	Nitrogen fractions				Degradability	
	1	2	3	4	Total N	Insoluble N
Orchardgrass						
Fecal N	-0.220	0.226	0.212	-0.251	-0.163	0.265
Urinary N	0.663**	-0.655**	-0.672**	0.599*	0.700**	-0.553*
Retained N	-0.604*	0.595*	0.615*	-0.534*	-0.654**	0.486
Alfalfa						
Fecal N	0.311	-0.314	-0.243	-0.337	0.312	-0.287
Urinary N	0.633**	-0.633**	-0.603*	-0.575*	0.629**	-0.628**
Retained N	-0.647**	0.648**	0.602*	0.602*	-0.644**	0.636**

* $p<0.05$, ** $p<0.01$. Nitrogen balance parameters are calculated as percent of nitrogen intake

1991). Results of this study also strongly confirm that there was a positive correlation between urinary N and fraction 1, and *in vitro* ruminal degradability of total N. In contrast, there was a negative correlation between N retention and those parameters for both forage silages. It is well known that soluble nitrogen in silage can only supply ammonia-N for rumen microbes for a short time after feeding because it is rapidly degraded in the rumen. Thereafter, insoluble nitrogen, which is slowly degraded in the rumen, is the main source of ammonia-N supply for growth of rumen microbes. The increase of ammonia-N supply during late phase might increase the efficiency of nitrogen utilization by rumen microbes, and subsequently by the ruminant. In agreement with this finding, there was a positive correlation between nitrogen utilization and fractions 2 and 3, and *in vitro* ruminal degradability of insoluble N for both forage silages in this study.

CONCLUSION

This study found that the proportion of fraction 1 increased while that of fraction 2 decreased as ensiling advanced in both forages. Of the overall increase of fraction 1 during 56 days of ensiling, about 70% occurred during the first 5 days, which could mainly be the result of the proteolytic activity of plant enzymes. This increased fraction 1 was thought to be the cause of the increase of total N degradability in the rumen, leading to the decreased tendency of nitrogen retention by sheep fed either solely orchardgrass silage or alfalfa silage.

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