The estimation of ruminal protein degradation parameters of various feeds using *in vitro* modified gas production technique

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Summary

This study was conducted to determine *in vitro* crude protein degradation (IVDP) parameters and effective crude protein degradability (EPD) of various feeds using the modified *in vitro* gas production (GP) technique. Feed samples were alfalfa hay, soybean meal, soybean, rapeseed meal, sunflower meal and fish meal. Rumen fluid was collected before the morning feeding from four rumen fistulated lambs (49.4 ± 3.5 kg, body weight). Approximately 90 ml of buffered rumen fluid (BRF), 400 mg of feed samples and carbohydrates (maltose, xylose and starch) at four concentrations (100, 200, 300, and 400 mg) were added to screw-cap bottles. Gas production (ml) and ammonia nitrogen concentration (mg) in each bottle were measured at 4, 8, 12, 16, 24, and 30 h post incubation and IVDP was calculated via estimated intercept of linear regression between GP (as main variable, X) and ammonia nitrogen (as dependent variable, Y) using the linear regression procedure. Feed, time and feed × time interaction had significant effect on IVDP ($P<0.001$). Estimated EPD values at the outflow rate of 0.06/h for alfalfa hay, soybean meal, soybean, rapeseed meal, sunflower meal and fish meal were 0.56, 0.77, 0.59, 0.45, 0.50 and 0.38, respectively.

Key words: Ammonia nitrogen, Degradability, Gas production, Protein

Introduction

Estimation of protein degradability in the rumen is one of the essential steps in feed evaluation systems to predict the nutritional requirements of ruminants (Hedqvist and Udén, 2006). Also, accurate estimation of ruminal protein degradability of feeds for optimization of the rumen system is necessary (Roe *et al*., 1991). Now, in situ techniques are widely used to determine ruminal protein degradability. However, this method ignores fine feed particles that are lost through bags pores (Dewhursta *et al*., 1995) and is inapplicable for soluble feed proteins (Hedqvist and Udén, 2006). Widespread use of *in vitro* gas production technique to evaluate ruminant feeds has largely been due to high analytical capacity and low cost (Bueno *et al*., 2005). Karlsson *et al*. (2009) based on Raab *et al*. (1983) developed a new gas production technique for estimation of ruminal protein degradability that rectifies problems of the common gas production technique for estimation of *in vitro* protein degradability. In this technique, *in vitro* ruminal protein degradability (IVDP) is estimated via linear regression between gas production (as main variable) and ammonia nitrogen emission (as dependent variable). It is assumed that the intercept of the regression shows the time that GP was zero and no microbial protein synthesis has occurred, thus it represents absolute ammonia nitrogen produced due to feed degradation. In fact, the modified GP technique uses a mathematical approach to eliminate the confounding effects of de novo protein synthesis during fermentation. In comparison to the original method (Raab *et al*., 1983), the modified gas production technique uses the fermentation units (rather than glass syringes) that facilitate to measure gas production and sampling of liquid phase from a single incubated feed sample at several times post incubation. This improvement causes the number of incubations required to be reduced and eliminates the use of different rumen inocula. Other modifications include drawing rumen fluid before morning feeding and conditioning it by pre-incubation procedures to reduce background ammonia levels. In addition, estimation of kinetic parameters of protein degradability and effective protein degradability (EPD) are possible by increasing sampling time points (Karlsson *et al*., 2009). However, the method is still sensitive to variation in rumen fluid and ammonia background as well as complications due to microbial turn overs in blanks and samples (Karlsson *et al*., 2009). Our objective was to estimate IVDP parameters and EPD of various feeds including alfalfa hay, soybean meal, soybean, rapeseed meal, sunflower meal and fish meal using modified *in vitro* GP technique.

Materials and Methods

Feed samples and animals

The feed samples used in our study were alfalfa hay, soybean meal, soybean, rapeseed meal, sunflower meal and fish meal. Rumen fluid was collected manually before the morning feeding from four fistulated lambs (49.4 ± 3.5 kg, body weight) fed a diet containing 300 g wheat straw, 200 g alfalfa hay and 250 g concentrate (40% barley, 30% corn and 30% wheat bran). Animals had free access to fresh water during the experiment.

Gas production technique

This study was conducted according to GP technique described by Karlsson *et al*. (2009). The Rumen fluid was filtered through four layers of cheesecloth and incubated with easily fermentable carbohydrates (3.5 g maltose, 1.8 g starch, 1.8 g xylose and 1.8 g pectin per L of strained rumen fluid) for 3 h at 39°C and was continuously flushed with CO_2. After pre-incubation, the rumen fluid was mixed with buffered mineral solution (Menke and Steingass, 1988) as 1:2 of rumen fluid to mineral buffer ratio (V/V). Then, 90 ml of buffered rumen fluid (BRF), 400 mg of feed samples, and four levels of carbohydrates including 100, 200, 300 and 400 mg comprised of maltose, xylose and starch with 2:1:1 ratio, respectively, were added to screw-cap bottles (Volume 250 ml) that facilitated to manually measure gas production and sampling from liquid phase (Fig. 1). Incubation of the feed samples with graduated amounts of carbohydrates will lead to different amounts of gas production and different levels of ammonia-N. This provides the possibility of establishing a linear regression between gas production (x-ml) and ammonia-N (y-mg). The incubations were performed in a water bath at 39°C for 30 h. Blanks contained only BRF and were treated in the same manner as the samples. Feed samples and blanks incubated simultaneously in three repeats and three runs. At 4, 8, 12, 16, 24 and 30 h post-incubation, gas production volume was recorded and liquid samples for ammonia nitrogen measurements were drawn from each bottle. The gas pressure built inside the bottles was measured manually by an electronic pressure transducer (Pressure Sensor, PSA-01, Autonics) and through a special duct for the measurement of the gas pressure (Fig. 1). The gas pressure was converted into volume using an experimentally calibrated curve (Jahani-Azizabadi *et al*., 2011). The liquid samples were drawn manually by a 5 ml plastic syringe and without opening the door of the screw-cap incubation bottles, through a separate special duct and a fixed fine plastic tube. Liquid samples were immediately cooled down using water-ice mixture, then centrifuged (3000 × g, for 10 min at 5°C) and supernatant was collected. All samples were stored in freezer at -20°C until chemical analysis.

Chemical analysis

Feed samples were ground (1 mm particle diameter) and chemical composition of test feeds was determined as follows. Dry matter (DM) was determined by drying at 65°C for 48 h. Total nitrogen was determined using Kjeldahl method according to AOAC (1990) by a Kjeltec Analyzer Unit (Kjeltec 2300 Autoanalyzer, Foss Tecator AB, Hoganas, Sweden). Crude fat was extracted with hexane solvent by Soxhlet extractor (Soxtec system HT, Tacator, Sweden) according to AOAC (1990). Neutral detergent fiber (NDF) was determined according to Van Soest *et al*. (1991). Ammonia nitrogen concentration was measured in supernatant using phenol-hypochlorite reaction (Weatherburn, 1967).

Fig. 1: A fermentation unit equipped with silicon junctions and three-way stopcock to facilitate sampling of liquid phase and measuring the amount of gas production at different times post-incubation

Biometric analysis

The IVDP values were calculated via estimated intercept of linear regression between GP (as main variable, x) and ammonia nitrogen concentration (as dependent variable, y) and assumed that regression equations intercept (b_0) is an indicator of total ammonia nitrogen that was released at zero gas production as discussed by Raab *et al*. (1983). Then, b_0 and related ammonia nitrogen concentration from blanks were replaced in the following equation (Raab *et al*., 1983) to calculate the amount of IVDP for each time point:

$$\text{IVDP} = \frac{b_0 - (\text{NH}_3 - \text{N from blanks})}{\text{Total nitrogen of feed sample}}$$

The kinetic parameters of rumen crude protein degradation were estimated by fitting the IVDP values to the following equation (Ørskov and McDonald, 1979) using SAS software (2001):

$$Y = a + b \times (1 - e^{-ct})$$

Where,
Y: The degraded part of crude protein at time t
a: The soluble part
b: Potentially degradable part
c: Rate of degradable b fraction of crude protein
EPD value was calculated via the equation of Ørskov and McDonald (1979):

$$\text{EPD} = a + \frac{b \times c}{k + c}$$

Where,
a, b, c: Defined above
k: Out flow rate that assumed to be 0.06/h
The effects of the feed and incubation time on the IVDP values were statistically determined using the MIXED procedure in SAS (2001) using the model:

$$Y_{ijkl} = \mu + \alpha_1 + \beta_1 + (\alpha\beta)_{ij} + c_k + e_{ijkl}$$

Where,
μ: The overall mean
α_i: The main effect of feed (i = 1-6)
β_j: The main effect of time (j = 1-6)
$(\alpha\beta)_{ij}$: The interaction between feed and time
c_k: The random effect of run (k = 1-3)
e_{ijkl}: The residual error
The effect of feed on CP kinetic parameters (a, b and c fractions, and EPD values) was evaluated by analysis of variance using the model:

$$Y_{ij} = \mu + \alpha_i + b_j + e_{ij}$$

Where,
μ: The overall mean
α_i: The main effect of feed (i = 1-6)
b_j: The random effect of run (j = 1-3)
e_{ij}: The residual error
In all of the analyses differences were considered significant if $P<0.05$.

Results

A brief description of chemical composition of feedstuffs used in the present experiment is given in Table 1. Fish meal and soybean meal had the highest CP and alfalfa hay had the lowest CP concentration. Among the plant protein sources, alfalfa hay had the highest and soybean meal had the lowest NDF content.

The pre-incubation treatment caused ammonia nitrogen levels in rumen fluid inoculants to reduce considerably ($P<0.001$). Concentration of ammonia nitrogen in the rumen fluid before pre-incubation was 10.33 and after treatment fell to 2.41 (SEM=0.60). Concentration of ammonia nitrogen in the rumen fluid after pre-incubation declined to an average of 23% of the initial value.

Table 1: Chemical composition (g/kg DM) of feedstuffs used in the present experiment

Feedstuffs	DM	CP	EE	NDF
Fish meal	920	524	151	-
Soybean meal	922	425	34	159
soybean	928	402	205	228
Rapeseed meal	935	384	11	276
Sunflower meal	950	305	23	381
Alfalfa hay	895	163	16	531

Estimates of IVDP were significantly affected by time, feed and time × feed interaction ($P<0.001$, Table 2).

Soybean meal and whole soybeans had the highest protein degradability while sunflower and rapeseed meals had the lowest IVDP values (Table 2). The IVDP values of soybean meal and soybean at 30 h post incubation were greater than one which indicates an over estimation of the IVDP. In general, *in vitro* ruminal protein degradation increased during the time post-incubation, but in some cases the IVDP values were smaller than values obtained at previous time points. For example, IVDP values of fish meal and sunflower meal at 4 h post-incubation were 0.35 and 0.25, respectively, while their IVDP values at 8 h post-incubation were 0.16 and 0.20, respectively.

The kinetic parameters and EPD were affected ($P<0.001$) by the feed sources evaluated (Table 3). Soybean meal and soybean had the highest amount of immediately degradable CP, while alfalfa hay and soybean meal had the highest amount of potentially degradable CP. Fish meal and alfalfa hay had the lowest amount of immediately degradable CP. Fish meal had the lowest amount of potentially degradable CP and EPD.

Table 2: *In vitro* degradable crude protein estimates (IVDP) of protein rich feeds at various times post-incubation by the modified gas production technique

Time/h	*In vitro* crude protein degradable (IVDP)						SEM	P-value		
	Protein feedstuffs									
	Fish meal	Soybean meal	Soybean	Rapeseed meal	Sunflower meal	Alfalfa hay		Feed	Time	Feed × time
4	0.35	0.09	0.20	0.34	0.25	0.04	0.03	0.001	0.001	0.001
8	0.16	0.46	0.42	0.36	0.20	0.10				
12	0.19	0.89	0.81	0.56	0.33	0.18				
16	0.56	0.77	0.70	0.44	0.41	0.35				
24	0.58	0.96	0.83	0.54	0.50	0.36				
30	0.66	1.10	1.19	0.57	0.39	0.69				

SEM: Standard error of the mean

Table 3: Estimates of kinetic parameters and effective crude protein degradability (EPD)

Parameter estimates	Kinetic parameters of CP						SEM	P-value[1]			
	Protein feedstuffs										
	Fish meal	Soybean meal	Soybean	Rapeseed meal	Sunflower meal	Alfalfa hay		a	b	c	EPD
a	0.07	0.17	0.15	0.1	0.09	0.06		0.001	0.001	0.05	0.001
b	0.63	0.91	0.77	0.66	0.74	1.09					
c	0.06	0.12	0.08	0.07	0.07	0.05					
EPD	0.38	0.77	0.59	0.45	0.50	0.56	0.04				

a: Immediately degradable fraction of protein, b: Potentially degradable fraction of protein, and c: Disappearance rate of b fraction.
SEM: Standard error of the mean. [1]: Effect of feed

Table 4: Dry matter disappearance after 30 h post-incubation

Feed	Fish meal	Soybean meal	Soybean	Rapeseed meal	Sunflower meal	Alfalfa hay	SEM	P-value
Dry matter disappearance on 30 h post incubation	0.41	-	0.96	0.79	0.67	0.70	0.01	0.001

SEM: Standard error of the mean

Dry matter disappearance was affected significantly ($P<0.01$) by the feed sources used in the present experiment (Table 4). The soybean had the highest dry matter disapearance, while fish meal had the lowest value.

Discussion

Present results showed a significant decrease in ammonia nitrogen after pre-incubation due to the use of ammonia and carbohydrates for microbial protein synthesis. This suggests that, favorable microbial activity existed in the rumen fluid immediately before the start of experiment. Broderick *et al*. (2004) and Karlsson *et al*. (2009) previously claimed that the *in vitro* pre-incubation step caused the microbial activity in the rumen fluid to increase.

In the present study, various protein feeds were evaluated to determine their IVDP values and to estimate kinetic parameters and EPD values. These feeds vary mainly in CP and NDF contents. The estimates of IVDP were in an acceptable range, but there were some inconsistencies among them. Although the blanks were used to correct the IVDP estimates, the estimated IVDP values of soybean and soybean meal at 30 h post incubation were greater than one. Also, the IVDP values of some feeds were smaller than the values of the previous time point. In the modified GP technique, protein degradability estimates were affected by the ammonia nitrogen concentration and gas production rate. Ammonia nitrogen is the main nitrogen derivate of protein degradation in rumen fluid (Raab *et al*., 1983) and its concentration was affected by fermentable energy availability, as was shown above in the pre-incubation step. In the modified GP technique the blanks were incubated with no added fermentable carbohydrate, while the feed samples were incubated with increasing levels of carbohydrates. It is expected that ammonia nitrogen concentration in the blanks increase much earlier than samples due to reduction or depletion of fermentable energy in the medium and initiation of microbial lysis (Cone *et al*., 1997). Therefore, in the earlier times post incubation, ammonia concentration in blanks is much higher than what is expected and the IVDP value estimates are low, while at the end times of incubation the IVDP values tend to be overestimated. Also, among rumen microorganisms, protozoa have higher activity in ammonia production than bacteria (Hino and Russell, 1985). Wallace and McPherson (1987) in an *in vitro* study on the breakdown of bacterial protein concluded that the predation of bacteria by small protozoa is the most important cause of bacterial protein turnover in the rumen. Lorenz *et al*. (2011) concluded that recycling of bacteria by protozoa is a severe shortcoming of the *in vitro* GP method for estimation of IVDP. They reported that reproducibility was improved for both GP and NH3 when defaunated rumen fluid was used.

In addition to ammonia, the gas production rate might also influence IVDP estimates. Higher rates of gas production at the early times post-incubation reduced regression coefficient and caused lower estimates of IVDP, while the reduction in gas production at the final time of incubation and increasing in ammonia production caused high IVDP estimates. As a basic assumption in modified GP technique, relationship of starch fermentation with gas production and ammonia production is linear and when gas production rate is greater than 90 ml/24 h the relationship between gas production and starch fermentation is not linear (Raab *et al*., 1983). According to Getachewea *et al*. (1998), relationship between gas production and starch fermentation until the amount of 400 mg starch is linear and thereafter is non linear. In our study, in the case of soybean meal at levels of 300 and 400 mg carbohydrate gas produced were 88.64 and 117.33 ml at the first 12 h post-incubation, respectively. This is higher than 90 ml/24 h which might affect IVDP estimates.

The modified GP technique used a blend of easily fermentable carbohydrates (maltose, xylose and starch) as fermentable energy source. These carbohydrates have different fermentation rates. Getachew *et al*. (1998) tested glucose, cellobiose, starch and cellulose to evaluate protein degradability of roughages and selected cellulose as a fermentable carbohydrate because of its slow and uniform fermentability. In our results the more reasonable increasing in IVDP estimates of alfalfa hay and other feeds that contain higher levels of NDF can be related to its structural carbohydrates such as cellulose.

Modified GP technique used in this study estimated IVDP values of alfalfa hay at 12 h and 16 h pos-incubation as 0.18 and 0.35, respectively. Danesh Mesgaran and Stern (2005) and Taghizadeh *et al*. (2005), reported CP disappearance of alfalfa hay after 12 h rumen in situ incubation of 0.55 and 0.51. This estimate of modified GP technique is considerably lower than similar in situ data. According to Dewhurst *et al*. (1995) this discrepancy may refer to some differences between in situ and *in vitro* method including relatively slower fermentation of fiber by *in vitro* systems, loss of fermentable materials through pores of in situ bags before fermentation and loss of unfermentable materials through pores. The starch and easily fermentable carbohydrates added to alfalfa hay can affect fiber degradability by increasing lag time (Mertens and Loften, 1980; Grant, 1994) and reducing exposure of fiber-linked proteins to degradation. Stern *et al*. (1985) reported nitrogen disappearance at 24 h for soybean meal and soybean by in situ technique of 0.965 and 0.993,

respectively. The differences in nitrogen disappearance might be due to heat treatment of soybean meal and resistance to microbial degradation. While IVDP values by modified gas production technique at 24 h for soybean meal and soybean were 0.96 and 0.83, respectively. None of the three *in vitro* methods evaluated by Roe *et al*. (1991) had a consistent relationship with in situ technique for protein degradation curves. Two of three *in vitro* methods estimated ruminal protein degradation of the soybean meal greater than raw soybean after 24 h of incubation. The ruminal degradability of soybean meal and soybean are very close together (Fathi Nasri *et al*., 2006), therefore, these two protein feeds are suitable for evaluating the GP technique accuracy.

In the present study, the EPD estimates of rapeseed meal and soybean meal were 0.45 and 0.77, respectively. Karlsson *et al*. (2009) reported EPD estimates of rapeseed meal and soybean meal of 0.36 and 0.67, respectively. These dissimilar results are due to different feed samples and rumen fluids. Our estimates of immediately degradable fraction of protein (a) in most cases are smaller than in situ estimates, while our estimates of potentially degradable fraction of protein (b) are considerably greater than similar in situ estimates (vide: Ganesh and Griever, 1990; Khan *et al*., 1998; Woods *et al*., 2003; Kamalak *et al*., 2005; Vakili *et al*., 2007). The main reason for this difference is probably the loss of particles through pores of nylon bags. One of the in situ method assumptions is that the solubility is equal to degradability. Olaisen *et al*. (2003) reported large differences between in situ measurements of water solubility and wash fraction for many of the feeds. These large differences also resulted in considerable differences between corrected and uncorrected estimates of EPD for particle loss of in situ wash fraction. The microbial contamination of residues also may associate with the differences between *in vitro* and in situ estimates of b fraction of protein (Beckers *et al*., 1995). Rotger *et al*. (2006) reported in situ b fraction of nitrogen in soybean meal and sunflower meal without correction (0.82 and 0.64, respectively) and corrected (0.90 and 0.72, respectively) for small particle loss. It is clear that the corrected estimates of in situ b fraction of nitrogen for loss of particles through bag pores are closer to modified GP estimates of b fraction of protein. Nonetheless, the kinetic parameters estimated via the modified GP technique depends on theoretical IVDP values and further investigations are needed to compare the modified GP technique and other methods.

Amount of dry matter disappeared at the end of the incubation was significantly influenced by the feed sources ($P<0.01$). *In vitro* dry matter disappearance of soybean was 0.96 at 30 h of incubation which was in agreement with Fathi Nasri *et al*. (2006) and Ganesh and Griever (1990) who reported in situ dry matter disappearance of soybean meal and raw soybean were 0.7 to 1 and 0.9 to 1, respectively. Dry matter disappearance of fish meal was 0.41 and was in agreement with Woods *et al*. (2002), who reported in situ dry matter disappearance of fish meal at 24 h and 36 h post incubation of 0.374 and 0.602, respectively.

In conclusion, the modified GP technique provides rapid estimates of IVDP with the possibility to calculate the kinetic parameters of CP degradability. Moreover, this method is simple, rapid and inexpensive and does not require to experiment on animals. Also, in this technique, errors related to the digestion of soluble proteins and undigested particles that leave the in situ bags are removed. In general, feedstuffs used in the present study had the different IVDP and dry matter disappearance values. Fish meal had the lowest EPD and dry matter disappearance, while soybean meal and soybean had the highest EPD and dry matter disappearance. Several factors including rumen fluid, gas production rate, microbial turnover and compounds of feed samples can affected the results. Therefore to overcome inaccuracies resulted from the above factors, further studies are necessary. Also, the investigations about reducing the number of incubations required to evaluate each feed sample could be very helpful.

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