Tropical Agricultural Research Vol. 11 1999, 199-210

Observations on Ruminal Protein Degradation Products Following *In vitro* Incubation of Some Concentrate Feeds

V.P. Jayawardena and K.E. Webb Jr.[1]

Department of Animal Science
Faculty of Agriculture, University of Peradeniya
Peradeniya, Sri Lanka

ABSTRACT. *The productions of NH_3, α-amino and peptide-N following in vitro incubation of soybean meal (solvent, SBM), fish meal (FM), dehydrated alfalfa (DA), corn gluten feed (CGF) and distillers dried grains with solubles (DDG) were studied using a mixed microbial culture prepared from the ruminal contents of cows. The concentrations of NH_3, α-amino and peptide-N measured in the cell free media at 0, 2, 4, 6 and 8 h of incubation had time × protein interaction ($P<0.05$). The concentrations of ammonia N increased linearly ($P<0.001$) with time. The peptide N concentrations increased linearly ($P<0.05$) in SBM, FM, DA and DDG. The amino acid profile of peptides (<3,000 MW) persisted at 8 h showed some specific patterns irrespective of the protein used; methionine and histidine contents were very low, while proline, glycine and alanine contents were high in this fraction. Peptide may accumulate in the ruminal fluid following incubation of SBM, FM, DA, CGF and DDG. Ruminal microorganisms can have a preference or resistance to certain peptides produced when proteins of SBM, FM, DA, CGF and DDG are hydrolysed in the rumen.*

INTRODUCTION

Dietary proteins consumed by ruminants are degraded extensively due to the microbial activity in the rumen. The ruminal microorganisms degrade proteins in the diet through a series of steps and synthesize microbial proteins or yield energy by fermentation (Russell *et al.*, 1991). Peptides, amino acids and ammonia are produced as either intermediates or end products during this process. But the early investigators assumed that the ruminal protein degradation occurs rapidly with no accumulation of peptides or amino acids in the rumen (Annison, 1956).

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[1] Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg VA 24061, USA.

Evidence indicates that the peptides (Chen *et al.*, 1987) and free amino acids (Broderick and Kang, 1980) can accumulate in ruminal fluid for a considerable time post feeding. Certain peptides (Yang and Russell, 1992) appear to be particularly resistant to ruminal microbial degradation; the size, structure and the composition of peptides can greatly influence this process. A careful study on the factors governing the peptide and amino acid production in the rumen will be very useful in planning future ruminant diet formulations as they can serve as important sources of N to the ruminants (Webb and Matthews, 1994). Thus, the patterns and possible mechanisms involved in peptide, free amino acid and ammonia productions during ruminal degradation of some proteins were studied (*in vitro*) in this experiment.

MATERIALS AND METHODS

Preparation of the mixed ruminal microbial culture

Ruminal contents were obtained from two cannulated lactating Holstein cows fed a hay-concentrate diet. Whole ruminal contents were collected from the bottom of the rumen approximately 2 h after feeding. Strained ruminal fluid (SRF) was obtained by squeezing the ruminal contents through eight layers of cheesecloth. The ruminal contents collected from both cows were processed similarly and the total volume of SRF collected was 3 l. The particle associated organisms were extracted by washing the remaining solid residue with an equal volume of buffer (pH 6.9, 39°C; Loper *et al.*, 1966) and filtering through cheesecloth. This buffer wash was combined with SRF and centrifuged at 5,000 × g (30 min at 4°C) to harvest mixed ruminal microorganisms (Luchini *et al.*, 1996). The supernatant consisted of soluble nitrogenous compounds that were present in the original ruminal fluid was discarded. The pellets containing mixed ruminal microorganisms were blended (30 sec) with 3 l of nutrient medium. The nutrient medium consisted of cellulose (4 g/l) and starch (1 g/l) plus the other constituents of the buffer. The harvested mixed ruminal microorganisms were transferred to a bottle containing the nutrient medium and allowed to grow free of O_2 at 39°C for 6 h in water bath (Luchini *et al.*, 1996).

In vitro incubation of dietary proteins

Five commonly used protein ingredients at the dairy farm of Virginia Polytechnic Institute and State University were collected for incubation. The proteins used and the crude protein contents were: Soybean meal (solvent,

48% CP), fish meal (Menhaden, 60% CP), dehydrated alfalfa (17% CP), corn gluten feed (19% CP), and distillers dried grains with soluble (25% CP). Air dried samples (1 kg) were ground to pass 1 mm screen using a Cyclotech mill and subsamples of 250 g were obtained.

Incubation was performed in plastic tubes (50 ml) as described by Tilley and Terry (1963). The incubation time periods, amounts of proteins and inoculant added to the tubes were all decided based on the preliminary experiments. Proteins equal to 0.50 mg N/ml of inoculant were used. Incubations began by dispensing 20 ml of the inoculant to each tube using a bottle top dispenser. The space above the liquid in each tube was flushed with CO_2. The tubes were then sealed with rubber corks and stirred on a vortex mixer. The time immediately after the inoculant was introduced to the tubes was considered as the 0 h. Duplicate tubes from five proteins plus the control treatment (without proteins) were allowed to incubate 0, 2, 4, 6 and 8 h in a constant temperature bath (39°C). Five ml of 25% trichloro acetic acid (TCA) was added to the tubes to stop fermentation at the end of incubation. The tubes were then held on ice (4°C) for 60 min and centrifuged (27,000 g, 25 min, 4°C) to harvest cell free supernatant containing soluble components including ammonia, amino acids, and peptides produced due to the degradation of proteins. Ten ml of the supernatant was drawn from each tube and stored at -20°C until analyzed. Four incubation runs were conducted on consecutive days.

Chemical analysis

The concentration of ammonia N in each supernatant was measured by the indophenol reaction (Chaney and Marbach, 1962). Total and free amino N concentrations were analyzed using the ninhydrin color reaction in hydrolyzed (6 N HCl, 24 h, 110°C) and nonhydrolyzed samples (Broderick and Kang, 1980). The concentration of peptide N was calculated as the difference between hydrolyzed and nonhydrolyzed samples. The amino acid profile of low molecular weight peptides persisted at 8 h was determined using a Pico Tag Amino Acid Analysis System (Waters Millipore Corp., Milford, MA) after filtering the supernatant by Centricon-3-microconcentrators of 3,000 MW cut-off (Amicon, Beverly, MA).

Statistical analysis

Data were analyzed as a completely randomized design. The effects of protein sources, time, and protein sources × time on ammonia, alpha amino and peptide-N concentrations were evaluated by analysis of variance using the GLM procedure of SAS (1988). The differences among protein sources on the mean concentration of ammonia, α-amino, and peptide-N concentrations at each time interval were compared using the Tukey's Student Range Test. The statistical significance of differences among individual amino acid concentrations between the protein treatments was assessed using the GLM procedure of SAS (1988), followed by Duncan's Multiple Range test.

RESULTS

Changes in NH_3, α-amino and peptide N concentrations with time

Table 1 represents the mean concentrations of ammonia, α-amino and peptide N (mg/l) following incubation of proteins. The values were obtained by averaging 40 observations for each protein incubated to 0, 2, 4, 6 and 8 h (eight replicates/time interval). Ammonia contributed to the largest fraction (between 62% in CGF and 78% in FM) of the total estimated ruminal degradation products. The concentrations of free amino N were lower ($P<0.05$) than NH_3 with all the proteins used. The α-amino N contributed between 2.4% (SBM) to 7.4% (CGF) of the total measured N. CGF and DA had the highest amino N concentrations. Peptides contributed to a considerable proportion of the total ruminally degraded N. The peptides contributed between 19% (FM) to 31% (CGF) of the total N produced. Corn gluten feed exhibited a relatively higher ($P<0.05$) peptide N concentration than the other proteins used. Differences ($P<0.05$) in NH_3, α-amino and peptide-N concentrations among proteins were noted.

Figure 1a represents the changes in concentration of ammonia N in the extracellular media during incubation of five proteins (DA, SBM, DDG, FM and CGF). The mean ammonia N concentration varied from 5.82 ± 3.22 at 0 h to 171.44 ± 11.28 mg/l at 8 h. The ammonia N concentration increased linearly ($P<0.01$) with time in all proteins, and there was a time *x* protein interaction ($P<0.01$). At 6 and 8 h of incubation, FM had the highest ($P<0.05$) ammonia N concentrations.

Presented in the Figure 1b are the changes in concentration of α-amino N in the extracellular media during incubation of proteins. The α-

Table 1. The concentrations of ammonia, free amino and peptide-N (mg/l) appeared in the extracellular media following *in vitro* ruminal incubation of soybean meal (SBM), fish meal (FM), dehydrated alfalfa (DA), distillers dried grains with soluble (DDG) and corn gluten feed (CGF).

N component	SBM	FM	DA	DDG	CGF	Mean
	◂---------- Concentration (mg/l)[1] ----------▸					
Ammonia	175.21^{c}	223.77^{a}	187.67^{c}	153.67^{d}	206.66^{b}	189.40^{q}
α - Amino	5.34^{d}	7.60^{cd}	13.23^{b}	8.95^{c}	24.73^{a}	11.97^{r}
Peptide	44.16^{b}	54.62^{b}	54.95^{b}	52.19^{b}	103.64^{a}	61.91^{s}

[1]Means of 40 observations incubated to 0, 2, 4, 6 and 8 h (8 replicates time interval^{-1} protein^{-1}).
$^{a, b, c, d}$ Means within a row lacking a common superscript letter differ at ($P<0.05$).
$^{q, r, s}$ Means within a column lacking a common superscript letter differ at ($P<0.05$).

amino N concentrations also had a time *x* protein interaction ($P<0.05$). Corn gluten feed showed the highest ($P<0.05$) α-amino N concentrations across all times. There was a decrease in α-amino N concentration in incubation with CGF and DA during the first 4 and 6 h. Concentration of α-amino N were influenced little by time for DDG, SBM and FM.

The data for peptide N concentration following incubation of proteins are presented in Figure 1c. There were differences among proteins in peptide production as indicated by time *x* protein interaction ($P<0.01$). Corn gluten feed had the highest peptide N concentration across all time intervals. The peptide N concentration of CGF declined initially before leveling off at about 4 h. A fair amount of peptides were originally present in this feed as indicated by the high initial concentration. The peptide N concentration increased linearly ($P<0.05$) during the incubation of other proteins.

The amino acid composition of peptides persisted in the media

The composition of peptide (<3,000 MW) amino acids persisted in the extracellular media following incubation (8 h) of DA, SBM, FM, CGF and

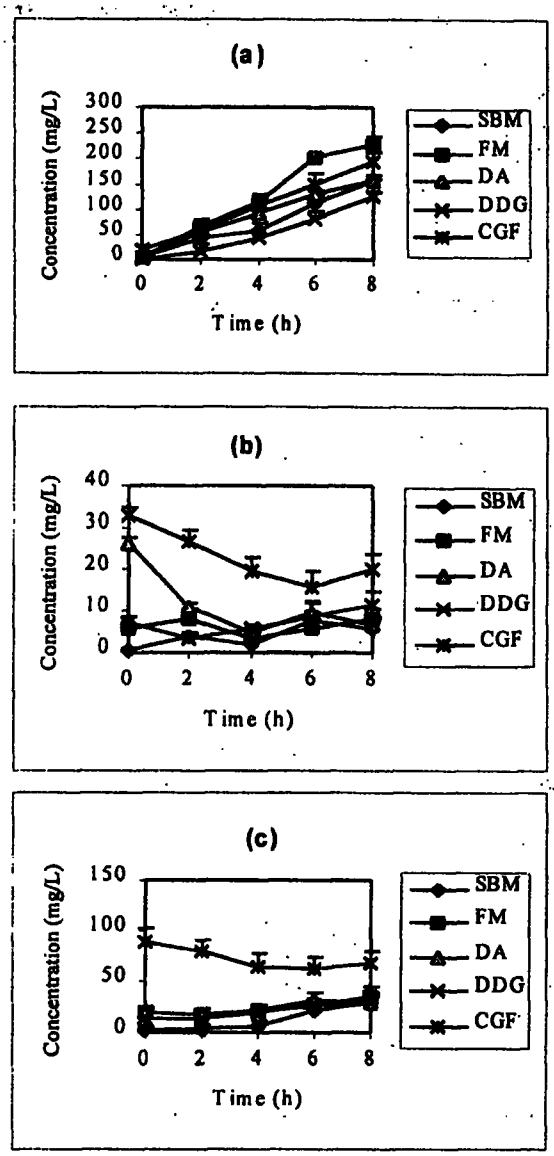

Figure 1. **Changes in concentration (mg/l) of (a) ammonia N, (b) α-amino N, and (c) peptide N in the extracellular media during *in vitro* ruminal incubation of soybean meal (SBM), fish meal (FM), dehydrated alfalfa (DA), distillers dried grains with soluble meal (DDG), and corn gluten feed (CGF).**

DDG are given in the Table 2. Asparagine, cysteine, glutamine and tryptophan are not included in the data as these amino acids are known to be severely affected during hydrolysis (Wallace *et al.*, 1993). The low molecular weight peptide (<3,000 MW) amino acids contributed between 34% (SBM) to 55% (CGF) of the total peptide-bound amino acids. Some similarity across treatments was observed in the appearance of certain amino acids within low molecular weight peptides. All proteins had a zero concentration of peptide-bound Met following 8 h incubation. Also, the concentration of histidine was very low (between 0 to 4% of total amino acids). The concentrations of peptide-bound (<3,000 MW) glutamine, proline, glycine and alanine tended to be high among all the proteins. Glutamate, proline, glycine and alanine in combine contributed between 46 (DA) to 55% (FM) of the total amino acids.

DISCUSSION

A broad objective of this experiment was to study the patterns and the possible mechanisms involved in peptide, amino acid and ammonia productions during ruminal degradation of SBM, FM, DA, CGF and DDG proteins. A mixed microbial culture prepared from the ruminal contents obtained from lactating dairy cows was used to simulate the ruminal microbial activity. The amount of protein added (0.5 mg/ml N of inoculation medium) to each incubation tube was decided based on the preliminary experimental results (data not shown) and reasonably compares with the level of protein usually present in the ruminal fluid soon after dietary protein supplementation. Additionally, (i) the conditions required for a normal ruminal microbial activity (Johnson, 1963) were maintained throughout incubations, (ii) the incubations were performed only for 8 h to prevent inhibition of microbial activity due to the accumulation of end products, and (iii) the presence of active ruminal microorganisms during incubations was confirmed by microscopic examinations. Therefore, the activities of the ruminal microorganisms can be assumed to occur similar to that under *in vivo* conditions. To eliminate the effects due to recycling of microbial proteins, and the residual N compounds of the inoculant, references were made using control treatments (without proteins). Therefore, the measurements of peptides, amino acids and ammonia should represent the net amounts produced due to the degradation of dietary proteins by ruminal microorganisms.

The protein utilization by ruminal microorganisms is known to occur in several distinct steps *i.e.*, solubilization, proteolysis, peptide hydrolysis,

Table 2. **Amino acid composition of peptides (<3000 MW) persisted in the extracellular media following *in vitro* incubation (8 h) of dehydrated alfalfa (DA), soybean meal (SBM), corn gluten feed (CGF), fish meal (FM) and distillers dried grains with soluble (DDG).**

Amino acid	DA	SBM	CGF	FM	DDG	Mean	SE
	Composition (g/100 g total amino acids)						
Arg	3.32	5.06	3.57	13.41	6.12	6.30	1.85
His	0.00	0.00	4.00	0.00	5.86	1.97	1.24
Ile	5.38	4.96	5.09	3.03	2.67	4.23	0.57
Leu	4.53	3.47	6.91	5.64	3.66	4.84	0.64
Lys	10.06	8.23	4.52	3.78	9.87	7.29	1.32
Met	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phe	3.25	4.23	2.53	4.90	1.97	3.38	0.54
Thr	9.42	6.07	7.48	2.22	7.19	6.48	1.19
Val	8.36	7.79	7.93	4.06	5.42	6.71	0.84
EAA	44.34	39.81	42.04	37.04	42.77	41.20	1.27
Ala	7.62	5.59	11.08	7.83	10.69	8.56	1.02
Asp	5.19	7.01	1.68	2.12	2.77	3.76	1.01
Glu	21.76	29.44	16.55	19.42	17.43	20.92	2.31
Gly	8.18	5.60	8.73	14.42	7.90	8.97	1.46
Pro	8.32	11.39	14.13	13.47	14.38	12.34	1.13
Ser	4.50	1.20	4.98	3.56	3.63	3.57	0.65
Tyr	0.08	0.00	0.83	2.14	0.43	0.70	0.39
NEAA	55.65	60.23	57.97	62.97	57.23	58.80	1.27

transport of peptide and amino acids into bacteria, fermentation and microbial protein synthesis (Russell *et al.*, 1991). A large accumulation of ammonia N was observed in all the proteins. This agrees well with previous measurements on ammonia N (Annison, 1956), which recognized ammonia

as the major end product of protein catabolism in the rumen. Accumulation of ammonia N during incubations indicates that the ruminal protein fermentation occurs extensively and the ammonia is produced in excess of the microbes' capacity to utilize them for protein synthesis.

The peptide N was produced to an intermediate level and there was a continuous build up in the concentration of peptide N during incubations. Accumulation of peptide N is in agreement with some previous observations on ruminal protein degradation products (Russell *et al.*, 1983; Chen *et al.*, 1987). The above observations indicate that the ruminal microorganisms degrade dietary proteins extracellularly to peptides, and that the subsequent steps in the degradation of proteins can occur at relatively slower rates than the rate of proteolysis. Therefore, the extracellular hydrolysis of peptides and/or peptide transport into the microbial cells could be the rate-limiting steps during protein utilization by ruminal microorganisms (Chen *et al.*, 1987). Also, it was reported that ruminal microorganisms can saturate their growth responses to peptides and amino acids at low concentrations such as 10 mg/l (Argyle and Baldwin, 1989), and some peptides are particularly resistant to further degradation in the rumen (Yang and Russell, 1992). As a result, ruminal microorganisms may not be utilizing large quantities of peptides produced due to proteolysis of SBM, FM, DA, CGF and DDG. Hence, the peptides can accumulate in the extracellular ruminal fluid during the degradation of these proteins in the rumen.

The concentrations of α-amino N were frequently low and the pattern of α-amino N appearance varied widely among proteins. Several other workers (Annison, 1956; Broderick and Wallace, 1988) also reported similar concentrations of α-amino N in the extracellular ruminal fluid. The low extracellular concentrations of α-amino acids could have occurred due to the rapid uptake of amino acids by ruminal microorganisms and/or hydrolysis of peptides to amino acids was mostly occurring intracellularly. When Broderick and Craig (1989) incubated casein and bovine serum albumin with mixed ruminal microorganisms, the intracellular free amino acid concentrations started to increase prior to the appearance of extracellular free amino acids. The above observation suggests that peptide uptake followed by intracellular hydrolysis would be the major route of amino acid absorption by ruminal microorganisms. The enhanced growth responses observed when amino acids were supplied as peptides in comparison to the free forms (Argyle and Baldwin, 1989) further substantiate that peptides are the preferred substrates for absorption by ruminal microorganisms. Thus, low concentrations of free amino acids can frequently appear in the extracellular ruminal fluid.

The differences ($P<0.05$) observed in the production of peptide N, α-amino N and ammonia N among proteins indicate that the variations among proteins can influence the multi-step process of ruminal protein metabolism. The differences in amino acid composition and the structure of the proteins may have a major influence at one or more of the above steps to produce different types and amounts of ruminal protein degradation products. The presence of specific patterns in the appearance of peptides (<3,000 MW) irrespective of the protein implies that the ruminal microorganisms can have preferences or resistance in the utilization of certain peptides. The results of the present study support the idea that ruminal microorganisms prefer methionine and possibly histidine containing peptides. Consequently, peptides containing those amino acids can be frequently lacking in the extracellular soluble N fraction of the ruminal digesta. Alternatively, the presence of relatively high proportions of glutamate, proline, glycine and alanine containing peptides were noted irrespective of the protein used. High glutamate contents could probably be due to the presence of high concentration of this amino acid in the proteins of all feeds. However, proline, glycine and alanine contents are generally not found in very high concentrations among the feed proteins used. The resistance of proline (Yang and Russell, 1992) and glycine (Broderick *et al.*, 1988) containing peptides to further degradation by ruminal microorganisms was demonstrated previously using synthetic peptides. Therefore, high proline, glycine and alanine contents in the low molecular weight peptide fraction indicate that the peptides containing those amino acids could be resistant to further degradation in the rumen.

CONCLUSIONS

Peptides can accumulate in the ruminal fluid following microbial degradation of SBM, FM, DA, CGF and DDG proteins. Ruminal microorganisms can exhibit some preference or resistance to certain peptides produced during the incubation of these proteins.

ACKNOWLEDGEMENTS

The authors wishes to thank John Lee Pratt Fellowship of Virginia Polytechnic Institute and State University, USA for providing financial assistance to conduct this research.

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