Metabolism of soluble rapeseed meal (*Brassica rapa* L.) protein during incubations with buffered bovine rumen contents in vitro

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ABSTRACT

Accurate quantitative information on the fate of dietary protein in the rumen is central to modern metabolizable protein systems developed to improve the efficiency of nitrogen utilization in ruminants. An in vitro method was developed to estimate the rate of soluble rapeseed meal (*Brassica rapa* L.) protein (SRMP) degradation. Unlabeled and ¹⁵N-labeled solvent-extracted rapeseed meal were incubated alone or as an equal mixture (125 mg of N/L) with buffered rumen contents and a mixture of carbohydrates formulated to provide a constant source of fermentable energy during the course of all incubations. Incubations were made over 0.33, 0.67, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, and 10.0 h. Enrichment of ¹⁴N and ¹⁵N isotopes in total N of ammonia (AN), soluble nonammonia (SNAN), and insoluble (ISN) fractions liberated during incubations with test proteins was determined. A model with 4 pools that accounted for both intracellular and extracellular N transformations was used to estimate the rate of SRMP degradation. Parameter values used in the model were adjusted based on the size of A¹⁴N, A¹⁵N, SNA¹⁴N, SNA¹⁵N, IS¹⁴N, and IS¹⁵N pools, measured at different time points during incubations with buffered rumen fluid. The mean rate of N degradation for SRMP was estimated at 0.126 (SD 0.0499) h⁻¹. No substantive difference in the rate of protein degradation or microbial protein synthesis was observed during incubations of labeled and unlabeled substrates with rumen fluid. In conclusion, combined use of data from incubations of unlabeled and ¹⁵N-labeled rapeseed protein with buffered rumen inoculum provided sufficient information to allow for estimation of parameter values in a complex dynamic model of soluble protein degradation. Results indicate the potential of the technique to evaluate the degradability of SNAN of other dietary protein sources and implicate ruminal escape of soluble

rapeseed protein as an important source of amino acids in ruminants.

Key words: rapeseed, nitrogen, ¹⁵N, soluble protein

INTRODUCTION

Considerable emphasis has been placed on improving the accuracy of protein feeding of the dairy cow because protein is typically the most expensive feed ingredient, and in excessive amounts increases the loss of N into the environment (Broderick and Clayton, 1997; Huhtanen and Hristov, 2009) and may impair reproductive performance (Butler, 1998; Shingfield et al., 1999). Accurate information on the proportion of dietary protein that is metabolized in the rumen and the quantity of microbial and undegraded dietary protein available for absorption is central to optimizing the amount and type of protein required in ruminant diets.

The in situ method (Ørskov and McDonald, 1979) has been widely used to assess both the rate and extent of protein degradability in the rumen, but this technique has an inherent limitation that the degradation of soluble N fractions cannot be measured. In practice, the rate of soluble N degradation is assumed to be infinite, but evidence from several investigations indicate that this assumption may not hold true (Choi et al., 2002; Volden et al., 2002; Reynal et al., 2007). Alternative in vitro methods for measuring ruminal protein degradation have been developed. These include measuring protein N solubility (Crooker et al., 1978) or ammonia release (Broderick, 1978), estimating microbial N synthesis from gas production (Raab et al., 1983; Karlsson et al., 2009), incubations of test proteins with inhibitors of ammonia and amino acid anabolism (Broderick, 1987), and the use of ¹⁵N-labeled ammonium to quantify microbial N synthesis (Hristov and Broderick, 1994). However, none of these techniques has been widely adopted for routine analysis.

The objective of the current study was to assess the rate of soluble rapeseed meal protein (SRMP) degradation of 2 intrinsically identical sources of SRMP, one labeled with ¹⁵N (L-SRMP) and the other unlabeled (U-SRMP), during incubations with rumen microbes.

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Both feed sources were investigated based on the assumption that the combined use of labeled protein in the presence of unlabeled ammonia and unlabeled protein in the presence of labeled ammonia during incubations with rumen fluid would yield more information for model development and parameter estimation relative to studies of labeled or unlabeled protein degradation alone. This approach extends earlier studies that developed the concept of using the ^{15}N isotope to estimate microbial uptake of ammonia liberated during protein degradation (Hristov and Broderick, 1994) and recent reports on the rate of ruminal degradation of rapeseed meal protein based on incubations with ^{15}N -labeled ammonia (Ahvenjärvi et al., 2009).

MATERIALS AND METHODS

Labeled and Unlabeled Rapeseed Meal

Labeled and unlabeled rapeseed meals were prepared from whole rapeseeds (*Brassica rapa* L. ssp. *oleifera* DC, variety Valo; Boreal Plant Breeding Ltd., Jokioinen, Finland) grown in 6 (10-m^2) experimental plots in Jokioinen, Finland (6049'0.120"N, 2328'0.120"E). Rapeseeds were sown on May 12, 2004, with 3 plots assigned for the production of unlabeled rapeseeds and 3 used for the production of ^{15}N -labeled rapeseeds. At sowing, each plot was fertilized with 6.6 kg of N, 26.7 kg of P, and 30.8 kg of K/ha, respectively. Twelve days after sowing, each plot was fertilized with 1,132 g of ammonium sulfate or ammonium sulfate enriched with 10% of N^{15} . Rapeseeds were collected from all experimental plots on September 15, 2004, using a dedicated mechanical harvester (Nursery-Master Elite; Wintersteiger AG, Reid, Austria).

Whole rapeseeds were ground with a mortar and pestle and then suspended in diethyl ether (1:3 wt/vol) in a glass bottle to extract and remove the seed oil. After the particles had been allowed to sediment for 6 h, the upper organic phase was removed by aspiration. Thereafter, fresh solvent was added and the extraction of lipid was repeated ($n = 9$). Lipid extracts removed by aspiration were mixed with water (10:1 vol/vol) and allowed to stand for 2 h to recover small particles. After removal of the upper organic phase, small particles suspended in water were recovered and combined with solvent-extracted rapeseed meal (RM) that had been stored overnight at room temperature to evaporate excess diethyl ether. Following drying RM at 60°C for 24 h, the RM was homogenized manually using a mortar and pestle.

For the preparation of SRMP, 12 g of RM was mixed with 240 mL of mineral buffer (Goering and Van Soest, 1970) in centrifugation tubes. The mixture was stirred

Table 1. Chemical composition and protein solubility of unlabeled and ^{15}N labeled rapeseed meal

Item	Rapeseed meal	
	Unlabeled	^{15}N Labeled
DM, g/kg	920	923
OM, g/kg	917	916
N, g/kg	60.7	63.1
Crude fat, g/kg	50	45
NDF, g/kg	266	271
CNCPS, ¹ g/kg of N		
A	218	250
B ₁	205	208
B ₂	483	440
B ₃	29	40
C	64	62

¹Characterization of protein based on the Cornell Net Carbohydrate and Protein System (CNCPS; Sniffen et al., 1992): A = NPN; B₁ = buffer-soluble true protein; B₂ = protein insoluble in buffer but soluble in neutral detergent; B₃ = protein insoluble in neutral detergent but soluble in acid detergent; and C = protein insoluble in acid detergent.

continuously for 1 h at 39°C and then centrifuged at $10,000 \times g$ for 15 min at 4°C . Buffer-soluble RM protein was removed by aspiration and passed through filter paper (Whatman filter paper no. 1). The chemical composition and protein solubility of unlabeled and ^{15}N -labeled rapeseed meal is given in Table 1.

Preparation of Rumen Inoculum

Equal volumes (750 mL) of rumen contents were collected 2 h after morning feeding from 2 Finnish Ayrshire cows (130 and 188 mean DIM for run 1 and runs 2 and 3, respectively) fitted with rumen fistulae. One cow received grass silage and the other was fed a mixture (2:1 wt/wt on DM basis) of grass silage and red clover silage. Cows were offered diets (60:40 forage:concentrate on a DM basis) to ensure 5 to 10% refusals and contained the same concentrate supplement comprising (% as fed) barley (45.0), molassed sugar beet pulp (22.5), RM (28.8) from a commercial source (Rehurasio Ltd., Raisio, Finland), and a mineral and vitamin premix (3.75; Onni-Kivennäinen; Melica Ltd., Vaasa, Finland).

Rumen contents from both cows (1.5 L) were processed under a constant stream of CO_2 (Hristov and Broderick, 1994), and then strained through 2 layers of cheesecloth to retain small particles. To enrich the rumen inoculum with microbes adhered to feed particles, 200 g of the solids retained on the cheesecloth were resuspended in 0.5 L of buffer (Goering and Van Soest, 1970) maintained at 39°C . Solids and buffer were mixed, and then strained through 2 layers of cheesecloth. Buffer containing detached bacteria was mixed with rumen fluid and warm buffer. The total volume of buffer used was equal to the volume of rumen fluid. Thereafter,

rumen inoculum was filtered through 8 layers of cheese-cloth and then maintained at 39°C under a constant stream of CO₂ for 30 min. During this time, small feed particles were allowed to rise to the top before being removed by aspiration (approximately 15% of total inoculum volume). Rumen inoculum was thoroughly mixed before incubations with L-SRMP and U-SRMP.

In Vitro Incubations

Incubations were carried out at 39°C in a temperature-controlled room using thirty 120-mL spinner flasks. Each series of incubations was done in triplicate. Flasks were assigned to 1 of 4 groups ($n = 3, 3, 12$, and 12 , for groups 1, 2, 3, and 4, respectively). Incubation flasks assigned to the first and second groups were used to determine the rate of gas production (blanks). Flasks assigned to groups 3 and 4 were used to assess the rate and extent of SRMP degradation. Group 1 comprised flasks containing buffered rumen inoculum, whereas group 2 comprised flasks containing buffered rumen inoculum, a mixture of carbohydrates, and an equal mixture (wt/wt) of L-SRMP and U-SRMP. A mixture of carbohydrates (mg) comprising citrus fruit pectin (60; P-9135; Sigma, Helsinki, Finland), potato starch (80), and grass silage neutral detergent extract (360) was used to supply a constant source of energy for the microbes over the 10-h incubations (Ahvenjärvi et al., 2009). The rate of energy supply available to rumen microbes was determined based on 24-h gas production for incubation vessels assigned to groups 1 and 2 using an automated system (Huhtanen et al., 2008b). The third group comprised 12 bottles, each treated with buffered rumen inoculum, carbohydrates, and L-SRMP. For the fourth group, incubations were made with 12 bottles, each treated with buffered rumen inoculum, carbohydrates, and U-SRMP.

Prior to inoculation, flasks were filled with 52.6 mL of buffer, 3.4 mL of reducing solution (Goering and Van Soest, 1970), and the mixture of carbohydrates. Both pectin and starch were dissolved in 3 mL of hot buffer before being transferred into incubation flasks. The mixture of added carbohydrates was allowed to hydrate for 1 h at 39°C before the addition of test protein (12.5 mg of N/100 mL) to flasks in groups 2, 3, and 4. Thereafter, 1.67 mg of unlabeled ammonium chloride dissolved in 1 mL of deionized water was added to flasks in group 3 (L-SRMP), whereas 1.67 mg of ¹⁵N-labeled ammonium chloride (98 atom% of ¹⁵N; Amersham International PLC, Amersham, UK) was added to flasks assigned to group 4 (U-SRMP). Incubations were initiated following the addition of 27 mL of rumen inoculum to each flask (total volume 100 mL). Incubations were performed for 0, 0.33, 0.66, 1.0, 1.5, 2.0, 2.5,

3.0, 4.0, 6.0, 8.0, and 10.0 h. Flask contents were mixed continuously using a magnetic stir bar.

Incubations were stopped at the designated time point by placing flasks in iced water. Flask contents were transferred to chilled centrifugation tubes. Residual material was recovered by rinsing with 10 mL of deionized water, which was also added to centrifugation tubes. Incubation contents were centrifuged at $10,000 \times g$ for 15 min at 4°C. Following pellet removal, centrifuge tubes were rinsed with deionized water, and the supernatant was collected and stored at 4°C for chemical analysis. Pellets were frozen at -81°C, freeze dried, and ground for chemical analysis using a ball mill (Retsch MM301; Retsch GmbH & Co. KG, Haan, Germany). Total N content of pellets was determined with an elemental analyzer (FP-428; Leco Corp., St. Joseph, MI). Enrichment of ¹⁵N (atom%) in total N was measured by isotope ratio mass spectrometry (SerCon Ltd., Crewe, UK) using standard methods (Esala, 1991).

The concentration of ammonia in the recovered supernatant was measured using standard procedures (McCullough, 1967). For the analysis of ¹⁵N in ammonia, samples were treated with 6 M NaOH to increase pH above 12 to liberate ammonia and allowed to stand for 22 h at room temperature. Released ammonia was trapped within a disk of glass wool treated with 8 µL of 1 M H₂SO₄, dried for 2 h at 60°C, and submitted for ¹⁵N analysis. For the determination of ¹⁵N in total N of liquid, samples were dried in tin capsules equipped with Chromosorb W 30–60 Mesh (SerCon Ltd.). Total N content of liquid N was determined by the Kjeldahl method on an automated system (Foss Kjeltac 2300 analyzer; Foss Tecator AB, Höganäs, Sweden) and copper as a digestion catalyst.

Metabolism of SRMP protein was evaluated based on observed changes in ¹⁴N and ¹⁵N pool sizes of ammonia-N (AN), soluble NAN (SNAN), and insoluble N (ISN) fractions over the course of 10-h incubations. The total N contents in flasks were fractionated into AN, SNAN and ISN fractions, and pool size of ¹⁴N and ¹⁵N isotopes in these N pools was determined (A¹⁴N, A¹⁵N, SNA¹⁴N, SNA¹⁵N, IS¹⁴N, and IS¹⁵N).

Modeling of Rapeseed Protein Degradation

A dynamic model was fitted to the measurement of temporal changes in the enrichment of isotopes in AN, SNAN, and ISN to describe the kinetics of SRMP metabolism during incubations with rumen inoculum (Figure 1). This approach is based on the concept of using information on the pool sizes of metabolites liberated during degradation, and the pool size of N incorporated into microbial N (MN) for both L-SRMP and U-SRMP to estimate model parameters. Model parameter values

were estimated using WinSAAM 3.0.7 software (version 3.0.7; New Bolton Center, Biostatistics Unit, University of Pennsylvania, Kennett Square; available at <http://www.winsaam.com>). The model assumes that SRMP is either incorporated directly into microbial N or is hydrolyzed and deaminated to AN. An additional pool between the SRMP pool and the AN pool was included to account for the delay between the disappearance of N from the SRMP pool and the appearance of N in the AN pool. Transfer between the delay pool and the AN pool was assumed to be 100%/h. The delay pool and the MN pool comprise the ISN pool (i.e., the delay pool described the fate of N associated with microbial cells that had not been metabolized to AN). Microbial N synthesis from the AN pool was predicted based on the rate of carbohydrate digestion estimated from the rate of gas production according to a 2-pool Gompertz model (Schofield et al., 1994) fitted to measured cumulative gas production using PROC of SAS (SAS Institute Inc., Cary, NC). The model included 2 adjustable parameters to estimate the empirical relationship between the gas production pools (fast and slow) and MN synthesis, assuming that MN synthesis is stimulated by ATP generated from the fermentation of added carbohydrates and that the synthesis of N compounds by rumen microbes is directly proportional to energy availability, which can be estimated from the measurements of gas production. The rate of degradation and lag time of fast and slow gas production pools were used to predict the synthesis of MN, without accounting for microbial maintenance energy requirements. Effective protein degradation (**EPD**) was calculated as

$$\text{EPD} = k_d / (k_d + k_p),$$

where k_d is the rate of protein degradation and k_p is the rate of liquid passage from the rumen. A passage rate of 0.16 h^{-1} for liquid passage from the rumen was assumed

based on earlier determinations of between 0.16 and 0.18 h^{-1} (Huhtanen et al., 1997; Hristov et al., 2003).

The proportion of MN at x h, where $x = 0, 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 4, 6, 8, \text{ or } 10$, was derived from measurements of the AN pool according to Atasoglu et al. (1998) with modifications:

$$\text{MN from NH}_3 \text{ at } h_x = 2 \times M^{15}\text{N enrichment } h_x / ({}^{15}\text{NH}_3 \text{ enrichment } h_0 + {}^{15}\text{NH}_3 \text{ enrichment } h_x),$$

where MN from NH_3 at h_x is the percentage of MN synthesis from AN at x h, $M^{15}\text{N}$ enrichment h_x is the ${}^{15}\text{N}$ enrichment in percentage of MN, and ${}^{15}\text{NH}_3$ enrichment h_0 and h_x are the ${}^{15}\text{N}$ enrichments in percentage of NH_3 at 0 and x h, respectively.

RESULTS

Degradation of Labeled Soluble Rapeseed Meal

The pool size of SNA^{14}N for L-SRMP decreased rapidly from 13.2 to 9.98 mg during the initial 60 min of incubation, but decreased linearly thereafter from 9.98 to 5.05 mg from 1 to 10 h of incubation (Figure 2). Similarly, the pool size of SNA^{15}N decreased rapidly from 245 to 200 μg during the first hour of incubation, with a much lower rate of decline (from 200 to 63 μg) between 1 and 10 h (Figure 3). Pool sizes of IS^{14}N during incubations with L-SRMP increased rapidly in a linear manner up to 4 h from 9.47 to 16.4 mg. Thereafter, the pool size of IS^{14}N increased at a slower rate, reaching 17.9 mg after 10 h (Figure 2). The IS^{15}N pool during incubations with L-SRMP increased from 79 to 163 μg between 0 and 4 h and from 163 to 175 between 4 and 10 h (Figure 3). Temporal increases in the ISN pools were closely associated with observed decreases in the ammonium pools, with the exception of initial increases

Figure 1. Dynamic model describing nitrogen transformations during the degradation of soluble rapeseed meal protein (SRMP).

Figure 2. Observed and predicted amounts of ^{14}N isotope in the ammonia-N (AN), insoluble N (ISN), and soluble NAN (SNAN) compartments over the course of 10-h incubations of ^{15}N -labeled soluble rapeseed meal with buffered rumen inoculum.

in the AN pools. A delay pool describing the exchange of N between the SNAN and AN pool had to be introduced in the model to account for this phenomenon. Thus, the delay pool is associated with the ISN pool. The A^{14}N pool increased from 16.0 to 16.7 mg during the first 60 min, decreased to 13.1 mg after 4 h, and increased again to 14.6 mg between 4 and 10 h (Figure 2). The A^{15}N pool increased slowly during the first 2.5 h of incubation from 71 to 83 μg , decreased during the next 1.5 h (76 μg), and then increased rapidly until the end of incubation, reaching a final pool size of 130 μg .

Degradation of Unlabeled Soluble Rapeseed Meal

The pattern of increases in the IS^{14}N pool for U-SRMP was essentially the same as that observed for L-SRMP and was characterized by a rapid linear increase up to 4 h from 10.1 to 16.3 mg, followed by a slower increase to a final pool size of 18.8 mg after 10 h (Figure 4). Temporal increases in the pool size of IS^{15}N during incubations with U-SRMP followed a sigmoidal type trend, increasing at a slow rate for the first 2 h (from 39 to 82 μg), a more rapid increase from 2 to 4

Figure 3. Observed and predicted amounts of ^{15}N isotope in the ammonia-N (AN), insoluble N (ISN), and soluble NAN (SNAN) compartments over the course of 10-h incubations of ^{15}N -labeled soluble rapeseed meal with buffered rumen inoculum.

Figure 4. Observed and predicted amounts of ^{14}N isotope in the ammonia-N (AN), insoluble N (ISN), and soluble N (SNAN) compartments over the course of 10-h incubations of unlabelled soluble rapeseed meal with buffered rumen inoculum.

h (from 82 to 150 μg), followed by a much lower rate of increase between 4 and 10 h (150 to 218 μg). During the first 60 min, the A^{14}N pool increased from 16.0 to 16.5 mg, decreased to 13.2 mg between 1 and 4 h, but then increased between 4 and 10 h to 14.8 mg. In contrast, the A^{15}N pool remained constant for the first 60 min, rapidly decreased from 482 to 358 μg between

1 and 4 h, and then decreased at a slower rate, reaching a final size of 312 μg after 10 h (Figure 5).

Model Performance

Despite small deviations between predicted and observed changes in N pools over time, the model resulted

Figure 5. Observed and predicted amounts of ^{15}N isotope in the ammonia-N (AN) and insoluble N (ISN) compartments over the course of 10-h incubations of unlabelled soluble rapeseed meal with buffered rumen inoculum.

in a good fit of experimental data, with a mean squared prediction error of 573.3 μg . However, the model overpredicted the size of the SNA^{14}N pool during the first 3 h of incubations with L-SRMP, and then underpredicted this pool size between 3 and 6 h, and from 6 to 10 h of incubation (Figure 2). In contrast, the model consistently underpredicted the SNA^{15}N pool during the first hour of incubation with L-SRMP (Figure 3). The magnitude of the IS^{14}N pool liberated during incubations with L-SRMP was underpredicted for incubations from 0 to 4 h, whereas the model overpredicted the size of this pool for incubations between 6 and 10 h, with the largest deviation between predicted and observed pool size at the end of 10 h (Figure 2). Model predictions of the IS^{15}N pool were lower than observed for the first 2.5 h, but higher than the measured pool size for subsequent incubation times (Figure 3). Furthermore, the model underpredicted the A^{14}N pool for incubations up to 2.5 h, and overestimated this pool size from 3 to 6 h, whereas for later incubation times, there was close agreement between predicted and observed values (Figure 2). For the ammonia- ^{15}N pool, model estimates were lower than determined values during the first 4 h, whereas the reverse was true for 6- to 10-h incubations (Figure 3).

For incubations with U-SRMP, the predicted SNA^{14}N pool was lower than observed for the first 20 min of incubation and between 1.5 and 10 h, but higher than measured values for incubations of between 0.33 and 1.5 h (Figure 4). The IS^{14}N pool was underpredicted for incubations of 0 to 3 h, but overpredicted toward the end of the 10-h incubation (Figure 4). For the IS^{15}N pool, model predictions were lower than observed val-

ues for the first 4 h of incubation, but higher than measured values for longer incubations (Figure 5). The A^{14}N pool was overpredicted for the entire 10-h period of incubation, with the exception of 0.66- and 1.5-h incubations, when the model underpredicted the size of the A^{14}N pool (Figure 4). Predictions of the A^{15}N pool were overestimates for the first 6 h of incubations with U-SRMP, but underestimates of this pool for 6 to 10 h incubations (Figure 5).

The mean rate of SRMP nitrogen degradation (Table 2) estimated using the model was 0.126 (SD 0.0499) h^{-1} , determined over 3 runs. Overall, 53% of MN was estimated to be derived from AN, with the rest from NAN. At the end of each incubation, the net amount of ISN following incubations with U-SRMP and L-SRMP was 8.86 and 8.51 mg (SD 0.25 mg), respectively. Comparison of the pool sizes of N fractions during incubations of L-SRMP and U-SRMP with buffered rumen inoculum did not indicate significant differences between the degradation rate of U-SRMP and L-SRMP or an obvious influence of the ^{15}N isotope on MN synthesis.

DISCUSSION

Unique features of this study included the incubations of intrinsically identical protein sources, either unlabeled or enriched with the ^{15}N isotope, whereas incubations of the unlabeled test protein were made in the presence of ^{15}N -labeled AN. Use of these complementary approaches allowed for both the extent of protein degradation and MN synthesis to be estimated, which is not possible when incubations are made with a single ^{15}N -labeled source. Discrimination of N into

Table 2. Estimated parameter values describing the degradation of soluble rapeseed meal protein (SRMP) during incubations with buffered rumen inoculum and predicted effective ruminal protein degradability

Item	Incubation							
	Run 1		Run 2		Run 3		Mean	SD ¹
	Mean	FSD ²	Mean	FSD	Mean	FSD		
Delay time, h	3.46	0.081	2.61	0.121	2.56	0.131	2.88	0.503
k_a , ³ h^{-1}	0.136	0.0980	0.070	0.0972	0.073	0.1011	0.093	0.0377
k_p , ⁴ h^{-1}	0.047	0.2862	0.028	0.2492	0.023	0.3311	0.033	0.0127
k_d , ⁵ h^{-1}	0.183	ND ⁶	0.098	ND	0.095	ND	0.126	0.0500
EPD, ⁷ g/g	0.534	ND	0.380	ND	0.373	ND	0.429	0.0908

¹Standard deviation between runs.

²Fractional standard deviation.

³Rate of transfer of protein N from the SRMP pool to the delay pool. Rates of protein N transfer from the delay pool to the ammonia-N pool was assumed as 100%/h (Figure 1).

⁴Rate of direct transfer of protein N from the SRMP pool into microbial N (Figure 1).

⁵Rate of protein degradation calculated as the sum of k_a and k_p .

⁶ND = not determined.

⁷Effective protein degradation (EPD) was calculated as $\text{EPD} = k_d / (k_d + k_p)$, where k_d is the rate of protein degradation and k_p is the rate of liquid passage from the rumen (assumed to be 0.16 h^{-1}).

multiple pools at regular time points allowed for quantitative measurements of the changes in the specific N fractions during the degradation of test proteins over an extended period, which, when used in conjunction with a multiple pool dynamic model, allowed for the rate of SRMP degradation to be estimated. Furthermore, conducting incubations with test proteins with natural or ^{15}N enrichment also provided an insight into possible isotope effects. Comparison of N pool sizes between labeled and unlabeled protein offered no support for discrimination against the heavy N isotope either during the degradation of rapeseed protein by rumen microbes or for MN synthesis as indicated by the small differences in the net ISN pool size following the incubation with L-SRMP and U-SRMP. Earlier investigations have provided evidence for ^{15}N as a suitable marker of N metabolism in vitro (Wattiaux and Reed, 1995) and demonstrated ^{15}N labeled ammonium sulfate as a reliable marker in vitro and in vivo (Hristov, 2002).

Incubations were based on buffered rumen inoculum obtained from 2 cows. The parameters estimated for incubation runs 2 and 3 had similar values, but the rates of degradation and EPD for the first run were much higher. The average gas production between runs also differed (141, 90, and 128 mL for runs 1, 2 and 3, respectively). Overall, these observations appear to be related to higher proteolytic activity of rumen contents used in the first set of incubations. Variation in the proteolytic activity of rumen inoculum between the first run compared with runs 2 and 3, but not between all runs, could be explained by the relatively long time between the first and subsequent incubations. After completing the first set of incubations, samples were analyzed and data computed, before embarking upon the second and third series of incubations. To minimize variation in the proteolytic activity of rumen inoculum, collection of rumen contents from more than 2 donor cows could be considered. Sampling of rumen contents before morning feeding, allowing more diverse diets to be fed, may also be advantageous, following reports that the microbial activity of rumen fluid collected just before morning feeding was higher than that collected 2 or 4 h afterward (Broderick et al., 2004). In future investigations, the use of a reference protein such as casein or, alternatively, a mixture of protein feeds (Edmunds et al., 2012), and measurements of protein degradation or gas production could be used to account for variation in the proteolytic activity of rumen inoculum. Even though differences in microbial activity may influence the validation of kinetic parameters, they did allow for an adequate test of method and model performance. In practice, when established in vitro methods are used, variations in gas production higher than 10%

serve to highlight that incubations should be repeated (Menke and Steingass, 1988).

Source of Rapeseed Protein

Rapeseed meals (Table 1) used in this experiment to prepare L-SRMP and U-SRMP were of similar chemical composition to those typically fed to ruminants (Ahvenjärvi et al., 1999). Characterization of rapeseed protein according to the Cornell Net Carbohydrate and Protein System (Sniffen et al., 1992) indicated a higher proportion of soluble N components (A and B₁) and a lower insoluble fraction than is typical for rapeseed meal (Ahvenjärvi et al., 1999). However, estimates of N solubility are known to vary both within and between protein feeds due to mechanical or chemical processing and the choice of buffer used in chemical analysis (Crooker et al., 1978; Blethen et al., 1990; Hedqvist and Udén, 2006), highlighting the challenges in measuring this variable.

Modeling of SRMP Degradation

Modeling of experimental data was based on the assumption that the degradation of soluble protein proceeds via extracellular and intracellular mechanisms. Extracellular processes are known to involve protein solubilization, adsorption, hydrolysis, and transportation (Russell et al., 1991). Solubilization is initiated upon ingestion, whereby protein is masticated and exposed to saliva, a process that is intensified during transit into the rumen. Following adsorption to the surface of rumen bacteria (Nugent and Mangan, 1981; Wallace, 1985), degradation of protein involves hydrolysis of adsorbed peptides. Small peptides and amino acids may also be absorbed and enter the intracellular space, an active site of peptide catabolism to amino acids (Russell et al., 1991). Amino acids may be used for the synthesis of microbial protein or deaminated to ammonia, which can also serve as a substrate for microbial protein synthesis (Nolan, 1993). Deamination also yields carbon substrates for the synthesis of odd- and branched-chain FA. In excess, intracellular AN and possibly amino acids are excreted into the extracellular space (Nolan, 1993).

The current investigation of SRMP degradation involved fitting a model (Figure 1) to simultaneously describe temporal changes in 11 different N pools; SNA¹⁴N, SNA¹⁵N, A¹⁴N, A¹⁵N, IS¹⁴N, and IS¹⁵N pools were liberated during the degradation of L-SRMP or U-SRMP and N pools were incorporated into MN during incubation with buffered rumen inoculum. The original objective was to use data for all 12 pools, but

due to the extremely small pool size it was not possible to include measurements of SNA^{15}N in the model of U-SRMP degradation. Measurements of the pool sizes of all 11 N fractions were assigned to 1 of 3 different pools in the dynamic model. The SNA^{14}N and SNA^{15}N pools were assigned to the SRMP pool and A^{14}N and A^{15}N were assigned to the AN pool, whereas all ISN pools were assigned to the MN pool. This approach was based on the implicit assumption that no inherent difference exists in the metabolism of U-SRMP and L-SRMP and that the incorporation of A^{14}N and A^{15}N into MN is in direct relation to the relative proportion of each isotope in the AN pool. Development of the dynamic model also assumed that MN synthesis is stimulated by ATP generated from the fermentation of added carbohydrates and that the synthesis of N compounds by rumen microbes is directly proportional to energy availability, which can be estimated based on gas production. To fit the observed data, a fourth pool describing the delay in transfer of N disappearing from the SRMP pool and appearance in the AN pool was introduced into the model. This pool, designated as the delay pool, accounts for the adsorption of dietary soluble protein onto the surface of rumen microbes before degradation can take place (Nugent and Mangan, 1981; Wallace, 1985). It is considered that the capacity for adsorption does not exceed 10 μg casein/mg of bacterial proteins (Wallace, 1985), with the implication that MN recovered by centrifugation in the current incubations contained no more than 1% soluble dietary protein. Extrapolation of these findings to current data suggests a maximal contribution of adsorbed N to the ISN pool during incubations with L-SRMP and U-SRMP of no more than 180 and 190 μg , respectively. Once adsorbed, protein is hydrolyzed to peptides and amino acids that are then either transported into the bacterial cell or released into the extracellular protein pool (Russell et al., 1991; Nolan, 1993).

The constant relationship between predicted and observed trends in the pool size of ^{14}N and ^{15}N isotopes in the AN and SNAN pools, combined with the small fractional standard deviation associated with the parameters estimated for the rate of SRMP degradation, provide evidence that the model accurately predicted the amount of N as SNAN and AN. Furthermore, the performance of the model in predicting the size of isotope ^{14}N and ^{15}N compartments in the ISN fraction also points toward an accurate prediction of MN synthesis. However, microbial maintenance energy requirements were not considered in the model due to the difficulties in estimating this parameter, which may account, at least in part, for the variance between observed and predicted values, particularly toward the end of incubations when the bacterial pool increased substantially.

An inherent assumption within the model is that a fraction of potentially degradable soluble feed protein is degraded to AN and another fraction can be incorporated directly into microbial protein. Earlier studies have also proposed direct utilization of soluble feed protein for microbial N synthesis (Steinhour et al., 1982; Russell et al., 1983; Nolan, 1993). It is generally accepted that the source of N used for protein synthesis differs between bacterial species. Bacteria that ferment structural carbohydrates are thought to use AN exclusively as a N source, whereas NSC-fermenting bacteria are able to utilize ammonia, amino acids, and small peptides for microbial protein synthesis (Russell et al., 1992; Van Soest, 1994). More recent investigations have provided evidence that even though N from ammonia alone can support the growth of bacteria capable of fermenting structural carbohydrates, microbial protein synthesis is stimulated in presence of both amino acids and peptides (Atasoglu et al., 2001). Furthermore, the relative proportion of microbial cell protein synthesis from AN and that is derived from the direct incorporation of amino acids is not constant, because the proportion of AN and total N differs according to diet and is known to vary over time (Wallace and McKain, 1990; Williams and Cockburn, 1991; Atasoglu et al., 1999). It follows, therefore, that techniques that account for both extracellular and intracellular mechanisms and the transformation of N in bacterial cells would provide a more reliable quantitative description of the fate of dietary protein in the rumen due to a more accurate estimation of the rate of ruminal SNAN degradation. Measuring the difference between isotope ^{15}N and ^{14}N pool sizes during incubations of L-SRMP and U-SRMP allowed the magnitude of both pathways in this investigation to be estimated. Through the use of labeled AN in one set of incubations and L-SRMP in another, 53% of microbial protein was estimated to originate from AN, whereas the remaining 47% was derived from direct incorporation of amino acids and small peptides. Previous reports indicated that 34% of bacterial protein was synthesized from AN and 66% from direct incorporation of AA and peptides originating from casein into bacterial cell protein (Russell et al., 1983). Other studies have estimated that the synthesis of microbial cellular protein from AN varies from 46 to 82% (Steinhour et al., 1982; Hristov and Broderick, 1996).

Calculations of the amount of MN originating from ammonia-N were found to increase with incubation time, such that the percentage of MN from ammonia-N would approach 100% if longer incubations had been made. Such a phenomenon can be explained by the enrichment of the MN pool increasing over time, whereas enrichment in the ammonia pool becomes depleted due to the release of ammonia during protein degradation

and the absorption of ammonia during MN synthesis. Such findings highlight the importance of incubation time on estimates of the amount of MN derived from ammonia-N in batch-culture systems. Rates of microbial N synthesis from N in the ammonia pool were related to the rate of carbohydrate degradation as determined by the rate of gas production. In the present study, a mixture of fermentable carbohydrates was used to provide a constant supply of energy for rumen microbes over incubations of up to 10 h (Ahvenjärvi et al., 2009).

Current measurements predicted the effective protein degradability of SRMP as 43% of N, which is in good agreement with earlier estimates of 44% (Hedqvist and Udén, 2006). There has been a widely held and long standing assumption that the soluble N fraction of dietary protein is rapidly and extensively degraded in the rumen (Ørskov and McDonald, 1979). However, several reports have indicated that a certain proportion of fractions corresponding to NPN (A) and buffer-soluble true protein (B₁) in the Cornell Net Carbohydrate and Protein System can escape the rumen (Volden et al., 2002; Reynal et al., 2007). Measurements of N flow at the omasum in single experiments (Ahvenjärvi et al., 1999) or the findings of meta-analysis of data derived from multiple investigations (Huhtanen et al., 2011) suggest that the potential extent of SNAN degradation in the rumen is similar to that of ISN. These observations are consistent with the analysis of data from 80 milk production trials highlighting that grass silage SNAN is of similar biological value as ISN, as adjudged on the basis of milk yield responses (Huhtanen et al., 2008a). Overall, the extent of SNAN degradation in the rumen suggests that this fraction may be more important in meeting the amino acid requirements of ruminants than predicted by current MP systems, highlighting the need to generate accurate estimates of feed protein SNAN content. The methods used in this investigation enabled the degradability of rapeseed SNAN to be determined, which could also be applied for the evaluation of other dietary proteins.

CONCLUSIONS

Investigations based on incubations of labeled and unlabeled rapeseed protein with rumen fluid provided sufficient information to allow for an accurate estimation of key parameters in a complex dynamic model of soluble protein degradation. Data suggest that the same approach could be applied in the evaluation of ruminal degradation characteristics of other feed proteins, and highlight ruminal escape of soluble rapeseed protein as an important source of amino acids in ruminants.

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