METHODS TO ANALYZE FEEDS FOR NITROGEN FRACTIONS AND DIGESTIBILITY FOR RUMINANTS WITH APPLICATION FOR THE CNCPS

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Deborah A. Ross

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METHODS TO ANALYZE FEEDS FOR NITROGEN FRACTIONS AND DIGESTIBILITY FOR RUMINANTS WITH APPLICATION FOR THE CNCPS

Deborah A. Ross, Ph. D.

Cornell University 2013

To improve the ability to predict amino acid flows in ruminants, which will enhance the efficiency of use of feed nitrogen (N), methods to better predict metabolizable protein (MP) and amino acid supply need to be developed. The objectives of this study were (1) to evaluate and improve the efficiency of precipitable true protein assay to understand the peptide profile that is chelated to better predict the supply of those components to support the MP supply of the cow; (2) to evaluate Streptomyces griseus assay as indicator for AA profile of MP supply; and (3) evaluate variation with published total digestion assays and make improvements to better estimate intestinal protein digestion of feeds for ruminants. Throughout, methods were evaluated or developed with commercial labs in mind so values could routinely be analyzed for use in the CNCPS.

The efficiency of the precipitated true protein (PTP) assay was improved with tungstic acid (TA) or stabilized TA filtered on 1 or 6 µm filters ($P < 0.05$) but was time consuming as were ultrafiltration devices. Nonprotein N as assayed contains substantial quantities of amino acids and peptides. The *Strep. griseus* assay will predict rumen degradation of N but will not predict AA supply or digest carbohydrates and fiber.

An *in vitro (IV)* assay to estimate intestinal protein digestion for ruminants was developed using an enzyme mix of trypsin, chymotrypsin, lipase and amylase at activities found in sheep digesta to replace pancreatin, reduced sample loss by

eliminating the use of bags by employing Erlenmeyer flasks, utilized small pore size filter papers to further improve recoveries of undigested feed N and corrects for microbial contamination with corn silage ND. Acid detergent insoluble (ADI) N is digestible; thus, undigested N from the *IV* intestinal assay more accurately represents the protein C fraction than does ADIN given the more physiological conditions of rumen incubation and enzyme exposure. The IV assay provides a method, adoptable by commercial labs, to evaluate intestinal digestibility of ruminant feeds to better predict the MP and amino acid supply for input into the CNCPS.

BIOGRAPHICAL SKETCH

Debbie was born on May 24, 1955 in Glens Falls, New York to E. Wendell Ross and Janet L. Ross. She began her education in the fall of 1960 at Chestertown Central School in Chestertown, New York and graduated in June of 1973 as a member of the last class from this school prior to consolidation. In the fall of 1973 Debbie entered the College of Agriculture and Life Sciences at Cornell University majoring in animal nutrition and graduated in January 1978. Throughout this period of her life, horses played a major role in her life. Between her junior and senior years at Cornell, Debbie took a semester off and showed her Quarter horse jumper, Fire Three, to a national championship. Upon returning to school, Debbie became involved in equine research projects and discovered the adventure of research. Following graduation she worked in Nutritional Sciences with Dr. Mary Morrison who introduced her to the world of amino acid analyses. When Dr. Morrison retired, Debbie returned to Animal Science, working with various faculty members. When the Department's amino acid analyzer was moved into Morrison, Debbie was given the responsibility for its operation. In the fall of 2000, working with Dr. Mike Van Amburgh, Debbie commenced a Master's program though the Employee Degree Program and completed it in August 2004. Intrigued with the ruminant digestive track and the current methods to predict metabolizable protein and amino acid supply, Debbie continued her graduated studies with Mike.

Dedicated to

my husband and family

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Thanks, Mom and Dad, for your support over the years and raising me to think on my own. I am grateful to my brother, Mike, who taught me to stick up for what I believed. In addition, I want to thank my friends and co-workers who encouraged me. Lastly, thank you Bill, my dear husband, for enduring life with me.

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LIST OF ABBREVIATIONS

AA	amino acid
AD	acid detergent
ADAAN	acid detergent alpha amino acid N
ADF	acid detergent fiber
ADIN	acid detergent insoluble nitrogen
AH	alfalfa hay
AM	amylase
ANVY	anchovy fish meal
AS	alfafla silage
BM	blood meal
C	chymotrypsin
CG	corn gluten
CM	canola meal
CNCPS	Cornell Net Carbohydrate and Protein System
CornBP	corn milling byproduct
CTAB	cetyltrimethylammonium bromide
CS	corn silage
Cxp	carboxypeptidase (A, B & Y)
CV	coefficient of variation
DDG	distillers grain
DM	dry matter
E	elastase
EAA	essential amino acid
EN	endogenous nitrogen
FAA	free amino acid
HCl	hydrochloric acid
HPLC	high pressure liquid chromatography
ID	intestinal digestion
IS	*in situ*
IV	*in vitro*
K method	Krishnamoorthy et al., 1982
L	lipase
L method	Licitra et al., 1996
mTSP	modified 3-step procedure
MP	metabolizable protein
N	nitrogen
ND	neutral detergent
aNDF	neutral detergent fiber assayed with heat-stable amylase
NDIN	neutral detergent insoluble nitrogen
NPN	nonprotein nitrogen
PC	perchloric acid
PCL	peptide chain length
PPT	precipitable
PTP	precipitable true protein
RF	rumen fluid
RT	room temperature
RUP	rumen undegraded protein
SBM	soybean meal
SD	standard deviation

SG	*S.griseus*
SSA	sulfosalicylic acid
STA	stabilized tungstic acid
T	trypsin
TA	tungstic acid
TCA	trichloroacetic acid
TN	total nitrogen
TP	true protein
TSP	3-step procedure

Chapter 1

LITERATURE REVIEW

On dairy farms, improving the efficiency of use of feed nitrogen (N) has become a central component of the ration formulation process for two reasons: the desire to be more environmentally friendly, and in some cases the ability to reduce feed costs or make best use of farm-specific feeds. New data and a re-evaluation of feed fractionation techniques are suggesting that how we currently characterize feed protein fractions and their associated degradation and passage rates might result in the overfeeding of protein to cows (Lanzas et al. 2007).

Soluble protein, non-protein nitrogen and feed value

Approximately 70 to 90% of the total nitrogen (TN) in fresh forage is present as protein since it is insoluble upon denaturation (Chase and Stone, 2004). Due to the activity of plant enzymes, the remaining 10 to 30% is soluble and contains nonprotein nitrogen (NPN) components and true soluble protein. The soluble protein and NPN fraction includes proteins, peptides, free amino acids, ammonia, amides, amines and nitrates (Chase and Stone, 2004). In well-fermented silages, free amino acids represent ~50 % of the NPN fraction (Brady, 1960; Hughes, 1970; Olmos Colmenero and Broderick, 2006;) while peptide N makes up a majority of the remaining soluble N. Data from Olmos-Colmenerao and Broderick (2006) (Table 1) demonstrate and reinforce observations made by Brady (1960) and Hughes (1970) that there are several components to the soluble and NPN fractions -- some of them are unidentifiable and should truly constitute NPN.

Peptides and free amino acids are nutritionally relevant components that are currently characterized in the NPN pool within the Cornell Net Carbohydrate and Protein System (CNCPS) feed library. This is significant because the model does not

differentiate ammonia N from the peptide and amino acid N in this fraction. There are now several data sets that demonstrate that the soluble pool of feeds contributes between 5 and 17% of the total amino acid flow to the duodenum of the cow (Hristov et al. 2001; Volden et al., 2002; Choi et al. 2002a,b; Reynal et al. 2007). The characterization of NPN within the current structure of the CNCPS reduces the feeding value of the fraction as a source of peptides and amino acids. This is a function of both how NPN has been measured and the degradation and passage rates currently assigned to it (Fox et al, 2004; Lanzas et al. 2007). With an assigned degradation rate of 10,000%/hr, any peptide and amino acid N is immediately converted to ammonia N, and the further implication is that ammonia N is instantaneously taken up by the microbial pool. To more accurately describe the soluble pool in future versions of the CNCPS, the true protein, peptides, and amino acids should be in a separate pool and the ammonia and other non-amino acid, non-protein constituents should remain in the NPN pool. For the new release of the CNCPS v6.1, we are adjusting the NPN pool size to reflect the peptide and amino acid content of the pool. Since the Protein B1 pool is determined by difference, the result is the model will predict a larger flow of soluble peptide N that was previously being converted to ammonia in the model and undervaluing the protein contribution of the soluble protein pool. Some examples of the changes in NPN values are found in Table 1.1.

Although a subtle change in some respects, when combined with other changes such as reduced rates of degradation of the pool and a change in assignment of passage rate from the solid to the liquid, the combined effect is significant. This is especially true for feeds like alfalfa silage where a significant portion of the feed can be soluble and be of high N content. A previous discussion of the insensitivity of the

predictions of the model when high quality alfalfa was fed can be found in Aquino et al. (2003)

Table 1.1. The nitrogen composition of the soluble and nonprotein nitrogen fractions of selected feeds.

	Alfalfa silage[a]	Corn silage[a]	Rolled HMSC[a]	SBM[b]	SoyBest[b]	-----Alfalfa--- silage[b]	hay[b]	Ryegrass silage[c]
Total N, % DM	3.66	1.16	1.35	8.17	7.76	3.47	3.31	1.93
% Total N								
Soluble	49.6[d]	56.5[d]	32.2[d]	5.69	5.34	67.95	27.71	52.85
NPN	46.3	55.9	31.3	1.56	2.73	28.42	8.61	52.85
NH3-N	6.5	8.7	3.6	--	--	--	--	--
FAA-N	25.9	36.3	20.3	0.9	0.99	28.4	2.96	40.55
Pep AA-N	--	---	--	0	0.07	17.86	6.86	
UnID NAN[e]	6.48	7.83	4.38	--	--	--	--	12.33
NPN, % Sol								
Updated values[f]	41	35	34	12	31	30	20	75
CNCPS v.5	50	100	88	53	--	50	50	95

[a]Olmos Colmenero and Broderick, 2006
[b]Ross, unpublished
[c]Brady, 1960
[d]Soluble value from Lanzas et al., 2007b
[e]UnID NAN, unidentified nonammonia N
[f]NPN, less total of free and peptide amino acids, as percent of soluble protein

The A pool is considered NPN and is determined as the buffer soluble portion of the feed that is not precipitable by either trichloroacetic acid (1.67 % final concentration; TCA) or tungstic acid ((1.18 % final concentration; TA) and is thus estimated by difference (Sniffen et al., 1992; Licitra et al., 1996). Licitra et al. (1996) published a paper standardizing the deproteinization method; however, their standardization created additional problems primarily with the saturation of binding sites due to conducting the precipitation in the presence of the entire feed sample and not just the soluble portion. This leads to an underestimation of the true protein measured in the soluble fraction. For a more thorough discussion of difficulties with chemical precipitation analyses, the reader is referred to Reynal et al. 2007. Those

authors suggest the application of ultra-filtration filters to characterize the soluble portions of feeds, instead of chemical deproteinization, to more clearly delineate the true molecular size of proteins and peptides that are either derived from the feed or flow out of the rumen. The data of Volden et al. (2002) and Choi et al. (2003) demonstrate *in vivo* that the rate-limiting steps in protein degradation are from proteins to peptides and peptides to AA; thus soluble proteins and some large peptides can escape rumen degradation and contribute to the protein supply of the cow. Therefore, being able to rapidly and efficiently identify those peptides should improve the feed characterization and the subsequent sensitivity of the model. We have made improvements in the efficiency of the TA precipitation assay of Licitra et al., (1996) through changes in reagents and filtering pore size; however, we are still testing the improvements against the suggestions of Reynal et al. (2007) to determine if the assay is adequately separating the peptides and the size of the peptide. To make the assay work more effectively, a much higher concentration of TCA or TA must be used than was recommended by Licitra et al. (1996) (~50% versus 1%) and centrifugation should also be employed, which is not a commercially viable step.

Pools and Rates of Degradation

In the CNCPS (v6.1), the carbohydrate (CHO) pools have been expanded to eight fractions: CA1 (acetic, propionic and butyric acids), CA2 (lactic acid), CA3 (organic acids), CA4 (sugars), CB1 (starch), CB2 (soluble fiber), CB3 (available NDF) and CC (unavailable NDF) to calculate rumen degradation and escape, as described by Lanzas et al. (2006). In previous versions of the CNCPS, carbohydrate fractions were categorized into four fractions: A, B1, B2 and C (Fox et al., 2004). The previous CNCPS fractionation system resulted in sugars, organic acids, and oligosaccharides

in the CHO A, and starch and soluble fiber compounds in the CHO B1. Organic acids, which can be quite high in forages, are used less efficiently for microbial growth compared to sugars, and lactate can be quite high in silages and, contrary to VFA, produces microbial protein (Doane et al., 1997; Molina, 2002). Lanzas et al. (2006) used published information to develop degradation rates for the CHO A fractions, soluble fiber, and starch, which varies with type of grain and processing method, and are used in CNCPS v6.1. Lanzas et al. (2007a) reported that the expanded CHO scheme provides a more biologically correct and appropriate feed description that more closely relates to rumen fermentation characteristics to account for variation in changes in silage quality and diet NFC composition.

This increase in the fractionation of the carbohydrate pools reduces the bias in energy predictions of the CNCPS, but alters the sensitivity of the model to changes in protein supply (Tylutki et al., 2008). Lanzas et al. (2007b) evaluated the CNCPS protein fractionation scheme relative to predicting flows of metabolizable protein and concluded there was bias in the prediction of the CNCPS possibly due to the current fractionation scheme. They suggested that collapsing the B2 and B3 protein pools improved the prediction of metabolizable protein (MP) supply, especially under low protein intake conditions (Lanzas et al. 2007). In the latest release of the model, we have linked the degradation rate of the Protein B3 pool (the neutral detergent (ND) insoluble proteins) to the available NDF degradation rate. Data generated by several groups clearly demonstrated that as the NDF carbohydrate was degraded, the associated proteins were also degraded, especially since they did not accumulate in the residue after microbial degradation. The end result is that less feed N flows from the insoluble portions of the feed and there is more feed N available for microbial utilization from the less soluble portions of the feed. Since most commercial laboratories are now generating rates of degradation for NDF in forages, this now

provides an update for two pools within the model. There are other potential offsets, within the current model framework that might improve the precision of the model.

The MP predictions of the CNCPS would be improved if the A and B1 pools were partitioned to reflect the amino acid and peptide content of the NPN separately from the ammonia and other NPN compounds (amines, amino acids and peptides). Peptides and amino acids are stimulatory to microbial growth (Chen and Russell, 1988) and this has been a consideration of the model for several iterations; however, by not partitioning them into different fractions, we underestimate the true pool size.

Other more immediate changes in the current CNCPS library that impact the prediction of soluble amino acid flows are the rates of digestion of the protein A and B1 pools. The latest version has updated rates for both the A and B1 pools and an example of those changes are found in Table 2. The previous version of the library characterized the degradation rate of the Prot A at 10,000%/hr, an instantaneous rate. As indicated earlier, the NPN fraction needs refinement to separate the ammonia and other NPN compounds from the true protein and amino acid N. The soluble proteins and peptides move with the liquid phase from the rumen to the small intestine and supply the cow with AA (Volden et al., 2002; Hedvquist and Uden, 2006; Reynal et al. 2007) whereas it appears the amino acids are degraded at a rapid rate (~200%/hr) (Volden et al. 2002). Accordingly, the data of Volden et al. (2002), Choi et al., (2002a,b) and Reynal et al., (2007) were used to update the Prot A rates of digestion as shown in Table1. 2. These rates were adjusted downward from 10,000%/hr to 200%/hr for all feeds.

Further, data also exist that demonstrate the rate of Prot B1 degradation as previously characterized has been too high (Broderick et al, 1989; VanStraalen and Tamminga, 1990; Peltkova and Broderick, 1996; National Research Council, 2001; Hedvquist and Uden, 2006; Lanzas et al. 2007; Reynal et al. 2007). The current data

suggest that the rate of protein degradation of the larger soluble proteins is much slower than originally considered (Sniffen et al. 1992). In previous versions of the model library, the Prot B1 levels were set between 120 and 350%/hr whereas the data used to update the Protein B1 rates range from 5 to approximately 50%/hr and more in line with the carbohydrate degradation rate data (Lanzas et al. 2006). Again, making these updates in isolation have a very negligible effect on the prediction of the model, but combined with the NPN pool and passage rates, has a significant impact on the overall prediction.

Table 1. 2. Rates of degradation (k_d) of protein A and B1 pools in CNCPS v5.0 and v6.1.

Feed	Prot_A_kd v.5.0	Prot_A_kd v.6.1 [1,2]	Prot_B1_kd v.5.0	Prot_B1_kd v.6.1
Barley Grain 55% PI	10000	200	300	22.7[4]
Beet Pulp Dry 35 NFC	10000	200	300	5[4]
Citrus Pulp Dry	10000	200	350	6.0[3]
Corn Grain Ground	10000	200	135	50[3]
Corn High Moisture 22%	10000	200	135	50[3]
Corn Hominy	10000	200	150	7.8[5]
Wheat Midds	10000	200	250	13.5[5]
Brewers Grain Dry	10000	200	150	50[3]
Soybean Meal 44	10000	200	230	46[5]
Soybean Meal 47.5	10000	200	230	46[3]
Corn Silage	10000	200	300	28[3]
Grass Silage	10000	200	200	49[3]
Alfalfa Silage	10000	200	150	28[3]

References: [1]Volden et al., 2002; [2]Choi et al. 2003; [3]Lanzas et al., 2007; [4]National Research Council, 2001; [5]VanStraalen and Tamminga, 1990;

Passage Rate Assignments

As indicated earlier proteins, peptides and free amino acids in the soluble pool can be rapidly degraded, but because they are in the soluble pool, move with the liquid phase from the rumen to the small intestine and supply the cow with AA (Volden et al., 2002; Reynal et al. 2007). There are now several data sets that demonstrate the soluble pool of feeds contributes between 5 and 15% of the total amino acid flow to

the duodenum of the cow (Hristov et al. 2001; Volden et al., 2002; Choi et al. 2002a,b; Reynal et al. 2007). In a paper evaluating protein fractionation schemes for models such as the National Research Council Nutrient Requirements of Dairy Cattle (NRC, 2001) and the CNCPS, Lanzas et al., (2007) pointed out that the CNCPS structure had the soluble pools, both protein and carbohydrate assigned to the solids passage rate. Given that liquid passage is 5 to 10 times faster than the solids passage rate, combined with the fast degradation rates assigned to the soluble protein pools, the end result was any constituents of the pool including the soluble carbohydrates were degraded in the rumen. This leads to several over and under estimations. The first over-estimation is the level of rumen ammonia production because all of the soluble proteins were degraded to ammonia, especially given the digestion rates previously assigned to those pools. Microbial yield will also be over-predicted because almost all of the soluble carbohydrates would be predicted to ferment in the rumen. In CNCPS v6.1 the soluble pools, both carbohydrate (CHO A) and protein (A and B1) have been re-assigned to the liquid passage rate equation to more appropriately reflect the biology of the system. Both the solid and liquid passage rate equations were recently updated and account for a greater amount of variation in liquid turnover than the equation found in v5.0 (Seo et al. 2006). This change in passage rate assignment increases the predicted outflow of soluble components, thus reducing microbial yield and estimated ammonia production and rumen N balance. These changes improve the sensitivity of the model to changes in feeds high in soluble carbohydrates and protein and reduce but don't eliminate, the under-prediction bias observed in a previous evaluation of the model (Tylutki et al., 2008).

The objective of this dissertation is to examine the current protein fractionation system and feed chemistry related to the N in feed, the behavior of these fractions in

the rumen, and describe changes in the model library and framework that improve the sensitivity of predictions to changes in feed N fractions using the Cornell Net Carbohydrate and Protein System (CNCPS).

The specific objectives are to 1) evaluate the efficiency of the precipitable true protein assay to determine if the efficiency and repeatability of the assay can be improved and to understand the peptide profile that is chelated in the assay to better describe how to better predict the supply of those components to support the metabolizable protein supply of the cow; 2) evaluate the *Streptomyces griseus* assay as an indicator for the amino acid profile of the feed MP supply and; 3) evaluate the variation associated with the three step procedure (Calsamiglia and Stern, 1995 and Gargallo et al., 2006) and make improvements if possible to better estimate intestinal protein digestion of ruminant feeds .

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Chapter 2

IMPROVING THE EFFICIENCY OF THE PRECIPITATED TRUE PROTEIN ASSAY TO REFLECT CONTRIBUTION OF AMINO ACIDS AND PEPTIDES TO SOLUBLE NITROGEN POOL

ABSTRACT

Protein fractionation in feeds for ruminants is separated into soluble and insoluble fractions based on solubility in the rumen (Krishnamoorthy et al., 1982). The soluble pool has been characterized by true protein (TP) and non-protein nitrogen (NPN) and standards have been described (Licitra et al., 1996). Ration formulation models calculate the ruminal and post-ruminal protein supply based on the relationship between the soluble and insoluble protein contents of feeds, and the relative degradation rates and passage rates. The objective of this study was to improve the efficiency of the precipitated true protein (PTP) assay by comparing three precipitating agents: tungstic acid (TA), TCA and a phosphate stabilized TA (STA) with three different pore size filter papers: 1, 6 and 20 µm tested under vacuum. The efficiency of the PTP assay was improved with TA or STA filtered on 1 or 6 µm filters ($P < 0.05$) using the procedures of Licitra et al. (1996) for all feeds evaluated except a fishmeal processed specifically to supply rumen escape protein. Further, the assay yielded more true protein when the buffer soluble component (Krnishnamoorthy et al., 1982) was precipitated with TA and filtered with 1 µm filters ($P < 0.05$). Subtle differences were observed in peptide lengths remaining in the soluble fraction of Trypticase, protein source for IV fermentation of rumen microbes, following precipitation with TA, STA, TCA, perchloric acid or sulfosalicylic acid, although significantly more true protein ($P < 0.05$) was precipitated with TA or STA than the other agents when filtered with 1 or 6 µm filters. Additionally, the PTP content of Trypticase obtained from TA filtered under vacuum on a 1 µm filter paper

was similar to filtration by gravity with similar lengths of the peptides in both NPN fractions.

Filtering rumen fluid (1 µm filter) or precipitating it with the common precipitating agents resulted in longer peptides remaining in the filtered and STA soluble fractions ($P < 0.05$) than the other treatments and longer peptides in the retentates from these two treatments than the filtrates following filtration through MW cut-off devices. Although TA precipitated more N as PTP in an alfalfa silage, peptide lengths in the NPN from TA, STA and TCA precipitation and the filtered water-soluble fraction were similar as only amino acids were present. Molecular weight fractionation using ultrafiltration provided more precise data but was not conducive for routine use. Given the variability in the assay and the difficulty achieving repeatable and consistent values, ammonia levels should be measured in the soluble feed fraction and true protein precipitated from the soluble fraction using TA filtered on 1 µm filters so the difference will more accurately reflect the amino acid and peptide contribution to the soluble nitrogen fraction.

Abbreviations: AA, amino acid; DM, dry matter; EN, endogenous nitrogen; FAA, free amino acid; K method, Krishnamoorthy et al., 1982; L method, Licitra et al., 1996; N, nitrogen; NPN, nonprotein nitrogen; PC, perchloric acid; PCL, peptide chain length; PPT, precipitation; precipitating; PTP, precipitable true protein; RF, rumen fluid; SBM, soybean meal; SSA, sulfosalicylic acid; STA, stabilized tungstic acid; TA, tungstic acid; TCA, trichloroacetic acid; TN, total nitrogen; TP, true protein.

INTRODUCTION

The Cornell Net Carbohydrate and Protein Model (Sniffen et al., 1992; Tylutki et al., 2008) partitions feed nitrogen into A, B and C fractions (Figure 2.1, Table 2.1; Pichard and Van Soest, 1977), based on rumen solubility and degradation. The

fractionation scheme is based on the solubility of N in water, borate-phosphate buffer or the detergent system and represents ruminal and post-ruminal degradation. Nonprotein nitrogen (NPN) represents the A fraction while true protein (TP), or the B fraction, is further divided into B1 (precipitable true protein; PTP), B2 and B3 with decreasing solubility. Soluble protein or the A and B1 fractions are assumed to be 100 % degraded by rumen microbes and not escape the rumen. Ruminant feeding models calculate the peptide supply and requirement for the non-fiber carbohydrate bacteria based on the true soluble protein in the feeds.

Various laboratory procedures have been employed to measure protein solubility including incubation in assorted mineral solvents and detergent (Krishnamoorthy et al., 1982). The soluble true protein of a feed is precipitated by various agents added to a feed in solution leaving peptides of varying sizes in the soluble NPN fraction. Knowledge of the soluble true protein and NPN contents of ruminant feeds is important in reducing the environmental concerns from overfeeding nitrogen (N) to dairy animals. Approximately 70 to 90 % (Chase and Stone, 2004) of the total nitrogen (TN) in fresh forage is present as protein being insoluble upon denaturation (Tamminga, 1986). Due to the activity of plant enzymes the remaining 10 to 30% is soluble and contains NPN components and true soluble protein. The soluble NPN fraction includes peptides, free amino acids, ammonia, amides, amines and nitrates (Chase and Stone, 2004). In 'good' silages the free amino acid N represents ~50 % of the NPN fraction (Brady, 1960; Hughes, 1970; Muck, 1987; Olmos Colmenero and Broderick, 2006; Ross, unpublished; Tamminga, 1986) while peptide N makes up a majority of the remaining soluble N.

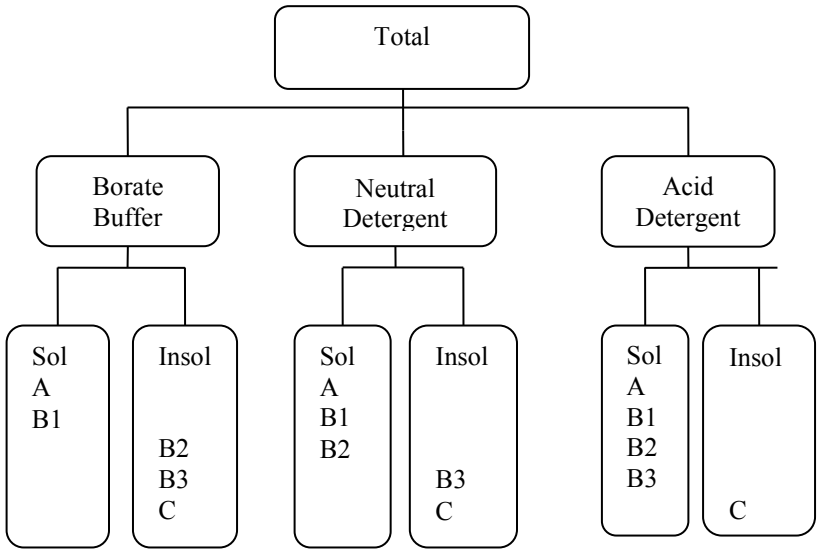

Figure 2.1. Analysis of protein fractions using borate-phosphate buffer, neutral detergent and acid detergent (Pichard and Van Soest, 1977).

Table 2.1. Protein chemical fractions for feeds (Pichard and Van Soest, 1977)

Fraction[1]	Definition
A	NPN + peptides
B1	True Protein, TPP – BIP
B2	BIP – NDIP
B3	NDIP – ADIP
C	ADIP

[1]NPN = Nonprotein nitrogen, TPP = True (tungstic or trichloroacetic acid) precipitable protein, BIP = Buffer insoluble protein, NDIP = Neutral detergent insoluble protein, ADIP = Acid detergent insoluble protein

Historically acids have been utilized to precipitate proteins from blood. Originally, the goal was to use an agent that would completely precipitate blood proteins but leave the peptides soluble (Munro and Fleck, 1969). From a solution of Witte's peptone, a partial protein digest containing polypeptides, tungstic acid precipitated all the peptides whereas 10, 5 and 2.5 % solutions of TCA left 38, 46 and 52 % of the

peptides, respectively, in the supernatant and 2.5 % TCA was considered the most suitable agent to precipitate proteins (Hiller and Van Slyke, 1922).

Tungstic acid precipitation requires the pH of the protein to be lower than its isoelectric point, 5.1 for blood (Berkman et al., 1954) and diphtheria antitoxin serum (Merrill, 1924), for the acid anion to bind with the cationic form of the protein. The WO_4^{-2} ion might also function as a chelating agent, attracting amino acids with cationic side chains, i.e., Lys and Arg. Tungstic acid being unstable is prepared fresh while stabilized TA is stable up to approximately 4 months (Martinek, 1964) by adding metaphosphoric acid, a strong precipitating agent for water-soluble proteins (Briggs, 1940), and sodium pyrophosphate, a buffering salt, which binds to potential sites.

Perchloric acid coagulates proteins but does not precipitate smaller peptides (Oser, 1965) and has been used to precipitate intact proteins and large peptides (>10 kDa) from intestinal digesta of rats to measure endogenous amino acid flow (Darragh et al., 1990; Moughan et al., 1990; Butts et al., 1991) and from rumen fluid to measure peptides (Chen et al., 1987a). Trichloroacetic acid (TCA) forms protein salts by altering the intramolecular shape of the protein due to the decrease in pH (Oser, 1965). Yvon et al. (1989) extensively examined the solubility of peptides prepared from trypsin, chymotrypsin and pepsin digests of α_{s1}-casein and β-casein, fractionated by reverse phase HPLC and precipitated with different final concentrations of TCA (2, 5, 8 or 12 %). Solubility decreased with increasing concentration of either TCA or peptide, suggesting a competition between water and TCA molecules due to electrostatic interactions between the three hydrophobic chloride ions in TCA and the basic AA, the hydrogen bonds of polar side chains in the peptides and the peptide backbones (Yvon et al., 1989). Results of this study suggested that TCA increased the hydrophobicity of peptides which caused

aggregation and led to precipitation (Yvon et al., 1989). Sivaraman et al. (1997) studied TCA precipitation over a wide-range of concentrations (0-70%) of three proteins and confirmed this mechanism.

Sulfosalicylic acid, frequently used in preparing blood/plasma samples for amino acid analysis, precipitates proteins via electrostatic interaction with the carboxyl group and hydrophobic interactions with the phenol group.

Plant protein precipitation is more complex due to the larger molecular weight proteins present, i.e., 100 kDa for SBM (Wolf, 1970), compared to blood proteins BSA (66 kDa) and globulin (56 kDa). Solubility of proteins in water generally increases at pH values further from its isoelectric point (Creighton, 1993) with electrostatic repulsion and hydration promoting solubility (Damodaran, 1996). In water, hydrophilic amino acids on the surface of the protein ionize, creating electrostatic interactions with the solvent and itself; thus, protein cations bind to the acid anion of the precipitating (PPT) agent. Differences in PTP obtained with various PPT agents may also be attributed to the hydrophobicity of the protein-PPT agent salt and the ability of the acid to chelate proteins. Acidic precipitating agents may also result in the partial hydrolysis of proteins and the release of peptides into the soluble fraction (Seal and Parker, 1998) which may or may not precipitate

Due to mechanistic differences among the PPT agents their ability to precipitate plant protein may be due to composition and processing of the protein. The tertiary structure of the protein and the extent to which AA in the protein and associated reactive groups (amino, hydroxyl, hydrogen, sulfur) are exposed to water and the PPT agent contribute to precipitation of the true protein. Also, size of the protein plays a role. Increasing the amount of TCA did not increase the amount of true protein from casein, hemoglobin, sunflower or SBM, each a mixture of proteins, some with MW > 100 kDa (Bhatty, 1972). Whereas TCA at higher concentrations, 15 %

versus 2.5 %, precipitated 49 % more rapeseed protein, a mixture of smaller proteins with MW in 10 – 25 kDa range (Bhatty, 1972).

There has been much debate on choosing a PPT agent to determine the size of peptides left in true soluble protein in the plant material fed to ruminants. Tungstic acid (TA) and trichloroacetic acid (TCA) are the agents commonly used for feedstuffs (Licitra et al., 1996). Greenberg and Shipe (1979) using TA and TCA to precipitate egg albumin and several proteins found that 69% and 79%, respectively, of the nitrogen remained in solution. Winter et al. (1964) observed that TA precipitated more nitrogen in ruminal fluid than TCA. Thus, these agents differ with respect to the peptide length that is chelated and precipitated or is considered NPN. Tungstic acid has been recommended and appears to be an acceptable PPT agent (Licitra et al., 1996); however, the filtering step uses Whatman 541 filter paper with no or very mild vacuum and this results in variability and a decrease in assay speed.

Krishnamoorthy et al. (1982) used a portion of the borate-phosphate solute, which could contain more solubilized protein due to the phosphate anion, to precipitate the true soluble protein (Figure 2.2b) whereas Licitra et al. (1996) solubilized the feed in water and added PPT agent to the entire contents (Figure 2.2a). Recently, Reynal et al. (2007) discussed the unreliability of methods commonly utilized to characterize the soluble N fraction of ruminant diets and concluded ultrafiltration devices with molecular weight cutoffs (Reynal et al., 2007; Butts et al., 1999; Moughan et al., 1990) may prove more reliable than chemical methods for this purpose. The true protein content of the soluble fraction requires further investigation because it appears the soluble fraction contains a significant amount of true protein that can flow with the liquid phase out of the rumen and supply the cow with up to 15% of the metabolizable protein supply (Reynal et al., 2007, Volden et al. 2002) which has

been underestimated by current field based ration formulation models (Tylutki et al. 2008; Van Amburgh et al. 2010).

The overall objective of this study was to improve the efficiency of the precipitated true protein (PTP) assay, separated into six sections: (1)The recovery of PTP from a variety of feeds using the standard procedure of Licitra et al. (1996) was compared to the method of Krishnamoorthy et al. (1982) using three precipitating agents and filters with three different size pores; (2) Trypticase®, was included as a standard to test the efficacy of five common PPT agents and three filter pore size combinations by examining the ratio of hydrolyzed to free amino acids in the NPN to determine the approximate peptide chain length unchelated by the precipitating agent; (3) The lengths of the peptides remaining in the buffer soluble were compared to those in the soluble NPN of several feeds using the method of Krishnamoorthy et al. (1982). (4) Peptide lengths in the soluble NPN remaining after precipitation of rumen fluid and (5) alfalfa silage with PPT agents and (6) peptide lengths in the buffer soluble filtrates of four common dairy feeds were determined via ultrafiltration fractionation.

MATERIALS and METHODS

Unless otherwise specified, all analyses were conducted on duplicate samples filtered flat in Buchner funnels using vacuum.

1. *True Protein – NPN Assays*

Samples: Fourteen feeds: two alfalfas (a silage and a hay), two grass silages, three soy products (solvent extracted soybean meal, a soy protein concentrate and SoyBest® (Grain States Soya, Inc., NE)), three feather meals, two cottonseeds and three fish meals (Omega Protein Corporation, Houston, Texas) were utilized. Forage samples were freeze dried (48-h with shelf temperature at 24º C; VirTus 20

SRC-X; The VirTus Co., Inc., Gardiner, NY). All samples were ground through a 1 mm screen in a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA).

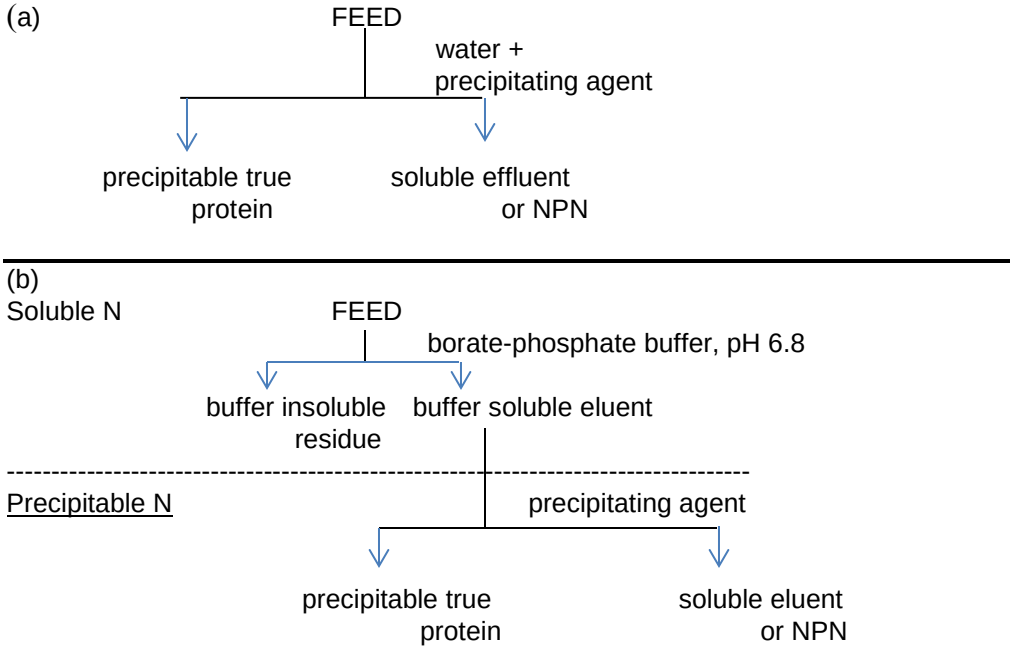

Figure 2.2. Flowcharts to determine (a) precipitable true protein and NPN (Licitra et al., 1996) and (b) soluble protein and precipitable true protein from the soluble fraction (Krishnamoorthy et al., 1982).

Incubations and Chemical analyses

Licitra et al. (1996) method: Half gram samples were incubated in 50-ml water plus the precipitating agent for the specified time following the standard procedure as shown in Fig 2.2a, filtered under vacuum with the PTP residue collected on filters and NPN filtrate captured in side-arm flasks. All assays were done at room temperature. The precipitating agents were as follows: tungstic acid (TA) as described by Licitra et al. (1996; 1.18% final concentration; 16-h incubation at room temperature), a

phosphate stabilized TA (STA; Martinek, 1964; 1.11 % final concentration; 16 hr) and trichloroacetic acid (TCA; 1.67 % final concentration; 1-h) filtered through filters of various pore sizes : 1 µm (Whatman GF-B; GE Healthcare Bio-Sciences Corp., Piscataway, NY, 6 µm (Whatman 3), and 20 µm (Whatman 541).

Krishnamoorthy et al. (1982) method: Soluble and insoluble N were separated by incubating half gram samples in 50-ml borate-phosphate buffer with sodium azide added for 3-h at room temperature with the insoluble captured on 20 µm filters and the soluble eluent collected in side-arm flasks. After weighing, a portion of the soluble eluent was precipitated with TA, STA and TCA with concentrations adjusted to yield similar final concentrations as above and filtered, as shown in Fig 2.2b.

Nitrogen (N) was determined by a modification of AOAC method 984.13 (1990) using boric acid in the distillation step (Pierce and Haenish, 1940) on the precipitable true protein and the soluble NPN. Although the precipitating agents precipitate protein, all results are expressed as nitrogen. Results from the Licitra assay were corrected for the insoluble N content by subtracting it from the PTP. Sample dry matter (DM) was determined at 106°C for 16-h.

2. *Trypticase® Peptide Chain Length*

Trypticase® (Becton Dickinson and Company, Sparks, MD), a casein trypsin digest composed of small peptides, was included as a standard to test the efficacy of the PPT agent and filter pore size combinations by examining the ratio of hydrolyzed to free amino acids in the NPN to determine an approximate peptide chain length (Seal and Parker, 1998; Chen et al., 1987) unchelated by a precipitating agent.
Incubations: Half gram incubations of Trypticase® were conducted in the precipitating agents as above except the NPN filtrate was weighed, a portion retained

for amino acid (AA) analyses and the remainder used for N determination. Additionally, the precipitating agents, perchloric acid (PCA; 0.6 M ≈ 5 % final concentration) and sulfosalicylic acid (SSA; 5 % final concentration) were also used to deproteinize Trypticase®. The PCA and SSA incubations were held overnight at 4ºC and centrifuged at 8500 x g in a Beckman JA14 rotor (Beckman-Coulter, Inc., Fullerton, CA) for 15 min at 4ºC prior to being filtered through 1 or 6 µm filters. The perchlorate in the PCA filtrate was precipitated with potassium hydroxide (1.5 times the molar concentration of perchlorate; Chen et al., 1987a) and removed by centrifugation at 8500 x g. The SSA filtrate was used directly. Trypticase® NPN from TA and STA precipitations following filtration by gravity on 20 µm filters and Trypticase dissolved in sample buffer without any filtration were also prepared but filtration on 20 µm filters under vacuum was omitted as the PTP was too small to be retained on the filter. All analyses were conducted on a weight basis.

Chemical analyses: Nitrogen was determined as above. The ratio of hydrolyzed to free AA yielded the approximate number of amino acid (AA) residues in the peptides, or the peptide chain length. For the free AA, filtrates were diluted 1:20 with sample buffer (0.05 M lithium hydroxide, 0.1415 M lithium chloride, 0.0457 M citric acid and 0.1% phenol, pH 2.8) filtered through a 0.2 µm nylon filter into a sample vial and frozen at -20ºC until analyzed. For hydrolysis a volume containing 0.5-mg N was mixed with an equal volume of concentrated HCl, flushed with N_2 gas, capped and hydrolyzed at 110ºC for 21-hr in a block heater (Gehrke et al., 1985). Hydrolysates were filtered on 20 µm filters and diluted to 50-ml with water. One ml aliquots were evaporated, re-dissolved in 1 ml water, evaporated again, repeated two more times to remove the acid and dissolved in 1 ml sample buffer for analysis. Tryptophan was included in the free AA total but not in the hydrolyzed total as alkaline hydrolysis was not performed and the sulfur AA contributions only represent the values obtained

from the acid hydrolysis as samples were not preoxidized with performic acid. Norleucine, an internal standard, was added to samples after chelation to yield ~62.5 nM/ml in the analyzed sample when diluted. All samples were treated similarly. *Amino acid analysis:* Amino acids were separated on a lithium cation exchange column using a three-buffer step gradient and column temperature gradient. Detection was at 560 nm following ninhydrin post column derivation on an HPLC System Gold with 32 Karat software (Beckman-Coulter, Inc., Fullerton, CA). Standards (250 nM/ml) for Asp, Thr, Ser, Glu, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe, NH_3, Lys, His, Arg and Cys (125 nM/ml) were prepared by diluting a purchased stock (Amino acid standard H, #20088; Pierce Chemical; Rockford, IL) with the sample buffer. Standards (250 nM/ml) for norleucine and tryptophan were prepared in sample buffer and combined with the others. The volume of samples and standards loaded on the column was 50 µl.

3. *Precipitation of soluble protein to determine peptide chain length in the buffer soluble and soluble NPN*

Samples and incubations: Seven feeds: three alfalfas (two silages and one hay), two corn silages (high in soluble protein contents) and two soy products (solvent extracted soybean meal and SoyBest®) were utilized. Using the soluble true protein scheme of Krishnamoorthy et al. (1982; Fig. 1.2b) half gram samples were incubated in phosphate-borate soluble buffer containing no sodium azide (Licitra et al., 1996) for 1-h, filtered on 20 µm paper filters and the buffer soluble eluent collected in a side-arm flask. Initially solubility was determined on six replicates of each feed. Nitrogen was determined on the soluble and insoluble fraction from two replicates. Aliquots of the buffer soluble effluent from remaining replicates were precipitated with STA as above (concentrations adjusted to yield equivalent final concentrations),

filtered on 6 µm paper filters and the soluble NPN collected. Nitrogen was determined on the precipitable true protein and soluble NPN fractions of two replicates. Analyses were conducted on a weight basis.

Chemical analyses: Nitrogen was determined as above. The ratio of hydrolyzed to free AA was used to determine the peptide chain length of the buffer solute. This was calculated from the borate-phosphate solubles and the soluble NPN after precipitation of the buffer solute with STA. To determine free AA, an aliquot equivalent to 40 µg N per ml was freeze dried (~ 4 hr with shelf temperature at 24º C; VirTus 20 SRC-X; The VirTus Co., Inc., Gardiner, NY) and reconstituted with an equal volume of sample buffer (as above) for amino acid analysis. For hydrolysis an aliquot containing 40 µg N per ml was hydrolyzed with an equal volume of concentrated HCl, gassed and hydrolyzed (as above) except the residue was dissolved in two-times the volume hydrolyzed. Norleucine was added as the internal standard, as above. Amino acid analyses were performed as described as above.

4. Ultrafiltration fractionation of rumen fluid

Rumen fluid: Rumen fluid (RF) from a lactating dairy cow receiving a 17 % crude protein diet was obtained approximately 2-h post feeding. The sample was filtered through 4 layers of cheesecloth and a 100 µm nylon cloth (PGC filter screen; Fisher #NC947764; Fisher Scientific; Pittsburgh, PA) and centrifuged at 27,000 x g in a Beckman JA14 rotor (Beckman-Coulter, Inc., Fullerton, CA) for 30 min at 4ºC to eliminate feed particles, protozoa and liquid- and particle-associated bacteria. Dry matter was determined at 106ºC for 16-h.

Incubations: Throughout, analyses were conducted on a weight basis. Half gram DM equivalents of centrifuged RF were incubated in the five precipitating agents (TA, PC, SSA, STA and TCA) used above with concentrations adjusted to yield final concentrations of 1.18, 5, 5, 1.11 and 1.67 %, respectively. Incubations were

terminated as above except only 1 µm filters were utilized. The N content of the residue remaining on the filters was measured as above and N content of the soluble NPN determined by difference. Additionally an aliquot of centrifuged RF was filtered on a 1 µm filter.

Ultrafiltration: The soluble N filtrates were fractionated with ultrafiltration devices using a modification of Reynal et al. (2007). An aliquot containing approximately 11.7 g of soluble N filtrate was pipetted into a tared 10 kDa molecular weight (MW) cut-off ultrafiltration filter with a cellulose membrane (Centriprep YM-10, no. 4321, Millipore, Billerica, MA). The filtration device was centrifuged at 3,000 x g for 30 min at 4ºC. Then, after weighing, the filtrate (<10 kDa) was decanted and the device centrifuged again at 3,000 x g for 30 min at 4ºC. The retentates (>10 kDa) and filtrates (<10 kDa) were weighed.

Approximately 8 g of the <10 kDa filtrate was pipetted into a tared 3 kDa MW cut-off ultrafiltration filter with a cellulose membrane (Centriprep-3, no. 4320, Millipore) and centrifuged at 3,000 x g for 45 min at 4ºC. After weighing, the < 3 kDa filtrate was decanted and the device centrifuged again at 3,000 x g for 20 min at 4ºC. The 10-3 kDa retentates and < 3 kDa filtrates were weighed. All retentates and filtrates were stored at -20ºC until analyzed for amino acids.

Chemical analyses: Nitrogen and dry matter were determined as above. As the N content of the rumen fluid was extremely low, overloading the amino acid column (usually 20 µg N/ml loaded) with any sample was unrealistic; however, finding dilutions to yield detectable amino acids was by trial and error. For FAA, a weighed aliquot was freeze dried (4-h with shelf temperature at 24º C; VirTus 20 SRC-X; The VirTus Co., Inc., Gardiner, NY) and diluted with less sample buffer, as above, than original aliquot, thus concentrating the sample. A weighed aliquot was hydrolyzed with an equal volume of concentrated HCl. Due to quantity, the entire hydrolysate

was evaporated, acid removed and the residue dissolved in sample buffer for amino acid analysis, as above. Norleucine was added as internal standard prior to freeze drying for the free AA or prior to hydrolysis for the hydrolysates.

5. *Peptide chain length in water-soluble alfalfa silage*

Sample and incubations: A water-soluble extract of third cutting alfalfa silage was prepared as follows: silage diluted with cold water (1:10 by weight; Muck, 1987), blended on high for 3 min. in a cold room, refrigerated for 0.5 h, blended again for 3 min., filtered through 4 layers cheesecloth and centrifuged (Huhtanen et al., 2008) at 27,000 x g in a Beckman JA14 rotor (Beckman-Coulter, Inc., Fullerton, CA) for 20 min at 4ºC. Nitrogen, DM and ammonia were determined on the extract, as above.

Half gram DM equivalents of the silage extract were incubated in the TA, STA and TCA precipitating agents used above with concentrations adjusted to yield equivalent final concentrations of each precipitating agent. Incubations were terminated as above except only 1 µm filters were utilized. The N content of the true protein residue remaining on the filters was measured as above and N content of the soluble NPN determined by difference. Additionally an aliquot of silage was filtered on a 1 µm filter.

Ultrafiltration: The soluble NPN filtrates were fractionated with ultrafiltration devices as above, except using only 10 kDa molecular weight (MW) cut-off filters. The >10 kDa retentates and <10 kDa filtrates were weighed.

Chemical analyses: Nitrogen was determined as above. Ammonia was measured by a modification of Chaney and Marbach (1962). Peptide chain lengths were determined as above.

6. Peptide chain length in soluble buffer feed filtrate

Samples, incubations and ultrafiltration: The buffer soluble eluents of three common high N feeds (SBM, SoyBest and Fishmeal 3) and one cottonseed meal, prepared as above in borate-phosphate buffer using 0.5 g samples, were further fractionated using the 3 and 10 kDa ultrafilters as above with the following modification. Filtrate was not taken sequentially from the 10 kDa to the 3 kDa device. Instead, eluent (13.7 g + 0.3 g of 14.6 mM B-Ala) was pipetted into a 3 and a 10 kDa ultrafilter and centrifuged as above. The >10 and > 3 kDa retentates and <10 and <3 kDa filtrates were weighed and peptide chain lengths determined as described above.

Statistical Analysis

The GLM procedure of SAS (SAS Inst., Inc., Cary, NC) was used to perform the analysis of variance on the percent of sample N recovered as PTP or NPN and the peptide chain lengths of Trypticase, rumen fluid or alfalfa silage following each precipitating agent and pore size combination, when appropriate. Least squares means within feeds were compared using contrasts when significance was observed in the analysis of variance test. Significance was considered when ($P < 0.05$). $Y_{ij} = \mu + A_i + F_j + \varepsilon_{ij}$, where Y_{ij} is the dependent variable, μ is the overall mean, A_i is the effect of precipitating agent i, F_j is the effect of filter pore size j and ε_{ij} is the residual error. The Proc TTEST procedure of SAS was used to determine differences in PTP between the Licitra and Krishnamoorthy assays; pooled or unequal variances were used depending on significance of equality of variances.

RESULTS

1. *True Protein -- NPN Assays*

The nitrogen contents of the whole and soluble feed fractions of the feeds used are presented in Table 2.2. With negligible soluble contents, the precipitable true

protein from the three feather meals and soy protein concentrate were 100 % of the soluble from all precipitating agent and filter pore combinations and were not included in any further analyses. Zero values represent no precipitated true protein

Table 2.2. The total and soluble nitrogen contents of experimental feeds. Results are expressed as % dry matter and % total nitrogen, respectively.

Feed	Total N % DM	Soluble N* % Total N
Alfalfa hay	3.30	36.55
Alfalfa silage 3	3.47	59.04
Cotton seed 1	4.24	27.59
Cotton Seed 2	3.48	23.56
Fish meal 1	10.84	20.18
Fish meal 2	10.92	26.86
Fish meal 3	10.60	11.62
Grass silage 1	3.25	65.00
Grass silage 4	3.21	61.00
Soybean meal	8.17	15.34
Soy Best	7.76	10.99
Soy protein concentrate	11.17	4.22
Feather meal 1	12.78	1.50
Feather meal 2	13.81	1.76
Feather meal 3	14.49	3.95

*Soluble N as described by Licitra et al. (1996)

in the soluble fraction, only NPN, i.e., all treatments of alfalfa silage 3 using the Licitra et al. (1996) method. Overall, precipitable true protein contents expressed as a percent of sample soluble N using the Licitra et al. (1996) method were significantly higher when precipitated with STA than TA or TCA and PTP levels were higher when filtered with either 1 or 6 µm filters than with 20 µm filters (Table 2.3). However, the three precipitating agents behaved differently in their ability to precipitate true protein among feeds and within a feed type. Stabilized tungstic acid precipitated significantly more true protein in the alfalfa hay than did TA or TCA whereas TCA precipitated more true protein in the cottonseed samples and TA the least. All acids precipitated equal amounts of protein in the SBM but for SoyBest, with a lower soluble N content but higher level of precipitable true protein than SBM,

TA precipitated the least true protein, STA the most and TCA intermediate. In grass silage1both TA and STA were significantly different from TCA but not each other whereas the three PPT agents precipitated equal amounts of true protein in the grass silage2. Tungstic acid precipitated more true protein in the three fishmeal sampels with STA trending toward precipitating significantly less; however both precipitated significantly more than TCA.

Overall, precipitable true protein levels between the 1 and 6 µm pore filters from the Licitra et al. (1996) assay were not significantly different, but both captured more PTP than the 20 µm pore (Table 2.3). As with the PTP agents, the three pore size filters exhibited differences in the PTP content within and among feeds. A 20 µm pore filter captured 50 % less TA precipitate from alfalfa hay than did the 1 or 6 µm filters while PTP from STA and TCA precipitation on 20 µm pore decreased yield only 6 and 11 %, respectively. In the fishmeal samples 1 and 2, 20 µm filters captured maximum TCA precipitated true protein with an increase of 75 % over the 1 µm in fishmeal 1. In summary, filtering TA precipitated TP on 1 µm filters yielded 16 % more PTP than using 20 µm filters as suggested by Licitra et al. (1996) while precipitating with STA on 1 or 6 µm filters generated 24 or 26.5 % more true protein, respectively.

The method of Licitra et al. (1996) yielded significantly more PTP than did that of Krishnamoorthy et al. (1982); however, exceptions were seen (Table 2.3). The approach by Krishnamoorthy yielded significantly more PTP from fishmeal 3 after TA precipitation filtered on 1 µm filters than the 'Licitra' method and showed a trend toward significance for TA precipitation filtered on 20 µm and STA filtered on 1 µm.

2. *Trypticase® Peptide Chain Length*

Overall, precipitating Trypticase with TA or STA yielded more true protein than did TCA and1 or 6 µm pore filters captured more than the 20 µm filters (Table 2.4).

Peptide chain lengths (PCL) remaining in the NPN were shortest filtered under vacuum on 1 µm pore filters or by gravity on 20 µm following TA precipitation (Table 2.4). True protein levels from SSA, PC or TCA precipitations filtered on 1 or 6 µm pore filters were equivalent to those obtained from TA or STA filtered with 20 µm pore filters filtered under vacuum (Table 2.4). The PCL for Trypticase in sample buffer was similar to that obtained with the PPT agents**;** however, TA filtered on 1 µm pore filters tended to chelate longer peptides allowing the shorter ones to pass into the NPN than did the 6 and 20 µm pore filters. Only the PCL in the soluble fraction resulting from TA and PC filtered with 1 µm pore filters were significantly shorter than those with 6 µm (Table 2.4). The TA and STA samples filtered by gravity with 20 µm pore filters yielded PCL similar to those filtered under vacuum with 1 µm filters. Filter pore size made no difference in the peptide chain length that was precipitated using STA to precipitate Trypticase.

3. *Precipitation of soluble protein to determine peptide chain length in the soluble buffer and soluble NPN*

The feeds used varied in their nitrogen, solubility and NPN contents (Table 2.5) with ranges of 1.3 to 8.2 % DM, 5.3 to 68 % total N (TN) and 3.2 to 67.5 soluble N as

Table 2.3. Comparing precipitable true protein (PTP) from Licitra et al. (1996) and Krishnamoorthy et al. (1982) methods using three precipitating agents filtered with three pore size filters. Results are expressed as percent of feed soluble N after adjusting for insoluble N in precipitate from Licitra method.

FEED[1]	M[2]	Overall[3]	PTP, % Sol N					
			TA[5]			STA		
			1[6]	6	20	1	6	20
AH	L	1.60	1.54[a]	1.49[a]	0.77[b]	1.94	1.86	1.83
	K	0.41	0.66	0.71	ND	0.21	0.19	ND
AS3	L	0	0	0	0	0	0	0
	K	0.07	0.08[a]	0.32[b]	ND	0	0.03	ND
CotS1	L	2.46	2.32[a]	2.06[a]*	1.23[b]*	2.65[+]	2.60	2.36
	K	0.24	0.23	0.12	ND	0.71[+]	0.03	ND
CotS2	L	2.01	1.59[a]	1.46[a]	0.87[b]	2.08	2.40[a]	1.80[b]
	K	0.52	1.77[a]	0.33[b]	ND	0.56[a]	0[b]	ND
Fish1	L	4.24	5.68	6.14[a]	4.89[b]	5.24*	4.99	4.26*
	K	0.35	1.11	0.21	ND	0.13	0.08	ND
Fish2	L	5.23	6.51	6.49	6.40	6.12[a]	6.59[a]	5.13[b]
	K	0.59	0.50	2.13	ND	0.39	0.35	ND
Fish3	L	4.31	6.05	5.44	6.09	5.01[+]	5.44[+]	5.01
	K	4.11	9.22[a]	6.31[b]	ND	5.90[a+]	2.38[b+]	ND
GS1	L	0.40	0.32	0.46	0.35	0.44	0.51	0.47
	K	0.16	0.67[a]	0.23[b]	ND	0	0.004	ND
GS4	L	0.04	0.004[a]	0.07[b*]	0.03[*]	0.08[+]	0.07[+]	0.05
	K	0.06	0.13[a]	0.23[b]	ND	0[+]	0.03[+]	ND
SBM	L	6.58	6.15	6.23[+]	5.09	7.49	7.37	7.02
	K	0.43	0.29	1.44[+]	ND	0.16	0.38	ND
SoyB	L	3.47	2.78	2.83	2.56	4.01	3.91	4.51
	K	0.57	0.49*	1.16*	ND	0.14	0.15	ND
Overall	L	2.76	3.00[a]	2.97[a]	2.57[b]	3.19[a]	3.25[a]	2.95[b]
	K	0.68	1.38	1.20	ND	0.75[a]	0.33[b]	ND

[1]AH, alfalfa hay; AS, alfalfa silage; CotS, cottonseed; GS, grass silage; Fish, fish meal; SBM, soybean meal; SoyB, Soy Best.
[2]M, method: K, Krishnamoorthy et al., 1982; L, Licitra et al., 1996.
[3]Least squares mean from SAS general linear model.
[4]Significance of precipitating agent (PPT) or pore size: NS, nonsignificant; S, P<0.05; T, trend, 0.05<P<0.10.
[5]precipitating agents: TA, Licitra tungstic acid; STA, stabilized tungstic acid; TCA, trichloroacetic acid.
[6]filter pore size, µm.
[ab]means with different letters within assay and precipitating agent (row) are significant, P<0.05.
*[†]Means with common superscript within feed show trends, 0.05<P<0.10.
All means between the 2 methods are significant, P< 0.05, unless noted:
[+]means with common superscript within feed (column) shows trend for method, 0.05<P<0.10,
ND, not determined.

Table 2.3 (Continued)

FEED[1]	M[2]	PTP, % Sol N			SEM	Significance of PPT agent or pore size[4]					
		TCA				TA vs. STA	TA vs. TCA	STA vs. TCA	1 vs. 6	1 vs. 20	6 vs. 20
		1	6	20							
AH	L	1.73	1.63	1.56	0.08	S	S	S	NS	S	S
	K	0.40*	0.29*	ND	0.06	S	S	S	NS	-	-
AS3	L	0	0	0	-	-	-	-	-	-	-
	K	0	0	ND	0.04	S	S	NS	S	-	-
CotS1	L	3.08*	2.68*†+	3.15†+	0.14	S	S	S	T	NS	T
	K	0.32	0.03	ND	0.11	NS	NS	NS	NS	-	-
CotS2	L	2.70	2.53	2.70	0.15	S	S	S	NS	S	S
	K	0.41*	0.03*	ND	0.18	S	S	NS	S	-	-
Fish1	L	1.62[a]*	2.48*	2.85[b]	0.37	S	S	S	NS	NS	T
	K	0.35	0.21	ND	0.15	NS	NS	NS	NS	-	-
Fish2	L	3.07	2.91*	3.82*	0.36	T	S	S	NS	NS	NS
	K	0.06	0.13	ND	0.3	NS	NS	NS	NS	-	-
Fish3	L	1.96+	1.85+	1.92	0.43	T	S	S	NS	NS	NS
	K	0.71+	0.16+	ND	0.99	S	S	S	S	-	-
GS1	L	0.56[a]	0.31[b]	0.21[b]	0.03	NS	NS	T	NS	NS	NS
	K	0	0.06	ND	0.07	S	S	NS	S	-	-
GS4	L	0	0.02	0.002	0.008	S	T	S	S	NS	S
	K	0	0	ND	0.03	S	S	NS	T	-	-
SBM	L	6.56	6.67	6.63	0.2	NS	NS	NS	NS	NS	NS
	K	0.21	0.09	ND	0.2	NS	NS	NS	NS	-	-
SoyB	L	3.45	3.89	3.32	0.18	T	T	T	NS	NS	NS
	K	1.05	0.44	ND	0.14	S	NS	S	NS	-	-
Overall	L	2.24	2.27	2.38	0.03	S	S	S	NS	S	S
	K	0.32	0.13	ND	0.04	S	S	S	S	-	-

% TN, respectively. The true soluble NPN in the alfalfa hay and alfalfa silage 3, prepared from a portion of the soluble protein fraction precipitated with STA and filtered through 6 µm, contained significantly more N (Table 2.5) than when prepared and calculated according to Licitra et al. (1996). Peptide chains were longer in the buffer soluble for the two soy products and the alfalfa hay than in the soluble NPN (Table 2.5) but no differences were observed between the buffer soluble and soluble NPN in one alfalfa silage and the corn silages. There was a trend toward significance in the second alfalfa silage with longer peptides in the soluble NPN than in the buffer soluble.

Table 2.4. Precipitable true protein and peptide chain length of Trypticase by precipitating agent and filter pore size combination, peptide chain length from TA and STA gravity filtered and sample buffer soluble. Results are expressed as percent of sample nitrogen and number of amino acid residues, respectively.

Precipitating agent[1]	Overall	Filter pore[2]				SEM
		1	6	20	20G	
Precipitable true protein						
TA	19.8	34.4^{aA}	23.1^{aA}	1.8^{b+}	ND	3.00
STA	21.3	31.0^{aA}	28.5^{aA}	4.4^{bA†}	ND	0.36
TCA	1.3	2.6^{a*B+}	0.8^{*B}	0.4$^{bB\dagger+}$	ND	0.19
SSA	1.4	1.9^{B}	0.8^{B}	ND	ND	0.45
PC	1.5	1.4^{B+}	1.6^{B}	ND	ND	0.09
Peptide Chain Length						
TA	3.6	3.0^{a}	4.3^{b}	4.2^{b}	2.8^{a}	0.15
STA	3.4	3.3	3.4^{+}	3.6	3.3	0.16
TCA	4.3	3.4*	4.3^{+}	5.0*	ND	0.25
SSA	4.1	4.2	4.0	ND	ND	0.15
PC	3.6	3.2^{a}	4.1^{b}	ND	ND	0.03
Sample buffer	3.8					0.002

[1]TA, tungstic acid; STA, stabilized; tungstic acid; TCA, trichloroacetic acid; SSA, sulfosalicylic acid; PC, perchloric acid. [2]filter pore size, µm; G, gravity. [3]PTP, precipitable true protein; PCL, peptide chain length. [ab]Means in same row and PPT agent with different letters are significant ($P < 0.05$). *Means in same row and PPT agent with common superscript show trend toward significance, $0.05<P<0.10$. [AB]Means in same column and pore size with different letters are significant ($P < 0.05$). [+†]Means in same column and pore size with common superscript show trend toward significance, $0.05<P<0.10$. ND, not determined.

Table 2.5. Feed nitrogen contents of whole, soluble and nonprotein nitrogen following stabilized tungstic acid precipitation of the buffer soluble comparing nonprotein nitrogen values from methods of Krishnamoorthy et al. (1982) and Licitra et al. (1996) for selected feeds and peptide chain lengths from the respective soluble fractions (mean ± SD). Results are expressed as a percent of dry matter, percent of total nitrogen, percent of soluble nitrogen and the number of amino acid residues in the peptide.

Feed[1]	Nitrogen Fractions[2]				Peptide Chain Length[3]	
			Method[4]			
	TN	SOL	K	L		
	% DM	%TN	NPN %TN		Buffer Sol[5]	Sol NPN[6]
AH	3.30	27.79	26.10[a]	15.84[b]	4.5[A]	3.4[B]
	0.06	0.35	0.67	1.00	0.3	0.1
AS1	3.54	32.23	32.65		1.4	1.3
	0.02	0.04	0.07		0.2	0.3
AS3	3.47	67.95	67.53[a]	62.02[b]	1.5*	1.9*
	0.03	0.15	0.56	1.18	0.2	0.1
CSB	1.39	50.08	48.16		1.7	1.5
	0.01	0.12	2.83		0.1	0.4
CSE	1.31	61.68	43.33		1.4	1.3
	0.04	0.07	0.86		0.2	0.1
SBM	8.17	6.67	3.16	1.51	4.6[A]	2.0[B]
	0.09	0.01	0.22	0.72	0.2	0.2
SoyB	7.76	5.34	5.00	5.45	12.4[A]	2.8[B]
	0.10	0.12	0.28	0.68	0.02	0.1

[1]AH, alfalfa hay; AS1 and AS3, alfalfa silages; CSB and CBE, corn silages; SBM, solvent-extracted soybean meal; SoyB, SoyBest®. [2]TN, total nitrogen;Sol, soluble nitrogen; NPN, nonprotein nitrogen. [3]peptide chain length represents the number of amino acid residues in peptide. [4]Method: K, Krishnamoorthy, Fig 1b; L, Licitra, Fig 1a, calculated using soluble NPN values in Sec 2.1.2. [5]Borate-phosphate buffer without sodium azide, incubated 1-hr. [6]NPN from Licitra method. [abAB]Means with different letters in same row and item are significant ($P < 0.05$). *Means in same row show trend towards significance ($0.05 < P < 0.10$).

4. *Ultracentrifugation fractionation of rumen fluid*

Filtering the centrifuged rumen fluid (RF) through a 1 µm filter removed 18 % of the N and reduced peptide size by 91 % (Table 2.6) compared to centrifugation. Tungstic acid and PC, representing the extremes, chelated 78 and 14 % of the remaining N, respectively. Stabilized TA precipitated less N than TA, but did not differ from SSA, STA or TCA. Centrifuged RF, with few free AA, contained the longest peptides. Peptides in the TA soluble NPN were shorter than those prepared

with PC, STA and TCA but similar to those prepared with SSA. The longest peptides were in the STA prepared soluble NPN which were not different than those in the 1 µm filtered centrifuged RF, with a large SD.

The filtered rumen fluid contained longer peptide lengths in the <10 kDa filtrate (fraction B) than any NPN fraction but there were no differences between NPN treatments, except a trend toward significance between the TA and PC treatments with a range of 3.6 to 6.5. The longest peptides in the >10 kDa retentate (fraction C) were in the STA prepared soluble NPN with no differences observed between the other precipitating agents although all soluble NPN recovered contained shorter peptides than the filtered rumen fluid. In the <3 kDa filtrate (fraction D) there was a trend toward shorter peptides using TA or SSA over TCA and TA while SSA precipitation yielded shorter peptides than just filtering the rumen fluid. The longest peptides in the 10-3 kDa retentate (fraction E) were from the STA precipitation while the shortest were in the filtered sample and the TA soluble with TCA being intermediate. Peptide lengths from TA precipitation were very similar in all fractions to those using SSA. No differences were observed between PC and SSA or PC and TCA in Fractions A though D.

With poor replication in the TCA treatment all fractions contained similar length peptides except peptides in the 10-3 kDa (E) retentate were 33 % longer than those in the >10 kDa (C) retentate. The lengths of the peptides remaining in the soluble NPN (fraction A) from all PPT agents except STA were similar to those in the <10 kDa filtrate (fraction B). Peptides were similar in length between the soluble NPN (fraction A) and the >10 kDa retentate (fraction C) for all PPT agents except TA and the filtered RF which left

Table 2.6. The nitrogen content of the soluble filtrates and peptide chain length in rumen fluid following protein precipitation and further fractionation with 10 and 3 kDa devices (means ± SD). Results are expressed as mg N per g DM and number of amino acid residues, respectively.

TRT[1]	N in Fil[2]	Peptide Chain Length ----------Fractions----------				
		A Sol Fil	B < 10 Fil	C > 10 Ret	D < 3 Fil	E 10-3 Ret
Cent	21.1[a]	132.9[a]	---	---	---	---
	0.3	21.1				
Fil	17.3[b]	24.7[b]	10.7[a]	51.1[a]	5.3[a]	10.2[a]
	0.3	13.1	0.1	9.2	0.3	0.2
Precipitating agent[3]						
TA	3.8[c]	3.3[c]	3.6[b]	8.2[b]	2.9[b]	5.5[a]
	0.1	0.4	0.04	0.1	0.2	0.3
PC	14.9[b]	8.7[c]	6.5[b]	6.5[b]	3.7[a]	ND
	2.5	0.3	3.1	0.1	1.7	
SSA	9.4[d]	5.3[c]	4.3[b]	4.0[b]	2.8[b]	ND
	0.4	0.1	0.5	1.4	0.3	
STA	7.8[d]	25.4[b]	4.7[b]	22.8[b]	4.0[a]	45.2[b]
	0.6	3.0	0.1	3.6	0.5	1.5
TCA	5.4[cd]	10.0[c]	3.9[b]	10.6[b]	4.8[a]	15.4[c]
	0.5	2.9	0.2	7.4	0.01	0.9

[1]Treatments: Cent, centrifuged only; Fil, centrifuged and 1 µm filtered. [2]N in soluble after Cent and Fil or Sol NPN after precipitation. [3]Precipitating agents: TA, tungstic acid; PC, perchloric acid; SSA, sulfosalicylic acid; STA, stabilized tungstic acid; TCA, trichloroacetic acid. [abcd]Means with different letters in same column are significantly different ($P<0.05$) from Cent or Fil. ND, not determined.

peptides longer than 10 kDa. Peptides were of similar length in the >10 retentate and the soluble filtrate after STA precipitation. The sum of the peptides in the > 10 kDa filtrate and < 10 kDa retentate for each PPT agent were not different from those in the respective filtrate (fraction A) except in the TA treatment indicating complete recovery of peptides except for TA.

Peptides were shorter in the <3 kDa filtrate than the soluble NPN when prepared with PC, SSA, STA or TCA but were of equal length prepared with TA. Peptide lengths were similar from the10 kDa device, filtrate and retentate. Peptide lengths from the 3 kDa filtrate were similar when precipitated with either PC or SSA.

However, peptide chains from the 10 kDa device filtrate were shorter than the retentates when using TA or STA. No difference in length of peptides from the 10 or 3 kDa filtrates with STA precipitation were observed but shorter peptides eluted from the 3 kDa device than the 10 kDa device following TA precipitation. Peptides in the 10-3 kDa retentates (E) were significantly shorter than the >10 kDa retentates (C) from filtered and TA precipitation but longer peptides were present in the STA and TCA treatments.

5. Peptide chain length in water-soluble alfalfa silage

The dry matter, ash and N components of the alfalfa silage are given in Table 2.7. Precipitation of the water soluble extract with TA left 34-35 % less N in the soluble NPN than did STA precipitation or filtering the extract through a 1 µm pore filter and 49 % less N than TCA precipitation (Table 2.8) while peptide chain lengths were similar in the filtered soluble extract and the precipitation treatments (Table 2.8). Although not significant, the TCA precipitation left peptides with 2 amino acid residues whereas the 1 µm filtered had 1.5 and TA and STA only free amino acids. Peptide lengths from the filtrate and retentate of the 10 kDa device were single amino acids. Amino acids and peptides constituted 47, 70, 57 and 49 percent, respectively, of the NPN from TA, STA, TCA and filtrate of this alfalfa silage, calculated using the N content of the AA remaining in the NPN.

Table 2.7. Composition of alfalfa silage. Values are expressed as percent of feed, dry matter or total nitrogen.

Component	Value
DM, % feed	30.48
Ash, % DM	10.82
Total N, % DM	4.33
Soluble, % TN	53.76
NH3, % TN	3.33
NDF, % DM	35.76
NDIN, % TN	12.04
ADF, % DM	27.72
ADIN, % TN	4.26

Table 2.8. The nonprotein nitrogen content and peptide chain length in the soluble fraction of water-extracted alfalfa silage following protein precipitation and further fractionation with a 10 kDa device (means ± SD). Results are expressed as percent of soluble nitrogen and number of amino acid residues, respectively.

	NPN	Fraction		
Trt[1]	% Sol N	NPN	<10 kDa Filtrate	>10 kDa Retentate
Cent	ND	1.4	---	---
		0.1		
Fil	83.6[a]	1.5	1.1	1.3
	0.6	0.4	0.1	0.4
Precipitating agent				
TA	48.4[b]	1.1	1.0	1.1
	0.5	0.2	0.2	0.05
STA	82.3[a]	1.2	0.9	1.9
	0.6	0.1	0.03	0.05
TCA	97.0[c]	2.2	1.1	1.2
	5.2	0.8	0.4	0.3

[1]Treatments: Cent, centrifuged only; Fil, 1 µm Filtered and centrifuged water-extract of alfalfa silage; TA, tungstic acid; STA, stabilized tungstic acid; TCA, trichloroacetic acid.
[abc]Means with different letters in same column are significant ($P<0.05$).

6. *Peptide chain length in soluble buffer feed filtrate*

The <10 kDa filtrates from the borate-phosphate buffer eluents from the four selected feeds were not sequentially centrifuged in the 3 kDa units; consequently, both devices fractionated the same peptides. Overall, peptide lengths in the buffer solubles were similar to those in the <10 and <3 filtrates while peptides from the >10 and >3 retentates were not different from the <3 filtrate (Table 2.9); however, individual feeds behaved differently. The peptides in the cottonseed meal soluble were longer than those in the <10 filtrate but shorter than those in the >10 retentate and both fractions from the <3 kDa device; however, both fractions from the <3 filtrate were similar. In the fishmeal the peptides from the < 3 filtrate were the shortest and those in the >3 retentate the longest while the soluble peptides were equal in length to those in the >10 retentate and those in the <10 filtrate the longest. The peptides in the >3 retentate from SBM were the longest while those in the soluble and <3 filtrate the shortest and those in the <10 filtrate and >10 retentate falling between the extremes. The peptides in the <10 filtrate of SoyBest were the shortest and those in the >3 retentate the longest with those in the >10 retentate and <3 filtrate being similar but a third shorter than the >3 retentate. The peptides after fractionation through the 10 and 3 kDa devices were shortest in the soluble fishmeal while the peptides in soluble SBM appeared longest.

Solubilizing SBM for 3 hours in buffer plus addition of sodium azide resulted in no further reduction in size of peptides remaining while 3 hour solublization of SoyBest reduced peptide length 25% over the 1 hour. Peptide lengths in the soluble NPN from stabilized TA from the 1 hour soluble eluent were 2 and 2.8 for SBM and SoyBest, respectively, while filtration through either 10 or 3 kDa ultrafilters generated longer peptides, 2 to 3 times in length.

Table 2.9. Peptide chain lengths in filtrates and retentates of selected feeds following fractionation of borate-phosphate soluble eluents with 10 or 3 kDa devices (n=2).

			Peptide Chain Lengths ----------fractions----------				
Feed[1]	TN[2] % DM	Sol N % TN	Sol eluent[3]	<10 Fil[4]	>10 Ret	<3 Fil	>3 Ret
CotS1	4.24	26.0	6.4[a]	3.0[b]	22.4[c]	13.6[d]	12.0[d]
Fish3	10.60	22.7	3.4[a]	4.8[b]	3.0[a]	1.8[c]	6.3[d]
SBM	8.17	12.2	3.3[a]	4.6[b]	13.2[c]	3.6[a]	17.2[d]
SoyB	7.76	14.3	7.1[a]	4.8[b]	8.5[c]	8.9[c]	12.4[d]
Overall	7.69	18.8	5.1[a]	4.3[a]	11.8[b]	7.0[ab]	12.0[b]
SEM			0.6	0.3	2.7	1.8	1.5

[1]CotS1, cottonseed meal1; Fish3, fishmeal 3. [2]TN, total N; Sol N, soluble N. [3]Sol eluent, phosphate-borate buffer + Na azide, 3 hr. [4]Fil, filtrate; Ret, retentate. [abcd]Means with different letters in same row are significant (P<0.05).

DISCUSSION

1. True Protein -- NPN Assays

From previous analyses in this lab, PC was only 50 % as efficient as STA at chelating TP in Trypticase and SBM when filtered with 1 µm filter. Given this result and the safety concern of using PC in a commercial lab setting, this study only tested TA, STA and TCA to precipitate true protein.

In the feeds tested, STA precipitated the greatest amount of true protein and TCA the least while TA was intermediate when filtered on either 1 or 6 µm filters using the assay as described by Licitra et al. (1996) after the insoluble N was subtracted. Originally, true protein was precipitated from the soluble fraction (Krishnamoorthy et al., 1982). Overall in this study, TA, filtered on 1 µm filters, precipitated more true protein from the soluble fraction, prepared according to Krishnamoorthy et al. (1982) than STA or TCA; however, the recovery of true protein was less than obtained from Licitra et al. (1996) for most of the feeds tested. This quandary raises two important questions with the methodology: should the entire sample be used to precipitate the

true protein or just the soluble fraction? Is protein solubility in borate-phosphate buffer more efficient and symbolic of the rumen environment than water? Given the concentration of precipitating agents like TA, precipitating the soluble components would be more efficient than trying to precipitate the soluble protein when all of the feed material is present. However it is another step in the process and an unlikely step to be conducted by a commercial feed laboratory and given the variability in feeds, this should be a feed fraction measured on a routine basis and not just a feed library lookup. An attempt was made to increase the concentration of a precipitating agent to improve the precipitation efficiency, however, as the concentration of TA increased, the entire liquid phase was transformed into a gelatinous mass that could not be filtered. Thus, this approach was abandoned and not discussed.

2. Trypticase® Peptide Chain Length

Trypticase, containing many short peptides, has been used by many (Licitra et al., 1996) to test the efficacy of the PTP agent. Tungstic acid and STA precipitated significantly more true protein than TCA, SSA and PC when filtered on 1 or 6 µm filters. Peptides were 3 amino acid residues in length in the TA and STA filtrates filtered through 1 µm filters and were not different from those filtered through 20 µm filters by gravity. Duplication using the Licitra et al. (1996) method has been a problem as PTP was pulled through the filter with the soluble NPN if filtered with too strong of a vacuum. The phosphate groups in stabilized TA appear to bind stronger to potential binding sites in feed proteins, thus, improving sample replication but leaving smaller peptides in the NPN fraction. The $-WO_4^{-2}$ ion may also function as a chelating agent attracting amino acids with cationic side chains, i.e., Lys and Arg, in Trypticase.

From the AA sequence of casein, trypsin and chymotrypsin digestion at the carboxyl end of lysine and arginine or phenylalanine, tyrosine and tryptophan, respectively, yielded peptides with 4 AA residues (Chen et al., 1987a). The ratio of hydrolyzed to free AA yielded a peptide length of 3.8 indicating the procedure used to estimate peptide bonds was within 5 % of the expected. Differences in precipitation mechanisms were observed in the study; however, the length of the peptides did not differ. No inferences can be made in the efficacy of precipitating agents and the length of peptides remaining in the soluble fraction from a protein, i.e., Trypticase, with peptide lengths of approximately 4 AA residues plus the precipitate salt or a MW < 800 in the soluble NPN,.

Data not shown indicated that the presence of polyamines (putrescine and mimosine) and carbohydrates (glucose, fructose and pectin) did not hamper the action of PPT agents to precipitate true protein in Trypticase. Also, performing sequential precipitations using TCA followed by stabilized TA or Licitra TA recovered 15 an 29 % more sample N, respectively, while doubling the concentration of the precipitation agents captured 19 and 25 % more sample N, respectively (data not shown).

3. *Precipitation of soluble protein to determine PCL in buffer soluble and soluble NPN.*

Since STA and TA precipitated equal amounts of true protein in the feeds but STA improved replication, the true protein contents in the soluble fractions from the Licitra et al. (1996) assay were precipitated with STA and filtered with 6 µm filters, since the 1 and 6 µm pore size filters yielded equal amounts of PTP for the feeds compared. In these samples STA had a cut off in molecular size of approximately 2 to 3 amino acids. The silage samples contained predominately free amino acids; experimental

error probably explained the trend toward significance in peptide length for one of the alfalfa silages. The differences in the N content of the soluble NPN obtained from the two methods were attributed to the borate-phosphate buffer (Krishnamoorthy et al., 1982) solubilizing more N than did water (Licitra et al., 1996) even though the feeds were only solubilized for 1-hr versus the usual 3. However, it should be noted that in the Licitra method, water and precipitating agent are added simultaneously; the feed protein is not solubilized in water first.

Differences in processing of the two soy products were evident in the peptide length in the buffer soluble fraction. SBM, fed to supply N to the rumen bacteria, has fewer AA in the peptide chain than does SoyBest. Free amino acids and small peptides contribute to the NPN, or Protein A fraction, in ruminant feeding systems and are assumed to be 100 % degraded in the rumen. Silages contain shorter peptides from proteolysis during ensiling while the peptides in SBM and alfalfa hay are longer.

4. Ultracentrifugation fractionation of rumen fluid

To prevent intracellular leakage of AA and peptides from the bacteria and protozoa, the rumen fluid (RF) was centrifuged to remove them and the precipitated true protein was filtered using 1 µm pore size to reduce variability. The lengths of the peptides present in the free amino acid concentrations in the various fractions were low, identification of some questionable and sample duplication poor; therefore, only the standard AA, except Met and Cys, (no ammonia or β-Ala) were included in the peptide chain length determinations and results were calculated on a dry matter basis to maximize concentrations. Free amino acids have been obtained after incubation in a dilute hydrochloric acid solution at room temperature (Heimbeck, W., personal communication) which would stop enzymatic hydrolysis but may cause acid hydrolysis.

Peptides with 3 and 10 AA residues remained in the soluble NPN following TA and TCA precipitation, respectively, concurring with published molecular cut-offs for these PPT agents (Licitra et al., 1996). The exposed side chains of free AA and small peptides in the RF did not bind to the STA which yielded long chain peptides in the soluble NPN and poor duplication.

The longest peptides were expected in the filtrates of the 1 µm filtered RF and the soluble filtrate from the respective PPT agents with the sum of the peptides in the < 10 kDa filtrate and >10 kDa retentate to approximately equal the peptides in the respective filtrate which were obvious in the STA treatment. The peptide lengths observed in the TA filtrate were significantly less than the sum of the 10 kDa filtrate and retentate. Although the free and hydrolyzed AA contents of the 1 µm filtered RF did not duplicate well, the increase in peptide length in the >10 kDa fraction over the < 10 kDa filtrate and the decrease seen in the 3 kDa fraction suggest that MW cut-off ultrafiltration did fractionate RF accordingly. Using an average of 119 for the MW of AA (Creighton, 1993), the MW of the peptides in the 1 µm filtrate, < 10 kDa filtrate, >10 kDa retentate and < 3 kDa filtrate were 2856, 1273, 6081 and 773, respectively. Due to lack of repeatability in the TCA treatment there were no differences in the size of peptides between the filtrate and any of the MW cut-off fractions whereas in the PC and SSA treatments, the respective filtrates contained longer peptides than the < 3 kDa filtrate, indicating the MW cut-off filters worked, but the peptide length in 10-3 kDa retentates were not determined due to lack of sufficient material.

5. Peptide chain length in water-soluble alfalfa silage

True protein was filtered using 1 µm filters since small peptides were expected. Tungstic acid precipitated smaller peptides/amino acids than did STA or TCA so less

N was recovered in the NPN fraction. As the soluble fraction contained single AA there were no differences in peptide chain lengths between any fractions. With single AA constituting the respective filtrates 10 kDa MW cut-off filters were not appropriate. The fermentation process solubilized approximately half of the TN in this silage and hydrolyzed the peptides to FAA with little ammonia.

6. Peptide chain length in soluble buffer feed filtrate

As both MW cut-off devices fractionated the same filtrate, the >3 kDa retentate would also contain the >10 kDa retentate or longer peptides and could be accounted for all feeds except the cottonseed meal. From the 10 kDa information it appears peptides in the soluble fraction of cottonseed are approximately 7 times longer than the < 10 kDa filtrate; however, peptide lengths were approximately equal from both the <3 kDa filtrate and >3 kDa retentate.

Using MW cut-off devices indicate that soluble feed fractions contain peptides of varying lengths. Soybean meal is fed as a source of rumen-degradable N while SoyBest is fed for rumen-undegradable but both soy products appear to have longer peptides in the >10 kDa fraction than in the soluble eluent or < 10 kDa filtrate. Fishmeal 3, processed to supply rumen by pass protein, had shorter peptides in the >10 kDa retentate than in the < 10 kDa filtrate, with the shortest occurring in the < 3 kDa filtrate. Assuming an average MW of 119 per AA, based on the average frequency of AA in 1021 proteins, (Creighton, 1993,), a peptide with 3 amino acid residues would have a MW of 357. A peptide with 13.2 residues would equal a protein with a MW of 1571, representing the peptides from > 10 kDa retentates of fishmeal and SBM, respectively . These peptides are not close to the exclusion limit which could affect their efficacy (Reynal et al., 2007; Butts et al., 1991).

7. *Application to ruminant feeding systems*

Protein and peptide degradation by ruminal microbes do not operate via precipitation mechanisms but are dependent upon composition, length and hydrophobicity. However, small peptides and free AA may stimulate microbial yield and fermentation (Argyle and Bladwin, 1989; Carro et al., 1999). For example, *Prevotella ruminocola* has been shown to utilize peptides with molecular weights of up to 2000 kDa (Pittman et al., 1967). Peptides containing Pro or Gly at the N-terminus degrade very slowly (Pittman et al., 1967; Broderick et al., 1988; Yang and Russell, 1992) due to the conformation of the AA side chains. Trialanine and oligopeptides of alanine degrade faster than di-alanine (Broderick et al., 1988). Hydrophobic peptides which are soluble in 90 % isopropyl alcohol and contain an abundance of phenolic and aliphatic AA are deaminated at half the rate of hydrophilic peptides which are precipitated by this concentration of alcohol and contain highly charged AA (Chen et al., 1987b).

Recent data sets (Hristov et al., 2001; Choi et al., 2002a,b; Volden et al., 2002; Reynal et al., 2007) demonstrate that part of the soluble dietary N is not degraded in the rumen and contributes between 5 and 17 % of the total AA flow to the duodenum of the cow (Van Amburgh et al., 2007). Based on data from MW cut-off devices, the omasal flow of AA from a diet with SBM as the protein source was 242 g/d (Reynal et al., 2007) or 35.67 g N/d, assuming an average of 0.1474 g N/g AA. If the NPN was measured by TCA precipitation, in keeping with other research from this group (Brito et al., 2007), then precipitating TP from the alfalfa and SBM with STA would reduce the AA N flow in the soluble fraction by 0.9 g while TA precipitation would reduce it by 0.8 g. Molecular weight cut-off fractionation generates more concise *in vivo* data and may be appropriate to detect the size of peptides in water or buffer solubles of feed but is not conducive to routine use.

To more accurately describe the soluble pool, amino acids and peptides should be in a separate pool and contribute to the protein supply while ammonia and other non-amino acid N remain in the NPN pool (Van Amburgh et al., 2007) in ruminant feeding systems, i.e., Cornell Net Carbohydrate and Protein Model (Sniffen et al., 1992; Tylutki et al., 2008). The contribution of endogenous N (EN) to the soluble pool flowing from the rumen must also be defined. Marini et al. (2008) noted that rumen microbes do not favor feed N or EN for microbial protein synthesis and 34.9 % of the total N entering the duodenum was from either free EN or microbial N that originated from EN. Ammonia levels should be measured in the soluble feed fraction and true protein precipitated from the soluble fraction using TA filtered on 1 µm filters so the difference will more accurately reflect the amino acid/peptide contribution to the soluble N pool.

CONCLUSIONS

The efficiency of the precipitated true protein (PTP) assay was improved with TA or STA filtered on 1 or 6 µm filters using the Licitra et al. (1996) method for all feeds tested except a fishmeal. The fishmeal has been processed specifically to supply rumen escape protein, which yielded more true protein from precipitation of the buffer soluble (Krnishnamoorthy et al., 1982) with TA and filtered with 1 µm filters. True protein precipitation and replication of Trypticase was improved using TA or STA filtered on 1 or 6 µm filters with similar peptide lengths in all treatments due to the chemical nature of Trypticase. Using MW cut-off devices to separate peptides in rumen fluid after filtration on 1 µm or STA precipitated exhibit differences in the filtrate and retentate while precipitation with TA, TCA, PC or SSA creates similar length peptides. However, fractionating the buffer or water soluble with a MW cut-off filter could provide an approximation of length of the peptide seen by the ruminal

microbes. Free amino acids compose ~50 % of the NPN from highly soluble feeds, i.e., alfalfa silage, while 18% of total N in high N feeds commonly fed to ruminants is comprised of short peptides in the soluble N pool. Although not as efficient for an analytical laboratory, precipitating the buffer soluble fraction with TA and filtering on 1 µm filters may better represent the NPN and amino acid/peptide pools that will more accurately reflect the supply of N sources for ruminal microbes and supply the cow with overlooked sources of metabolizable protein.

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Chapter 3

COMPARING DIGESTIBILITY OF FEED AMINO ACIDS, DRY MATTER AND FIBER AFTER *IN SITU* OR *IN VITRO* RUMINAL FERMENTATION WITH *S. Griseus* INCUBATIONS AND IN VITRO INTESTINAL DIGESTION OF ACID DETERGENT RESIDUE FOLLOWING IN SITU OR IN VITRO RUMINAL DIGESTION IN BAGS OF THREE PORE SIZES

ABSTRACT

The rumen degradability of feed proteins has been measured using *in situ* (IS), *in vitro* (IV) and protease procedures but without comparative amino acid (AA) contents of the residues, while the acid detergent (AD) residue is considered indigestible. As ruminant feed analysis schemes move forward, assays employed should provide residues that result in appropriate pools to predict AA availability including those in the acid detergent insoluble nitrogen (ADIN) fraction. Thus, the objectives of this study were (1) to evaluate the AA contents of the residues of twelve feeds following 24-h IS or 24-h IV fermentations and a comparative *S.griseus* (SG) incubation to correspond with N degraded in IV and (2) to assess the intestinal digestibility of AA content of AD following 12- or 24-h IS or IV ruminal digestion in three pore size bags. Parallel IV and SG incubations without feed were run and analyzed for AA content which were subtracted from the respective incubations in an attempt to correct for microbial and protease contributions, respectively. To correct for microbial contamination in the IS and IV residues purine bases were analyzed by HPLC and used to adjust the AA content. Residues from these incubations were analyzed for AA content using HPLC after acid hydrolysis. The N content (g/g DM**;** mean ± SD) of the feeds were as follows: alfalfa forages, 0.0359 ± 0.0032; corn

silages, 0.0127 ± 0.0014; soy products, 0.0791 ± 0.0039. The results demonstrated significant differences in DM, N and AA values area obtained when IS, IV and SG incubations were performed on the same samples, even with the variation within feeds. It was apparent that protease cleaved the N specifically without degrading the cell wall which resulted in a different ratio of AA to carbohydrate and other dry matter in the residues. This effect was demonstrated by lack of change in digestions of neutral detergent (aNDF) and acid detergent fiber (ADF) by the SG incubations from the un-incubated feeds. This observation was very prominent in the corn silages in which the protease degraded 57.6 % of the N but only 17.7 % of the DM while the IV incubation degraded more DM than N. This relationship also held for the alfalfa and soy products. However, the IS incubation degraded more N than DM in the alfalfa and corn silage while the reverse was observed in the soy products. Based on these results we conclude that the *S.griseus* procedure is of limited value if the AA profile of the residue is required to improve our ability to predict the flow of AA in the ruminant.

Keywords: *in situ; in vitro; S. griseus*; amino acids; protein degradation.

Abbreviations: AA, amino acid; ADF, acid detergent fiber; ADIN, acid detergent insoluble nitrogen; DM, dry matter; EAA, essential amino acid; IS, *in situ;* IV, *in vitro* ; N, nitrogen; aNDF, neutral detergent fiber assayed with heat-stable amylase ; NDIN, neutral detergent insoluble nitrogen; SAS, Statistical Analysis Systems; SG, *S.griseus;* TN, total nitrogen.

INTRODUCTION

Meeting protein requirement of ruminants is more complex than just supplying crude protein (CP) as two requirements must be satisfied: first for rumen microbial synthesis and second for the animal. Dietary CP yields rumen degraded protein (RDP) for microbial synthesis and rumen undegraded protein (RUP) which eludes microbial breakdown and escapes to the small intestine (SI) where it is digested or appears in the fecal material (NRC, 2001).

The proportion of CP that results in RDP and RUP in any feedstuff depends on the properties of the feed and on the characteristics of the animal consuming the protein (Broderick, 1995). Proteins are large molecules that differ in size, solubility, structure, amino acid (AA) composition and function (NRC, 2001). The cross linkages of disulfide bonds that form both within and between protein molecules and covalent bonds between proteins and carbohydrates as a result of processing or heating, or those occurring naturally, have a profound effect on the tertiary structure which affects solubility and degradability.

The metabolizable protein (MP) or metabolizable AA feeding standard for cattle and sheep, as presented by Burroughs et al (1971, 1974ab, 1975), was developed as a result of the problems generated with the increased feeding of urea to beef cattle. The MP represents the 'true' protein reaching the SI for digestion and absorption and deals directly with the requirements of the animal body for AA and the fulfillment of those requirements by absorbable AA arising from RUP, microbial protein escaping the rumen and endogenous CP from enzymes, sloughed intestinal cells and saliva (Burroughs et al., 1974a). Ruminant feeding systems that balance for MP (NRC, 2001; Sniffen et al., 1992) are restricted by the limited information available in determining the portions of RDP and RUP of feedstuffs. The amount of MP reaching the SI is dependent on the rate and extent of digestion of the feedstuffs

in the rumen. The rate of feedstuff degradation in the rumen can have a profound effect on fermentation and end products: 1) if the rate of protein degradation exceeds the rate of carbohydrate fermentation, large quantities of nitrogen (N) can be lost as ammonia; 2) if the rate of carbohydrate fermentation exceeds protein degradation, microbial protein production can decrease; 3) if degradation rate is slow, some of the feed may escape ruminal fermentation and pass directly to the SI; or 4) if feedstuffs are degraded slowly, rumen fill will decrease intake (Nocek and Russell, 1988).

The relative rates of fermentation and passage compete to determine the fate of a feedstuff. It is assumed that the fermentation rate is an inherent property of the feedstuff (Russell et al., 1992). Passage rate, or transit time of undigested residues through the digestive tract (Van Soest, 1994), is a function of feed and water intake, feed processing and the type of feed consumed. Passage of potentially degradable substrates from the rumen can affect nutrient availability and can have a profound effect on the balance of fermentation and products (Russell et al., 1992).

The efficiency of converting RDP to microbial protein is considered to be 100% by most feeding systems, but this estimate includes the recycling of N via urea in saliva and portal blood, sloughed mucosal cells and ruminal secretions (Jones et al., 1996). Using data from lactating dairy cows, Lapierre and Lobley (2001) examined the ratio of urea-N synthesis to digestible N and achieved 'recoveries' close to, or in excess of, 100% (43 to 123%; mean 88%) which demonstrates that endogenous N must be contributing to the N supply. Lapierre and Lobley, (2001) stated that as much as 40% of the ammonia absorbed from the gut is derived from endogenous urea-N and forms part of a cycle that conserves N within the body. In another study, endogenous N secretions represented 30 % of total digestive tract protein synthesis but were not affected by of the level of dietary fiber (Ouellet et al., 2002). Recent data suggests

that recycled N is relatively constant even with substantial changes in N intake; however, the efficiency of use of recycled N is a function of total N intake (Marini and Van Amburgh, 2003).

The true value of protein in feedstuffs is the AA content reaching the SI as free AA or small peptides which are available for absorption. Due to the complexity of protein digestion in ruminants there is no clear-cut procedure that represents AA availability from ruminant feeds. Research has focused mainly on the quantity of protein undegraded in the rumen using *in situ* or *in vitro* techniques with little emphasis on the availability of individual AA in the feed ingredients.

Pichard and Van Soest (1977) divided protein into five fractions: A, B1, B2, B3 and C with the fractionation scheme based on the solubility of N containing compounds in water, borate-phosphate buffer or the detergent system. It is thought that this scheme adequately represents both ruminal and post-ruminal feed behavior (Pichard and Van Soest, 1977). The solubilities of the five fractions are shown in Figure 3.1. Fraction A is nonprotein nitrogen (NPN). The NPN (ammonia, peptides and AA) is rapidly converted to ammonia in the rumen and is soluble in borate-phosphate buffer. The B fraction or true protein is fractionated into three parts based on the relative rates of ruminal degradation. B1, true soluble protein, is rapidly degraded in the rumen. Some of B2 is fermented in the rumen and some passes to the lower tract. B3 is slowly fermented in the rumen and is digested mostly in the SI. Fraction C is considered unavailable or bound and lost in the feces.

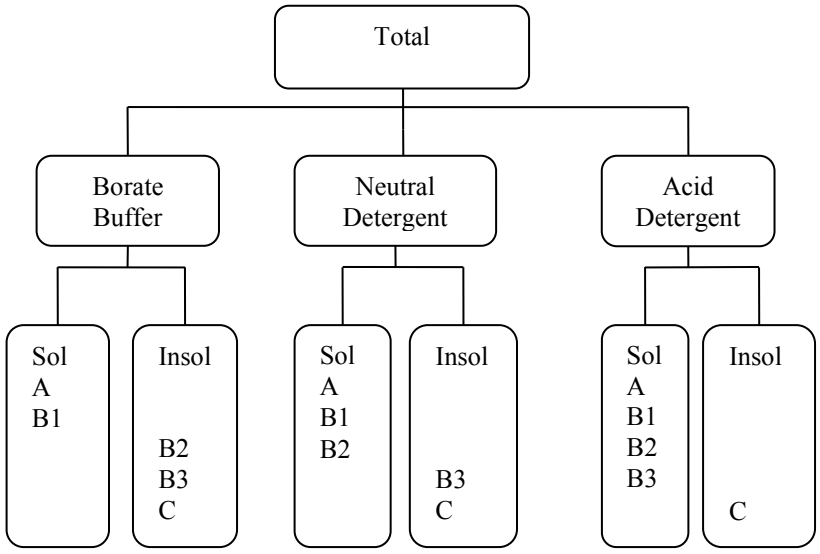

Figure 3.1. Analysis of protein fractions using borate-phosphate buffer, neutral detergent and acid detergent (Pichard and Van Soest, 1977).

Various methods have been utilized to determine the availability of protein and AA to the cow including the *in situ* bag technique, ^{15}N-labeled procedures using $^{15}NH_3$ of roughage grown with ^{15}N-fertilizer and various *in vitro* and enzymatic procedures. For the *in situ*, or mobile bag,technique, an expensive and labor intensive undertaking, feed samples are placed in nylon or polyester mesh bags and incubated in the rumen of fistulated cows for specified times, with emphasis on the degradablility of dry matter (DM) or CP but not AA (Nocek, 1988). More recently, investigators using animals with ruminal and duodenal cannulas, placed the ruminally incubated samples in the duodenum and collected the bag and degraded feed residue in the feces to determine whole tract protein or AA degradation (mobile bag technique; Boila et al., 1992, 1995; England et al., 1997; Erasmus et al., 1994; King et al., 1990; O'Mara et al., 1997; Von Keyserlingk et al., 1996, 1998).

Data obtained using the mobile bag technique must be critiqued carefully. Many sources for variation exist, including sample size to bag surface area ratio, bag porosity, feed particle size, order of bag insertion, number of animals used, basal diet of the fistulated animals and bacterial contamination (Vanzant et al., 1998; Nocek, 1988), making standardization of the procedure almost impossible. Bacterial contamination would underestimate feed digestibility while small particles washing out of the bag would overestimate it.

The mobile bag study of Erasmus et al. (1994) included several feeds with high RUP. If the RUP was associated with the cell wall, or the B3 fraction, and not degraded in the SI, then the protein was bound and unavailable to the animal. They emphasized that feeding systems must take this into account in calculating the quantity of protein digested from RUP sources. However, ruminant feeding systems (NRC, 2001; Sniffen et al., 1992) assign an 80% digestibility in the SI to the B3 protein fraction as it is considered to be slowly digested. Additionally, the Erasmus et al. (1994) data indicated branched-chain AA seemed to be more resistant to microbial degradation in the rumen while methionine degradation in the rumen appeared to be dependent on the feedstuff. The group observed that, within feedstuffs, rumen degradation influenced which specific AA were presented to the SI for absorption more than did the SI digestion process. They concluded that feeding systems should incorporate the AA values for the RUP fraction or the absorbable AA profile into their program since the RUP can influence the AA profile in the SI digesta. Did the diaminopimelic acid (DAPA) correction adequately account for the microbial contamination in the rumen and fecal residues in their study? Crooker et al. (1987) compared the AA composition of several feeds to the residue after a12-h incubation in the rumen and detected some DAPA present in the original feedstuffs which had never been exposed to the microbes in the rumen.

Using the mobile bag technique, O'Mara et al. (1997) found that the intestinal disappearance of AA ranged from 84 to 93% depending upon the feed and confirmed that the AA profile of feed residue reaching the SI is different from the original feed AA composition while the N recovered as AA N in the original feedstuffs ranged from 73 to 84 %. Von Keyserlingk et al. (1998) evaluated nineteen grass silages to determine the AA content of the *in situ* residue and found no reliable relationships between the concentration of individual AA in the silages and the concentrations of AA in the 12-h rumen residue, the mean rumen retention time of grass silages in a high producing dairy cow. Thus, AA in grass silage were degraded at different rates making the AA composition of the residues different from AA composition of the original silage which indicates the mix of AA is not homogeneous but heterogeneous. Miranda et al. (2003b) reached the same conclusion with the AA content of tropical forages and their residues after incubation in the rumen for 18 h.

Calsamiglia and Stern (1995) used the *in situ* bag technique as a first step and proceeded with two additional *in vitro* steps to closely simulate physical conditions in the ruminant as an alternative to duodenal cannulated animals for intestinal digestion. The dried *in situ* residues were subjected to a hydrochloric acid (HCl) and pepsin predigestion prior to pancreatin digestion. This three-step assay was validated and optimized against duodenal samples obtained from an *in vivo* intestinal digestion study using the same protein sources. The results from the pancreatin digestion assay were highly correlated ($n = 34$; $r = 0.91$, $P < 0.001$) to estimates of the *in vivo* intestinal protein digestion (Calsamiglia and Stern, 1995). Using the three-step procedure Mirandi et al. (2003a) studied the intestinal digestibility of CP and AA from tropical forages. The results demonstrated that the intestinal digestibility of the CP did not always predict the intestinal digestibility of individual AA. Rumen incubation increased the amount of CP digested for some proteins while it

had no effect on others indicating that there are characteristics of some protein sources that are refractory to microbial activity. Recently, Gargallo et al. (2006) utilized the Daisy^{II} incubator (Ankom Technology, Macedon, NY) for *in vitro* intestinal digestion to simplify the three-step procedure.

Besides the three-step (Calsamiglia and Stern, 1995) and the modified three-step procedures (Gargallo et al., 2006), ruminant intestinal digestion of AA in various soy products, distillers dried grains, and fishmeals were recently estimated using the precision-fed cecectomized rooster bioassay (Boucher et al., 2009a,b,c) and immobilized digestive enzyme assay (IDEA; Boucher et al., 2009c) compared intact feed and rumen undegraded residue. In the rooster bioassay true digestibility was determined. However, by comparing the rumen undegraded residue with the intact feed it was apparent that a protein fraction in these feeds was intestinally digested only after ruminal incubation. The IDEA (Novus International, Inc., St. Louis, MO) measured protein digestibility, and AA indirectly, in soybean meal, meat and bone meal or poultry by-products after solubilization, hydrolysis and quantification with o-phthaldialdehyde. Amino acid digestibility was calculated from equations supplied by Novus International, Inc. to determine digestibility of Lys. Digestibilities of several AA (Arg, Cys, Ile, leu, met, Thr, Trp and Val) were then calculated based on predicted Lys digestibility (Boucher et al., 2009a)

Feed degradation has also been estimated using *in vitro* anaerobic fermentation with rumen fluid in Erlenmeyer flasks in a 39°C water bath (Goering and Van Soest, 1970). The Daisy^{II} incubator (Ankom Technology, Macedon, NY) offered another variation for *in vitro* ruminal digestion. An alternative *in vitro* procedure used limited substrate (England et al., 1997; Broderick, 1978; 1987) and soaked proteins in dialyzed rumen fluid which removed most of the background NH_3 and soluble AA. The inhibitors, 1.0 mM hydrazine and 30 ug chloramphenicol/ml, were added to

suppress bacterial uptake of NH_3 and total AA allowing quantitative recovery of protein escaping ruminal degradation. However, when compared with uninhibited ruminal inoculum Using $^{15}NH_3$ to quantify microbial uptake of protein breakdown products for growth, mean degradation rates averaged 28% greater (Hristov and Broderick, 1994) and RUP estimates for alfalfa hay and silage were 18 to 59 % greater (Peltekova and Broderick,1996) demonstrating the *in vitro* inhibitor method did not behave physiologically.

Other investigators (Pichard and Van Soest, 1977; Roe et al., 1990; 1991; Krishnamoorthy et al., 1983; Licitra et al., 1998; 1999; Coblentz et al., 1999) used *in vitro* protease assays to determine the RDP or soluble protein fraction of feeds. Using this procedure Pichard and Van Soest (1977) showed the B fraction has two degradation rates. Krishnamoorthy et al. (1983) increased the pH of the buffer from 6.5 to 8, used a fixed sample weight (0.5 g) with 0.33 enzyme units/ml incubation media and stopped the incubation at 18 h for grains and after 48 h for forages, which represent mean retention times in the rumen, for 12 mixed diets. Roe et al. (1990; 1991) used a constant substrate-to-enzyme ratio (0.2 g CP to 0.33 enzyme units/ml buffer) and decreased the incubation time for forages to 30 h. When compared to *in situ* data on the same diets, the protease *in vitro* data could not accurately predict protein degradability, apparently due to insufficient degradation by the *S. griseus* enzyme (Roe et al., 1990; 1991).

Licitra et al. (1998) maintained the ratio of enzyme to true protein constant at 13.4 enzyme units/true protein by adding the same amount of sample (0.5 g) and varying the amount of enzyme. When compared, the methods of Krishnamoorthy et al. (1983) and Roe et al. (1990; 1991) were significantly different than those of Licitra et al. (1998). The degradable N obtained from the constant ratio of enzyme to true protein in the sample was significantly higher ($P > 0.01$) than those obtained from the

fixed enzyme while the effect of buffer pH (6.7 vs. 8) was significantly different at P > 0.05 with pH 8 yielding higher degradable N (Licitra et al., 1998). In another experiment, Licitra et al. (1999) examined the effects of enzyme concentration (0.33 to 6.6 enzyme units/ml) and incubation time (0 to 48 h) on protein degradation. Most of the enzyme-time combinations gave high correlations with the *in situ* values on the same feeds but high enzyme concentration and longer times tended to over digest while low concentrations and shorter times to under digest.

Other assays have been evaluated to determine a procedure to predict the MP supply from feeds. Coblentz et al. (1999) compared *in vitro* protease assays with *in situ* to compare RDP using a single time point of 48 h with an enzyme concentration of 0.066 enzyme units/ml media and the RDP estimates from the *in vitro* assay closely agreed with those obtained from the *in situ*. Further, Cone et al. (2002) compared the RUP in 26 concentrate feeds from *in situ* to those from an *in vitro S. griseus* protease assay and found the highest correlation was observed after 1 h of incubation (R^2 = 0.77). Recently Cone et al. (2004) determined the RUP in six grasses and 16 grass silages using *in situ* and an *in vitro* protease assay and observed the highest correlation occurred at 24 h incubation (R^2 = 0.71). It should be noted however that 24 h was their longest time point. The *in vitro* protease assays do not biologically represent rumen conditions as they do not account for a lag time, keep pH approximately constant and lack a passage rate but under the right assay conditions can represent the RDP of feeds.

Use of the *in situ*, either alone or in combination with an *in vitro* assay, measuring the partitioning of ^{15}N labeled forages and the *in vitro* protease assay to measure soluble (degradable) protein all are met with many advantages and disadvantages. To date no method exists to readily measure protein digestion of a feedstuff to

provide estimates of AA for absorption in ruminants. As mentioned earlier, the C fraction, or acid detergent fiber, content of feed is considered undigested; however, do the amino acids in the ADIN remain intact following ruminal and intestinal digestion? The objectives of this study were two-fold. (1)To compare the AA digestion of alfalfa, corn silage and soy feeds following 24-hr *in situ* or *in vitro* fermentation with rumen fluid to assays using *S. griseus* incubation. Additionally, the residues were analyzed for neutral detergent (aNDF) and acid detergent (AD) fiber plus the corresponding insoluble nitrogen (NDIN, ADIN) to compare the remaining matrix of carbohydrate and protein. (2)To assess the effect of *in vitro* intestinal digestion on amino acid digestibility of feed ADIN following 12- or 24-h ruminal exposure either *in situ* or *in vitro,* using a Daisy^{II} incubator, of animal and soy proteins.

MATERIALS AND METHODS

Unless specified otherwise, all analyses were conducted on duplicate samples.

1. In situ or in vitro fermentation versus S. griseus incubation

Samples: Twelve feeds: four alfalfas (3 silages and a hay), four corn silages and four soy products (solvent extracted soybean meal, SoyPlus (West Central Soy, Ralston, IA), AminoPlus (Ag Processing, Inc., Hastings, NE) and SoyBest (Grain States Soya, Inc., NE)), were utilized. Forage samples were freeze dried (48 h with shelf temperature at 24º C; VirTus 20 SRC-X; The VirTus Co., Inc., Gardiner, NY). All samples were ground through a 1 mm screen in a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA).

Incubations: Parallel blank incubations were carried through IV and SG incubations and samples adjusted accordingly.

In situ (IS): *In situ* bags (5 x 10 cm nitrogen-free polyester with 50 ± 15 micron pore size; Ankom R-510; Ankom Technology, Macedon, NY) containing 5 g sample were placed in two mesh bags and inserted into the rumen of nonlactating cow (~600 kg BW consuming a good-quality grass hay) for 24-h. Residues in bags were rinsed in tap water until runoff was clear and dried in a 55ºC oven for 48-h.

In vitro (IV): Fermentations of half gram samples were held under anaerobic conditions at 39°C for 24-h in a buffered medium(Goering and Van Soest, 1970) containing Trypticase and rumen fluid from cow used for IS strained through 4 layers of cheesecloth, glass wool and a 100 µm cloth (#34-1800-04; PGC Scientifics, Frederick, MD). The IV residues were transferred quantitatively with water to 250-ml centrifuge bottles and centrifuged in a Beckman JA14 rotor (Beckman-Coulter, Inc., Fullerton, CA) at 11,100 x g, 10°C for 20 min; the supernatant was discarded. The residues were rinsed with 75-ml water, centrifuged and the rinse repeated. The residues were then transferred quantitatively with water to preweighed crucibles containing a known amount of acid-washed celite and dried at room temperature until a constant weight was obtained. After weighing, the residue and celite were ground with motor and pestle to obtain a homogeneous mix.

S. griseus (SG): The procedure of Licitra *et al.* (1998) was followed. The assay utilized a phosphate-borate buffer (pH 6.7) and 1 EU enzyme (Sigma P5147; Sigma Chemical Co., St. Louis, MO) per ml based on true protein content of feed (Licitra et al., 1996). The time points for the enzyme assay were selected to correspond to the protein degradation observed in the IV fermentation. The incubation times for the protease at 39°C were predetermined to be 0.5-h for the forages and 10-h for the soy products, the time point closest to the 24-h *IV* nitrogen degradation. The residues after SG incubation were filtered into crucibles as the IV, rinsed with 250-ml water and dried as the IV residues.

Chemical analyses: Dry matter (DM) and N were obtained on all samples. Dry matter was determined at 106ºC in a forced-air oven for 48 h and N was measured by a modification of AOAC method 984.13 (1990) using boric acid in the distillation step (Pierce and Haenish, 1940). Purines (Makkar and Becker, 1999) were used to correct for microbial AA (Clark *et al*., 1992) contamination in the IS and IV residues with recoveries of yeast RNA averaging 95 %.

Neutral detergent with amylase (aNDF) and acid detergent (ADF): Residues from IV and SG incubations were transferred quantitatively with aNDF solution to beakers while aliquots of the dry IS residue corresponding to half gram of the original sample were weighed into beakers and 50 ml aNDF or ADF solution added. *S. griesus* residues for ADF were filtered into preweighed crucibles with celite and boiled in ADF solution. IV residues for ADF were centrifuged as above and then transferred to beakers with ADF reagent. The aNDF with α-amylase and ADF (Van Soest et al., 1991) residues were analyzed for insoluble N.

Amino acid hydrolyses: Two-mg N aliquots of each residue were acid hydrolyzed at 110ºC for 21-hr in a block heater (Gehrke et al., 1985) with 5-ml 6 M HCl after flushing with N_2 gas. Hydrolysates were filtered on Whatman 541 filters and diluted to 50-ml with water. One ml aliquots were evaporated, redissolved in 1 ml water, evaporated again, repeated two more times to remove the acid and dissolved in 2 ml sample buffer for analysis. Tryptophan and sulfur AA were not determined. Norleucine, an internal standard, was added to samples prior to hydrolysis to yield ~62.5 nM/ml in the analyzed sample. All samples were treated similarly.

Amino acid analysis: Amino acids were separated on a lithium cation exchange column using a three-buffer step gradient and column temperature gradient. Detection was at 560 nm following ninhydrin post column derivation on an HPLC System Gold with 32 Karat software (Beckman-Coulter, Inc., Fullerton, CA).

Standards (250 nM/ml) for Asp, Thr, Ser, Glu, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe, NH_3, Lys, His, Arg and Cys (125 nM/ml) were prepared by diluting a purchased stock (Amino acid standard H, #20088; Pierce Chemical; Rockford, IL) with the sample buffer. Norleucine standard (250 nM/ml) for was prepared in sample buffer and combined with the others. The volume of samples and standards loaded on the column was 50 µl.

Calculations: Digestibility was calculated as a fraction of component (DM, ND, AD) in whole feed using the following equation: digestible fraction = 1- (residue/whole feed). For N digestibility of the respective fractions, the following equation was used and adjusted for the DM digestibility of the respective component: digestible N = 1 – ((residue DM * N) / (Feed N, NDIN or ADIN). If residue to whole fraction was greater than one, digestibility was zero. Amino acid digestibility was expressed as a percent of the amino acid content of the feed.

2. *In vitro intestinal digestion of amino acids in ADIN following 12- or 24-h ruminal digestion*

Samples: Five commercial feeds: Poet HP-DDG and Basic DDG (Venture Milling Co., Inc, Seaford, DE), Mercer Soy (Mercer Landmark, Inc., Coldwater, OH), SoyBest (Grain States Soya, West Point, NE) and ruminant fishmeal (Sea-Lac; Omega Protein, Houston, TX) plus a corn silage and alfalfa silage were utilized. The silages were freeze dried and ground as above; the commercial feeds were used as received and not ground. Half gram samples were weighed into prepared bags and sealed.

Incubation bags: Three pore size (µm) bags: *in situ*, 50 ± 15 (Ankom Technology, Macedon, NY); Fiber, 25 (Ankom F57 material; Ankom Technology) and Mesh, 15 (Ankom PETP monofilament; Ankom Technology) were utilized for all incubations. Bags were 5 x 5 cm from respective materials. The sample size to bag surface area

of 10 mg feed per cm^2 was within the range (10-20) recommended by Mertens (2004). Vanzant et al. (1998) recommended ~10 mg per cm^2 for *in situ* while Adesogan (2005) utilized a range of 10.1 to 11.4. Fiber bags were rinsed with acetone (4 min) and air dried prior to adding sample to remove the surfactant which inhibits microbial fermentation

Incubations: To control for within feed bias in the *in vitro* ruminal and intestinal digestions, all bags for a feed were incubated at the same time with the 4 jars in an incubator split between the two time points and bags for a time point divided between 2 jars or separated by ruminal digestion and time point, respectively. Empty bags for each pore size were included for both incubations and time points to correct to correct DM (N) and AD for microbial contamination. The number of bags per incubation was in line with Ankom specifications.

Ruminal degradation: Ruminal incubations were performed simultaneously *in situ* and *in vitro* using a Daisy^{II} incubator for 12 and 24 hours.

In situ (IS): Sample bags were split between two lactating cows with each time point in separate mesh bags but inserted simultaneously. Both cows consumed the standard farm TMR.

In vitro: For each feed the sample bags to measure 12-h degradation were divided between two vessels of a Daisy and the 24-h bags were placed in the other two. The procedure was a modification of the Ankom protocol. Briefly, the buffered medium used in Part 1 was utilized and left to reduce with CO_2 purging while the rumen fluid was collected and the IS bags inserted. Rumen fluid from the cows used for IS was strained as in Part 1 and 400 ml added to each vessel. Vessels were capped with CO_2 purging, placed in incubator at 39°C and rotated for the appropriate time.

After completion of ruminal digestion, bags were removed, rinsed in cold tap water until it ran clear (De Boer et al., 1987) and drained. Bags for ruminal

degradation determination were dried overnight at 106°C and weighed. Bags for sequential intestinal degradation were frozen at -20°C by feed.

Intestinal digestion: Thawed bags from one feed were divided among four Dairy jars and intestinal digestion was performed following the method of Gargallo et al. (2006). Briefly, bags were incubated in 0.1 N HCl (2L) containing pepsin (1 g/ L; P7000, Sigma, St. Louis, MO) for 1-h with constant rotation at 39°C. Fluid was drained, bags rinsed with tap water followed by incubation in prewarmed potassium phosphate buffer (pH 7.75) containing pancreatin (3g/L; P7545, Sigma) and 50 ppm thymol for 24-h. Bags were rinsed until tap water ran clear and dried overnight at 106°C, except those for AA were dried at 55ºC for 24-h.

Acid detergent fiber: Acid detergent (AD) was determined in bags using the Ankom Fiber apparatus (Ankom^{200}, Ankom Technology Corporation, Macedon, NY) and analyzed by feed. Acid detergent insoluble N (ADIN) was measured following hot weighing duplicate bags at 106ºC. Amino acid contents were determined on residues after drying at 55ºC as above.

Chemical analyses: Residual nitrogen (N) was measured by block digestion and steam distillation with automatic titration (Tecator Digestor 20 and Kjeltec 2300 Analyzer, Foss Analytical AB, Höganäs, Sweden; AOAC 2001.11). Results were expressed as percent of N to avoid assuming all protein contains 16% N.

Amino acid hydrolysis and analysis: Hydrolysis and analysis were done as above. Only hydrochloric acid hydrolysis was performed; consequently, methionine, cystine and tryptophan digestions were not determined.

Calculations: Digestibility was calculated as a fraction of component (DM and AD) in whole feed as in Section 1 except ruminal and total or cumulative (ruminal plus intestinal) degradations were calculated for each point. As above, DM digestibility of the respective fractions was used to adjust for the N digestibility of the respective

component. Additionally, intestinal pool size was calculated as follows: Intestinal pool size = ((Total dig – Rum dig) / (1-Rum dig)). If the ratio, residue to whole fraction was greater than one, digestibility was considered zero. Amino acid digestibility was expressed as a percent of the amino acid content of the feed or feed AD.

Statistical Analysis: Data were analyzed using the GLM procedure of SAS version 9.1 (SAS Inst., Inc., Cary, NC): $Y_i = \mu + T_i + \varepsilon_i$, where Y_i is the dependent variable, μ is the overall mean, T_i is the effect of treatment i and ε_i is the residual error. Least squares means within feed group were compared using contrasts when significance was observed in the analysis of variance test. Significance was considered when ($P < 0.05$). DUNCAN's multiple range comparison was used to test significance of bag pore size of feed N and feed ADIN α-amino N following ruminal and intestinal digestion.

RESULTS

1. In situ or in vitro fermentation versus S. griseus incubation

The aNDF, ADF, total nitrogen (TN), NDIN, ADIN and essential amino acid, except methionine and tryptophan, contents of the feeds are listed by feed group (mean ± SD) in Table 3.1 with results expressed as percent of dry matter or TN, respectively. Although variation of total nitrogen within a feed group was small, neutral and acid detergent insoluble nitrogen as a percent of the total N exhibited considerable variation in the three feed groups.

Table 3.1. The chemical composition of alfalfa, corn silage and soy (n = 4 per group; mean ± SD). Results are expressed as percent of dry matter or total nitrogen.

	Alfalfa	Corn silage	Soy products
aNDF[1], % DM	33.87 ± 6.34	47.38 ± 2.61	17.67 ± 6.43
ADF, % DM	25.01 ± 3.14	26.43 ± 3.06	9.18 ± 1.30
TN, % DM	3.59 ± 0.32	1.27 ± 0.14	7.91 ± 0.39
NDIN, % TN	26.57 ± 11.70	18.4 ± 2.44	66.06 ± 32.87

ADIN, % TN	8.86 ± 2.43	14.39 ± 2.95	33.79 ± 16.56
EAA, % DM			
Thr	9.59 ± 0.17	2.57 ± 0.12	22.49 ± 1.15
Val	11.60 ± 0.51	3.73 ± 0.15	21.13 ± 1.58
Ile	9.61 ± 0.23	2.89 ± 0.13	20.09 ± 1.61
Leu	16.53 ± 0.29	6.60 ± 0.18	40.00 ± 2.36
Phe	10.32 ± 0.17	2.56 ± 0.24	25.28 ± 1.55
Lys	10.42 ± 0.75	1.62 ± 0.11	31.30 ± 1.85
His	4.05 ± 0.24	0.77± 0.06	13.04 ± 0.82
Arg	9.91 ± 0.38	1.23± 0.12	37.80 ± 2.64

[1]aNDF, neutral detergent fiber with amylase; ADF, acid detergent fiber; TN, total nitrogen; NDIN, neutral detergent insoluble nitrogen; ADIN, acid detergent insoluble nitrogen.

Dry matter and nitrogen digestibility: Overall, DM digestibility (Table 3.2) was highest following *IS* incubation with approximately 100 % more digested than by SG. The aNDF digestibility was similar between *IS* and IV incubations which was 8 times greater than SG. Acid detergent fiber digestibilities were similar between the IS and IV treatments but were 300 % greater than SG. Total N digestibility was similar between all incubations. Digestion of NDIN was highest in the IS treatment and the IS and IV incubations digested more NDIN than did SG. The highest ADIN digestion occurred in the SG, and the SG and IV treatments digested more ADIN than did the IS incubation In the individual feed groups dry matter digestion was similar between IS and IV incubations for corn silage and soy, being 300 and 140 %, respectively, greater than SG. Neutral detergent fiber digestion was 200 % greater in the IS treatment compared to the IV incubation of alfalfas with no difference in aNDF

Table 3.2. Fraction of feed dry matter, neutral detergent and acid detergent fiber, nitrogen, neutral detergent and acid detergent insoluble nitrogen in alfalfa, corn silage and soy digested by *in situ, in vitro* or *S.griseus* incubations. Results are expressed as g digested per g of the feed item.

Feed	Item[1]	n	Overall	Incubation[2]			SEM	Contrasts[3]		
				IS	IV	SG		IS vs. IV	IS vs. SG	IV vs. SG
ALL	DM	72	0.572	0.711	0.636	0.369	0.023	S	S	S
	ND	70	0.318	0.456	0.451	0.057	0.029	NS	S	S
	AD	71	0.387	0.508	0.530	0.127	0.030	NS	S	S

	TN	72	0.765	0.785	0.791	0.720	0.015	NS	S	S
	NDIN	70	0.890	0.913	0.895	0.863	0.013	NS	S	S
	ADIN	71	0.938	0.871	0.963	0.973	0.015	S	S	NS
Alfalfa	DM	24	0.567	0.738	0.591	0.371	0.032	S	S	S
	ND	22	0.242	0.450	0.305	0.006	0.044	S	S	S
	AD	24	0.293	0.461	0.367	0.053	0.038	S	S	S
	TN	24	0.786	0.863	0.812	0.683	0.021	NS	S	S
	NDIN	22	0.915	0.969	0.907	0.875	0.012	S	S	NS
	ADIN	23	0.941	0.913	0.958	0.948	0.008	S	T	NS
Corn	DM	24	0.454	0.601	0.583	0.179	0.042	NS	S	S
	ND	24	0.260	0.356	0.425	0	0.041	T	S	S
	AD	24	0.285	0.350	0.481	0.025	0.043	S	S	S
	TN	24	0.684	0.767	0.633	0.651	0.021	S	S	NS
	NDIN	24	0.765	0.787	0.782	0.726	0.015	NS	NS	NS
	ADIN	24	0.881	0.741	0.931	0.972	0.040	S	S	NS
Soy	DM	24	0.696	0.794	0.736	0.557	0.027	NS	S	S
	ND	24	0.445	0.562	0.606	0.166	0.053	NS	S	S
	AD	23	0.590	0.744	0.743	0.303	0.050	NS	S	S
	TN	24	0.824	0.726	0.926	0.820	0.028	S	NS	T
	NDIN	24	0.992	0.990	0.996	0.989	0.001	T	NS	S
	ADIN	22	0.998	0.996	0.998	0.999	0.0005	S	S	NS

[1]Degraded: DM, dry matter; ND, neutral detergent fiber; AD, acid detergent fiber; TN, total nitrogen; NDIN, neutral detergent insoluble nitrogen; ADIN, acid detergent insoluble nitrogen. [2]Incubations: IS, *in situ*; IV, *in vitro*; SG, *S. griesus*. [3]S, significant at $P < 0.05$; NS,nonsignificant; T, trend toward significance, $0.05 < P < 0.10$.

digestion between these two incubations in corn silage and soy. The *S.griseus* incubation was ineffective in digesting aNDF in alfalfa silage and corn silage but digested approximately one-third of the ND in the soy compared to IS or IV incubation. The ADF digestion was equal between IS and IV incubations of soy but was 25 % greater in IS versus IV for alfalfa and just the reverse in corn silage. The *S.griseus* enzyme digested only half as much ADF in soy as did IS or IV and was ineffective in digesting any ADF in alfalfa and corn silage.

The highest TN digestibility was observed in IV incubation of soy while IV incubation yielded the lowest TN digestion in corn. However, SG incubation yielded only 26 and 18 % reductions in the TN degradation of alfalfas when compared to IS and IV, respectively. Digestion of NDIN was lowest in corn with no differences

among the incubations and highest in soy while the IS incubation of alfalfa yielded higher NDIN digestion than the IV and SG incubations, which were similar. ADIN digestion was highest in SG incubation of all feeds although no differences were observed between SG and IV incubations while IS incubation digested the least ADIN.

Essential amino acid digestibility: Overall, degradation of the feed EAAs (Table 3.3) were similar between the IS and SG incubations for Thr, Val, Ile and Leu with a trend toward higher digestibility in the IS bags for Phe, Lys and His and significantly higher Arg degradation in the IS bags. Significantly more AA degraded or was removed through the IS bags than the IV while IV and SG degraded equal amounts of Leu, Phe, His and Arg with SG degrading more Val, Ile and Lys than IV. The individual feeds varied widely from this. In alfalfa, IS incubation gave the highest AA digestibilities while IV and SG degraded equally. In corn, IS incubation also digested more EAA than the others with SG digesting more than IV except for Leu. With soy, SG digested the highest percent of AA while IS and IV incubations were similar but significantly lower than SG.

Table 3.3. Digestibility of essential amino acids, except methionine and tryptophan, in alfalfa, corn silage and soy following *in situ, in vitro* or *S. griseus* incubations. Results are expressed as percentages of feed amino acid content.

Feeds	Amino Acid	Overall	Treatment[1]			SEM[2]	contrasts[3]		
			IS	IV	SG		IS vs. IV	IS vs. SG	IV vs. SG
All	Thr	29.6	49.7	7.1	32.0	5.7	S	NS	T
	Val	35.9	54.8	13.3	39.6	5.3	S	NS	S
	Ile	36.0	50.9	12.5	44.7	5.3	S	NS	S
	Leu	28.3	46.6	12.0	26.4	5.4	S	NS	NS
	Phe	28.3	49.7	7.8	27.4	5.8	S	T	NS
	Lys	31.1	53.5	7.2	32.6	5.8	S	T	S
	His	41.8	61.6	23.3	40.5	5.0	S	T	NS
	Arg	37.4	65.9	12.8	28.8	6.1	S	S	NS
Alfalfa	Thr	17.8	35.2	5.4	12.9	4.9	S	S	NS

	Val	30.7	48.9	23.3	19.8	5.2	S	S	NS
	Ile	28.5	38.1	18.2	29.1	4.1	S	NS	NS
	Leu	17.5	31.1	10.9	10.6	3.9	S	S	NS
	Phe	15.1	32.4	5.8	7.0	4.8	S	S	NS
	Lys	16.9	34.1	4.0	12.4	5.2	S	T	NS
	His	34.4	54.6	26.5	22.1	5.8	S	S	NS
	Arg	28.3	66.8	7.3	10.8	8.8	S	S	NS
Corn Silages	Thr	37.2	18.1	0	58.2	13.5	S	S	S
	Val	45.5	77.2	5.3	66.4	12.1	S	S	S
	Ile	46.4	52.5	6.1	69.2	12.1	S	S	S
	Leu	36.6	63.5	12.2	47.1	13.1	S	S	S
	Phe	34.8	57.4	0.8	45.6	13.9	S	S	NS
	Lys	37.1	0.0	0.0	47.8	13.6	S	S	S
	His	45.5	71.2	12.0	46.2	11.8	S	S	S
	Arg	33.3	41.5	0	29.2	14.2	S	S	.
Soy	Thr	33.7	14.0	15.9	71.4	9.3	NS	S	S
	Val	31.6	15.7	11.3	67.8	8.9	NS	S	S
	Ile	33.2	14.6	13.1	71.8	9.4	NS	S	S
	Leu	30.7	11.3	12.8	68.1	8.9	NS	S	S
	Phe	35.0	16.7	16.6	71.6	8.9	NS	S	S
	Lys	39.4	26.5	17.5	74.2	8.9	NS	S	S
	His	45.5	30.2	31.3	74.9	7.8	NS	S	S
	Arg	45.9	31.0	31.1	75.6	8.1	NS	S	S

[1]Treatments: IS, *in situ*; IV, *in vitro*; SG, *S. griseus*. [2]SEM, standard error of least squares mean. [3]S, significant at $P < 0.05$; T, trend toward significant, $0.05<P<0.10$; NS, nonsignificant.

2. In vitro intestinal digestion of amino acid in ADIN following 12- or 24-h ruminal digestion

The DM, total N, ADF, ADIN and amino acid contents, except methionine, cystine and tryptophan, of the feeds used are listed in Table 3.4 with results expressed as percent of dry matter or total N and the α-amino N contents of the feeds calculated as a percent of TN. The amino acid contents, except methionine, cystine and tryptophan, of the acid detergent fiber residue, as percent of DM, are presented in Table 3.5 with the α-amino N contents of the residues calculated as a percent of the ADIN and TN. Total N ranged from 1.33 to 13.73 (% DM) while feed α-amino N ranged from 42 to 72 percent

Table 3.4. Composition of feeds. Values are expressed as percent of feed, dry matter or total nitrogen.

Component	ProVaal	Poet HP DDG	Mercer Soy	Ruminant Fishmeal	Corn silage	Alfalfa silage	Soy Best
DM, % feed	92.96	92.20	95.17	93.82	93.06	91.76	93.28
Total N, % DM	13.73	6.39	7.31	10.53	1.33	3.81	7.51
ADF, % DM	11.39	20.10	13.19	6.91	31.30	23.90	8.35
ADIN, % TN	6.32	11.16	1.65	2.12	7.58	5.75	1.64
EAA, % DM							
THR	3.00	1.51	1.87	3.14	0.23	0.80	1.70
VAL	5.02	1.50	1.63	2.57	0.35	1.04	1.59
ILE	1.07	1.10	1.58	2.04	0.21	0.72	1.51
LEU	7.67	4.50	3.20	4.20	0.49	1.37	2.91
PHE	4.52	1.70	2.12	2.27	0.24	0.82	1.74
LYS	5.11	0.87	2.63	5.30	0.14	0.95	2.66
HIS	2.66	0.64	0.90	1.06	0.03	0.21	0.85
ARG	3.33	1.20	1.50	3.61	0.14	0.85	2.39
NEAA, % DM							
ASP	4.81	2.22	4.01	5.62	0.36	2.22	4.62
SER	4.85	1.84	2.34	2.56	0.22	0.83	1.97
GLU	6.23	6.15	8.12	7.91	0.63	1.67	7.11
PRO	3.37	3.03	1.97	2.98	0.37	1.35	1.69
GLY	3.63	1.10	1.85	4.37	0.26	0.91	1.61
ALA	5.04	2.71	1.89	3.87	0.55	1.11	1.69
TYR	1.67	1.34	1.42	1.82	0.12	0.53	1.28
α-Amino N, % TN	64.04	62.42	66.14	72.29	42.10	54.54	64.10

of TN. ADIN ranged from 1.65 to 11.16 (% TN) with α-amino N contributing 0.8 to 10.5 (% TN). Alpha-amino N in the ADIN ranged from 10% in the corn silage to 90 % in Poet HP DDG.

Table 3.5. Amino acid content, of feed acid detergent residues without Met, Trp and Cys,. Results are expressed as fraction of dry matter.

Component	ProvAAl	Poet HP DDG	Mercer Soy	Ruminant Fishmeal	Corn silage	Alfalfa silage	Soy Best
EAA, % DM							
THR	0.099	0.157	0.005	0.050	0.004	0.018	0.007
VAL	0.173	0.302	0.008	0.058	0.007	0.038	0.013
ILE	0.058	0.179	0.008	0.044	0.004	0.025	0.010
LEU	0.228	0.813	0.015	0.082	0.011	0.035	0.020

PHE	0.152	0.287	0.009	0.058	0.005	0.026	0.012
LYS	0.082	0.063	0.006	0.063	0.002	0.026	0.012
HIS	0.026	0.109	0.003	0.017	0.002	0.010	0.005
ARG	0.158	0.187	0.007	0.051	0.003	0.016	0.012
NEAA, % DM							
ASP	0.183	0.267	0.017	0.080	0.007	0.040	0.024
SER	0.147	0.279	0.008	0.039	0.004	0.032	0.014
GLU	0.281	1.308	0.023	0.162	0.011	0.035	0.037
PRO	0.189	0.596	0.007	0.031	0.008	0.030	0.015
GLY	0.102	0.177	0.007	0.035	0.005	0.030	0.009
ALA	0.119	0.476	0.008	0.053	0.006	0.022	0.012
TYR	0.052	0.229	0.005	0.037	0.002	0.017	0.008
α-Amino N							
% ADIN	32.224	90.050	15.502	51.452	10.337	25.823	21.116
% TN	2.037	10.553	0.244	1.092	0.799	1.410	0.375

Feed AD was analyzed in the three pore size bags using the Ankom Fiber analyzer with the degraded residues in addition to the traditional crucible fitted with a 1.5 µm filter (Whatman 934-AH, GE Healthcare Bio-Sciences Corp., Piscataway, NY 08855) with results (mean ± SD) shown in Table 3.6. Overall, crucibles plus filter yielded more consistent and higher AD yields but did not differ from those obtained using the 25 µm bag. The 15 and 50 µm bags yielded similar but lower levels of AD. The 25 µm bags retained the highest ADIN levels with the most variation but were similar to the crucible values while the 15 and 50 µm bags were lower but comparable.

Table 3.6. Acid detergent (AD) and acid detergent insoluble nitrogen (ADIN) contents of feeds using three pore size bags and crucible with filter. Results are expressed as gram per gram dry matter or total nitrogen (mean ± SD).

	AD				ADIN			
Feed	C1[1]	15	25	50	C1	15	25	50
ProvAAI	0.114[a]	0.089*	0.118[a]	0.049[b†]	0.063[†]	0.080	0.096*	0.057[†]
	0.013	0.003	0.003	0.006	0.011	0.000	0.003	0.009
PoetHPDDS	0.201[a]	0.132	0.173	0.109[b]	0.112	0.046	0.116	0.081
	0.006	0.001	0.046	0.007	0.006	0.030	0.030	0.002
MercerSoy	0.132[a]	0.091[b]	0.119[c]	0.071[d]	0.017	0.051	0.030	0.012
	0.003	0.001	0.001	0.001	0.000	0.037	0.002	0.002
RumFishMeal	0.069[a]	0.016[bc]*	0.031[c†]	0.002[b]	0.021[a]	0.006[b]	0.018[a]	0.001[b]

	0.002	0.005	0.003	0.000	0.001	0.000	0.002	0.0002
CornSilage	0.313	0.307	0.321	0.275	0.076[a]	0.065[ab]	0.111[c]	0.056[b]
	0.002	0.002	0.001	0.018	0.002	0.003	0.004	0.002
Alfalfa Silage	0.239[a]	0.220[b]	0.237[a]	0.193[c]	0.058*	0.045[†]	0.059*	0.046
	0.002	0.000	0.005	0.001	0.003	0.003	0.003	0.000
SoyBest	0.084[a]	0.073[b]	0.084[a]	0.060[c]	0.016	0.011[b]	0.024[a]	0.012[b]
	0.000	0.001	0.003	0.002	0.001	0.000	0.004	0.003
OVERALL	0.172[a]	0.132[b]	0.155[a]	0.108[b]	0.053[ab]	0.043[a]	0.068[b]	0.039[a]
	0.020	0.025	0.024	0.025	0.008	0.009	0.012	0.007

[1]Pore size, µm; C1, crucible with 1 µm filter. [a,b,c,d]Means within row and item with different superscripts differ (P < 0.05). *†Trend toward significance in same item and row with different superscripts (0.05<P<0.10).

Dry matter and nitrogen digestibility: Overall, ruminal DM digestibility (Table 3.7) was significantly higher *in situ* than *in vitro* in the Daisy incubator with no difference between 12-h and 24-h incubations although a trend toward higher digestibility in 24-h Daisy versus the 12-h. The 24-h ruminal digestion was higher than the corresponding Daisy. The 15 and 50 µm pore bags yielded higher ruminal, cumulative and intestinal pool DM digestibility than did the 25 µm. Ruminal N dry matter digestion was significantly higher in the Daisy than *in situ* with both time points showing significant differences between the fermentations although it varied by bag as the 15 µm bag *in situ* digested more N than in the Daisy after 24-h. Feed in 50 µm bags showed the highest N digestion in the rumen while 25 µm bags yielded consistently lower digestibilities *in situ* even though the bags were rinsed with acetone to remove surfactants. The 24-h incubation for both fermentation methods gave higher ruminal N digestibility than did the 12-h.

Total DM digestibility, or cumulative, was higher *in situ* than in the Daisy with 15 and 50 µm bags yielding similar values which were higher than those obtained with 25 µm bags. No difference was observed between fermentations in digesting total N*DM although more was digested after 24-h except with the 15 µm bags *in situ*. Being calculated, the DM and N*DM intestinal pools showed no differences between any of the treatments.

Ruminal AD digestion (Table 3.8) was highest after 24-h *in situ*. No difference was observed in the Daisy between 12- and 24-h although variations among bag types were observed. Digestibility in 50 µm bags decreased at 24-h by 7 % over the 12-h while ruminal AD digestion in the 15 and 25 µm bags increased 5-8 % over the 12-h. *In situ* fermentation for 24-h increased AD digestion 28 to 30 % over the 12-h in all bags with the lowest digestion occurred in the 25 µm bags. Overall, ruminal ADIN digestibility was higher with Daisy fermentation with no time effect with less digested after 24-h, although not significant. *In situ* 24-h in 50 µm bags yielded the highest ADIN digestibility while digestion in 25 µm bags the lowest.

Overall, after *in vitro* intestinal digestion, cumulative AD digestion was higher from *in situ* fermentation than *in vitro* which represented 37 and 30 %, respectively, more AD digested but ranged from 0 % in the IS24, 15 µm bags to 95 % in the IS12,15 µm bags while AD digestion during IV fermentation in these bags increased 75 and 67 %, respectively. Cumulative AD digestion in the 50 µm bags increased the least, 14 and 16 % in the IV 12- and 24-h, respectively, and 15 and 5 % in the IS 12- and 24-h, respectively. Fraction of feed dry matter and nitrogen digested in rumen or totally with intestinal pool fractions calculated following ruminal digestion either in situ or in vitro for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g DM or N in the feed (n=7).

Table 3.7 Fraction of feed dry matter and nitrogen digested in rumen or totally with intestinal pool fractions calculated following ruminal digestion either in situ or in vitro for 12- or 24-h and IV intestinal digestion using three pore size bags.

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		DM	N*DM	DM	N*DM	DM	N*DM
IV12	15	0.42	0.67	0.62	0.93	0.77	0.98
IV12	25	0.39	0.66	0.62	0.89	0.76	0.97
IV12	50	0.47	0.71	0.66	0.93	0.81	0.98
IV24	15	0.45	0.7	0.65	0.92	0.81	0.98
IV24	25	0.41	0.65	0.6	0.92	0.76	0.97
IV24	50	0.52	0.76	0.69	0.94	0.84	0.99
IV		*0.44*	*0.69*	*0.64*	*0.92*	*0.79*	*0.98*
IS12	15	0.47	0.64	0.69	0.98	0.84	1
IS12	25	0.33	0.46	0.64	0.91	0.78	0.96
IS12	50	0.49	0.67	0.65	0.89	0.84	0.97
IS24	15	0.56	0.73	0.64	0.94	0.85	0.99
IS24	25	0.41	0.55	0.66	0.93	0.82	0.97
IS24	50	0.63	0.83	0.65	0.91	0.89	0.98
IS		*0.48*	*0.65*	*0.65*	*0.92*	*0.84*	*0.98*
	Overall	0.46	0.67	0.65	0.92	0.81	0.98
	SEM	0.01	0.01	0.02	0.01	0.01	0
	N	163	162	155	152	159	156
Contrasts[3]							
Digestion and time							
IV vs. IS		S	S	NS	NS	S	NS
12-h vs. 24-h		NS	NS	NS	S	NS	S
IV12 vs. IV24		T	S	T	S	NS	NS
IS12 vs. IS24		NS	S	NS	NS	S	T
IV12 vs. IS12		NS	S	S	T	S	NS
IV24 vs. IS24		S	S	NS	NS	NS	NS
Pore, µm							
15 vs. 25		S	S	S	S	S	S
15 vs. 50		NS	S	NS	NS	NS	S
25 vs. 50		S	S	T	NS	S	NS

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$

Table 3.8. Fraction of feed acid detergent and acid detergent insoluble nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in situ or in vitro for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g AD or ADIN in the feed (n=7).

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		AD	ADIN	AD	ADIN	AD	ADIN
IV12	15	0.30	0.90	0.31	0.68	0.53	0.97
IV12	25	0.34	0.89	0.38	0.62	0.49	0.95
IV12	50	0.57	0.93	0.23	0.61	0.65	0.97
IV24	15	0.33	0.89	0.25	0.67	0.55	0.98
IV24	25	0.36	0.88	0.24	0.54	0.47	0.95
IV24	50	0.53	0.92	0.29	0.59	0.62	0.96
IV		*0.40*	*0.90*	*0.29*	*0.62*	*0.40*	*0.96*
IS12	15	0.40	0.82	0.70	0.92	0.77	0.99
IS12	25	0.25	0.74	0.26	0.75	0.43	0.94
IS12	50	0.57	0.91	0.21	0.60	0.66	0.96
IS24	15	0.62	0.95	0.20	0.56	0.61	0.97
IS24	25	0.39	0.87	0.42	0.87	0.62	0.97
IS24	50	0.80	0.98	0.33	0.39	0.84	0.99
IS		*0.50*	*0.88*	*0.35*	*0.67*	*0.65*	*0.97*
Overall		0.45	0.89	0.32	0.65	0.60	0.97
SEM		0.03	0.01	0.03	0.03	0.03	0.01
N		157	157	150	150	156	155
Contrasts[3]							
Digestion and time							
IV vs. IS		S	S	NS	T	S	S
12-h vs. 24-h		S	NS	S	NS	S	NS
IV12 vs. IV24		S	NS	NS	S	T	NS
IS12 vs. IS24		NS	S	S	S	S	NS
IV12 vs. IS12		NS	NS	NS	S	S	S
IV24 vs. IS24		S	S	T	S	NS	NS
Pore, µm							
15 vs. 25		S	T	S	NS	S	S
15 vs. 50		S	NS	T	NS	NS	NS
25 vs. 50		S	S	NS	NS	S	S

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$

25 µm bags increased more *in situ* than *in vitro*, almost opposite the 15 µm bags. Cumulative, or total, ADIN digestion was similar to total AD, being higher with *in situ* incubation than the Daisy. ADIN digestion in 25 µm bags at 12- and 24-h IV yielded similar results to that obtained in IS24-h and 18 % higher than was digested after 12-h IS.

Digestion of DM and N for the individual feeds are given in Tables 3.9 to 3.15 and digestion of ADF and ADIN in Tables 3.16 to 3.22. Overall 50 % of the DM in ProvAAL (Table 3.9) digested in the rumen and 96 % digested following IV intestinal digestion with 76 % of the N degraded in the rumen (range of 50 to 93 %) and 99 % overall. Dry matter digestibility of Poet HP DDG (Table 3.10) averaged 39 % with a wide range 24 to 76 % and highest digestibility in 50 um bags; N digestion followed DM. The 15 µm bags in the IS24 treatment of Mercer soy (Table 3.11) fell apart so data are not available for that comparison. Ruminal dry matter and N digestion averaged 50 % and 69 %, respectively. Total DM digestion ranged from 80 to 98 % while N digestion was 99 %.

The 15 µm bags in the IS24 treatment of the ruminant fish meal (Table 3.12) came apart so no data. Ruminal DM digestion averaged 30 % with the highest occurring in 15 um bags IS12. However, there was concern about the patency of the bags, so these values might not reflect actual digestibility. The IS12, 15 um treatment also had the highest total DM digestion. Nearly 100 % of the N in all treatments was degraded following intestinal incubation but only 50 % following ruminal digestion ranging from 0.30 to 0.64. A longer fermentation increased digestion of total DM and ruminal N of the corn silage (table 3.13). The 15 µm bag treatments of corn silage are incomplete due to bags coming apart. *In situ* fermentation of the alfalfa silage (Table 3.14) appeared to degrade more of the parameters than IV fermentation. The 25 µm bags *in situ* yielded lower digestibilites

for ruminal parameters of SoyBest (Table 3.15) but all treatments yielded similar results (100 %) following IV intestinal degradation.

Acid detergent and corresponding N of ProvAAL (Table 3.16) was 100 % digested in rumen of the IS-24, 50um treatment. Ruminal AD degradation of the DDG (Table 3.17) was 41 % with the lowest digestion in 25 µm bags and highest in 50 µm but total AD digestion was similar among the treatments (0.90 to 0.98) while ADIN digestion in rumen was broad 0.14 to 0.89 but nearly 99 % digested following intestinal digestion. Ruminal AD digestion of Mercer Soy (Table 3.18) ranged from 24 to 94 % and ADIN degradation from 48 to 99 %. Total AD digestion ranged from 41 to 100 % with 98 % of the respective N fractions were degraded. Ruminal AD digestion of ruminant fish meal (Table 3.19) ranged from 85 to 98 % in all except for IS12 15 µm where only 53 % digested. Intestinal exposure digested no more AD except for IS12 15 µm treatment. Nearly ~ 100 % of the ADIN degraded in rumen.

Corn silage acid detergent digestion (Table 3.20) did not increase significantly following IV intestinal digestion except for IS24, 25 µm treatment. Total ADIN digestion appeared to increase. Ruminal AD digestion of alfalfa silage (Table 3.21) varied from 0 % in IV12, 15 um bags to 63% for IS24, 50um. Increasing digestion time did not increase parameter digestion with IV fermentation. The 15 µm bags favored total AD digestion. In situ 24-h fermentation yielded higher total AD digestions of SoyBest (Table 3.22) than 12-h counterparts with broad range in ruminal AD digestion: 0 % (IS12, 25 µm) to 71 % (IS24 50 µm).

Table 3.9. Fraction of ProvAAl dry matter and nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in vitro or in situ for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g DM or TN in the feed.

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		DM	N*DM	DM	N*DM	DM	N*DM
IV12	15	0.64	0.88	0.91	1	0.97	1
IV12	25	0.50	0.76	0.90	0.99	0.95	1
IV12	50	0.71	0.92	0.93	1	0.98	1
IV24	15	0.60	0.84	0.88	0.99	0.95	1
IV24	25	0.58	0.82	0.90	0.99	0.96	1
IV24	50	0.74	0.93	0.91	1	0.98	1
IV		*0.63*	*0.86*	*0.91*	*1*	*0.97*	*1*
IS12	15	0.37	0.60	0.95	1	0.97	1
IS12	25	0.30	0.50	0.90	0.99	0.93	1
IS12	50	0.47	0.71	0.94	1	0.97	1
IS24	15	0.43	0.65	0.92	1	0.95	1
IS24	25	0.32	0.52	0.93	1	0.95	1
IS24	50	0.51	1	0.98	0.98	0.99	1
IS		*0.40*	*0.66*	*0.94*	*1*	*0.96*	*1*
Overall		0.52	0.76	0.92	0.99	0.96	1
SEM		0.03	0.03	0.01	0	0	0
n		22	22	21	21	23	23
Contrasts[3]							
Digestion and time							
IV vs. IS		NS	S	T	S	S	S
12-h vs. 24-h		S	S	S	S	NS	NS
IV12 vs. IV24		S	S	NS	S	S	S
IS12 vs. IS24		S	S	S	S	S	S
IV12 vs. IS12		S	S	S	S	NS	NS
IV24 vs. IS24		S	S	NS	S	S	S
Pore, µm							
15 vs. 25		S	S	S	S	S	S
15 vs. 50		S	S	NS	S	NS	S
25 vs. 50		S	S	S	S	S	S

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.10. Fraction of Poet HP distillers dry matter and nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in vitro or in situ for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g DM or TN in the feed.

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		DM	N*DM	DM	N*DM	DM	N*DM
IV12	15	0.28	0.41	0.61	0.98	0.72	0.99
IV12	25	0.33	0.44	0.55	0.97	0.7	0.98
IV12	50	0.33	0.49	0.65	0.98	0.77	0.99
IV24	15	0.36	0.49	0.6	0.98	0.74	0.99
IV24	25	0.31	0.4	0.55	0.97	0.69	0.98
IV24	50	0.39	0.55	0.64	0.98	0.78	0.99
IV		*0.33*	*0.46*	*0.6*	*0.98*	*0.73*	*0.99*
IS12	15	0.43	0.54	0.69	0.98	0.82	0.99
IS12	25	0.25	0.28	0.59	0.96	0.69	0.97
IS12	50	0.39	0.5	0.75	0.99	0.85	1
IS24	15	0.48	0.59	0.76	0.99	0.87	1
IS24	25	0.37	0.42	0.68	0.98	0.8	0.99
IS24	50	0.76	0.91	0.68	0.99	0.92	1
IS		*0.45*	*0.54*	*0.69*	*0.98*	*0.83*	*0.99*
	Overall	0.39	0.5	0.65	0.98	0.78	0.99
	SEM	0.03	0.03	0.01	0	0.02	0
	n	24	24	24	24	24	24
Contrasts[3]							
Digestion and time							
	IV vs. IS	S	S	T	T	S	S
	12-h vs. 24-h	S	S	NS	NS	S	NS
	IV12 vs. IV24	S	S	S	NS	NS	NS
	IS12 vs. IS24	NS	S	S	NS	S	NS
	IV12 vs. IS12	NS	NS	S	T	S	NS
	IV24 vs. IS24	S	S	S	NS	NS	T
Pore, µm							
	15 vs. 25	S	S	S	S	S	S
	15 vs. 50	S	S	S	NS	NS	NS
	25 vs. 50	S	S	S	S	S	S

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.11 Fraction of Mercer soy dry matter and nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in vitro or in situ for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g DM or TN in the feed.

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		DM	N*DM	DM	N*DM	DM	N*DM
IV12	15	0.46	0.61	0.83	1	0.91	1
IV12	25	0.42	0.66	0.69	0.99	0.82	1
IV12	50	0.52	0.74	0.75	1	0.88	1
IV24	15	0.51	0.72	0.92	1	0.92	1
IV24	25	0.46	0.68	0.64	0.99	0.81	1
IV24	50	0.54	0.76	0.75	0.99	0.88	1
IV		*0.49*	*0.70*	*0.76*	*1*	*0.87*	*1*
IS12	15	0.51	0.62	0.96	1	0.98	1
IS12	25	0.38	0.42	0.80	0.99	0.89	1
IS12	50	0.59	0.73	0.79	1	0.91	1
IS24	15	NA	NA	NA	NA	NA	NA
IS24	25	0.52	0.63	0.78	0.99	0.89	1
IS24	50	0.70	0.84	0.87	1	0.96	1
IS		*0.54*	*0.65*	*0.84*	*1*	*0.93*	*1*
Overall		0.52	0.69	0.79	1	0.89	1
SEM		0.02	0.02	0.02	0	0.01	0
n		22	22	19	19	21	21
Contrasts[3]							
Digestion and time							
IV vs. IS		NS	S	NS	NS	NS	NS
12-h vs. 24-h		NS	S	NS	NS	NS	NS
IV12 vs. IV24		T	S	NS	NS	NS	NS
IS12 vs. IS24		NS	S	T	T	S	T
IV12 vs. IS12		NS	S	NS	S	T	S
IV24 vs. IS24		S	S	T	NS	T	NS
Pore, µm							
15 vs. 25		S	S	S	S	S	S
15 vs. 50		NS	T	S	S	S	S
25 vs. 50		S	S	NS	NS	T	NS

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.12. Fraction of ruminant fish meal dry matter and nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in vitro or in situ for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g DM or TN in the feed.

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		DM	N*DM	DM	N*DM	DM	N*DM
IV12	15	0.26	0.45	0.98	1	0.98	1
IV12	25	0.23	0.43	0.96	0.94	0.97	1
IV12	50	0.33	0.56	0.99	1	0.99	1
IV24	15	0.27	0.49	0.98	1	0.99	1
IV24	25	0.21	0.41	0.93	1	0.94	1
IV24	50	0.38	0.62	0.97	1	0.98	1
IV		*0.28*	*0.49*	*0.97*	*0.99*	*0.98*	*1*
IS12	15	0.51	0.65	0.99	1	1	1
IS12	25	0.18	0.3	0.92	1	0.93	1
IS12	50	0.3	0.52	0.97	1	0.98	1
IS24	15	NA	NA	NA	NA	NA	NA
IS24	25	0.18	0.33	0.97	1	0.97	1
IS24	50	0.36	0.61	0.92	1	0.95	1
IS		*0.31*	*0.48*	*0.95*	*1*	*0.97*	*1*
	Overall	0.31	0.51	0.96	1	0.97	1
	SEM	0.03	0.03	0.01	0	0.01	0
	n	23	22	21	20	21	20
Contrasts[3]							
Digestion and time							
IV vs. IS		S	S				
12-h vs. 24-h		S	NS				
IV12 vs. IV24		S	NS	S	NS	S	NS
IS12 vs. IS24		S	NS				
IV12 vs. IS12		S	NS	NS	NS	NS	NS
IV24 vs. IS24		NS	NS				
Pore, µm			NS	NS	S	S	S
15 vs. 25		S	NS				
15 vs. 50		S	NS				
25 vs. 50							

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.13. Fraction of corn silage dry matter and total nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in vitro or in situ for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g DM or TN in the feed.

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		DM	N*DM	DM	N*DM	DM	N*DM
IV12	15	0.30	0.70	0.23	0.77	0.46	0.30
IV12	25	0.28	0.77	0.23	0.46	0.45	0.28
IV12	50	0.33	0.62	0.24	0.70	0.49	0.33
IV24	15	0.35	0.78	0.28	0.65	0.53	0.35
IV24	25	0.28	0.68	0.20	0.65	0.42	0.28
IV24	50	0.46	0.83	0.09	0.50	0.5	0.46
IV		*0.33*	*0.73*	*0.21*	*0.62*	*0.48*	*0.90*
IS12	15	0.35	0.62	0.01	NA	0.35	0.35
IS12	25	0.34	0.56	0.10	0.61	0.41	0.34
IS12	50	0.39	0.59	0.14	0.62	0.48	0.39
IS24	15	0.46	0.65	0.21	0.76	0.57	0.46
IS24	25	0.35	0.55	0.12	0.64	0.43	0.35
IS24	50	0.52	0.71	0.11	0.60	0.58	0.52
IS		*0.40*	*0.61*	*0.12*	*0.65*	*0.47*	*0.87*
Overall		0.37	0.67	0.17	0.63	0.48	0.37
SEM		0.02	0.02	0.02	0.21	0.01	0.02
n		24	24	22	20	22	24
Contrasts[3]							
Digestion and time							
IV vs. IS		NS	NS	S		S	
12-h vs. 24-h		NS	S	S		S	
IV12 vs. IV24		NS	S	NS	S	S	NS
IS12 vs. IS24		NS	S	S		S	
IV12 vs. IS12		NS	S	NS	S	NS	NS
IV24 vs. IS24		NS	S	S		S	
Pore, µm							
15 vs. 25		S	S	T	NS	S	NS
15 vs. 50		NS	S	NS		NS	
25 vs. 50		S	NS	S		S	

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.14. Fraction of alfalfa silage dry matter and nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in vitro or in situ for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g DM or TN in the feed.

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		DM	N*DM	DM	N*DM	DM	N*DM
IV12	15	0.45	0.76	0.34	0.87	0.63	0.97
IV12	25	0.43	0.76	0.33	0.82	0.62	0.96
IV12	50	0.48	0.79	0.33	0.81	0.65	0.96
IV24	15	0.44	0.75	0.37	0.87	0.65	0.97
IV24	25	0.43	0.75	0.35	0.83	0.63	0.96
IV24	50	0.48	0.79	0.42	0.84	0.70	0.97
IV		*0.45*	*0.77*	*0.36*	*0.84*	*0.65*	*0.97*
IS12	15	0.62	0.85	0.32	0.90	0.74	0.98
IS12	25	0.47	0.66	0.40	0.90	0.68	0.97
IS12	50	0.73	0.93	0.15	0.69	0.77	0.98
IS24	15	0.77	0.95	0.19	0.84	0.81	0.99
IS24	25	0.61	0.80	0.44	0.93	0.78	0.99
IS24	50	0.77	0.96	0.34	0.84	0.85	0.99
IS		*0.66*	*0.86*	*0.31*	*0.85*	*0.77*	*0.98*
	Overall	0.56	0.81	0.33	0.85	0.71	0.97
	SEM	0.03	0.18	0.02	0.01	0.02	0
	n	24	24	24	24	24	24
Contrasts[3]							
Digestion and time							
	IV vs. IS	S	S	NS	NS	S	S
	12-h vs. 24-h	S	S	S	S	S	NS
	IV12 vs. IV24	S	NS	NS	S	S	S
	IS12 vs. IS24	S	S	S	NS	S	S
	IV12 vs. IS12	S	S	S	NS	S	S
	IV24 vs. IS24	S	S	S	NS	S	S
Pore, µm							
	15 vs. 25	S	S	T	NS	S	S
	15 vs. 50	NS	NS	NS	S	NS	S
	25 vs. 50	S	S	NS	NS	S	S

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.15. Fraction of SoyBest dry matter and nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in vitro or in situ for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g DM or TN in the feed.

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		DM	N*DM	DM	N*DM	DM	N*DM
IV12	15	0.55	0.79	0.71	1	0.87	1
IV12	25	0.54	0.78	0.65	0.98	0.84	1
IV12	50	0.62	0.84	0.71	0.99	0.89	1
IV24	15	0.60	0.84	0.69	0.99	0.88	1
IV24	25	0.59	0.83	0.67	0.98	0.87	1
IV24	50	0.63	0.86	0.76	1	0.91	1
IV		*0.59*	*0.82*	*0.70*	*0.99*	*0.88*	*1*
IS12	15	0.51	0.62	0.84	1	0.92	1
IS12	25	0.43	0.50	0.86	1	0.92	1
IS12	50	0.59	0.73	0.86	1	0.94	1
IS24	15	0.61	0.75	0.89	0.94	0.96	0.98
IS24	25	0.49	0.59	0.84	1	0.92	1
IS24	50	0.70	0.85	0.83	0.99	0.95	1
IS		*0.56*	*0.67*	*0.85*	*0.99*	*0.94*	*1*
Overall		0.57	0.75	0.78	0.99	0.91	1
SEM		0.02	0.02	0.02	0.01	0.01	0
n		24	24	24	24	24	24
Contrasts[3]							
Digestion and time							
IV vs. IS		S	S	NS	NS	NS	NS
12-h vs. 24-h		NS	S	S	NS	T	NS
IV12 vs. IV24		S	S	S	NS	S	NS
IS12 vs. IS24		S	S	S	NS	S	NS
IV12 vs. IS12		S	S	S	NS	S	NS
IV24 vs. IS24		S	S	S	NS	S	NS
Pore, µm							
15 vs. 25		S	S	NS	NS	NS	NS
15 vs. 50		NS	NS	NS	NS	NS	NS
25 vs. 50		S	S	NS	NS	S	NS

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.16. Fraction of ProvAAL acid detergent and acid detergent insoluble nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in situ or in vitro for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g AD or ADIN in the feed (n=7).

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		AD	ADIN	AD	ADIN	AD	ADIN
IV12	15	0.33	0.93	0.70	0.95	0.80	0.97
IV12	25	0.56	0.96	0.70	0.92	0.87	1
IV12	50	0.83	0.99	1	1	1	1
IV24	15	0.40	0.93	0.68	0.95	0.81	1
IV24	25	0.70	0.98	0.60	0.85	0.88	1
IV24	50	0.96	1	1	1	1	1
IV		*0.63*	*0.97*	*0.78*	*0.95*	*0.89*	*1*
IS12	15	0.33	0.92	0.69	0.94	0.80	1
IS12	25	0.17	0.89	0.63	0.9	0.68	0.99
IS12	50	0.72	0.98	NA	NA	NA	NA
IS24	15	0.52	0.96	0.35	0.81	0.70	1
IS24	25	0.14	0.86	0.73	0.95	0.77	0.99
IS24	50	1	1	0	0	1	1
IS		*0.48*	*0.94*	*0.48*	*0.72*	*0.79*	*1*
	Overall	0.55	0.95	0.68	0.88	0.84	1
	SEM	0.06	0.01	0.06	0.05	0.03	0
	N	22	22	22	22	23	23
Contrasts[3]							
Digestion and time							
	IV vs. IS	NS	NS				
	12-h vs. 24-h	S	S				
	IV12 vs. IV24	S	S	NS	S	S	S
	IS12 vs. IS24	S	S				
	IV12 vs. IS12	S	S	S	NS	S	S
	IV24 vs. IS24	S	S				
Pore, µm							
	15 vs. 25	NS	NS	NS	S	S	T
	15 vs. 50	S	S				
	25 vs. 50	S	NS				

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.17. Fraction of Poet HP distillers acid detergent and acid detergent insoluble nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in situ or in vitro for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g AD or ADIN in the feed (n=7).

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		AD	ADIN	AD	ADIN	AD	ADIN
IV12	15	0.32	0.88	0.39	0.95	0.59	0.99
IV12	25	0.26	0.23	0.49	0.93	0.63	0.95
IV12	50	0.50	0.91	0.50	0.97	0.76	1
IV24	15	0.43	0.93	0.30	0.91	0.60	0.99
IV24	25	0.33	0.89	0.44	0.92	0.62	0.99
IV24	50	0.46	0.91	0.6	0.97	0.78	1
IV		*0.38*	*0.79*	*0.45*	*0.94*	*0.66*	*0.99*
IS12	15	0.38	0.86	0.55	0.97	0.71	1
IS12	25	0.14	0.75	0.44	0.94	0.52	0.98
IS12	50	0.42	0.87	0.73	0.98	0.84	1
IS24	15	0.50	0.89	0.59	0.97	0.80	1
IS24	25	0.37	0.85	0.54	0.95	0.72	0.99
IS24	50	0.80	0.98	0.67	0.97	0.94	1
IS		*0.44*	*0.87*	*0.59*	*0.96*	*0.76*	*1*
	Overall	0.41	0.88	0.52	0.95	0.71	0.99
	SEM	0.03	0.01	0.03	0.01	0.03	0
	N	24	24	22	22	23	23
Contrasts[3]							
Digestion and time							
IV vs. IS		S	S	NS	NS	S	S
12-h vs. 24-h		NS	S	S	T	S	NS
IV12 vs. IV24		S	S	NS	NS	S	NS
IS12 vs. IS24		S	S	S	S	NS	NS
IV12 vs. IS12		NS	S	NS	S	T	NS
IV24 vs. IS24		S	S	NS	NS	S	S
Pore, µm							
15 vs. 25		S	S	NS	NS	S	S
15 vs. 50		NS	S	S	NS	S	NS
25 vs. 50		S	S	S	S	S	S

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.18. Fraction of Mercer Soy acid detergent and acid detergent insoluble nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in situ or in vitro for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g AD or ADIN in the feed (n=7).

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		AD	ADIN	AD	ADIN	AD	ADIN
IV12	15	0.37	0.95	0.17	0.81	0.48	0.99
IV12	25	0.24	0.85	0.27	0.83	0.45	0.97
IV12	50	0.60	0.97	0.03	0.78	0.61	0.99
IV24	15	0.34	0.95	0.11	0.72	0.41	0.99
IV24	25	0.29	0.88	0.22	0.74	0.44	0.97
IV24	50	0.55	0.97	0.27	0.86	0.67	1
IV		*0.40*	*0.93*	*0.18*	*0.79*	*0.51*	*0.99*
IS12	15	0.91	1	1	1	1	1
IS12	25	0.26	0.48	0.28	0.93	0.48	0.98
IS12	50	0.72	0.97	0.18	0.88	0.77	1
IS24	15	NA	NA	NA	NA	NA	NA
IS24	25	0.53	0.86	0.45	0.9	0.75	0.99
IS24	50	0.94	1	1	0.50	1	1
IS		*0.67*	*0.86*	*0.58*	*0.84*	*0.80*	*0.99*
Overall		0.52	0.90	0.36	0.81	0.64	0.99
SEM		0.06	0.04	0.74	0.05	0.05	0
N		21	21	21	21	22	22
Contrasts[3]							
Digestion and time							
IV vs. IS							
12-h vs. 24-h							
IV12 vs. IV24		S	NS	S	NS	S	S
IS12 vs. IS24							
IV12 vs. IS12		NS	NS	NS	NS	S	NS
IV24 vs. IS24							
Pore, µm							
15 vs. 25		S	NS	NS	NS	S	S
15 vs. 50							
25 vs. 50							

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.19. Fraction of ruminant fish meal acid detergent and acid detergent insoluble nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in situ or in vitro for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g AD or ADIN in the feed (n=7).

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		AD	ADIN	AD	ADIN	AD	ADIN
IV12	15	0.94	1	0.17	0.55	0.95	1
IV12	25	0.99	1	1	1	1	1
IV12	50	1	1	0	0	1	1
IV24	15	0.94	1	0.02	0.24	0.94	1
IV24	25	0.96	1	0.21	0.81	0.97	1
IV24	50	1	1	0	0	1	1
IV		*0.97*	*1*	*0.23*	*0.43*	*0.98*	*1*
IS12	15	0.53	0.50	0.93	1	0.97	1
IS12	25	0.86	0.97	0.5	0.91	0.93	1
IS12	50	1	1	0	0	1	1
IS24	15	NA	NA	NA	NA	NA	NA
IS24	25	0.92	0.99	0.53	0.94	0.96	1
IS24	50	1	1	0	0	1	1
IS		*0.86*	*0.89*	*0.39*	*0.57*	*0.97*	*1*
Overall		0.93	0.95	0.28	0.39	0.98	1
SEM		0.03	0.03	0.08	0.09	0.01	0
N		24	24	24	24	24	24
Contrasts[3]							
Digestion and time							
IV vs. IS		S	S	NS	NS	S	T
12-h vs. 24-h		S	S	S	S	S	NS
IV12 vs. IV24		S	NS	S	S	S	NS
IS12 vs. IS24		S	S	S	S	NS	NS
IV12 vs. IS12		NS	NS	S	S	NS	NS
IV24 vs. IS24		S	S	S		S	NS
Pore, µm			S	S	S	NS	S
15 vs. 25		S	S	S	S	S	NS
15 vs. 50		S	NS	S	S	S	NS
25 vs. 50		S	S	NS	NS	S	T

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.20. Fraction of corn silage acid detergent and acid detergent insoluble nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in situ or in vitro for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g AD or ADIN in the feed (n=7).

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		AD	ADIN	AD	ADIN	AD	ADIN
IV12	15	0.05	0.84	0	0.27	0.05	0.88
IV12	25	0.06	0.77	0.02	0.20	0.09	0.82
IV12	50	0.20	0.78	0.05	0.44	0.25	0.88
IV24	15	0.07	0.70	NA	NA	NA	NA
IV24	25	0.05	0.67	0.02	0.40	0.06	0.8
IV24	50	0.20	0.77	0.02	0.35	0.21	0.85
IV		*0.11*	*0.76*	*0.02*	*0.33*	*0.13*	*0.85*
IS12	15	0.02	0.65	NA	NA	NA	NA
IS12	25	0.02	0.69	0.02	0.26	0.04	0.77
IS12	50	0. 21	0.71	0.03	0.48	0.23	0.85
IS24	15	NA	NA	NA	NA	0.20	0.82
IS24	25	0.07	0.57	0.02	0.60	0.23	0.85
IS24	50	0.48	0.89	0	0.08	0.48	0.90
IS		*0.15*	*0.70*	*0.02*	*0.36*	*0.24*	*0.84*
Overall		0.12	0.73	0.02	0.33	0.16	0.84
SEM		0.03	0.02	0	0.04	0.03	0.01
N		18	18	15	15	18	17
Contrasts[3]							
Digestion and time							
IV vs. IS							
12-h vs. 24-h							
IV12 vs. IV24		S	NS	S	NS	T	NS
IS12 vs. IS24							
IV12 vs. IS12		S	S	NS	NS	NS	T
IV24 vs. IS24							
Pore, µm							
15 vs. 25		S	T	NS	S	NS	NS
15 vs. 50							
25 vs. 50							

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.21. Fraction of alfalfa silage acid detergent and acid detergent insoluble nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in situ or in vitro for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g AD or ADIN in the feed (n=7).

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		AD	ADIN	AD	ADIN	AD	ADIN
IV12	15	0	0.75	0.72	0.72	0.72	0.93
IV12	25	0.04	0.79	0.05	0.32	0.10	0.86
IV12	50	0.20	0.83	0.06	0.44	0.25	0.91
IV24	15	0.04	0.79	0.36	0.79	0.38	0.96
IV24	25	0.04	0.77	0.11	0.48	0.15	0.88
IV24	50	0.17	0.81	0.08	0.45	0.23	0.9
IV		*0.08*	*0.79*	*0.23*	*0.53*	*0.31*	*0.91*
IS12	15	0.31	0.89	0.67	0.71	0.77	0.97
IS12	25	0.25	0.79	0	0.49	0.25	0.90
IS12	50	0.46	0.85	0.10	0.57	0.51	0.94
IS24	15	0.44	0.93	0.04	0.31	0.46	0.95
IS24	25	0.31	0.87	0.2	0.78	0.45	0.97
IS24	50	0.64	0.96	0.02	0.45	0.64	0.98
IS		*0.40*	*0.88*	*0.17*	*0.55*	*0.51*	*0.95*
Overall		0.24	0.84	0.2	0.54	0.41	0.93
SEM		0.04	0.01	0.05	0.04	0.05	0.01
N		24	24	24	24	24	24
Contrasts[3]							
Digestion and time							
IV vs. IS		S	S	NS	NS	NS	NS
12-h vs. 24-h		S	S	NS	NS	NS	NS
IV12 vs. IV24		S	NS	S	T	S	T
IS12 vs. IS24		S	S	NS	NS	S	NS
IV12 vs. IS12		S	S	NS	NS	S	T
IV24 vs. IS24		S	NS	S	NS	S	NS
Pore, µm							
15 vs. 25		S	T	S	S	S	S
15 vs. 50		S	NS	S	NS	S	S
25 vs. 50		S	NS	NS	NS	S	T

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Table 3.22. Fraction of SoyBest acid detergent and acid detergent insoluble nitrogen digested in the rumen or totally with intestinal pool fractions calculated following ruminal digestion either in situ or in vitro for 12- or 24-h and IV intestinal digestion using three pore size bags. Results are expressed as g digested per g AD or ADIN in the feed (n=7).

		Digestion Parameter[1]					
Digestion time[2]	Pore µm	Rumen		Intestinal Pool		Total	
		AD	ADIN	AD	ADIN	AD	ADIN
IV12	15	0.12	0.98	0.03	0.54	0.14	0.99
IV12	25	0.20	0.99	0.10	0.11	0.29	0.99
IV12	50	0.48	0.98	0.03	0.73	0.50	1.00
IV24	15	0.10	0.96	0.06	0.41	0.15	0.97
IV24	25	0.14	0.96	0.07	0.33	0.19	0.98
IV24	50	0.40	0.98	0.06	0.54	0.43	0.99
IV		*0.24*	*0.98*	*0.06*	*0.44*	*0.28*	*0.99*
IS12	15	0.10	0.86	0.29	0.92	0.36	0.99
IS12	25	0.00	0.67	0.12	0.93	0.12	0.98
IS12	50	0.49	0.97	0.22	0.68	0.60	0.99
IS24	15	0.61	0.99	0.00	0.39	0.61	0.99
IS24	25	0.29	0.96	0.27	0.88	0.48	0.99
IS24	50	0.71	1	0.26	0.34	0.79	1.00
IS		*0.37*	*0.91*	*0.19*	*0.69*	*0.49*	*0.99*
Overall		0.30	0.94	0.13	0.57	0.39	0.99
SEM		0.05	0.02	0.02	0.06	0.04	0
N		24	24	24	24	24	24
Contrasts[3]							
Digestion and time							
IV vs. IS		S	S	NS	NS	S	S
12-h vs. 24-h		S	S	S	NS	S	NS
IV12 vs. IV24		S	T	NS	T	S	NS
IS12 vs. IS24		NS	S	S	S	S	NS
IV12 vs. IS12		S	NS	NS	NS	S	S
IV24 vs. IS24		S	S	S	S	S	T
Pore, µm							
15 vs. 25		NS	S	S	NS	T	NS
15 vs. 50		S	S	NS	NS	S	NS
25 vs. 50		S	S	S	S	S	S

[1]DM, dry matter; N, nitrogen. [2]IV, *in vitro* Daisy incubator; IS, *in situ*.
[3]Significance: NS, nonsignificant; S, $P < 0.05$; T, trend, $0.05 < P < 0.10$.

Amino acid digestibility: Total digestibility of the essential amino acids (EAA), except methionine and tryptophan, in the feed residue and the AD residue following IV or IS ruminal fermentation and IV intestinal digestion are given in Table 3.23 with the nonessential amino acids (NEAA), except cystine, in Table 3.24. Overall, digestibility of the AA in the intestinal residue was significantly higher than digestion of the amino acids in the feed acid detergent residue with no differences observed among the methods or lengths of rumen fermentation and the bag pore size. Essential AA digestibility ranged from 88.1 % for His to 93.6 % for Leu in the intestinal residue and from 58.3 % for Lys to 76.2 % for Phe in the acid detergent residue. Nonessential AA digestibility ranged from 84.3 % for Gly to 94.4 % for Tyr in the intestinal residue and 63.3 % for Pro to 78.5 % for the acid detergent residues. However it should be noted that EAA and NEAA digestibilities between the intestinal and feed acid detergent residues using 15 µm bags for 12-h *in situ* were very similar, except for Pro and Ala which behaved like the other treatments exhibiting higher digestibility in the intestine. Additionally, the IS12-h 15 µm bag treatment yielded the highest AD amino acid digestion.

Overall, more α-amino nitrogen (AAN) was digested in the intestinal residue than in the acid detergent fiber of feed intestinal residue (Table 3.25), 91.3 ± 11.7 and 71.1 ± 32.2 (mean ± SD), respectively, with no differences observed among bag pore sizes in the respective residue due to high standard errors. However, variation in feeds was seen. Digestibilities of AAN in both residues and all bags were similar in ProvAA. Digestibility of AAN in corn silage AD using 15 µm bags was negligible but increased to 29 % with larger pore size bags while the AA digestibility of the SoyBest AD residue following IV intestinal digestion was 50 % that of the intestinal residue.

Table 3.23. Digestibility of essential amino acids, except methionine and tryptophan, in intestinal digesta and its acid detergent residue following rumen digestion of feeds (n = 7) either in situ (IS) or in vitro (IV; Daisy incubator). Results are expressed as percent of amino acids in feed or the acid detergent fiber of the feed on dry matter basis.

Dig Time[1]	Pore µm	THR	VAL	ILE	LEU	PHE	LYS	HIS	ARG	EAA
Intestinal residue, % feed AA										
IV12	15	92.51	94.05	93.58	94.86	93.87	92.16	89.20	93.84	93.01
	25	92.22	93.43	93.27	94.32	93.92	92.22	89.97	93.30	92.83
	50	90.15	92.53	92.13	93.30	92.17	87.84	85.97	85.69	89.97
IV24	15	91.07	92.93	92.37	93.51	92.63	88.24	87.95	89.11	90.98
	25	91.05	92.18	92.27	93.40	92.93	91.22	88.74	91.77	91.70
	50	92.21	93.60	93.46	94.48	93.56	90.28	88.35	88.61	91.82
IS12	15	88.07	90.79	89.83	91.70	90.34	87.35	85.57	89.37	89.13
	25	88.00	89.63	88.03	90.91	89.80	87.58	84.61	87.56	88.27
	50	91.11	92.81	91.91	93.90	92.98	89.33	89.28	86.56	90.99
IS24	15	92.07	92.60	92.61	93.80	93.77	91.71	90.03	90.97	92.20
	25	87.95	90.91	88.88	91.33	89.89	87.44	83.90	88.23	88.57
	50	97.47	97.81	97.46	98.18	97.95	97.33	96.61	97.06	97.48
	Mean	91.10	92.73	92.09	93.59	92.73	90.09	88.17	90.17	91.33
	SEM	1.45	0.97	1.12	0.95	1.19	1.80	2.19	1.93	1.45
Acid detergent residue of in vitro intestinal digestion assay, % ADIN AA										
IV12	15	60.44	61.12	61.88	63.13	67.23	56.44	61.44	69.47	62.64
	25	62.93	65.58	67.96	68.16	71.26	54.01	61.72	71.56	65.40
	50	65.53	75.30	72.27	77.07	75.39	64.33	73.72	71.00	71.83
IV24	15	67.89	65.42	68.31	69.79	74.26	52.01	64.70	72.77	66.89
	25	55.20	57.68	62.87	65.74	70.80	42.61	58.74	67.46	60.14
	50	76.51	76.42	78.88	80.93	84.12	58.58	76.53	79.74	76.46
IS12	15	87.23	88.87	90.31	90.18	91.86	83.29	85.62	92.88	88.78
	25	72.25	78.42	78.28	78.14	81.71	69.59	74.62	85.12	77.27
	50	72.12	80.67	79.50	83.20	83.17	68.11	78.48	73.77	77.38
IS24	15	60.41	64.31	67.86	68.17	70.46	53.79	57.05	71.05	64.14
	25	64.97	68.78	70.75	69.58	72.38	59.66	60.22	73.74	67.51
	50	70.34	76.81	76.03	78.40	78.17	70.19	82.81	80.30	76.63
	Mean	68.00	71.44	72.89	74.27	76.16	58.35	67.03	73.59	70.22
	SEM	4.27	3.97	4.01	3.72	3.62	4.84	4.27	4.04	4.09

[1]IV, *in vitro* (Daisy incubator); IS, rumen *in situ*; 12-h; 24-h. [2]Amino acids in residue after *in vitro* intestinal (Int) digestion or acid detergent (AD) residue of the intestinal digesta. [3]Level of significance between amino acid content of intestinal versus acid detergent residues.

able 3.24. Digestibility of nonessential amino acids, except cystine, in intestinal digesta and its acid detergent residue following ruminal digestion either *in situ* (IS)or *in vitro* (IV; Daisy incubator) of feeds (n = 7). Results are expressed as percent of amino acids in feed or the acid detergent fiber of the feed on dry matter basis.

Dig Time[1]	Pore µm	ASP	SER	GLU	PRO	GLY	ALA	TYR	NEAA
Intestinal residue, % feed AA									
IV12	15	94.32	91.59	94.91	94.10	89.47	94.76	96.01	93.59
	25	94.38	91.86	94.96	93.66	89.28	94.19	95.38	93.39
	50	96.28	88.12	92.23	87.85	75.29	91.19	94.49	89.35
IV24	15	97.59	89.10	92.92	91.36	80.59	92.71	95.03	91.33
	25	93.77	90.31	93.88	92.68	87.01	93.08	94.28	92.14
	50	93.17	89.92	93.21	90.36	77.78	92.79	95.35	90.37
IS12	15	93.11	87.31	91.75	92.47	85.43	92.37	92.63	90.72
	25	95.26	87.01	91.44	90.13	83.40	91.00	91.17	89.92
	50	92.64	89.72	93.26	91.24	79.13	92.70	93.71	90.34
IS24	15	94.01	91.05	94.20	90.25	83.54	92.39	95.04	91.50
	25	91.00	87.36	91.31	91.96	84.99	92.00	91.93	90.08
	50	98.21	96.87	97.93	97.17	93.52	97.53	98.08	97.04
	Mean	94.47	89.98	93.48	91.96	84.25	93.06	94.39	91.66
	SEM	1.21	1.52	1.04	1.14	2.54	0.94	0.89	1.33
Acid detergent of in vitro intestinal digestion assay, % ADIN AA									
IV12	15	67.95	59.54	70.18	56.73	60.55	61.29	61.17	62.49
	25	70.33	62.04	72.35	54.81	64.62	66.15	66.22	65.22
	50	85.24	67.80	71.16	67.07	50.90	66.02	77.31	69.36
IV24	15	70.52	63.66	72.35	55.74	63.44	64.09	68.84	65.52
	25	67.94	54.06	69.10	47.79	56.95	57.32	62.54	59.39
	50	85.18	73.44	83.24	68.91	76.65	73.52	79.39	77.19
IS12	15	93.50	86.82	92.77	84.62	87.03	88.66	90.10	89.07
	25	86.32	72.21	84.97	72.32	73.65	76.84	81.18	78.21
	50	86.72	69.74	82.70	73.76	56.72	68.65	79.40	73.96
IS24	15	75.89	57.14	71.23	48.71	48.39	64.16	63.63	61.31
	25	77.00	64.98	73.45	59.79	65.50	67.55	70.73	68.43
	50	75.35	74.28	76.35	75.01	75.17	71.28	78.07	75.07
	Mean	78.54	66.94	76.80	63.30	64.92	68.83	72.99	70.33
	SEM	3.67	4.28	3.69	4.51	4.51	4.29	3.87	4.12

[1]IV, *in vitro* (Daisy incubator); IS, rumen *in situ*; 12-h; 24-h. [2]Amino acids in residue after *in vitro* intestinal (Int) digestion or acid detergent (AD) residue of the intestinal digesta. [3]Level of significance between amino acid content of intestinal versus acid detergent residues.

The AA N from intestinal digestion using 25 µm bags yielded the lowest digestibility in the Poet HP DDG but the variation was also high. Large variation also negated differences among the bags in the Mercer Soy.

Table 3.25. Degraded α-amino nitrogen in the intestinal residue and acid detergent fiber of intestinal residue of feeds (n=7) following ruminal and intestinal digestion in three pore size bags. Results are expressed as a percent of the α-amino nitrogen in the feed for Intestinal and the feed acid detergent fiber for AD (mean ± SD).

FEED	Bag pore, µm						Overall Int vs. AD[1]
	15		25		50		
	Int	AD	Int	AD	Int	AD	
ProvAAl	98.5	99.1	98.0	98.6	99.2	99.3	NS
	0.3	0.4	1.3	2.2	0.6	0.6	
Poet HP DDG	95.9^{b}	98.8^{ab}	90.0^{c}	97.7^{ab}	96.6^{ab}	99.6^{a}	S
	0.5	0.7	4.8	1.0	1.4	0.4	
Mercer Soy	96.7	89.2	97.6	87.6	98.0	90.6	S
	5.1	9.8	0.8	11.8	1.3	0.7	
R.Fish Meal	99.6	NA	98.8	NA	NA	NA	--
	0.3		1.2				
CSB	68.6^{a}	1.2^{c}	68.8^{a}	3.4^{c}	69.2^{a}	29.4^{b}	S
	9.0	1.7	31.2	5.8	16.2	16.9	
AS2	89.9^{ab}	71.0^{c}	89.1^{ab}	55.7^{d}	91.8^{a}	79.3^{bc}	S
	3.6	6.0	1.2	11.3	2.5	9.7	
SoyBest	98.8^{a}	45.5^{b}	98.2^{a}	44.6^{b}	99.1^{a}	58.7^{b}	S
	0.3	23.3	0.6	14.4	0.1	19.9	
Overall	91.4^{a}	71.1^{b}	90.3^{a}	67.2^{b}	92.3^{a}	75.2^{b}	S
	11.6	34.4	11.5	34.1	12.4	28.3	

[1]Significance between digestibility of intestinal (Int) versus acid detergent (AD) amino acid: S, P<0.05; NS, nonsignificant. [abcd]Means with different letters within common row are significant, P<0.05 (Duncans). NA, not available

DISCUSSION

1. In situ or in vitro fermentation versus S. griseus incubation

Dry matter and nitrogen digestibilities: Rumen fluid contains a mix of proteases and cellulases to hydrolyze peptide and cellulose bonds, thus, providing a source of protein and energy for microbial and host use while *Streptomyces griseus* (SG) is a blend of at least 10 proteases which include serine-type proteases, zinc endopeptidases, zinc leucine aminopeptidases and a carboxypeptidase and is nonspecific but yields extensive or complete protein degradation, digesting casein so

>70 % is reduced to free amino acids (Sigma product information; Nomoto, 1960) but contains no cellulolytic activity. The time points of 0.5- and 10-h for forages and soy, respectively, were selected so the amount of N degraded in the SG assay equaled that digested in the IV incubation; however, SG digested the N in corn silage quicker and a shorter incubation, i.e., 0.25-h, should have been used. Dry matter digestions, and consequently, aNDF and ADF digestion, were diminished with SG degradation. In contrast nitrogen-dry matter digestions – total, NDIN and ADIN – were not impeded by SG incubation to the same extent as DM alone due to the extensive N digestion. Consequently, SG degradation does not represent ruminal digestion but did adequately predict crude protein degradation.

In situ incubation is ideal in that energy should not limit ruminal microbial production, thus, not depressing N degradation (Clark et al., 1992) while energy in the IV incubations may have been deficient and depressed N degradation. However, microbial fermentation requires blank analysis to correct residue for their contribution. Nonetheless, as reviewed by Nocek (1988) all ruminal protein degradation schemes have limitations.

Essential amino acid digestibilities: The limitations of methods used to estimate ruminal protein digestion are exemplified in AA degradation of the feeds using the three incubations. *In situ* incubation digested 100 % of the AA in corn silage while IV digested none. The N, and consequently the AA, content is low in corn. Did IS incubation overestimate AA digestion due to small feed particles flowing through the pores of the bag? The IS and IV incubations were corrected for microbial AA content using book values (Clark et al., 1992). The soy products, with a higher N content and less cell wall in the plant structure, was treated similarly and IS and IV incubations yielded comparable AA degradations and residue AA contents; however, a 10-h SG incubation resulted in higher AA digestions and consequently, lower residual AA

contents. Although the time point for the SG assay of soy was taken to yield approximately the same N digestion as observed in 24-h IV incubation, AA degradation proceeded at a faster rate. The undegraded amino acid to DM ratio of feeds from SG incubations differed from that obtained after ruminal fermentation, either IS or IV, (Table 3.26), reflecting higher N digestion but less DM.

Table 3.26. The essential amino acid content in residues from all feeds following in situ, in vitro or S. griseus incubations. Results are expressed as grams AA in residue per g undigested dry matter.

Amino acid	Incubation[1]		
	IS	IV	SG
	----- g AA / g residue ----		
Thr	0.032	0.036	0.007
Val	0.030	0.038	0.008
Ile	0.029	0.032	0.006
Leu	0.059	0.062	0.015
Phe	0.035	0.037	0.009
Lys	0.035	0.044	0.008
His	0.013	0.012	0.003
Arg	0.037	0.037	0.009

[1]*IS, in situ; IV, in vitro*; SG, *S. griseus*.

2. In vitro intestinal digestion of amino acids in ADIN following 12- or 24-h ruminal digestion

Dry matter and nitrogen digestibility: The industry considers the 50 µm bags the standard pore size for *in situ* incubations. Using the IS24, 50 µm bags as the standard, Table 3.27 shows the relative value of each digestion parameter for the other treatments. Total N, DM and ADIN values of the other treatments were similar to the IS24, 50 µm but the remaining digestion parameters are less than 1, raising questions. When comparing 12- with 24-h, higher digestibility would be expected after a longer period in the rumen, or slower rate of passage, 6.25 versus 4.17 % h^{-1}, respectively. The 12-h *in vitro* incubation in the Daisy appeared to degrade total N

and ADIN to the same extent as 24-h *in situ* but slightly underestimated DM and AD digestion. Or, did the higher digestion of DM and AD in the IS24 50 µm bags result from loss of feed particles through the pores? The lower digestibility in the 25 µm bags is not understood and was observed throughout the study, suggesting the bags created a barrier to microbial movement or function that affected the digestibility values.

Adesogan (2005) observed higher DM digestibility of ryegrass, ryegrass hay and corn silage in 50 µm pore size bags than 25 µm using the Daisy incubator but concluded the 25 µm bags gave a more standardized predication of digestibility parameters. Further, Gargallo et al. (2006) and Boucher et al. (2009c) also obtained lower digestibilities using the 25 µm pore size bags and recommended 50 µm pore bags.

Table 3.27. Fraction digested relative to IS24, 50 µm.

		Digestion parameter							
Dig/time[1]	Pore µm	Rum DM	Rum N*DM	Rum AD	Rum ADIN	Tot DM	Tot N*DM	Tot AD	Tot ADIN
IV12	15	0.67	0.81	0.38	0.92	0.87	1.00	0.63	0.98
	25	0.62	0.80	0.43	0.91	0.85	0.99	0.58	0.96
	50	0.75	0.86	0.71	0.95	0.91	1.00	0.77	0.98
IV24	15	0.71	0.84	0.41	0.91	0.91	1.00	0.65	0.99
	25	0.65	0.78	0.45	0.90	0.85	0.99	0.56	0.96
	50	0.83	0.92	0.66	0.94	0.94	1.00	0.74	0.97
IS12	15	0.75	0.77	0.50	0.84	0.94	1.00	0.92	1.00
	25	0.52	0.55	0.31	0.76	0.88	0.98	0.51	0.95
	50	0.78	0.81	0.71	0.93	0.94	0.99	0.79	0.97
IS24	15	0.89	0.88	0.78	0.97	0.96	1.00	0.73	0.98
	25	0.65	0.66	0.49	0.89	0.92	0.99	0.74	0.98
	50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

[1]IV, *in vitro* Daisy; IS, *in situ*; 12- and 24-h.

Problems were encountered sealing the 15 and 50 µm bags and it appears the bags were not able to release trapped fermentation gasses easily, causing many of the

bags to burst. The 25 µm bags were easier to handle and seams held but digestion of DM, N and AD appeared lower in these bags with more microbial contamination on the outside making blank correction imperative. However, feed AD determination in 25 µm bags yielded similar values to those obtained using a crucible with 1 µm filter.

The CNCPS (Sniffen et al., 1992; Tylutki et al., 2008) assumes rumen undegraded protein to be 80 % digested in the small intestine (SI) while the NRC (2001) assumes a digestibility of 67 % for metabolizable protein. In the present study N-dry matter digestibility in the SI averaged 92 % with the corn and alfalfa silages at 63 and 84 %, respectively, and the animal and soy proteins at 98 % while Calsamiglia and Stern (1995) obtained digestibilities of 89.9 and 87.3 % for soybean meal (SBM) and lignosulfonate-treated SBM and were highly correlated ($r = 0.91$, $P < 0.001$) to *in vivo* digestion. This suggests that values in the library should be dynamic and be analyzed on a routine basis to improve the prediction of intestinal digestibility of ruminant protein feeds.

Acid detergent insoluble N is considered unavailable (Van Soest et al., 1991) with no ruminal or intestinal digestibility (Sniffen et al., 1992). Some (Muscato et al., 1983; Miranda et al., 2003; Tedeschi et al., 2001) have adjusted the insoluble AA pool for the acid detergent bound AA by subtracting the AA of the AD residue from it. However, in this study the ADIN in the animal and soy proteins appeared to be highly digestible following ruminal and intestinal digestion while the corn silage ADIN was essentially unavailable and digestibility of the alfalfa silage between these extremes.

Amino acid digestibility: Total tract digestibility of the feed amino acid was 91 % and agrees with the 93 % obtained after *in vivo* incubation by O'Mara et al. (1997). Digestibility of the AD α-amino N followed that of ADIN with 71 % of the AA digested

in the total tract. The high percentage of α-amino N in the AD residue of Poet HP DDG reflects the heating and processing but it appeared to be highly digestible.

It appears that the intestinal digestion obtained in this study may be too high. To estimate intestinal digestion, the three-step method (Calsamiglia and Stern, 1995) digested 15 mg residual N following rumen incubation with 10 mg pepsin, yielding a ratio of 1.5 mg N per mg pepsin, while Akeson and Stahmann (1964) used a ratio of 6.4 mg sample N per mg pepsin. In the modified three-step (Gargallo et al., 2006) incubated up to 30 bags, each containing 5 g sample initially, in Daisy Jar with 2 g pepsin, or a ration close to 1.5 for mg residual N to mg pepsin. In the present study, half gram samples were incubated in the bags, making the N to enzyme ratio 0.02 for corn silage to 0.2 for ruminant fish meal, far less than the 1.5, which may have caused higher digestibility. Consequently, the pancreatin level was also 10-fold overloaded, but the amino acid intestinal digestibility was in line with Boucher et al. (2009a,b,c).

An *in vitro* intestinal digestion procedure requires standardization to the *in vivo*. For ruminants, *in vivo* requires an animal with ruminal and duodenal fistulas and recovery of mobile bags in the fecal material, at considerable expense and time (O'Mara et al., 1997). The precision-fed cecectomized rooster bioassay (Boucher et al., 2009a,b,c) provides intestinal true digestibility but requires surgical intervention plus crop intubation; does it represent intestinal digestion in ruminants? Since the intestinal digestion in the three-step procedures is based on assays developed with rats would the rat, with hind-gut fermentation, be more representative of ruminal intestinal digestion. Digestibility studies would entail feeding diets with the protein supplied by the ruminal residue of a feed ingredient for 2-3 days (adaptation) and 5-7 days for total fecal collection (Keith and Bell, 1988 ; Sarwar and Peace, 1986). Dry matter, nitrogen and amino acid digestion would be determined in the fecal material

with metabolic fecal N and AA determined in a group of rats fed a protein-free diet to yield true digestibility. Ideally, aliquots of a ruminal feed residue would undergo parallel digestions via rat bioassay, the original and modified three-step procedures and *in situ* mobile bag in the ruminant. If intestinal digestion in ruminants is estimated using the modified three-step procedure, the amount of pepsin used should be adjusted to maintain the ratio of sample N to pepsin at approximately 1.5 as in the original three-step method (Calsamiglia and Stern, 1995), especially if 5 g of sample is not used. Since purchased enzymes vary, a standard feed should be included with each digestion as an internal standard and used for standardization.

CONCLUSIONS

S. griseus incubation can be used to predict rumen degradation of the crude protein pool in ruminant feeds. However, if the goal is to obtain residues that can be used to further our ability to predict AA supply in ruminants, *S. griseus* does not appear to adequately accomplish this due to the inability of the enzyme blend to digest carbohydrate. This effect results in ratios of AA in the residues that do not parallel those found after *in situ* and *in vitro* incubations. Feedstuffs vary in the proportion of their AA and the acid detergent AA that are intestinally digested and should be incorporated into the feeding systems used for ruminant animals. The ADIN and corresponding AA appear to be highly digestible in animal and soy proteins while those of forage feeds may be more digestible than previously thought, although, the intestinal digestion with physiological enzyme levels may need repeating. Inclusion of these increased digestibilities will allow the ruminant to be fed closer to requirement, thus reducing contamination of environment from excess N excretion and cost to the farmer**.** Ruminal digestion in 50 µm pore size bags appears to be appropriate to allow microbes access to feed particles. Intestinal

digestion in 25 µm pore size bags may prevent loss of particles. Incubation in the Daisy is sufficient for DM and N digestion but microbes may become limiting for maximal AD digestion. Intestinal digestion in ruminants needs additional refining

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Chapter 4

DEVELOPMENT OF AN IN VITRO INTESTINAL DIGESTIBILITY ASSAY FOR RUMINANT FEEDS THAT MINIMIZES SAMPLE LOSS AND STANDARDIZES ASSAY CONDITIONS AND COMPARISON WITH ACID DETERGENT INSOLUBLE NITROGEN AS AN UNAVAILABLE PROTEIN FRACTION

INTRODUCTION

Current cattle diet formulation models rely on estimates of intestinal digestibility of proteins and carbohydrates to adequately predict metabolizable energy (ME) and protein (MP) supply (NRC, 2001; Fox et al., 2004; Tylutki et al., 2008). As the models become more accurate and precise in the prediction of nutrient supply and evaluation of requirements and nutrient balance, greater scrutiny will be placed on inputs currently relegated to static library values. For example, work from Recktenwald (2010) and Higgs et al. (2012) demonstrated levels of milk production in dairy cattle in excess of 40 kg milk per day fed diets with CP content below 15.5%. With diets lower in CP, estimates of intestinal digestibility (ID) of protein and amino acids are important to ensure an adequate supply of those nutrients. Application of feed library values under all feeding conditions can lead to under- and over-estimations of MP and amino acid supply, resulting in variation from expected production.

Methods to estimate *in vivo* digestibility have been described by Tilly and Terry (1963) and have been further refined by Calsamiglia and Stern (1995) and Gargallo et al. (2006). More recently, Boucher et al. (2009a,b,c; 2011) has published

additional methodological approaches in an attempt to provide a robust, commercially adaptable assay for estimating intestinal protein and amino acid digestibility.

Tilley and Terry (1963) developed a two-step *IV* procedure, combining ruminal digestion with acid pepsin to determine digestibility of forage crops. A review by Stern et al. (1997) detailed the development of an assay and calculated intestinally absorbable dietary protein from specific feeds as ruminally undegraded protein multiplied by intestinal protein digestion and stated ”It was evident that an *IV* technique to estimate protein digestion should include enzymes with activity and specificity similar to those found in the digestive tract of the animal” and ”be correlated with intestinal digestion and not total tract digestion”. Concurrently, a three-step procedure (TSP, Calsamiglia and Stern,1995) utilizing in situ (IS) ruminal digestion followed by two additional *IV* steps to closely simulate physical conditions in the ruminant was developed as an alternative to duodenal cannulated animals for intestinal digestion (Calsamiglia and Stern, 1995; Gargallo et al. 2006).

Calsamiglia and Stern (1995) used the *IS* bag technique as a first step and proceeded with two additional *IV* steps in centrifuge tubes. Dried *IS* residue was subjected to a hydrochloric acid (HCl) and pepsin pre-digestion prior to pancreatin digestion, similar to the procedure of Akeson and Stahmann (1964). This three-step assay was validated and optimized against duodenal samples obtained from an *in vivo* intestinal digestion study using the same protein sources. The results from the pancreatin digestion assay were highly correlated ($n = 34$; $r = 0.91$, $P < 0.001$) to estimates of the *in vivo* intestinal protein digestion (Calsamiglia and Stern, 1995). However, digestion was terminated by addition of trichloroacetic acid (TCA) and protein content determined on the soluble fraction. Thus, determining the digestibility of amino acids was impossible.

The three step procedure was modified by Gargallo et al. (2006) (mTSP) where the intestinal digestion step was performed in an IV system (Daisy incubator; Ankom Technology, Macedon, NY) and then placing the IV undigested residue in the enzymes to estimate intestinal digestion while eliminating the use of TCA. Recently, Boucher et al., (2009a,b,c) utilized the precision-fed cecectomized rooster bioassay, deemed to be an appropriate technique to estimate small intestinal (SI)digestion of amino acids in cattle by Titgemeyer et al. (1990), and an immobilized digestive enzyme system to determine intestinal true digestibility of rumen undegraded protein. However, despite the sensitivity of this approach, it is not easy to apply to a commercial feed analyses system for routine analyses.

The current methods for estimating RUP rely on polyester bags with a pore size greater than 75 µm as the first step of the assay in either an *IS* or *IV* system. This step potentially creates problems on two levels. The use of the bag in either approach creates a microbial barrier to feed access and microbial attachment, which artificially prolongs the lag phase of digestion. The subsequent issue is the possibility of loss of highly soluble feeds from the bag prior to digestion, and loss of particles as digestion progresses. Work describing NDF digestibility demonstrated significant loss of sample from the bag during *IV* digestion and through the bottom of the sintered glass crucible, implying that the digestibility of sample measured in the bag is only a measure of what is retained and possibly not the sample of interest (Uden, 2006; Raffrenato, 2011).

For the assay to provide adequate estimates of digestibility, the enzyme profiles and activities must be properly described and characterized. Fermentation of cellulosic material by the microbes requires time; thus, the digestive process of the ruminant is a continuous flow of digesta with a continuous secretion of enzymes and digestive juices (Hill, 1965). In the abomasum, pepsin, an endopeptidase, hydrolyzes

only peptide bonds between amino acids (AA) with phenyl groups (phe, tyr) and a dicarboxylic acid (asp, glu) but not amide or ester linkages (http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Product_Information_Sheet/1/p7012pis.Par.0001.File.tmp/p7012pis.pdf; accessed Oct 15, 2010) and preferentially cleaves the C-terminal of Phe and Leu and to a lesser extent Glu linkages and does not cleave at Val, Ala, or Gly (http://www.worthington-biochem.com/pm/default.html; accessed Oct 15, 2010), thus, reducing approximately 15-20 % of dietary protein to AA and small peptides (Kutchai, 1998). Even though bovine and porcine pepsins have similar structures, bovine pepsin has only ~60-70 % of the activity of porcine pepsin with hemoglobin as substrate (Lang and Kassell, 1971) while Chu and Nakagawa (1981) reported bovine pepsin activity to be only 22 % that of porcine using this substrate. It should be noted that porcine pepsin is generally used in the first step of *IV* intestinal digestion assays to measure ruminant intestinal digestion (Calsamiglia and Stern, 1995; Gargallo et al., 2006).

Beside pepsin and hydrochloric acid, the ruminant abomasum secretes lysozymes. Lysozymes, or 1,4-β-N-acetylmuramidases, usually have an immune function, hydrolyzing the peptidoglycan cell wall backbone of foreign bacteria, but aid in digestion of microbes in the abomasum. Bovine digestive lysozyme has a lower optimum pH than chicken lysozyme, 7.65 vs. 10.7, respectively; thus, the pH optimum of bovine lysozyme is 5, not 7, making it resistant to pepsin. Furthermore, bovine lysozymes lyse gram-negative bacteria in addition to gram-positive which the chicken lysozyme does not (Dobson et al., 1984; Protection of plants against plant pathogens: http://www.patentstorm.us/patents/5422108/description.html; accessed Nov 1, 2010)

In rats, trypsin activity represents ~80 % of total protease activity in the pancreas and ~74 % in the duodenum and jejunum (Fauconneau and Michel, 1970) while in

ruminants it represents 15 % of pancreatic juice protease activity while chymotrypsin makes up 43 % (Keller et al., 1958). The calculated activities (U/ml) of trypsin and chymotrypsin in intestinal contents from 5 month old calves (Gorrill et al., 1968) were 19.48 U/ml and 15.9 U/ml, respectively using p-toluene-sulfonyl-L-arginine methyl ester (TAME) and benzoyl-L- tyrosine-ethyl ester (BTEE), respectively, as substrates. In sheep, the activities of trypsin, chymotrypsin and carboxypeptidase A increased from the pylorous to 7 m beyond with maximum specific activities of 24, 150, and 35 µM of respective substrates (benzoyl-L-arginine-ethyl ester (BAEE), acetyl- L-tyrosine-ethyl ester (ATEE), hippuryl-DL-phenyl-lactic acid) per minute per ml digesta, and then decreased (Ben-Ghedalia et al.,1974). Sklan and Halevy (1985) found maximal activities of pancreatic enzymes in the proximal segments of the ovine SI at 1 m distal to the pylorous and then relatively constant ratios of enzyme levels (trypsin, chymotrypsin, elastase, carboxypeptidases A & B) to cerium-141, an unabsorbed reference, of 0.065, 0.053, 0.015, 0.05 and 0.045, respectively, 1.5 to 9 m distal to the pylorous.

No other *in vivo* activities for bovine pancreatic proteolytic enzymes were found. Luchini et al. (1996) tested an *IV* system as a substitute for ruminal digestion with final concentrations of 116.6, 0.5, 2.5, and 0.5 EU/mL for trypsin, carboxypeptidase B, chymotrypsin, and carboxypeptidase A, respectively.

When comparing the activity of the pancreatic proteolytic enzymes among papers, the units are dependent upon the substrate (a protein or ester) hydrolyzed in addition to the wavelength used, and among the studies reviewed, this data varies considerably and is not standardized. For example with pepsin, one Fédération Internationale Pharmaceutique/International Pharmaceutical Federation unit (FIP) is the amount of standard preparation, which upon incubation at 25.0 ± 0.1 °C for one minute with a suitable preparation of pure hemoglobin will cause the decomposition

of the hemoglobin to such an extent that the amount of hydroxyaryl substances liberated will, upon reaction with Folin-Ciocalteus-Phenol-Reagent, result in the formation of a colored solution of equal intensity to that resulting from the reaction of 1 micromole, or milli-equivalent of tyrosine with the reagent (Anson, 1958), (www.biozym.de/datasheets/pepsin.php; accessed, Nov 2, 2010). One Worthington unit x 0.0071 equals 1 FIP unit. Further, Worthington defines pepsin activity in terms of releasing 0.001 A_{280} as TCA soluble hydrolysis products from denatured hemoglobin or casein per minute at 37°C (http://www.worthington-biochem.com/PM/cat.html), (http://www.worthington-biochem.com/PM/cat.html; accesed Nov 2, 2010). Pepsin activity is also reported as digestion capacity NF XII which digests not less than 3000 and not more than 3500 times its weight of coagulated egg albumin, or bovine pepsin which digests 2500 times its weight of coagulated egg albumin. In older papers pepsin activity used for digestion studies was 1 g/l containing 2000 FIP units per g (Antoniewicz et al., 1992; Steg et al., 1994; Van Staalen et al., 1997) which equals 282 Worthington units per mg.

The current IV intestinal proteolytic assays (Calsamiglia and Stern, 1995; Gargallo et al. 2006; Borucki Castro et al., 2007; Boucher et al., 2009a,b,c) use 3 g per L of pancreatin, after an IV abomasal digestion with 1 g L^{-1} of porcine pepsin in 0.1 N HCl N at pH 1.9 or 2. Pancreatin always contains amylase and lipase but over time the proteolytic enzyme has been changed from trypsin to many enzymes, including trypsin, ribonuclease and protease (specifications for P7545; www.sigmaaldrich.com/catalog/product/sigma/p7545?lang=en. accessed , Nov 10, 2010) and specific units of enzymatic activity are not provided. From the specification sheet (Sigma Chemical Company; St. Louis, MO) pancreatin will convert 25 times its weight of potato starch into soluble carbohydrates within 5 minutes in water at 40°C, will release greater than 2 uEq of acid from olive oil per min per mg

pancreatin and will digest 25 times its weight of casein in 60 min. For abomasal digestion, one mg of porcine pepsin contains 200 to 625 units with pH between 1.5 and 2.5, optimum for pepsin activity however, a 0.1 N solution of HCl is more acidic than this range. Further, lipase activity is essentially nonexistent in bovine pancreatic juice (Keller, 1958) but is high in the saliva. Calsamiglia and Stern (1995) attributed the increase in digestion of their proteins over those obtained using the multi-enzyme system of Hsu et al. (1977) to the presence of amylase and lipase in pancreatin.

The objective of this study was to further develop and standardize an *IV* intestinal digestion assay for proteins in the bovine using methods that minimize sample loss, provide adequate sample size, standardize enzyme activity and concentration and can be used on intact commercially available feeds with manufacturing characteristics that are designed to resist rumen degradation. Once developed, the assay was used to develop data on digestibility and compared to acid detergent insoluble nitrogen as an unavailable protein fraction. A further objective was to develop an assay that could be adopted by commercial laboratories for routine analyses of samples in an effort to better provide inputs for nutrition models.

MATERIALS AND METHODS

Unless specified otherwise, all analyses were conducted on duplicate samples. Dry matter was determined at 105ºC in a forced-air oven overnight. Residual nitrogen (N) was measured by block digestion and steam distillation with automatic titration (Application Note, AN300; AOAC Official method 2001.11; Foss, 2003; Tecator Digestor 20 and Kjeltec 2300 Analyzer, Foss Analytical AB, Höganäs, Sweden; AOAC 2001.11). The development of the assay evolved through three

stages for sample analyses initially for the IV step but subsequently for all steps: bags, then tubes and finally Erlenmeyer flasks. In place of the *in vivo* step, all feeds went through rumen fermentation via IV methods prior to digestion with enzymes representing abomasal and intestinal digestion. The paper follows the steps that were evaluated and modified starting with the use of sample bags for the IV and subsequent steps as described by Gargallo et al. (2006) and continues through each subsequent phase to a final assay description. In this study, bags were found to contribute to variation due to sample loss, tubes were found to be inconsistent due to a decrease in microbial activity and enzyme function and Erlenmeyer flasks were found to provide the most robust analyses and repeatability.

1. Evaluation of the use of sample bags

In the initial evaluation, two blood meals, a ring dried blood meal (BM0) and a highly digestible and soluble blood meal (BM1) were heat treated for 6 hr at 165°C which resulted in four different samples: BM0, BM1, BM2 (heat damaged BM) and BM3 (heat damaged BM1)). The four samples plus a lignosulfonate treated SBM (SOY1) were used for the initial studies. Samples were not ground and were used as received primarily because commercially available manufactured feeds are designed to be more rumen stable, thus any grinding can invalidate the conditions the developers were trying to achieve. In addition, grinding will exacerbate any potential loss from the sample bag if not standardized or understood, thus not grinding samples provides a more robust test of sample loss from the bags

Table 4.1. Description of feeds used in this study. Chemical composition will be described in the subsequent tables with analyses.

Abbr.	description
ANVY	Anchovy fish meal (Boucher et. al., 2011)
AS	Alfalfa silage
BM0	ring dried blood meal

BM1	highly soluble blood meal
BM2	heat damaged blood meal 0 (165°C, 6 h)
BM3	Heat damaged blood meal 1 (165°C, 6 h)
BM4	blood meal 4 (Boucher et. al., 2011)
BM5	blood meal 5 (Boucher et. al., 2011)
BM11-26	Ring dried blood meals
CM	Canola meal
CG1	Corn gluten feed 1
CG2	Corn gluten feed 2
CornBP	Corn milling byproduct
Com1-6	Commercial feeds high in escape protein
CS	Corn silage
SBM0	Soybean meal, untreated
SBM1	Heat damaged SBM0 (150°C; 3.5-h)
Soy1	Lignosulfonate treated soybean
Soy2	Modified expeller soybean product
Soy3	Solvent extracted soybean product
Soy4	Commercial soybean product
Soy11-15	Commercial expeller soybean products

Evaluation of type of incubation bag: Two pore size bags: *in situ*, 50 ± 15 µm (Ankom Technology, Macedon, NY) and Fiber 25 (Ankom F57 material; Ankom Technology; rinsed with acetone to remove surfactant.) were utilized for ruminal digestion while only the fiber bags were used for the intestinal digestion. Bags were 5 x 10 and 5 x 5 cm, respectively, with 5 and 1 or 0.5 g of sample added, respectively.

In situ versus in vitro fermentation: Fermentations of 0.5 g samples were performed simultaneously *in situ* in two lactating dairy cattle with ruminal cannulas fed diets formulated for 42 kg milk per day, and *IV* using a Daisy^{II} incubator (Ankom Technology; Macedon, NY) under anaerobic conditions at 39°C for 16-h in a buffered medium (Goering and Van Soest, 1970) containing Trypticase and rumen fluid strained through 4 layers of cheesecloth, glass wool and a 100 µm nylon cloth (#NY1H00010; EMD Millipore; Billerica, MA). Both bag types were utilized for the *in situ* incubation but only fiber bags were used *IV*. After completion of ruminal

fermentation, bags were removed, rinsed in cold tap water until the water ran clear (De Boer et al., 1987) and drained. Bags were placed overnight in either a freeze dryer with a shelf temperature of 25°C (VirTus 20 SRC-X; The VirTus Co.,Inc., Gardiner, NY), and then desiccated for AA digestion or a 105°C oven (Hotpack Corp., Philadelphia, PA) for DM disappearance, and weighed. Residues were composited by fermentation and bag type and analyzed for N.

Evaluation of intestinal digestion using bags with an enzyme mixture or pancreatin: Fifteen mg of dry IV sample residual N was weighed into a F57 fiber bag (Ankom Technology) and subjected to a 1-h abomasal digestion in a beaker containing 10 ml per bag of 0.6 g of pepsin L^{-1} (~224 U ml^{-1}; Van Staalen et al., 1997) in pH 2 HCl (~0.013 N), in a shaking 39°C water bath (Precision Dubnoff metabolic shaking incubator, Precision Scientific Co., Chicago, IL). After one hour, the bag was rinsed with cold tap water, then placed in beakers containing the enzymes (see Table 4.2) in 13.5 ml of 0.5 KH_2PO_4 (pH 7.75) per bag for a 24-h intestinal digestion in the shaking 39°C water bath, rinsed, dried as above and weighed for DM disappearance before analysis of residue for N content. The abomasal conditions for all studies were based on the activity of the bovine enzymes at approximately 60 % of the activity of porcine enzymes.

Study 1: Intestinal protein digestion using trypsin, chymotrypsin and carboxypeptidase (Cxp) A & B after abomasal digestion was compared to the original TSP and mTSP using pancreatin (3 g L^{-1}) except ruminal residues were not rinsed in a washing machine. The study also compared *in situ* and *IV* ruminal digestion in 25 and 50 µm bags. Maximum activities of trypsin, chymotrypsin and Cxp A found in sheep digesta by Ben-Ghedalia et al. (1974) were added for the intestinal assay. However the activity of chymotrypsin measured in this study was 20 uM/ml using benzoyl-tyrosine ethyl ester as substrate compared to 150 in the sheep digesta with

acetyl- L- tyrosine-ethyl ester as substrate. Activity of Cxp B was calculated by applying the ratio of Cxp B to A from the study of Sklan and Halevy (1985) to the Cxp A value. Units of enzyme activity are given in Table 4.2.

Study 2: Carboxypeptidase Y, an exopeptidase with the ability to release all AA from carboxy-terminus, was substituted for Cxp A, which was available in very limited quantities and increasingly cost prohibitive. Based on the previous literature, Cxp A and B are most likely the enzymes that degrade microbial cell wall and other microbial components, thus maintaining them or an analogous enzyme was important as long as the cost was not prohibitive and there was adequate availability.

Study 3: Elastase, an endopeptidase, specifically hydrolyzes elastin by cleaving glycine, alanine and valine from the carboxy end, was also evaluated as a substitute for Cxp A.

Study 4: In the work by Calsamiglia and Stern, (1995) they attributed the higher intestinal digestibility of feeds by pancreatin to the presence of amylase and lipase, assuming the enzyme activity and specificity of pancreatin was standard among sources. Addition of amylase and lipase was evaluated using levels observed in bovine digesta (Sir ElKhatim et al., 1983; Kreikemeier et al., 1990) and in sheep (Johnson et al., 1974), respectively.

Table 4.2. Final assay activity of pancreatic enzymes added (mg or U ml^{-1}) by study. Intestinal digestion with pancreatin was always assayed along side the enzymatic mix.

	Study			
	1	2	3	4
Pancreatin mg ml^{-1}	3	3	3	3
Enzyme, U ml^{-1}				

Trypsin	24	24	24	24
Chymotrypsin	20	20	20	20
Carboxypeptidase				
A	18	18	-	-
" B	17	17	17	-
" Y	-	18	-	-
Elastase	-	-	0.18	
			0.33	
			0.67	
			1.0	-
Amylase				50
Lipase				4

Characterization of enzymes: Pepsin (P7000), trypsin (T9201), chymotrypsin (C4129), carboxypeptidase A (C0261) and pancreatin (P7545) were obtained from Sigma Chemical Co. (St. Louis, MO), carboxypeptidase Y (12758) from Affymetrix, Inc. (Santa Clara, CA), carboxypeptidase B (LS001724) and elastase (LS002274) from Worthington Biochemical Corp. (Lakewood, NJ), amylase (E-BLAAM100) from Megazyme (Wicklow, Ireland) and lipase (18485) from USB Corporation (Cleveland, OH). Company enzyme activities were used except for amylase. Amylase activity was measured using maltose (SSSTAR01; Sigma Chemical Co., St. Louis, MO) and activity observed is shown in Table 4.2. Pepsin activity was confirmed using hemoglobin (SSHEMO01, Sigma Chemical Co., St. Louis, MO). Lipase was corrected for dry matter. Units of enzyme activity are provided in Table 4.1.

Calculations: Intestinal digestibility was calculated as a fraction of component DM and N using the following equation:

intestinal digestion (ID_{DMTN}) fraction = [1- (undigested rumen residue DM fraction) * (undigested rumen residue N/TN)].

2. Alternatives to bags: Nalgene tubes

Due to observed sample loss, an alternative to incubation bags were necessary. The first assay vessels evaluated were Nalgene tubes that could be used in the water bath and would hold a stopper so they could be flushed and maintained with CO_2 to

remain anaerobic. Seven feeds were used in this evaluation: BM0, BM1, BM3, BM4 and BM 5, Soy1, Soy2, Soy3, and DDG were utilized. Samples were weighed to provide ~15 mg N of RUP for the intestinal digestion (for amount see Table 4.3.) into six - fifty ml tubes (Nalgene 3110-0500; Nalge Nunc International; Rochester, NY) with two tubes for RUP determination and two additional tubes for ID using the enzyme mix and pancreatin.

Table 4.3. Approximate weight of sample for fermentation to yield 15 mg N for intestinal digestion

	~ g N g^{-1} DM	g sample
Blood meal	0.16	0.10
Soy products	0.07	0.18

Ruminal fermentation: In vitro anaerobic fermentation was performed using 20 ml of buffered medium, pH 6.8, and 5 ml rumen fluid (prepared as above) per tube. After 16-h all tubes were centrifuged at 8,160 x g in Beckman JA-17 rotor (Beckman Coulter, Brea, CA) and the supernatant aspirated. For RUP, pellets were quantitatively transferred with room temperature distilled water to pre-tared filters (55 mm; 1.5 µm pore, Whatman 934-AH, GE Healthcare Bio-Sciences Corp., Piscataway, NY) and dried overnight for DM, if appropriate, before N analysis.

Intestinal digestion (ID): With ~15 mg N in the pellet, the abomasum digestion was performed as previously described except 10 ml of pH 2 HCl containing 0.6 g pepsin L^{-1} was pipetted into each tube. After 1-h, the acid was neutralized with ~0.057 ml 2 N NaOH (the pH of some samples was checked) to raise pH to ~5. The enzyme mix containing trypsin, chymotrypsin, amylase, lipase and pancreatin, as shown in Study 4 of Table 3.1, was added in 13.5 ml potassium phosphate buffer, pH 7.75 and tubes

incubated for 24-h in shaking water bath. Tubes were centrifuged, supernatant aspirated, pellets filtered, dried and analyzed for N, as done for the RUP samples. *Calculations*: Intestinal digestion was expressed as a fraction of RUP digested (ID_{RUP}): Fraction of RUP digested = [(RUP,TN – Und N, TN) $(RUP,TN)^{-1}$], but RUP in this set of studies was not corrected for microbial contamination.

3. Alternatives to bags and Nalgene tubes: Erlenmeyer Flasks

Samples: In addition to feeds used previously, another commercially produced expeller soy with modifications for rumen stability (Soy4), an untreated SBM, a heat damaged SBM (150°C, 2 hr), canola meal, two corn gluten meals, a corn milling byproduct, corn silage, alfalfa silage, sixteen ring dried blood meals (BM11-26), five expeller soy meals (Soy11-15) and six other commercial feeds high in rumen escape protein (Com1-6) were obtained. Silages were ground to 1 mm (Wiley Mill, Arthur H. Thomas Company, Phil. PA) but other feed products were used as is. Half gram samples were added to 125-ml glass Erlenmeyer flasks. Each feed required six fermentation flasks: two for RUP determination and two each for ID with enzyme mix or ID with pancreatin.

Ruminal fermentation: In vitro anaerobic fermentation was performed using 40 ml of buffered medium, pH 6.8, and 10 ml rumen fluid (prepared as above) per flask for 16-h in a bath at 39°C (Precision Model 170, Precision Scientific Co., Chicago, IL; MW1140A-1, Blue M, Blue Island, IL;). For RUP determination samples were filtered on a 90 mm filter to increase the filtration speed (1.5 µm; Whatman 934-AH), rinsed with boiling distilled water, dried overnight at 105°C for DM digestibility and analyzed for N. Shaking the fermentation flasks did not change RUP (Ross, unpublished).

Intestinal digestion: After fermentation flasks for ID determination were placed in shaking water bath set for 39 °C (Precision Dubnoff metabolic shaking incubator,

Precision Scientific Co., Chicago, IL; VWR Model 1227reciprocating water bath, VWR International, Inc., West Chester, PA), acidified with ~2 ml 3 M HCl (check pH -- ~1.9 - 2); vigorously mixed for 1 min prior to abomasal digestion: 2 ml pepsin (16.6 mg $ml^{-1}$of pepsin with activity of 351 U mg^{-1} to yield ~224 U ml^{-1}) in 0.013 M HCl (~pH 2) were added and incubated with shaking for 1 h. The mix was neutralized to pH 5 with ~2 ml 2 M NaOH (check) and vigorously mixed for 1 min. Then enzymes were added in 10 mls of 1.8 M potassium phosphate buffer containing either 12.04 mg pancreatin per ml or a mix of 168 U trypsin, 140 U chymotrypsin, 705 U amylase and 28 U lipase per ml to account for the dilution (~70 ml) so total volume contained 0.5 M potassium phosphate with either 1.72 mg pancreatin (Gargallo et al., 2006) per ml or mix of 24, 20, 50 and 4 U per ml, respectively. Flasks were incubated with shaking for 24-h at which time samples were removed and filtered, as above for RUP, prior to N analysis.

After fermenting half gram samples, homogeneity of the samples became a concern due to the small amount of material remaining and also the relatively small amount of feed material used at the beginning of the assay. To evaluate the homogeneity of the feed residues for repeatability of the assay, the IV residue of four soy products (Soy 1 - 4) and a corn milling byproduct were determined as duplicates, quadruplicates and six replicates divided between two 39°C baths, filtered and rinsed with hot water and analyzed for N. A second rep was conducted the following day using the same 2 cows for rumen fluid.

Use of positive and negative controls to evaluate IV step and intestinal digestibility step: Positive and negative controls for both the fermentation and intestinal digestibility steps were included. It was decided that a control to assess the rumen fermentation portion of the assay should be included. To evaluate the fermentation phase, a corn silage sample with a known NDF digestibility was used as a control

and the NDF digestion was run concurrently. A heat damaged blood meal (above) was included throughout as a negative control. Another feed, i.e., a soy product or blood meal, with similar digestibility as samples was included. A blood meal with >90 % rumen escape and known high intestinal digestibility was included as a positive control for the ID assay.

Calculations: To estimate an RUP from the first step of the assay, the following equation was used:

ID_{RUP} = [(RUP,TN – Und N, TN) $(RUP,TN)^{-1}$] which required representative RUP value without microbial contamination.

Correction of in vitro residues for microbial contamination

As the assay was being developed, questions were generated about microbial contamination affecting the estimates of the ID measurement and also affecting the estimate of RUP, especially for samples lower in N content. The original objective of the assay was to estimate ID and not RUP, however, it became apparent that in either case, microbial contamination should be accounted for if possible to ensure less bias in the ID determination. Therefore a series of approaches were evaluated to provide a quantifiable and repeatable indication of microbial contamination.

Labeling with ^{15}N

Half gram samples of corn silage (CS) and Soy1 were fermented in IV bags (F57; Ankom Technology) using a Daisy incubator (Ankom Technology) and in flasks in a 39°C bath in two reps using fermentation buffer containing 0.02 ape $^{15}N_2$-urea (98%+; NLM-233-0; Cambridge Isotope Laboratories, Inc., Andover, MA) in 50 ml, Following the 16-h fermentation, all bag residues were rinsed with tap water and then some with methylcellulose while flask residues were rinsed with boiling water, methylcellulose, neutral detergent (ND) solution, acid detergent (AD) solution or sequential ND then AD.

Washing with methylcellulose: For samples in bags, fermentation mix was dumped, bags rinsed until water ran clear and then 0.1 % methylcellulose (Whitehouse et al., 1994) was added to jar, incubated at 39°C for 30 min and dumped. Following fermentation in flasks, an equal volume of 0.2 % methylcellulose, double the concentration of Whitehouse et al. (1994), was added to each flask, to yield 0.1 %, and incubated as above in shaking water bath prior to filtering using boiling water to transfer.

Washing in neutral detergent (ND) solution with amylase: Fifty mls of ND solution without sulfite (Van Soest et al., 1991) plus 200 µl heat stable amylase (FAA; Ankom Technology) were added to fermented sample, boiled for 1-h, filtered and rinsed with boiling water.

Washing in acid detergent (AD) solution: Fifty mls of 2X AD solution (2 N H2SO4 + 4 % cetyltrimethylammonium bromide (CTAB)) were added to fermented samples, boiled for 1-h, filtered and rinsed as above.

Washing in a sequential neutral detergent/acid detergent solution: ND was performed as above except after boiling for 1-h, 100 ml 2X, filtered and then AD was added, boiled for another 1-h, filtered and rinsed as above.

Nitrogen was analyzed as above. ^{15}N analyses were performed by The Cornell Isotope Laboratory (COIL) on a Thermo Delta V isotope ratio mass spectrometer (IRMS) interfaced to a NC2500 elemental analyzer loading ~20 ug residue N, which was conducted on residue for all samples except the ND, AD and ND-AD, due to the quantity and particle size, and those contained filter material and residue.

Calculations: For these calculations it was assumed that all ^{15}N was bacterial contamination and the N content of bacteria was 77 g kg^{-1}.

- ape = ((AT%-blk)/(AT%-blk+1))

- Corrected RUP = [residue N – (corrected ape * residue N)] (sample total N content)$^{-1}$, g N g^{-1} DM

Using purine derivatives as markers of microbial contamination

Microbial purines were measured in the dry residues using the HPLC procedure of Makkar and Becker (1999). Approximately 100 mg residue and the internal standard, allopurinol (100 ul of 6 mM) were hydrolyzed in 2.5 ml 2 M perchloric acid at 95°C for 60 min. Recoveries were checked using yeast (~0.8 mg) and averaged 84 % with lower recoveries attributed to possible weighing error.

Calculations were based on the following assumptions: (1) all purine bases were microbial contamination, (2) microbes contained 11.02 uM purine bases (100 mg)$^{-1}$ (Makkar and Becker, 1999) and (3) microbial N content was 77 g kg^{-1} (Clark et al., 1992).

Microbial N = [(uM guanine g^{-1} + uM adenine g^{-1}) x (110.2 uM purine base g^{-1} microbes)$^{-1}$] * .077 g N g^{-1} microbes

Corrected RUP = (residue N – microbial N) x (sample total N content)$^{-1}$, g N g^{-1} DM

Evaluation and adaptation of other fermentation substrates low in nitrogen content for estimating microbial contamination

The primary factors considered for this step were substrates low in N content with the potential to grow adequate levels of bacteria. In the first evaluation, cellobiose was used as a substrate for microbial growth but bacteria did not attach to it readily due to its solubility, thus there was little microbial protein to use for correction and cellobiose was deemed an inappropriate substrate. Subsequently, isolated straw NDF was prepared by grinding it through a 2 mm screen using a Wiley Mill, washing it in hot ND solution with amylase, rinsing and washing again with an ammonium sulfate (1

M) solution overnight (18 h) in a 39°C water bath (Doane et al., 1997) filtered, rinsed with distilled water and dried as above. Washing with ammonium sulfate is done to remove sodium lauryl sulfate which will inhibit microbial growth. The straw ND residue was considered a good substrate for evaluating contamination since it was essentially devoid of N, however it did not support adequate microbial growth. Another alternative was the use of ND residue from corn silage. Fresh corn silage was obtained from the Cornell University Teaching and Research Farm (Harford, NY), freeze dried and ground to 2 mm particle size. Bulk ND residue was prepared in several batches as follows:

Combined 120 g CS + 120 g sodium sulfite + 24 ml heat-stable amylase + 3 L ND solution; boiled 1.25-h, filtered (Whatman 541; 24 cm), rinsed with boiling water and dried overnight at 105°C. All ND residues were composited. Again, to prevent ruminal bacterial inhibition, the ND residue was washed in an ammonium sulfate (1 M) solution overnight (18 h) in a 39°C water bath (Doane et al., 1997) filtered, rinsed and dried. The N content was determined on the ND residue.

To evaluate the level of microbial growth and attachment that could be obtained with this isolated corn silage NDF residue, two replicates of twenty 0.5 g samples were fermented with and without rumen fluid from 2 cows, separately, with samples split between two (39°C) baths, filtered using boiling water and analyzed for N. This evaluation was conducted in Erlenmeyer flasks. Concurrently, feed samples underwent intestinal digestion of corrected RUP, as outlined above.

Calculation: Using Kjeldahl, the microbial correction was calculated as volume of acid (ml) g^{-1} DM: [Δ ml acid g^{-1} DM = (ml acid from CS ND + rumen fluid/g DM) – (ml acid from CS ND w/out rumen fluid/g DM)] multiplied by sample weight, DM and subtracted from RUP residue acid along with volume of acid from assay blank prior to calculation of fraction of feed N undigested.

An evaluation of the assay was conducted in the following manner: Sixteen ring dried blood meals (BM11-26), five expeller soy meals (Soy11-15) and six commercial feeds high in escape protein (Com1-6) were obtained and analyzed for RUP and ID of the RUP estimated using the enzyme mix or pancreatin with microbial contamination of RUP corrected using the corn silage ND as outlined above.

Comparison of intestinal digestion with the acid detergent insoluble protein amino acids

Within the current structure of many contemporary nutrition models the acid detergent insoluble nitrogen (ADIN) represents the unavailable N component of the feed (NRC, 2001; Tylutki et al., 2008) however, the NRC for Dairy Cattle (2001) provides for some digestibility of the ADIN fraction of up to 5%. The implication is that the ADIN fraction is not completely unavailable to the animal. Accordingly, the ID assay as outlined was utilized to ascertain whether the amino acids in the ADIN are indigestible. For this analyses, 0.5 g samples of nine feeds: anchovy fish meal (ANVY; AS, CS, BM0, CM, DDG, SBM0, Soy1 and Soy2 and BM2 HDBM; 165°C, 6 hrs), were fermented IV for 16-h followed by ID) in Erlenmeyer flasks with either the enzyme mix or pancreatin after abomasal degradation with pH 2 hydrochloric acid and pepsin. Amino acid digestion (only hydrochloric acid hydrolysis) plus DM and N disappearance were followed on the feeds and feed ADIN residue. Acid detergent residues following IV fermentation and ID were obtained using an equal volume of 2X AD solution, as described above. Consequently, each feed or blank required twenty-four flasks. Assay blanks were included. Incubation of corn silage ND with and without rumen fluid was utilized to correct for microbial contamination of the RUP fraction during the ID step. Feed N loss after fermentation, abomasal and ID represents 'R and SI' digestion unless calculated as $ID_{RUP,}$ or intestinal digestion of RUP.

Effect of day and cow:

Throughout the assay development, variation was observed among days and cows. To evaluate the source of the variation, a study was conducted using ten of the feeds in Table 4.1, and rumen fluid from two cows was used using Erlenmeyer flasks, the enzyme mix or pancreatin and the assay was conducted in duplicate on two separate days.

Statistical analysis. For all comparisons of interest, means and standard deviations were calculated using SAS (SAS Inst., Inc., Cary, NC). The GLM procedure of SAS was used to perform the analysis of variance on the ID outcome. Means within enzyme treatment (enzyme mix or pancreatin) were compared using Duncan's method when significance was observed in the analysis of variance test. Differences were considered significant when ($P < 0.05$).

RESULTS AND DISCUSSION

The results will follow a similar order to the development of the assay and the evaluation of the various steps associated with the assay. In some instances, there are interactions between the various steps, however, as observations were made and evaluated, the primary factors affecting the variability of the assay were addressed and resulted in the final assay conditions. For example, to eliminate any losses due to the use of bags and to optimize the fermentation, both bags and Nalgene tubes were abandoned in favor of Erlenmeyer flasks as the fermentation and ID assay vessel.

Use of bags for assay conditions

When using bags it is difficult to distinguish bag loss from digestion or solubility. Incubating Soy1 in 1, 25 and 50 µm pore bags in distilled water (with sodium azide added to inhibit microbial growth) for 1 h at room temperature resulted in more loss ($P < 0.05$) of DM from the 50 µm bags than the 1 µm bags (29 versus 22 %, respectively) while N increased due to less material remaining. Feed samples in 25 µm pore bags incubated in water at room temperature lost varying amounts of DM, ranging from less than 1 to 28 %, while N loss ranged from 0 to 8 % for BM5 (Table 4.4). The apparent loss from the bags prior to any analyses indicates that the sample remaining in the bag might not represent the sample of interest and could potentially bias the outcome of the assay analyses. The loss could be due to either particle loss from the bag or from the solublization of feed components that wash out of the bag.

Table 4.4. Fraction of DM and N loss from 25 um bags after soaking in water for 1-h at room temperature

Item	BM0	BM1	BM3	BM4	BM5	Soy1	Soy3
DM	0.0087	0.0120	0.0098	0.0100	0.1194	0.2776	0.2623
N	0.0077	0.0173	0.0096	0.0000	0.0799	0.0000	0.0474

Table 4.5. Percent digestion of different sample weights of blood meal (BM0) in 25 and 50 µm bags in situ and in vitro fermentation. Results represent means (SEM).

Fermentation	Bag pore size (µm)	sample size (g)	BM0
IS	50	5	24.9 (0.8)
		1	8.5 -
		0.5	8.7 (0.0)
IV	25	0.5	9.1 (0.3)

The digestibility of a blood meal (BM0) was evaluated *in situ* and IV

using different bags and different sample weights (Table 4.5). In this case the highest digestibility was observed in the *in situ* bag with 5 g of blood meal, however, an additional observation was that sample was moving through the bag and could be observed on the outside of the bag and on the counter under the bag. This suggests that samples can migrate out of the 50 µm bag if it is overfilled. The comparison was similar between the *in situ* and IV bags at the lower inclusion rates in this particular evaluation. The observation of significant sample loss from the bag under a few different assay conditions lead to the conclusion that the use of the in situ and IV bags were not acceptable to provide robust sample retention for accurate and precise measurement of digestibility.

Study 1. Comparing the enzyme mix and pancreatin, the pancreatin digested roughly 100 % of the DM and N in the rumen residues of BM0 and BM1 fermented in bags while most combinations of pancreatic enzymes yielded significantly lower digestibilities in the three untreated samples than did pancreatin (Table 4.6). When the enzymes native to the ruminant intestine were included (trypsin (T), chymotrypsin (C) and Cxp A and B), at levels observed in sheep digesta (Ben-Ghadalia et al., 1974), the assays yielded higher digestibility than pancreatin, 84 and 78 % for BM0 and BM1, respectively, versus 98 %; however, Cxp A became unavailable since it was harvested from calves and not produced recombinantly. The lack of availability and extreme cost of the available enzyme precluded further inclusion and evaluation of the enzyme and an alternative was explored.

Carboxypeptidase B alone or in combination with Cxp Y or multiple levels of elastase was evaluated as a substitute for Cxp A. The combination of T, C, CxpY, Cxp B digested roughly the same amount of BM0 but significantly less BM1 as did the pancreatin but with greater variation while 0.2 units of elastase with and without

amylase and TC plus amylase and lipase digested Soy1 to a similar extent as did pancreatin. However, Soy1, a soybean product designed by the manufacturer to be more rumen inert required the presence of amylase and lipase to adequately digest in the enzyme mix. In the presence of trypsin, chymotrypsin, Cxp A and Cxp B without elastase, only digested 23 % of the product digested, and with the addition of amylase and lipase to the mixture, ID increased to 74 %. Elastase alone only digested about 10 % of Soy1. These observations were consistent with those of Calsamiglia and Stern (1995) where they associated increased digestibility of their system compared to Hsu et al., 1977 to the presence of lipase and amylase in the pancreatin.

Rumen fermentation *IV* in either 25 or 50 µm pore bags (mT25 and mT50) yielded comparable intestinal digestions as *in situ* in the mSTP (Gargallo et al., 2006). Heating BM1 and BM0 for 1-h at 140°C resulted in 42 and 80 % less intestinal dry matter-nitrogen digestion using pancreatin, respectively, with very little, or no digestion using T, TC or TCAB; however the mTSP method yielded even less digestion (60 and 92 %, respectively). Since elastase is not naturally present in the intestine and Cxp Y is not present in measurable quantities, trypsin and chymotrypsin plus amylase and lipase became the intestinal enzyme combination adopted for the assay. Pancreatin was co-assayed for comparison. Porcine lipase which Julien et al. (1972) observed to have four times the activity compared to bovine was used in these studies while the amylase was heat-stable from bacterial source *(B. licheniformis*).

Table 4.6. Fraction of intestinal feed dry matter and nitrogen digested (ID_{DMTN}) using pancreatin or combinations of trypsin, chymotrypsin, carboxypeptidase A, B or Y, elastase, amylase and lipase. Values are mean and standard error.

	BM1	BM3	BM0	BM2	Soy1	SEM
trt†	ID_{DMTN}*	ID_{DMTN}	ID_{DMTN}	ID_{DMTN}	ID_{DMTN}	
mT50	0.994^{a}	0.574	0.999^{a}	0.196		0.001
mTSP	0.979^{ab}	0.384	0.983^{ab}	0.065	0.666^{a}	0.003
mT25	0.985^{a}	--	0.999^{a}			0.002
TCYB	0.856^{cde}	--	0.954$^{ab\ c}$			0.012
TC1E	0.863^{cde}	--	0.876^{cd}		0.294^{bc}	0.016
TC1EB	0.872^{cd}	--	0.864^{cd}		0.285^{bc}	0.018
TC.6EB	0.837^{cde}	--	0.886^{bcd}			0.016
TC.2EAmL	0.883^{bc}	--	0.830^{def}		0.774^{a}	0.025
TCAmL	0.897^{abc}	--	0.809^{defg}		0.737^{a}	0.025
TC.6E	0.812^{cde}	--	0.847^{de}			0.021
TC.2E	0.860^{cde}		0.792^{defg}		0.692^{a}	0.003
TCAB	0.840^{cde}	0	0.779^{defgh}	0	0.226^{bc}	0.028
TC.3E	0.806^{cde}		0.806^{defg}			0.035
TC.3EB	0.805^{cde}		0.749^{efgh}			0.048
TCY	0.861^{cde}		0.680^{h}			0.124
TCB	0.762^{de}		0.741^{efgh}		0.177^{bc}	0.045
TC	0.767^{de}	0	0.736^{fgh}	0.026	0.355^{b}	0.017
1E	0.760^{e}		0.707^{gh}		0.097^{e}	0.053
T	0.373^{f}	0.055	0.348^{i}	0.021		0.100
n=	55	10	54	10	24	

*ID_{DMTN}: intestinal digestion calculated as fraction of dry matter and total nitrogen; SEM, standard error of the mean. †intestinal enzyme: mTSP, modified 3-step with pancreatin; mT24, 50, pancreatin but fermentation in Daisy using 25, 50 µm bag; T, trypsin; C, chymotrypsin; A, B & Y, carboxypeptidases; E, elastase preceeded by level; Am, amylase; L, lipase. ^{abcdefghi}Means within same column with different superscripts differ ($P < 0.05$).

Feed was placed in plastic tubes to yield ~15 mg N following *IV* fermentation with rumen fluid for intestinal digestion with the enzyme mix and pancreatin. Fermentation in plastic tubes yielded high and variable amounts of RUP for BM0 (Table 4.4.7). Boucher et al. (2011) reported a RUP of 0.80 for BM4 while fermentation in a tube yielded 0.98. Fermentation of BM5 in a plastic tube yielded a

RUP of 0.60 versus 0.58, *in situ* in a bag (Boucher et al., 2011). In the plastic tubes, the RUP values for Soy1, Soy3 and DDG were 100 %. Meanwhile, duplication of the ID of the IV residues by either the enzyme mix or pancreatin was also variable, especially for the BM0 and DDG . However, pancreatin digested more feed N overall in all except the Soy3 and Soy4. Increasing centrifugal force to separate undigested feed residue did not solve the issue. Sayre and Van Soest (1972) noted the incompatibility of plastic tubes to maintain rumen bugs.

Table 4.7. Fraction of feed RUP intestinally digested by enzyme mix and pancreatin using ~15 mg N in plastic tubes. Values are mean and standard error.

Feed*	RUP	Intestinal digestion of RUP by	
		Enzyme Mix**	Pancreatin
BM0	0.916	0.320[a]	0.579[b]
BM4	0.984	0.546[a]	0.709[b]
BM5	0.603	0.588[a]	0.763[b]
DDG	1.00	0.481[a]	0.835[b]
Soy1	1.00	0.531[a]	0.685[b]
Soy3	1.00	0.805	0.857
Soy4	0.769	0.771	0.805
Overall	0.896	0.577[a]	0.747[b]
SEM	0.040	0.043	0.026

*feed: BM, bloodmeals; DDG, distillers grain; Soy products.
**Enzyme mix: trypsin, chymotrypsin, amylase and lipase.
[ab]Means in same row with different superscripts differ ($P < 0.05$).

In an attempt to decrease variability due to sample possible heterogeneity 0.5 g samples were fermented for 16-h in 125-ml Erlenmeyer glass flasks, which were not inhibitory to rumen bugs, contents acidified, pepsin added to simulate abomasum digestion for 1-h, contents neutralized prior to addition of enzyme mix (trypsin,

chymotrypsin, amylase and lipase) or pancreatin for 24-h intestinal digestion. Enzymes were added so activities reflected the dilution effect of previous steps and enzyme activities in the final assay were as reported in Table 4.2. However, as the assay was being developed questions about microbial attachment were being asked relative to contamination and effects on any estimate of RUP and the subsequent ID estimation.

Microbial contamination correction of RUP

Microbial contamination was more pronounced in low N, high fiber feeds, i.e., corn silage, than in high N, low fiber feeds, i.e., soy products. Methylcellulose, 0.1 % for 30 min at 39°C, with and without methanol and Tween 80 added, has been the 'gold standard' (Whitehouse et al., 1994; Gargallo et al., 2006; Boucher et al., 2009abc; 2011) to remove microbial contamination in addition to measurement using purine bases and ^{15}N incorporation. Estimates of corn silage RUP were reduced by approximately 3 % using methylcellulose and the associated additives, suggesting that the contamination by microbial N was still too high to adjust the ID calculation (data not shown).

The residue rinsed with either water or methylcellulose (MC) was similar to the RUP calculated after IV fermentation of corn silage and soy products with $^{15}N_2$-urea in buffer solution (Table 4.8). The estimated RUP from Erlenmeyer flask fermentations was significantly higher than from bags for both feeds, again raising issues about bag losses. Methylcellulose stripped significantly more bacteria from the CS residue in flasks than did water but only a trend toward significance of soy residue in the flasks; however, the MC treatment of soy in flasks did not replicate well but duplicates within each replicate did. No difference was observed between water and MC with either feed for RUP estimation in the bags due to no detectable difference in measurement of microbial contamination. Purine bases were analyzed but the

measured values did not correct for microbial attachment of either feed, nor was any difference observed between rinsing with water or MC of either feed. From previous studies, when assayed in bags, significantly lower estimates of RUP for either feed were observed. The ND residue from both feeds also had bacterial contamination present. and some detectable ^{15}N was still present when residue underwent sequential ND-AD. No ^{15}N was detected in the AD of either feed. The amount of undigested N in the AD fraction in the flask incubations was similar to the calculated RUP values from the incubated samples in bags. This suggested that the material remaining in the bags was more associated with the AD fraction of the feeds and might not represent the rumen escape feed and protein expected of the assay.

Table 4.8. Correction of estimated RUP of a corn silage (CS) and a soy product for microbial contamination using $^{15}N_2$-urea after 16-h *IV* fermentation in flasks or incubator with rinsing, neutral and acid detergent. Results are expressed as g undigested N g^{-1} total feed N, DM (means (SEM); n=2, 2 rep).

feed	Rumen incubation	Treatment*	^{15}N corrected RUP
CS	Flask	Rinse	0.6775^{a} (0.0301)
	Daisy	Rinse	0.1242^{b} (0.0073)
	Flask	MC	0.5100^{c} (0.0135)
	Daisy	MC	0.0846^{b} (0.0051)
	Flask	ND	0.1243^{b} (0.0200)
	Flask	ND-AD	0.0426^{d} (0.0002)
	Flask	AD	0.0387^{d} (0.0011)
Soy1	Flask	Rinse	$1.000^{A\dagger}$
	Daisy	Rinse	0.5650^{B} (0.0010)
	Flask	MC	$0.8380^{\dagger}$ (0.0835)
	Daisy	MC	0.5442^{B} (0.0103)
	Flask	ND	0.5013^{B} (0.0075)
	Flask	ND-AD	0.0410^{C} (0.0012)
	Flask	AD	0.0196^{C} (0.0024`)

*Treatment: rinse with boiling water; MC, methylcellulose after boiling water; ND, neutral detergent; ND-AD, sequential neutral detergent then acid detergent; AD, acid detergent. ^{abcdABCD}Means in same feed with different superscripts differ ($P < 0.05$). †Means in same feed show trend toward significant ($0.05 < P < 0.10$)

The ^{15}N analysis should be a viable option for correction of microbial contamination since the only labeling should be fiber digesting bacteria that have utilized the $^{15}N^{15}N$ urea, incorporated it into their cell and remained attached to the feed. However, it is costly and not for routine testing, especially by commercial labs. Further, the analyses of purine base is time consuming and based on the evaluation conducted in this study, does not accomplish the objective of quantifying microbial residue associated with the IV residue in a quantitative and repeatable manner. Buffer blanks are used to correct for microbial attachment for the calculation RUP (Table 4.9) since there should be little accumulation of N because there are no bacteria present at this step. Correcting for microbial attachment using the washed CS NDF lowered the calculated CS RUP values by 67-73 % compared with buffer alone and 89 % compared with straw demonstrating the significant difference in microbial growth between the CS and straw (Table 4.9). The differences in bacterial contamination of the IV residue of the various feeds was more evident with the low protein, high fiber feeds having greater measurable protein post assay than the higher protein feeds..

Correcting the RUP calculation with CS ND which has undergone exposure to buffer solution with and without rumen fluid can vary among days and this variation is due to several factors. Variation among cows and days are given in Table 4.10 where twenty samples of CS ND were inoculated from each of two cows. No difference between cows was observed on day 1 but the two cows were different from each other on day 2 and both cows were higher in estimated RUP on day 1 with no differences observed between the 2 water baths. No difference was observed between days in samples without rumen fluid. It is also possible that a pipetting error may have also contributed to the variation observed on day 2.

Table 4.9. Estimated fraction of rumen escape protein following correction for microbial contamination using assay buffer and straw or corn silage neutral detergent fiber (ND; n=2).

	Day 1		Day 2		
Feed	Assay Blank	Straw ND	Assay Blank	Straw ND	CS ND
correction**	3.05	0.75	1.46	1.02	2.60
units	mg N	mg N g-1 DM	mg N	mg N g-1 DM	mg N g-1 DM
AS	0.616	0.596	0.678	0.651	0.610
CornBP	0.563	0.545	0.550	0.526	0.488
CG1	0.843	0.825	0.818	0.793	0.755
CG2	0.807	0.788	0.831	0.805	0.764
CS1	0.800	0.736	0.807	0.719	0.585
CS2			0.591	0.518	0.405
CS3	1	1	0.990	0.890	0.734
DDG	0.954	0.943	0.952	0.936	0.912
Soy1	0.946	0.936	0.964	0.950	0.930
Soy4			0.743	0.730	0.709
Overall	0.816	0.796	0.778	0.743	0.689
SEM	0.039	0.041	0.030	0.031	0.035

**correction to correct RUP for bacterial contamination. Assay blank, mg N ; Straw and corn silage ND, mg N g^{-1} sample DM

Table 4.10. Milligrams of microbial nitrogen remaining in corn silage neutral detergent fiber after 16-h fermentation/solubilization with and without rumen fluid from 2 cows on 2 days in 2 water baths (n=10). Results are expressed as mg nitrogen g^{-1} DM corn silage neutral detergent (SD).

Cow*	Bath	Day	
		1	2
0	1	3.14^{a} (0.32)	3.63^{a} (0.31)
	2	3.18^{a} (0.15)	2.98^{a} (0.28)
1	1	7.82^{b} (0.67)	8.81^{c} (0.92)
	2	7.89^{b} (0.61)	10.15^{d} (1.30)
2	1	8.34^{b} (0.68)	10.21^{d} (1.38)
	2	8.19^{b} (0.70)	10.39^{d} (0.86)

*cow 0, no rumen fluid added. ^{abcd}Means with different superscript differ ($P < 0.05$).

Another ten feeds were fermented with combined rumen fluid from two cows along with the CS ND samples and then the IV residues underwent ID with either the enzyme mix and pancreatin over two days (Table 4.11). The estimated RUP values were corrected for microbial contamination and for most feeds were similar between days except the CS which was approximately 33% lower on day 2 compared to day 1 which also carried through the ID with both enzymes. The ID of CS with the enzyme mix was higher than with pancreatin, but both enzymes digested less CS IV residue on day 2. Also on day 2 pancreatin digested less alfalfa silage. Overall, the enzyme mix and pancreatin digested equal amounts of IV residue, but pancreatin digestion was different between the two days primarily due to the day 2 CS fermentation. The RUP of BM2, the heat damaged blood meal, was not digested by either intestinal enzyme preparation. A significant observation associated with variation measured between the two days was the timing of feeding of the cows. It appeared less microbial mass was in the rumen fluid on Day 1, possibly due to cows being sampled too soon post feeding. To optimize the IV step in the procedure, the rumen fluid sampling should be done at least 4 to 6 hr after the first major feed delivery and cattle meal. The optimum IV digestibility and ID assay results were obtained when rumen fluid was obtained approximately 6 hr after feed delivery. This was determined after assay repeatability was significantly decreased for several weeks resulting in unusable values for both the IV and ID assays which resulted in a system wide evaluation of all steps. The water bath temperatures, buffer pH, CO_2 concentration, rinsing water temperatures, filter integrity and many other factors were investigated and after a comprehensive review, we learned the farm changed the timing of feed delivery. After establishing a similar interval between feeding and sampling, the variation in both assays decreased. Rumen fluid sampling immediately after the cattle were fed most likely resulted in more dilute rumen fluid and low

microbial concentrations, whereas after 4 to 6 hr, the microbial population had increased, thus improving the digestibility of the feed. Establishing a standard operating procedure for time of feeding the lactating dairy cows and time of sampling rumen fluid was important in reducing variation due to cow and day and it is suggested rumen fluid be sampled approximately 6 hr after the major morning meal to reduce variability in assay conditions and outcome.

Table 4.11. Fraction of feed RUP and intestinal digestion of feed RUP using an enzyme mix of trypsin, chymotrypsin, amylase and lipase or pancreatin on 2 days. RUP was corrected for microbial contamination using corn silage neutral detergent fiber. Values represent means (n=2)

		RUP		Intestinal digestion of RUP by				SEM	P value all
				Enzyme Mix		Pancreatin			
Feed	day->	1	2	1	2	1	2		
AS		0.494^{a}	0.495^{a}	0.542^{a}	0.546^{a}	0.541^{a}	0.388^{b}	0.018	0.007
BM0		0.983^{a}	0.943^{ab}	0.815^{c}	0.870^{bc}	0.869^{bc}	0.904^{abc}	0.019	0.028
CM		0.528^{a}	0.594^{a}	0.609^{ab}	0.704^{c}	0.685^{bc}	0.738^{c}	0.014	0.006
CS		0.667^{a}	0.440^{b}	0.504^{c}	0.430^{b}	0.562^{d}	NA	0.031	0.000
DDG		0.907^{ab}	0.949^{a}	0.710^{d}	0.771^{cd}	0.858^{b}	0.845^{bc}	0.025	0.003
SBM		0.431^{a}	0.456^{a}	0.840^{b}	0.810^{b}	0.897^{c}	0.914^{c}	0.066	<0.000
Soy1		0.917	0.944	0.887	0.904	0.922	0.935	0.007	0.207
Soy2		0.739^{a}	0.782^{b}	0.884^{c}	0.889^{c}	0.875^{c}	0.903^{c}	0.019	0.000
Soy3		0.787^{a}	0.780^{a}	0.872^{b}	0.902^{bc}	0.917^{c}	0.938^{c}	0.019	0.000
Soy4		0.659^{a}	0.736^{b}	0.860^{c}	0.912^{d}	0.905^{d}	0.913^{d}	0.030	<0.000
BM2*		0.954	0.950	0	0	0	0		
	overall	0.711	0.712	0.752	0.776	0.794	0.759		
	SEM	0.042	0.045	0.033	0.043	0.032	0.054		

*heat damaged blood meal not included in overall mean. ^{abcd}Means with different supscripts differ (P<0.05).

Homogeneity of feed is also a concern and could explain some of the variation in feed digestibility observed among assay runs and replicates. With other factors held constant, the estimated RUP values of four soy products and a corn byproduct were estimated after IV digestion of 2, 4 and 6 samples on 2 consecutive days (Table 4.12) after correcting for microbial contamination of 0.010 and 0.0072 g N g^{-1} DM on days

1 and 2, respectively, using CS ND. Overall the estimated RUP values on day 1 were lower than day 2. Quadruplicates on day 1 were lower than the others while duplicates were the lowest on day 2. The coefficient of variation of Soy2, assuming no difference between days or reps, was 8.7 % while Soy4 was 32.5 %, with an overall CV of 22.6 %. Thus, sample homogeneity could be an issue. With low or high N feeds, i.e., corn silage and soy products, respectively, triplicates probably should be analyzed for RUP, if an accurate estimation of this value is desired since the recommendation is to not grind feeds other than forages, especially if it is a commercial protein source.

Intestinal digestion of several feed IV residues, corrected with CS-ND and digested using the enzyme mix yielded lower ID compared to pancreatin digestion (Table 4.13); however, no difference was observed for several blood meals (BM18-22), and some of this lack of difference was due to the large variation observed in a particular sample (BM18). Physiologically, the enzyme mix is more comparable to *in vivo* digestion based on the enzyme activity described by (Keller et al., 1958; Gorrill et al., 1968; Ben-Ghedalia et al., 1974 and Sklan and Halvey, 1985). The 24-h ID incubation might be longer than *in vivo* but has been used extensively and has not been questioned in prior assay development descriptions (Boucher et. al., 2011; 2009abc; Gargallo et al., 2006; Calsamiglia and Stern, 1995). The range in the estimated blood meal RUP values illustrates how handling and treatment of the feed prior to analyses affect the RUP and also demonstrate that the assay is sensitive enough to capture those differences. Heating SBM improved escape N but did not affect ID when evaluated using either enzyme preparation.

In manufacturing feeds that escape ruminal degradation, companies have reduced particles to a size that will flow with the rumen fluid; thus, the IV residues are not always captured on the small pore filter paper, despite the small pore size (1.5

μm). Consequently, in the case that known water soluble components are present that can pass prior to being fermented the filtration eluent has to be captured, the N analyzed and then the freeze dried eluent has to be added back to the filtered residue. This process became necessary when we recognized that several of the samples provided for analyses had components that solubilized immediately upon addition to water and when immediately filtered could not be recovered on the filter paper. Or, the entire IV fermentation mix can be freeze dried, analyzed for N and corrected for microbial contamination using corn silage ND with and without rumen fluid. Using this procedure, after freeze drying the entire IV fermentation mix of Com 3, 4 and 6 (Table 4.13) the calculated RUP values were 0.96, 0.95, and 0.68, respectively. With updated processing methods designed to enhance rumen escape of feed N, the manufacturer needs to disclose the behavior of the feed and the manufacturing process in order to appropriately characterize the feed.

Table 4.12. Mean calculated rumen undegraded protein values of four commercial soy products and a corn milling byproduct showing variation associated with n=2, 4 or 6 on 2 days (g RUP g^{-1} feed nitrogen). The RUP values were corrected for microbial contamination using corn silage neutral detergent fiber.

	Day 1			Day 2			SEM	P value	CV*
n=	2	4	6	2	4	6			
Soy1	0.936[a]	0.829[b]	0.953[ac]	0.954[ac]	0.983[ac]	0.998[c]	0.013	<0.0001	0.179
Soy2	0.703[a]	0.621[b]	0.744[ac]	0.809[c]	0.806[c]	0.804[c]	0.016	<0.0001	0.087
Soy3	0.732[ab]	0.680[a]	0.792[bc]	0.784[bc]	0.836[c]	0.823[c]	0.014	0.0021	0.201
Soy4	0.616[a]	0.523b	0.686[c]	0.672[ac]	0.737[c]	0.720[c]	0.017	<0.0001	0.325
CornB	0.545	0.492[†]	0.510[‡]	0.593	0.624[†]	0.612[‡]	0.017	0.0302	0.235
overall	0.724[a]	0.629[b]	0.737[ac]	0.762[cd]	0.797[e]	0.791[de]	0.014	<0.0001	0.226
SEM	0.045	0.030	0.029	0.042	0.028	0.024			
RUP corr**	0.0101			0.0072					

*CV: (range difference)/average. **g/g^{-1}DM

Table 4.13. Estimated fraction of feed nitrogen digested by compartment comparing intestinal digestion using an enzyme mix of trypsin, chymotrypsin, amylase and lipase with pancreatin. Feed rumen undigested nitrogen was corrected for microbial contamination using corn silage neutral detergent fiber. Values are mean and standard error.

Feed*	*in vitro*	Intestine by*		R to SI** digestion by	
	fermentation	Enzyme Mix	Pancreatin	Enzyme mix	Pancreatin
BM2	0.021	0.016	0.020	0.037	0.041
SBM1	0.073	0.749	0.833	0.822[a]	0.906[b]
Soy1	0.006	0.878	0.908	0.884[†]	0.914[†]
Soy2	0.224	0.640	0.627	0.864	0.851
Soy11	0.312	0.579	0.613	0.891	0.925
Soy12	0.281	0.580	0.629	0.861	0.910
Soy13	0.260	0.560	0.653	0.820	0.913
Soy14	0.252	0.616	0.669	0.869	0.921
Soy15	0.271	0.612	0.630	0.883	0.901
BM11	0.089	0.739	0.784	0.828	0.873
BM12	0.046	0.804	0.887	0.850[†]	0.933[†]
BM13	0.076	0.690	0.745	0.766[a]	0.821[b]
BM14	0.065	0.742	0.805	0.807	0.870
BM15	0.072	0.677	0.756	0.749	0.828
BM16	0.066	0.731	0.807	0.797	0.873
BM17	0.363	0.500	0.536	0.863[a]	0.899[b]
BM18	0.354	0.505	0.550	0.859	0.904
BM19	0.358	0.533	0.556	0.891	0.914
BM20	0.292	0.595	0.602	0.887	0.894
BM21	0.340	0.565	0.576	0.905	0.917
BM22	0.286	0.583	0.620	0.869	0.906
BM23	0.032	0.857	0.912	0.889	0.945
BM24	0.043	0.828	0.914	0.871	0.957
BM25	0.040	0.802	0.913	0.842[a]	0.953[b]
BM26	0.050	0.851	0.895	0.900	0.944
Com1	0.104	0.605	0.743	0.709[a]	0.847[b]
Com2	0.206	0.320	0.427	0.526[†]	0.633[†]
Com3	0.579	0.344	0.372	0.923[a]	0.951[b]
Com4	0.342	0.503	0.560	0.845[a]	0.902[b]
Com5	0.202	0.653	0.701	0.855	0.903
Com6	0.529	0.329	0.346	0.858[†]	0.875[†]
overall	0.206	0.634	0.687	0.840[a]	0.893[b]
SEM	0.020	--	--	0.010	0.008

*Intestine calculated, difference between (R to SI) and Rumen. **R to SI equal digestion following rumen fermentation, abomasal digestion with pepsin in HCl and intestinal digestion. [ab]Means in same row with different superscripts are significant at <0.0001, paired ttest in SAS; †in same row show trends toward signifance 0.05 < P < 0.10.

Digestion of acid detergent insoluble nitrogen and amino acids

Throughout the manuscript, heat damaged blood meal values are not included in the overall means to avoid bias since those feeds were designed as negative controls. The feeds utilized in the assay development exhibited a wide range of feed total N, ADF and ADIN contents (Table 4.14). Further, the detergent system was not designed to provide an evaluation of the N availability and digestibility of ruminant feeds, it was designed to remove interfering feed components so that the carbohydrates could be better described although there is reference to protein availability (Van Soest et al., 1991).

Table 4.14. Feed total nitrogen, acid detergent fiber and insoluble nitrogen values as percent of dry matter.

	% DM		
Feed	Total N	AD	ADIN
ANVY	11.54	0.97	0.15
AS	3.81	23.19	0.23
BM0	16.21	5.69	0.76
CM	6.48	20.9	0.41
CS	1.39	3.02	0.13
DDG	6.39	27.00	2.09
BM2	16.14	1.43	0.28
SBM	7.60	8.99	0.51
Soy1	7.70	9.77	0.50
Soy2	7.33	17.38	0.58

Compartmentalization of N digestion between the IV and ID by either (a) the enzyme mix or (b) pancreatin with ID calculated by difference of ten feeds is shown in Figure 4.1. Abomasal digestion with pepsin in hydrochloric acid preceded ID. The enzyme

mix digested significantly less total N than pancreatin in the bloodmeal, DDG and Soy1. Total N digestion (IV fermentation, abomasal plus ID) of bloodmeal was roughly 84 % using the enzyme mix and 92 % with pancreatin of which 5.5 % was consumed by the rumen bacteria while total N digestion of DDG was 72 and 86 %, respectively, after 6 % disappeared during fermentation and Soy1 was 91 and 96 %, respectively, after microbes consumed 7%. Roughly 60 and 48 % of the corn and alfalfa silages, respectively, fermented and 12 and 29 % digested in the intestine by enzyme mix and pancreatin, respectively. Total N digestion of the soy products was similar; however, rumen protection differences of the soy products were observed with 67 % of the untreated SBM N degraded in the IV fermentation step but only 7 and 28 % of Soy1 and Soy2, respectively, with remainder of N digested intestinally, and finally the enzyme mix digested less N in Soy1 than pancreatin. The N fraction of canola was degraded 55% during the IV fermentation step and 84% and 88% after ID with the enzyme mix and pancreatin, respectively. Fermenting a treated canola resulted in only 35 % of the N being degraded during the IV step (data not shown). The N in BM2 (heat damaged blood meal ; 165°C, 6 hrs) was digested 7% by *IV* fermentation with no further degradation by either intestinal treatment.

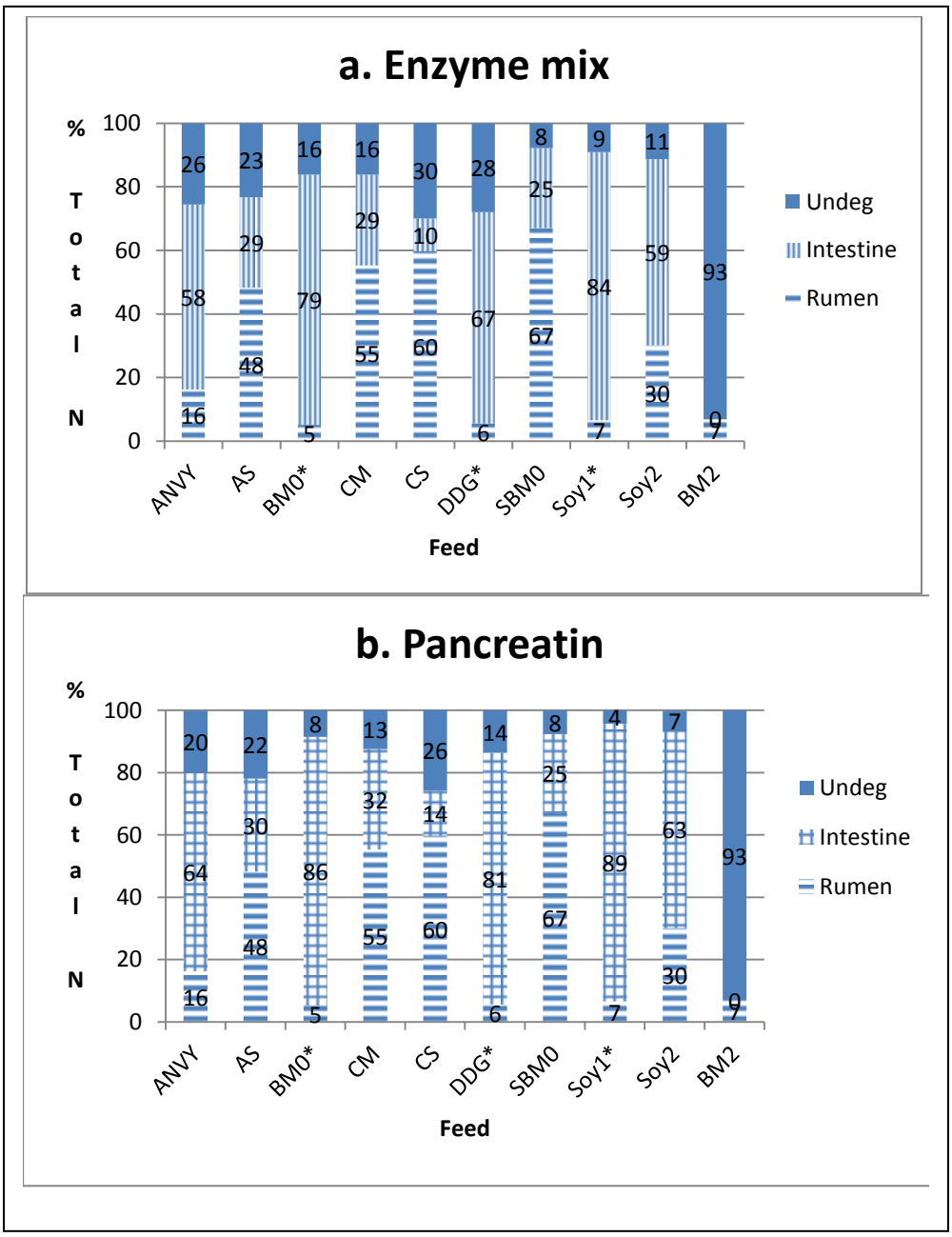


Figure 4.1. Description of the feed nitrogen digested by assay compartment by in vitro fermentation and intestinal digestion using (a) enzyme mix of trypsin, chymotrypsin, lipase and amylase or (b) pancreatin, preceded by abomasal digestion with pepsin in hydrochloric acid. Intestinal digestion calculated by difference. *Nitrogen digestion in these feeds differs by enzyme treatment ($P < 0.05$).

Table 4.15. Percent of feed acid detergent insoluble nitrogen and acid detergent amino acid nitrogen digested following 16-h *in vitro* ruminal fermentation and totally digested after intestinal digestion of fermentation residues with either an enzyme mix of trypsin, chymotrypsin, lipase and amylase or pancreatin. Results are expressed as percent of total feed nitrogen.

Feed	N Fraction[1]	% Total N	% feed acid detergent insoluble nitrogen digested after			SE
			in vitro fermentation	Fermentation + Intestinal digestion with		
				Enzyme mix	Pancreatin	
Overall	ADIN	8.1	4.2[a]	6.2[b]	6.8[b]	1.2
	ADAAN	3.8	2.7[a]	3.8[b]	4.8[c]	0.5
ANVY	ADIN	1.3	0	0	0	-
	ADAAN	0.9	0	0	0	-
AS	ADIN	6.1	0[a]	1.5[b]	2.5[c]	0.5
	ADAAN	1.9	0	0	0	0.6
BM0l	ADIN	4.7	0.6[a]	4.0[b]	4.6[c]	1.8
	ADAAN	2.4	0[a]	2.4[b]	2.4[b]	1.0
CM	ADIN	6.3	1.7[a]	1.7[a]	2.3[b]	0.1
	ADAAN	2.5	2	2.4	1.9	0.2
CS	ADIN	9.2	0	0	0	-
	ADAAN	2.2	0	0	0	-
DDG	ADIN	32.7	23.5[a]	29.6[b]	31.4[c]	1.5
	ADAAN	12.5	8.7[a]	12.5[b]	12.5[b]	1.3
SBM0	ADIN	6.7	6.1	6.3	6.5	0.1
	ADAAN	5.1	5.1	5.1	5.1	-
Soy1	ADIN	6.5	4.0[a]	5.6[b]	6.5[b]	0.5
	ADAAN	5.4	4	5.4	5.4	-
Soy2	ADIN	7.9	2.3[a]	6.8[b]	7.2[b]	1.0
	ADAAN	4.2	2.3	4.2	4.2	-
BM2	ADIN	1.8	0	0	0	-
	ADAAN	1.1	0	0	0	-

. [1]N fraction as percent of total feed nitrogen: ADIN, acid detergent insoluble nitrogen; ADAAN, acid detergent alpha amino acid nitrogen. [abc]Means with different superscripts in same row differ ($P < 0.05$) using Duncans Multiple Range test (SAS).

Historically, ADIN is expressed as a percent of feed N and has represented the unavailable fraction of feed protein. The percent of feed ADIN and respective amino acid content as percent of feed N digested during fermentation and digested following abomasal and intestinal digestion by either (a) the enzyme mix of trypsin, chymotrypsin, lipase and amylase or (b) pancreatin are given in Table 4.15 with compartmentalization of the ADIN digestion with intestinal digestion calculated by difference shown in Figure 4.2 and were highly correlated ($r=0.89$, $P < 0.0001$). The ADIN content of the feed ranged from 1.3 to 32 % of the feed N in anchovy and DDG, respectively. Overall, 4.2 % of the ADIN fermented and 6.0 and 6.8 % was totally digested following fermentation and intestinal digestion with either the enzyme mix or pancreatin, respectively, with pancreatin degradation being significantly greater; however, acid detergent amino acid N degradation was not different between fermentation and either intestinal treatment.

Acid detergent insoluble N was undigested in the anchovy, corn silage and heat damaged bloodmeal. Fermentation digested no alfalfa silage ADIN but pancreatin digested 2.5 % ADIN while enzyme mix digested less (1.5 %); however, alfalfa silage AD amino acid nitrogen remained undigested. Fermentation digested 91 % of the untreated SBM ADIN, 61 % of Soy 1 and 29% of Soy 2; while the intestinal plus abomasal treatments digested 32 and 60 % of Soy 1 and Soy2 ADIN, respectively, but only 4 % of the SBM. Consequently, 85 to 97 % of the soy ADIN and ~100 % of the acid detergent alpha amino acid N (ADAAN) digested. Overall digestion of DDG and bloodmeal ADIN was higher with pancreatin intestinal digestion than the enzyme mix and ranged from 85 to 96 % while 100 % of the ADAAN digested post fermentation. As illustrated by the feeds processing of the

feed to escape ruminal fermentation was also reflected in the site of ADIN digestion. Acid detergent insoluble N has historically been the measure of the undigested protein fraction of feeds; however, some of it is digested. Additionally, it does not represent the undegraded N in all feeds. Following ID, 7.7, 6.7, 8.9 and 28 % of the SBM, Soy1, Soy2 and DDG total nitrogen remained undigested which was similar with the ADIN feed values 6.7, 6.5, 7.9 and 32.7%, respectively. The estimated intestinal digestibility of the soy ADIN followed the value indicated by the National Research Council (2001) of 5 %. However, the undigested N of alfalfa silage and blood meal measured in the assay was four times the ADIN content. For corn silage and canola the assay indigestible N was three times the ADIN value while for the sample of anchovy analyzed, it was twenty times the ADIN value. One of the negative control samples for the ID assay, a heat damaged blood meal had an ADIN of 1.8 %, whereas the amount of N that remained indigestible based on the ID assay was 93 % of the total feed N. This was a significant difference in estimated protein availability and if the ADIN was applied to a diet formulation, the MP supply would be greatly over-estimated compared to using the ID assay value.

The alpha-amino N in the ADIN of bloodmeal, canola, DDG and the three soy products essentially digested following *IV* fermentation and ID with either the enzyme mix or pancreatin; however, the digestibility of the amino acid content of ADIN as a percent of feed total N often resulted in more being digested than present in the feed. For example, the digestibilities of the SBM, Soy1 and Soy2 ADINs were 12 % of the total N after incubation in the ID assay; however, the content of acid detergent AAN in the feed only ranged from 6.5 to 7.9.

The digestibility of amino acid N followed feed N and were highly correlated ($r=0.93$, $P < 0.0001$). However, roughly 35 % of the feed N was digested after IV fermentation while only 10.5 % of the feed amino N (Table 4.16) were digested after the same exposure but it should recognized that these residues were not adjusted for microbial contamination. Overall, the enzyme mix digested 81 % of feed N and 78 % of amino acid N in conjunction with microbial fermentation while pancreatin digested 87 and 80 %, respectively. Pancreatin digested more total N than the enzyme mix but no difference was observed in digestion of amino acid N. However, differences in individual amino acid digestibility between ID enzymes were observed with pancreatin digesting more than the enzyme mix except for Pro, Ala, Ile and Val.

Individual feed ID's of nitrogen (TN) and total amino acid N (TAAN) after fermentation and intestinal digestion with either the enzyme mix or pancreatin are shown in Table 4.17. As shown above, less N was digested by the enzyme mix of trypsin, chymotrypsin, lipase and amylase than pancreatin in the bloodmeal, DDG and Soy1. The enzyme mix digested less amino acid N than pancreatin in DDG, Soy1 and Soy2, but not blood meal due to variability. Variability also contributed to the higher digestibility of TAAN than TN of anchovy with the enzyme mix. Digestibility of amino acid N during fermentation could not be ascertained since residues were not corrected for microbial contamination, resulting in 0 % TAAN digestibilities in Table 4.17. In the future, corn silage ND samples with and without rumen fluid, should be assayed with the feeds as performed for N digestion correction, since the assay reagent blank alone does not correct for

microbial contamination. The amino acid contents of the feeds and feed ADF are given in Appendix 1 and 2 and results are expressed as percent of total feed N digested following IV fermentation and abomasal digestion with pepsin in dilute hydrochloric acid and intestinal digestion by either an enzyme mix of trypsin, chymotrypsin, lipase and amylase or pancreatin of 16-h fermentation residues.

Table 4.16. Overall percentage of feed nitrogen (TN) and feed amino acid nitrogen (TAAN) digested following 16-h *in vitro* l fermentation and total digested after intestinal digestion of fermentation residues with either an enzyme mix of trypsin, chymotrypsin, lipase and amylase or pancreatin. Results are expressed as percent of feed total nitrogen.

Item*	*In vitro* fermentation	Rumen to SI Digestion** After fermentation, abomasal & Intestinal digestion with		SE
		Enzyme mix	Pancreatin	
TN	34.9[a]	80.7[b]	86.6[c]	3.9
TAAN	10.4[a]	78.4[b]	80.2[b]	4.9
Amino acids				
ASP	11.3[a]	78.9[b]	81.1[c]	5.0
THR	6.8[a]	77.0[b]	80.6[c]	5.0
SER	12.6[a]	80.2[b]	82.7[c]	4.9
GLU	12.5[a]	82.5[b]	84.7[c]	5.2
PRO	31.0[a]	90.5[b]	86.9[b]	4.9
GLY	10.7[a]	70.0[b]	77.1[c]	4.8
ALA	10.0[a]	78.2[b]	79.4[b]	4.7
VAL	7.4[a]	75.7[b]	79.7[c]	4.8
ILE	3.9[a]	73.4[b]	74.9[b]	4.9
LEU	7.1[a]	76.5[b]	81.5[c]	5.0
TYR	6.2[a]	79.0[b]	84.3[c]	5.2
PHE	6.1[a]	75.8[b]	79.9[c]	5.1
LYS	8.9[a]	78.0[b]	80.0[b]	5.2
HIS	10.5[a]	72.3[b]	76.5[c]	5.6
ARG	11.6[a]	80.2[b]	81.0[b]	5.3

*Item: TN, percent feed nitrogen; TAAN, feed alpha amino nitrogen as percent of feed nitrogen. **Rumen to small intestine digestion. [abc]Means with different superscripts in same row differ ($P < 0.05$) using Duncan's Multiple Range test (SAS).

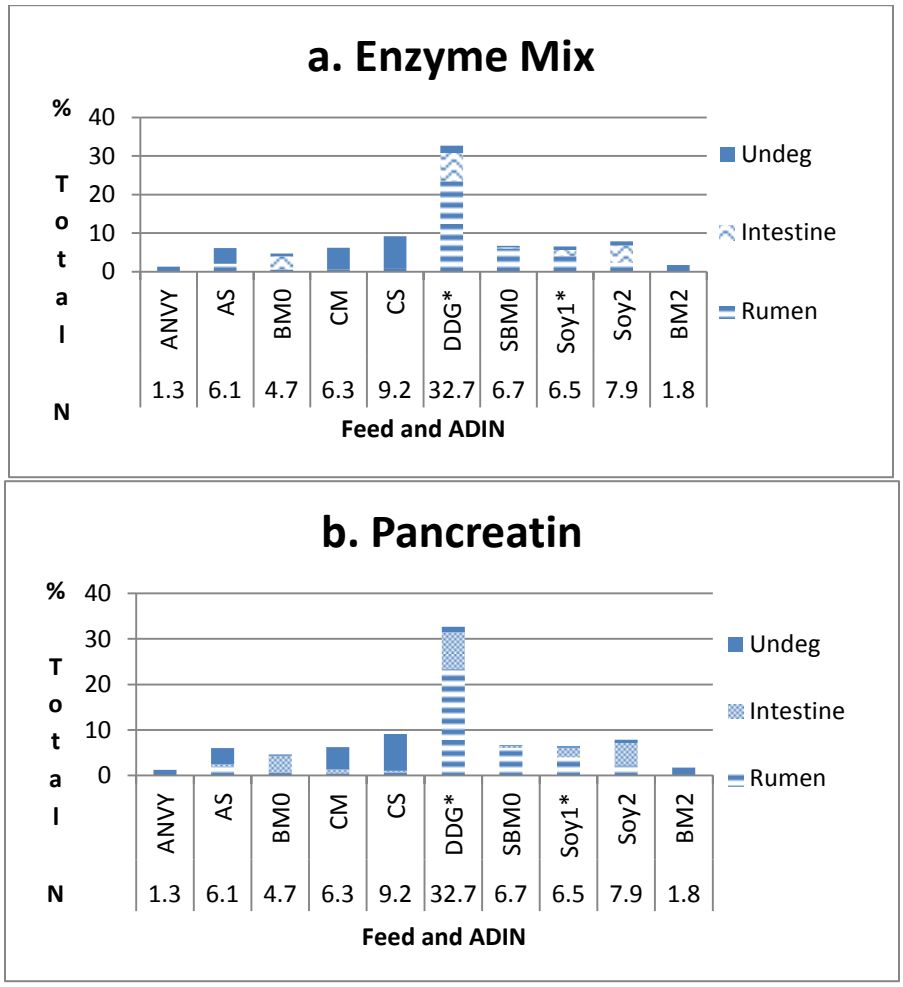
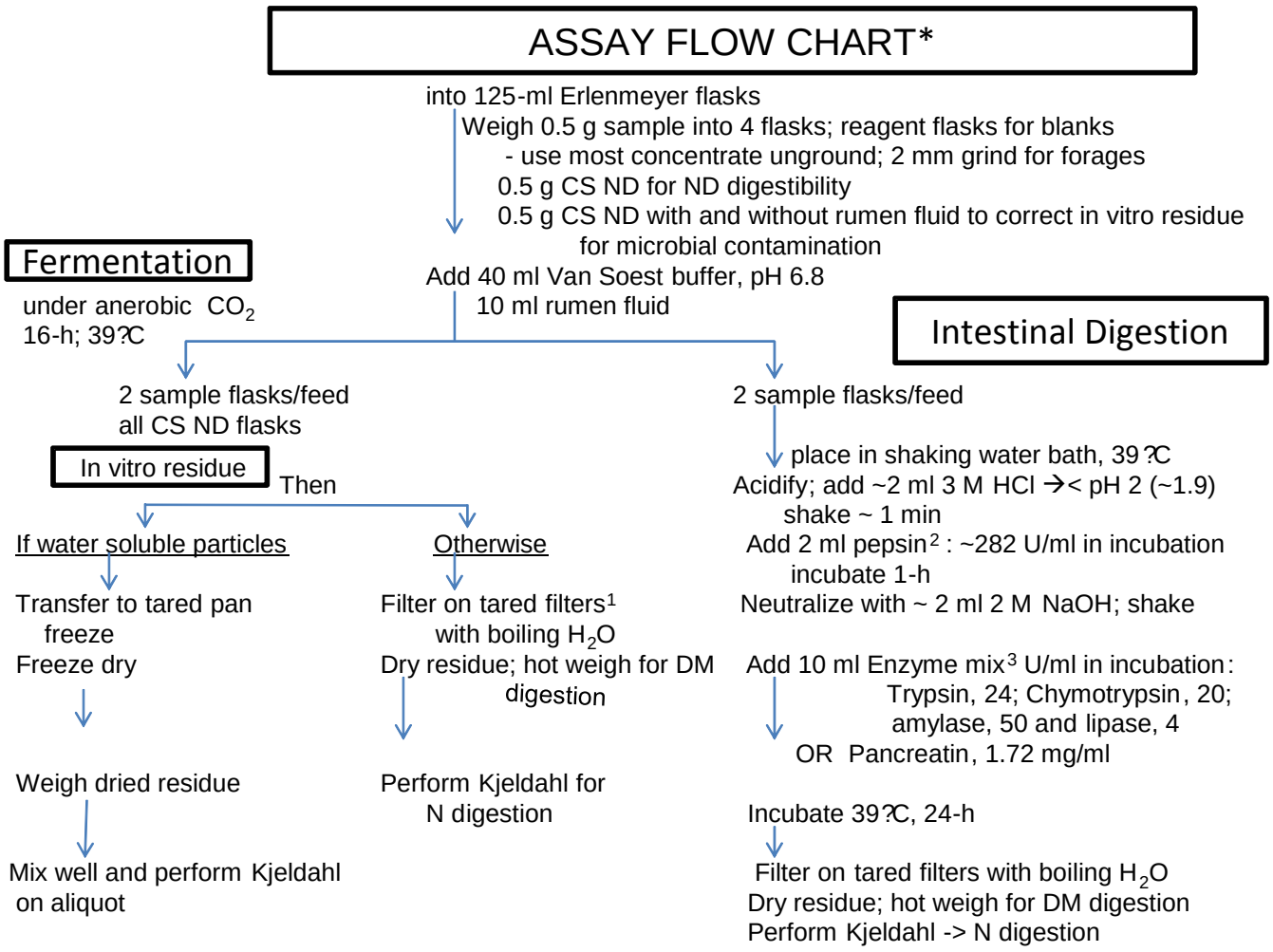


Figure 4.2 Compartmentalization of feed acid detergent insoluble nitrogen digestion as a percent of feed nitrogen by *in vitro* fermentation and intestinal digestion using (a) enzyme mix of trypsin, chymotrypsin, lipase and amylase or (b) pancreatin preceded by abomasal digestion with pepsin in hydrochloric acid. Intestinal digestion calculated by difference. *Feeds ADIN digestibility differ between intestinal treatments ($P < 0.05$).

Table 4.17. Percent of individual feed nitrogen (TN) and feed amino acid nitrogen (TAAN) digested following 16-h *in vitro* fermentation and total digested after intestinal digestion of fermentation residues with either an enzyme mix of trypsin, chymotrypsin, lipase and amylase or pancreatin. Results are expressed as percent of feed total nitrogen.

Feed	Fraction[1]	TN, %DM TAAN, % TN	% feed nitrogen digested after			SE
			In vitro fermentation	Fermentation, abomasal + Intestinal digestion with		
				Enzyme mix	Pancreatin	
ANVY	TN	11.5	16.3[a]	74.5[b]	79.9[b]	12.9
	TAAN	76.5	0[a]	78.4[b]	75.8[b]	16.3
AS	TN	3.8	48.3[a]	76.8[b]	78.1[b]	6.2
	TAAN	54.5	16.3[a]	75.4[b]	74.8[b]	12.4
BM0	TN	16.2	5.5[a]	77.1[b]	92.0[c]	16.9
	TAAN	73.3	0[a]	90.0[b]	89.3[b]	21.9
CM	TN	6.5	55.3[a]	83.8[b]	87.5[b]	6.5
	TAAN	66.3	28.2[a]	82.2[b]	82.8[b]	11.7
CS.	TN	1.4	59.8[a]	70.0[ab]	74.1[b]	3.0
	TAAN	40.2	10.0[a]	51.8[b]	49.1[b]	8.6
DDG	TN	6.4	6.5[a]	72.1[b]	86.4[c]	15.6
	TAAN	62.4	0[a]	64.3[b]	84.2[c]	16.1
SBM0	TN	7.6	67.1[a]	92.2[b]	92.4[b]	5.1
	TAAN	79.5	39.4[a]	90.7[b]	88.8[b]	10.8
Soy1	TN	7.7	7.3[a]	91.0[b]	95.7[c]	18.2
	TAAN	63.3	0[a]	90.5[b]	93.2[c]	19.4
Soy2	TN	7.3	30.2[a]	88.9[b]	93.4[b]	12.9
	TAAN	71.9	0[a]	87.7[b]	83.9[c]	18.1
BM2	TN	16.1	6.9	0	0	0.4

[1]Fraction: TN, feed nitrogen as percent of dry matter; TAAN, feed alpha amino nitrogen as percent of feed nitrogen. [abc]Means with different superscripts in same row differ ($P < 0.05$) using Duncan's Multiple Range test (SAS).


ASSAY FLOW CHART*
into 125-ml Erlenmeyer flasks
Weigh 0.5 g sample into 4 flasks; reagent flasks for blanks
- use most concentrate unground; 2 mm grind for forages
0.5 g CS ND for ND digestibility
0.5 g CS ND with and without rumen fluid to correct in vitro residue for microbial contamination
Add 40 ml Van Soest buffer, pH 6.8
10 ml rumen fluid
Fermentation
under anerobic CO2
16-h; 39?C
Intestinal Digestion
2 sample flasks/feed
all CS ND flasks
In vitro residue
Then
If water soluble particles
Transfer to tared pan
freeze
Freeze dry
Weigh dried residue
Mix well and perform Kjeldahl on aliquot
Otherwise
Filter on tared filters1
with boiling H2O
Dry residue; hot weigh for DM digestion
Perform Kjeldahl for N digestion
2 sample flasks/feed
place in shaking water bath, 39?C
Acidify; add ~2 ml 3 M HCl →< pH 2 (~1.9)
shake ~ 1 min
Add 2 ml pepsin2 : ~282 U/ml in incubation
incubate 1-h
Neutralize with ~ 2 ml 2 M NaOH; shake
Add 10 ml Enzyme mix3 U/ml in incubation:
Trypsin, 24; Chymotrypsin, 20;
amylase, 50 and lipase, 4
OR Pancreatin, 1.72 mg/ml
Incubate 39?C, 24-h
Filter on tared filters with boiling H2O
Dry residue; hot weigh for DM digestion
Perform Kjeldahl -> N digestion

Flow Chart, continued

[1]Filters: 90 mm; Whatman 934AH, 1.5µm.
[2]Pepsin in pH 2 HCl: 16.6 mg/ml.
[3]Enzyme mix and Pancreatin prepared daily in 1.8 M KH2PO4. Enzyme mix prepared to contain the following U in 10 ml: Trypsin, 1680; chymotrypsin, 1400; amylase, 7050, and lipase, 280. If using pancreatin, prepared to contain 120.4 mg in 10 ml. *NOTE: Quantitatively transfer all residues

SUMMARY

An *IV* assay to estimate intestinal protein digestion for ruminants was developed using an enzyme mix of trypsin, chymotrypsin, lipase and amylase at activities found in sheep digesta to replace pancreatin. The assay was developed to reduce sample loss by eliminating the use of bags and employing Erlenmeyer flasks and utilizing small pore size filter papers to further improve recoveries of undigested feed N. The procedure was modified when necessary to account for soluble components of feeds and the calculations described. The assay is a modification of published methods but the enzyme levels are standardized, sample loss minimized, works for all types of ruminant feeds, with sufficient residue from half gram samples after the 16-h fermentation, abomasal digestion with pepsin in hydrochloric acid and intestinal digestion for further analyses, ie., acid detergent fiber and amino acids. Concurrent samples of corn silage ND with and without rumen fluid are assayed to correct for microbial contamination. A complete assay description follows in Appendix 3. The protease and lipase activities are similar between the two intestinal treatments but the amylase activity in the enzyme mix is double that of pancreatin (Appendix 3). The assay provides an opportunity to evaluate intestinal digestibility of ruminant feeds and is designed in a manner that would allow adoption by commercial laboratories.

Acid detergent insoluble N does degrade. The undigested N from the *IV* assay more accurately represents the protein C fraction than does ADIN given the more physiological conditions of rumen incubation and enzyme exposure.

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Chapter 5

Future Studies

The development of the IV intestinal digestion assay has created an opportunity to provide an analysis procedure that allows nutritionists to analyze feeds for intestinal digestibility to further refine the nitrogen efficiency of cattle. This has significant implications as nutrition models like the CNCPS are improved to be more mechanistic at defining the ruminal and post-ruminal protein and amino acid supply and requirements. The current version of the CNCPS model (v6.1) has been refined to better predict the most limiting nutrient (ME or MP) with greater accuracy and precision and this is allowing nutritionists and producers to reduce the amount of N fed to cattle and improve overall N efficiency and improve income over purchased feed costs. However, the model is limited by the information in the feed library. The feed library currently uses the ADIN value as the unavailable N fraction (Protein C fraction) and also assigns a digestibility for the other fractions ranging from 80 to 100%. From literature data, field experience and the data generated in the previous chapter, the intestinal digestibility of many of these feeds varies considerably and even if the assay is not absolutely correct, it provides a proportionate direction to the digestibility that is currently lacking in both the feed library and also in commercial laboratory assays.

Thus, to implement the assay information appropriately, all of the feeds in the CNCPS feed library will need to be reanalyzed to develop a better estimate of intestinal digestibility. This will be more difficult than just analyzing the samples and putting the values into the library because the library was developed based on the detergent system of fraction by Van Soest et al. (1991) and this assay works across all of those pools and in essence ignores the detergent fractionation approach. This means it will be a little difficult to implement the new digestibility values and link them

to the current digestion and passage rate equations to provide a reasonable estimate of the apparent and true digestibility of the feeds. Application of these values might entail the redevelopment of the CNCPS rumen sub-model to better integrate the two approaches and this is being considered.

Another objective should be the conduct of experiments with high producing lactating cattle to test the validity and capacity to formulate diets based on intestinal digestibility. An experimental design could entail feeding protein sources or the same protein source with varying levels of predicted intestinal digestion to lactating dairy cows consuming isonitrogenous diets at total nitrogen intake levels that would provide adequate sensitivity to detect a difference in protein availablility. This approach would be one step closer to formulating a ruminant diet in a similar manner to a monogastric animal and could further provide an opportunity to evaluate the concept of balancing for digestible amino acids in ruminants, something that has not truly been tested in a large study.

Feedstuffs vary in the proportion of their AA and the acid detergent AA that are intestinally digested and should be incorporated into the feeding systems used for ruminant animals. The ADIN and corresponding AA appear to be highly digestible in animal and soy proteins while those of forage feeds may be more digestible than previously thought and do not represent protein C fraction. Inclusion of these increased digestibilities will allow the ruminant to be fed closer to requirement, thus reducing contamination of environment from excess N excretion and cost to the farmer.

APPENDICES

1. Feed amino acid values as g AA N, % feed N. Only acid hydrolysis performed – no sulfur amino acids or tryptophan.

	anchovy	alfalfa silage	Blood meal	canola	corn silage	DDG	SBM	Soy1	Soy2	HDBM
ASP	7.32	6.12	4.12	2.68	2.73	3.66	6.17	3.70	3.42	3.01
THR	3.23	2.46	3.04	2.87	1.91	2.78	2.95	2.82	2.47	3.13
SER	3.18	2.91	3.57	3.18	2.12	3.83	4.59	3.99	3.48	3.27
GLU	7.92	4.17	4.13	9.87	4.34	9.16	11.50	10.79	9.25	4.14
PRO	2.39	4.31	1.45	3.78	3.20	5.77	1.43	3.00	2.46	1.41
GLY	6.51	4.46	4.53	5.63	3.55	3.21	4.96	4.73	4.13	4.69
ALA	5.91	4.59	7.63	3.93	6.21	6.66	4.37	4.18	3.65	7.82
VAL	3.78	3.25	6.04	3.54	2.97	2.82	3.07	3.43	2.80	6.31
ILE	2.96	2.03	0.25	2.37	1.65	1.83	2.67	2.95	2.54	0.19
LEU	5.10	3.85	7.99	4.30	3.73	7.51	5.34	5.07	4.34	8.38
TYR	1.71	1.08	1.37	1.21	0.66	1.62	1.71	1.64	1.44	1.17
PHE	2.35	1.82	4.06	1.91	1.44	2.26	2.72	2.65	2.28	3.79
LYS	8.13	4.77	8.79	5.33	1.88	2.60	7.52	5.65	5.39	8.11
HIS	3.67	1.52	9.63	3.96	0.53	2.70	4.41	3.99	3.57	9.33
ARG	12.37	7.21	6.74	11.77	3.31	6.04	16.04	13.34	12.06	6.78
TAAN	76.53	54.54	73.33	66.33	40.21	62.44	79.45	63.26	71.92	71.55

2. Feed acid detergent insoluble nitrogen amino acid values as g AA N, % feed N. Only acid hydrolysis performed -- no sulfur amino acids or tryptophan

	anchovy	Alfalfa silage	Blood meal	canola	corn silage	DDG	SBM	Soy1	Soy2	HDBM
ASP	0.07	0.16	0.18	0.18	0.14	0.49	0.50	0.38	0.55	0.08
THR	0.04	0.08	0.09	0.14	0.11	0.38	0.17	0.15	0.21	0.05
SER	0.04	0.15	0.05	0.16	0.13	0.74	0.29	0.25	0.30	0.04
GLU	0.09	0.12	0.20	0.27	0.23	2.22	0.63	0.55	0.67	0.08
PRO	0.02	0.10	0.02	0.16	0.15	0.97	0.12	0.12	0.17	0.01
GLY	0.06	0.20	0.16	0.19	0.21	0.48	0.29	0.25	0.33	0.07
ALA	0.07	0.12	0.23	0.16	0.21	1.40	0.33	0.27	0.37	0.11
VAL	0.05	0.14	0.26	0.17	0.14	0.62	0.29	0.24	0.34	0.10
ILE	0.04	0.08	0.01	0.11	0.09	0.47	0.25	0.21	0.27	0.01
LEU	0.07	0.13	0.26	0.18	0.21	1.83	0.44	0.36	0.48	0.12
TYR	0.02	0.04	0.03	0.05	0.04	0.40	0.12	0.10	0.12	0.01
PHE	0.04	0.07	0.18	0.09	0.10	0.49	0.23	0.18	0.23	0.06
LYS	0.09	0.21	0.27	0.22	0.11	0.27	0.39	0.31	0.36	0.13
HIS	0.04	0.11	0.13	0.12	0.09	0.59	0.20	0.18	0.27	0.10
ARG	0.12	0.15	0.31	0.29	0.22	1.11	0.81	0.63	0.72	0.11
T.AAN	0.86	1.86	2.38	2.50	2.17	12.46	5.07	4.18	5.38	1.07

3. A description of the intestinal protein digestion assay for ruminants

Materials Needed

- 4-place balance
- Erlenmeyer flasks, 125 ml
- *in vitro* bath (39°C; Precision Model 170, Precision Scientific Co., Chicago, IL 60647; MW1140A-1, Blue M, Blue Island, IL) & CO2 system
- shaking water bath (39°C; Precision Dubnoff metabolic shaking incubator, Precision Scientific Co., Chicago, IL 60647; VWR Model 1227reciprocating water bath, VWR International, Inc., West Chester, PA 19380)
- 2-piece glass filter holders (90 mm) & manifold (C-02923-30 & C-02924-30, respectively; Cole-Parmer Instrument Co., Vernon Hills, IL 60061)
- Whatman 934-AH glass filters (90 mm; GE Healthcare Bio-Sciences Corp., Piscataway, NY 08855)
- drying oven (105°C; Hotpack Corp., Philadelphia, PA 19135) or desiccator
- vacuum
- boiling water
- Kjeldahl or combustion unit
- rumen fluid from 2 lactating cows on lactating cow TMR, ~6-h post feeding, no dry cows: mix but do not use blender, filter through 4 layers cheesecloth, 100 um nylon filtration cloth (34-1800-14, PGC Scientifics, Frederick, MD) and glass wool

Enzymes & Reagents

- Enzymes diluted as needed
- pepsin (porcine; P7000; Sigma-Aldrich Corp., St. Louis, MO). Confirm activity using Sigma's hemoglobin assay for pepsin (SSHEMO01)
- trypsin (bovine; T9201; Sigma-Aldrich Corp.)
- chymotrypsin (bovine; C4129; Sigma-Aldrich Corp.)
- amylase (*B. licheniformis* E-BLAAM100; Megazyme Company, Wicklow, Ireland). Confirm acitivity as uM maltose reduced in 3 minutes (Dahlqvist, 1962; Sigma SSSTAR01)
- lipase (porcine; 18485; USB Corp., Cleveland, OH)
- pancreatin (P7545; Sigma-Aldrich Corp.)
- HCl: 0.013 (pH 2) & 3 M
- NaOH: 2 M
- KH_2PO_4: 1.8 M, pH 7.75 with solid KOH
- Van Soest IV buffer, pH 6.8; pH just before use

- corn silage ND residue washed with ammonium sulfate to remove detergent and used to correct microbial contamination of RUP
    - ND digestibility to check fermentation
    - and as background for microbial contamination of RUP

Enzyme activity in Assay contains

	Pancreatin	Enzyme Mix
Amylase	25.8	50
Protease*	0.72	0.42
Lipase	3.44	4

*U/ml per min wt of CN

Calculations using Kjeldahl.

Fraction feed nitrogen digested following fermentation = {[mls titrant for residue - mls titrant for assay blank – (Δ mls for CS ND * sample wt, DM)*nitrogen factor]/sample wt, DM} / g feed nitrogen / g dm, where Δ mls for CS ND = (mls titrant for CS ND + rumen fluid per g DM) – (mls titrant for CS ND – rumen fluid per g DM).

Fraction feed nitrogen digested following fermentation and intestinal digestion = [(mls titratant for residue – mls titrant for assay blank)/sample wt, DM] / g feed nitrogen / g DM.

If an aliquot of the residue is analyzed for N, the weight must be converted to an original weight equivalent