Comparison of In Vitro Techniques to the In Situ Technique for Estimation of Ruminal Degradation of Protein

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ABSTRACT

The accuracy with which the *Streptomyces griseus*, ficin, and neutral protease with amylase in vitro methods could be used to predict in situ protein degradation of concentrate feeds was evaluated. Four types of soy products and two types of distillers grains were incubated using each of the four techniques for .5, 1, 2, 4, 8, 12, 24, and 48 h. The percentages of initial CP that remained undegraded over time were determined. Comparison of the degradation curves and contrast analysis of the data indicated that the in vitro methods generated degradability estimates in conflict with those obtained by the in situ method. However, the neutral protease with amylase method ranked the test feeds according to the extent of CP degradation at 24 h most similar to that in situ. Regression equations developed with the neutral protease with amylase degradability estimates explained 78, 76, and 74% of the variation in the in situ estimates obtained after 12, 18, and 24 h of incubation, respectively. At least 69% of the variation in the 18-h in situ estimates could be explained by the neutral protease with amylase estimates obtained after 1, 2, 4, 8, and 12 h of incubation. Little relationship was found between the ficin and *S. griseus* versus in situ results. Although none of the in vitro methods resulted in degradation curves consistently related to those generated by the in situ technique, relationships were found between protein degradability estimates obtained by the neutral protease with amylase method at specific time points and those obtained by the in situ technique.

(**Key words**: enzymes, protein, degradability)

Abbreviation key: DD = dark distillers grains, **LD** = light distillers grains, **RSB** = roasted soybean, **SB** = raw soybeans, **SBM** = soybean meal, **SP** = Soy-PLUS.

INTRODUCTION

Diet formulation systems have been developed that attempt to balance diets for both ruminal protein degradability and carbohydrate availability with the goals of maximizing microbial protein synthesis, minimizing protein wastage as ammonia, and supplementing the small intestine with dietary protein that escapes ruminal fermentation (10, 16, 18, 19). For commercial laboratory analysis, it would be beneficial to define a procedure for estimating ruminal protein degradability of feedstuffs that is simple to conduct, relatively rapid, repeatable, and reasonable in cost.

The extent to which feed protein is degraded in the rumen is complicated by numerous factors, which include the nature of the feed protein, the individual cow, the total diet being fed, and the feeding method used. The protein degradation rate of a feed will vary depending on its proportions of NPN, true proteins that degrade at variable rates, and unavailable protein, or fractions A, B, and C, respectively (20, 23). Thus, feeds each have a characteristic protein degradation curve during ruminal incubation depending on protein fraction content. The rate of feed passage through the rumen is a competing force against the rate of protein degradation (9, 27). Tamminga (27) increased the proportion of undegraded dietary N entering the small intestine from .26 to .42 by increasing feed intake from 8.6 to 12.9 kg DM/d. Finally, the type of fermentation occurring in the rumen

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can influence the extent of ruminal protein degradation (14, 24). Bartle et al. (3) used an in vitro rumen fluid procedure and found that soybean meal protein degradation responded to pH in a quadratic manner with degradation highest at pH 6.0 to 6.5 and lower at pH 5.5 and 7.0.

Numerous methods currently are available for estimating ruminal protein degradation of feedstuffs. The in situ (dacron bag) technique involves the placement of nondigestible bags containing a feedstuff into the rumen of a fistulated animal for various time intervals and the measurement of the amount of DM or N removed from the bags over time (17, 26). This procedure is not practical for commercial laboratories.

In the *Streptomyces griseus* method, feeds are incubated in a borate-phosphate buffer at pH 7.8 to 8.0 to facilitate optimum enzyme activity, and enzyme is added at a level of 6.6 units of enzyme/g sample DM (13). *Streptomyces griseus* is a broad spectrum, commercially available protease that has both exopeptidase and endopeptidase activity, as in the rumen. It has a mode of action similar to that of *Bacteroides amylophilus* (11). The *S. griseus* method, using a 1-h incubation period and regression equations for prediction of 16-h in situ ruminal protein degradation, has been recommended in France (10).

Ruminal protein degradation also has been estimated by the ficin method, which breaks down protein by proteolytic ficin (*Ficus glabrata*) used at a level of 8.24 units/g of sample DM in a phosphate buffer at pH 6.5 (21). The ficin enzyme has endopeptidase activity (4).

Finally, the neutral protease with amylase method makes use of an endopeptidase (4), *Bacillus subtilus*, at 9.6 units/g sample DM, in a sodium citrate buffer (pH 6.5), in addition to an enzyme that contains endo-β-glucanase and α-amylase activities (2).

Some researchers have questioned the use of any commercial protease for estimation of ruminal protein degradation due to differences in specificity and mode of action of proteases (6, 15). Broderick (6) proposed an in vitro system that utilized strained ruminal liquor and an inhibitor of amino acid and ammonia metabolism. Mahadevan et al. (15) prepared protease from mixed rumen microorganisms to estimate ruminal protein degradability. At present, these two methods are not practical for commercial laboratories.

The purpose of our project was to compare the results of three in vitro procedures currently in use for estimating ruminal protein degradation of feedstuffs against degradability results obtained by the in situ procedure in order to determine if an in vitro assay will simulate in situ disappearance of protein over time and to determine whether an in vitro method at various periods of incubation can predict the in situ degradability of proteins.

MATERIALS AND METHODS

Samples

Four types of soy products were selected for this project in order to test the sensitivity of the three enzymes to detect differences in protein type and processing. They were soybean meal (SBM; solvent extracted, dehulled); Soy-PLUS (SP; an expeller processed soybean meal, West Central Cooperative, Ralston, IA); raw soybeans (SB; Cornell Food Science Department, Ithaca, NY); and roasted soybeans (RSB; Cornell Food Science Department) in addition to a dark (DD) and a light (LD) sample of distillers grains without solubles (Cornell University Teaching and Research Center).

Each of the samples was evaluated for nutrient content prior to in situ and in vitro incubation (Table 1). Approximately 1 g of each sample was dried at 100°C for 24 h, and the amount of change in sample weight was used for DM determination. Crude protein analysis was conducted using a Buchi digestion and steam distillation apparatus (Buchi AG, Flawil, Switzerland), a micro-Kjeldahl that agrees with macro-Kjeldahl standards (1). The acid detergent insoluble CP content of the samples was determined by the procedure of Goering and Van Soest (8). Additionally, the samples were analyzed for soluble protein (12).

In Situ Trial

Two ruminally cannulated Holstein cows averaging 50 d in milk and producing 39.3 kg of milk (3.25% fat) were utilized to determine the rate of in situ protein degradation in the selected samples. All samples were incubated

TABLE 1. Composition of concentrate feed samples used for in situ and in vitro trials.

Ingredient	DM	CP	Protein fraction	
			Sol CP[1]	ADF-CP[1]
	(%)	(% DM)	(% CP)	(% CP)
Soybean meal	88.4	53.9	18.5	4.8
Soy-PLUS	88.3	52.7	17.6	3.6
Raw soybean	90.3	38.8	38.9	8.8
Roasted soybean	93.0	38.1	11.7	8.4
Light distillers grains	89.6	29.6	24.5	13.5
Dark distillers grains	91.1	25.9	6.5	39.0

[1]Sol CP = CP soluble in borate-phosphate buffer (12); ADF-CP = CP insoluble in acid detergent solution (8).

in both cows. The cows were fed a total mixed ration containing 38.8% corn silage, 16.6% alfalfa-grass silage, 24.9% high moisture shelled corn, 17.3% soybean meal, and 2.4% minerals and vitamins on a DM basis (Table 2) ad libitum once each day at 1100 h.

The bags (Ankom Products, Spencerport, NY) were constructed of 53 plus or minus 10-μ pore size polyester material, and the seams were heat sealed. Each bag contained 5 g of sample. After closing the bags with 20-cm nylon clamp ties, 10- × 20-cm bag surface area remained, providing approximately 12.5 mg DM/cm^2 surface area. Quadruplicate sets of bags were attached to a 225-g weight and 91 cm of 36-kg test monofilament fish line (for outside attachment) and inserted into the rumens for .5, 1, 2, 4, 8, 12, 24, or 48 h (17).

Prior to ruminal incubation, all bags were soaked in lukewarm water for 15 min for the purpose of hydrating the samples and removing water-soluble material (5, 17). Sets of bags were inserted into the rumen at different times according to designated incubation periods. All bags were removed together, rinsed with lukewarm water, and dried at 60°C for 48 h. Two quadruplicate sets of each sample were soaked and rinsed without ruminal incubation to determine degradation at 0 h within the rumen.

Bags with their remaining contents were weighed, and the loss in weight as a result of the incubation was computed. The contents of each quadruplicate set of bags were combined, mixed, and ground to pass a 1-mm screen. A composite sample was analyzed for residual CP, and the degree of CP degradation over time was determined. Microbial contamination of the bag residues was not estimated. Microbial N should have had no detectable effect in the soybean meal samples; however, due to the higher fiber content in distillers grains, it may have had a minimal effect in these samples (17).

In Vitro Trials

The *S. griseus* (13), ficin (21), and neutral protease with amylase (2) methods were used to determine extent of protein degradation of duplicate feed samples after 0 (solubility), .5, 1, 2, 4, 8, 12, 24, and 48 h of incubation. For the *S. griseus* method, a .5-g DM sample was incubated in 40 ml of borate-phosphate buffer for 1 h, and then 10 ml of protease solution (330 × 10^{-3} units/ml protease type XIV from *S. griseus* (Sigma Chemical Co., St. Louis, MO)

TABLE 2. Analysis of herd ration fed throughout the in situ digestion trial (DM basis).

Nutrient	Composition
CP, %	19.3
Sol CP,[1] %	7.1
DIP,[2] %	13.8
UIP,[3] %	5.5
ADF, %	17.5
NDF, %	29.3
NE_L, Mcal/kg	1.7
TDN, %	73.9
NSC,[4] %	41.4
Fat, %	3.0
Ca, %	.9
P, %	.5

[1]Sol CP = CP soluble in borate-phosphate buffer (12).
[2]DIP = Rumen degradable protein (13).
[3]UIP = Rumen undegradable protein (13).
[4]NSC = Nonstructural carbohydrate.

TABLE 3. Comparison of feedstuff ranking order based on the percentage of initial CP remaining undegraded after 24 h of incubation by in situ and in vitro methods.

	Procedure[1]							
	In situ		*S. griseus*		Ficin		Neutral protease	
Ingredient[2]	% Remaining	Ranking	% Remaining	Ranking	% Remaining	Ranking	% Remaining	Ranking
DD	72.3	1	53.9	2	33.1	1	94.4	1
SP	42.9	2	34.1	5	8.6	6	61.2	3
LD	26.8	3	47.4	4	18.7	4	72.2	2
SBM	12.2	4	26.7	6	16.5	5	33.6	5
RSB	7.5	5	56.5	1	21.7	3	58.7	4
SB	.7	6	47.8	3	32.5	2	27.7	6

[1]Degradability methods: in situ (17); *Streptomyces griseus* (13); ficin (21); neutral protease with amylase (2).

[2]DD = Dark distillers grains without solubles; SP = Soy-PLUS; LD = light distillers grains without solubles; SBM = soybean meal; RSB = roasted soybean; SB = raw soybean.

were added. For the ficin procedure, a .6-g DM sample was incubated in 10 ml of in vitro macromineral buffer for 1 h, and then 10 ml of ficin solution (1.4 g of ficin in 1 L containing 200 ml of phosphate buffer and 6.1 g of cysteine hydrochloride) were added. For the neutral protease with amylase procedure, a .5-g DM sample was incubated with 25 ml of HCl citrate buffer (82.2% .1 *M* citrate, 17.8% .1 *M* HCl) and .2 ml of Biase [Biocon (US) Inc., Lexington, KY]. Then, the remaining sample was incubated with 49.8 ml of sodium citrate buffer [53.7% .1 *M* citrate (with .01% sodium azide), 46.3% .1 *M* NaOH] and .2 ml Neutrase (Novo Laboratories Inc., Danbury, CT). Enzymatic activity was not tested for the ficin and neutrase enzymes; however, activities were thought to be 8.24 units/g sample DM and 9.6 units/g sample DM, respectively (2, 21).

Interpretation of the Data

The percentage of initial CP that remained undegraded after each period of incubation was computed for each of the methods. Due to the nature of the protein sources containing numerous protein fractions, each degrading at a different rate (5), it was necessary to plot the results using each technique on the same graph for each feed in order to compare the degradation curves. The results of duplicate test samples were averaged for analysis. Test feed ranking according to protein remaining after 24 h of in vitro incubation was compared with the 24-h in situ ranking.

The general linear models procedure of SAS (22) was used for contrast analysis between the percentage of initial CP remaining undegraded after in situ and in vitro incubations. Interactions were compared by least squares procedures using the model:

$$\% \text{ CP} = \mu + H_i + N_j + T_k + (H_iT_k) + (N_jT_k) + (H_iN_j) + (H_iN_jT_k) + e_{ijk},$$

where:

% CP = percentage CP remaining undegraded,
H_i = hours of incubation,
N_j = feed sample tested, and
T_k = treatment (degradability method).

Treatment differences were compared using orthogonal contrasts (in vitro versus in situ methods).

The regression procedure of SAS (22) was used to regress in vitro on in situ CP remaining undegraded over time for each test feed and for regression of in vitro incubation degradability results of all test feeds on in situ values at various time points throughout incubation.

RESULTS AND DISCUSSION

In Situ (Dacron Bag) Trial

After 24 h of incubation, the DD sample had 72.3% of the original CP remaining undegraded; SP, 42.9%; LD, 26.8%; SBM, 12.2%; RSB, 7.5%; and SB, .7% (Table 3). This ranking order was expected due to the larger percentage

of hydrophobic prolamine in corn and the more degradable albumin and globulin proteins present in dicotyledons (5, 28). Additionally, the effect of heat denaturation on solubility was evident.

Comparison of the Protein Degradation Curves

As knowledge in computer modeling has progressed, the need to predict ruminal protein degradation for dairy cows with diverse rates of ruminal passage has become increasingly apparent. For this reason, attention initially was focused on the degradation curves rather than simply on the extent of degradation after a given incubation period.

The results of each technique were plotted on the same graph for each feed (Figure 1). In all of the graphs shown on this figure, with the exception of the DD and SP, the asymptotic curve generated by the in situ data can be clearly distinguished from among the flatter curves obtained with the in vitro data.

The curves generated by the *S. griseus* method seem to correspond with the in situ curves for SP and the distillers samples but not with curves for RSB and SB. Two possible factors that could be involved in the explanation of these results are 1) the alkaline buffer may have promoted the solubilization of glutelins present in the distillers grains (5) in addition to the denatured protein side-chains formed during the processing of SP (7), and 2) the SB and perhaps also the RSB contain an antitrypsin factor that may inactivate the *S. griseus* enzyme inside the flask (28).

All of the ficin degradation curves, with the exception of that for SB, had a very rapid initial degradation rate followed by an almost complete termination of all protein degradation, indicating the presence of a residual protein fraction that resisted degradation by the ficin enzyme. This result also may be due to either end-product inhibition of enzyme or an insufficient supply of enzyme to continue protein degradation (13). The antitrypsin factor likely was the cause for the dissimilar enzyme activities observed with SB.

Other researchers have observed maximum protein solubilization with similar protein types within 1 h of incubation with the ficin enzyme (11). The protein degradation curves generated by the ficin method often were clearly uncorrelated with those from the in situ method due to this rapid initial degradation of protein.

The neutral protease with amylase method consistently degraded feed protein to a lesser extent than that of in situ protein degradation. Additionally, most of the protein digestion occurred in the first few hours of incubation rather than a steady breakdown of protein throughout the entire 48 h of incubation. The limited effect of the neutral protease on protein degradation especially is evident in the distillers grains samples. Those factors mentioned as possible limitations in the ficin method also may be a hindrance to the action of the neutral protease. The beneficial effect of the carbohydrate-digesting enzyme used prior to the protease was not evident in our study because the test samples did not have a high starch content that could inhibit proteolytic activity (2).

Because ruminal protein degradation is not a zero-order reaction, a constant enzyme:protein substrate ratio must be defined in any enzymatic procedure. Krishnamoorthy (11) determined the proteolytic activity of rumen fluid, assumed that ruminal protein degradation was a combination of first-, second-, and zero-order reactions depending on the type of protein, feeding frequency, and microbial activity, and then determined the appropriate enzyme concentration required to estimate protein degradation in the test samples. However, a constant enzyme:protein substrate ratio was not specified. Poos-Floyd et al. (21) recommended a ratio of substrate to enzyme activity of 3:1 for the ficin method but did not indicate attempts to approximate ruminal proteolytic activity. The neutral protease with amylase procedure did not specify a constant enzyme:protein substrate ratio (2).

Mahadevan et al. (15) prepared protease from mixed rumen microorganisms to estimate ruminal protein degradability. A ratio of substrate feed protein to enzyme protein of about 6:1 and an amount of enzyme equal to that which would hydrolyze 5.0 mg azo-casein/h was determined to approximate rumen proteolytic activity.

Ranking Order of the Test Feeds

Of the three in vitro methods tested, the neutral protease with amylase method ranked the test feeds, in terms of the percentage of CP

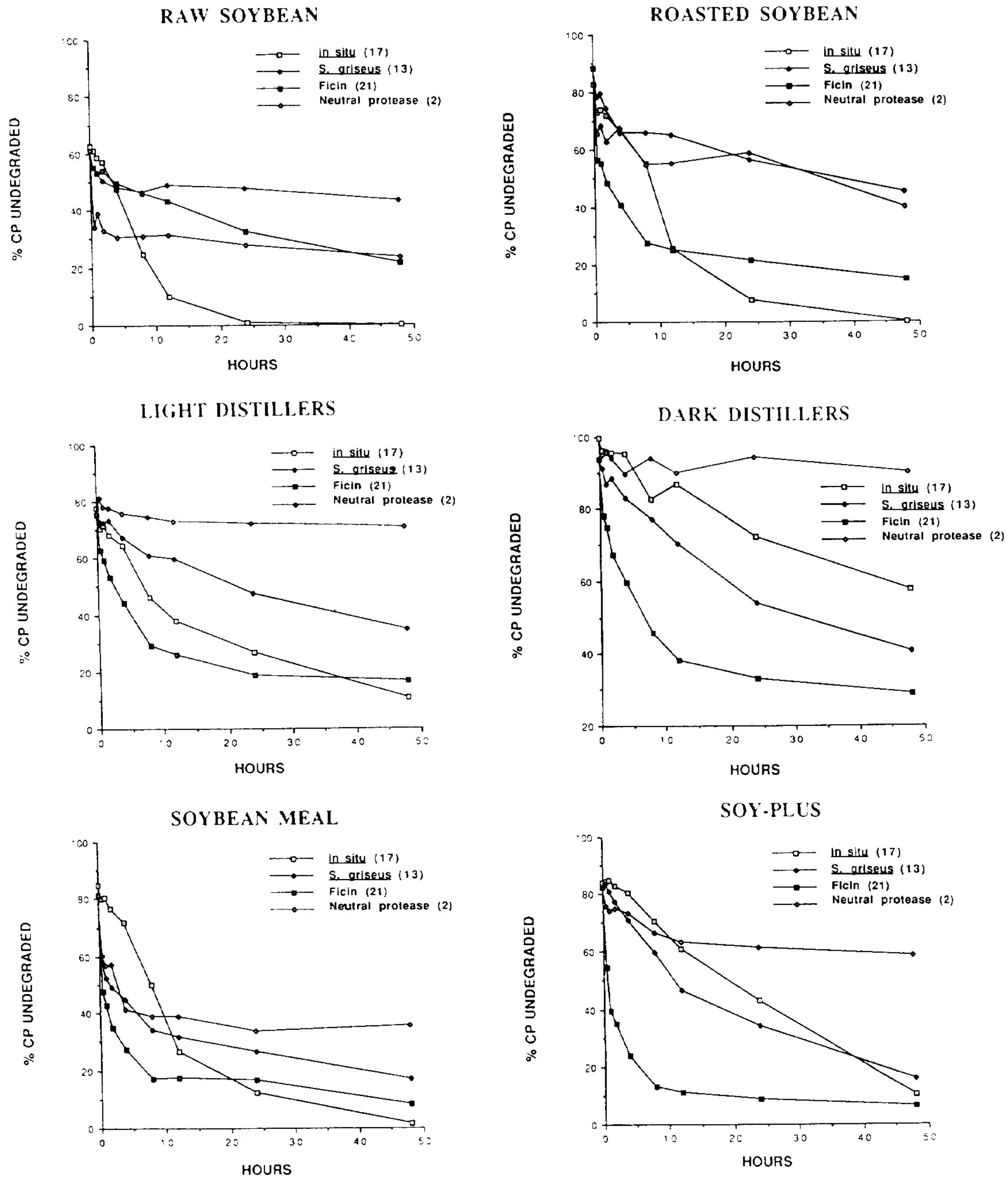

Figure 1. Protein degradation curves (percentage CP remaining undegraded versus incubation time) for each of the test feeds constructed using data generated by the in situ and in vitro methods (*Streptomyces griseus*, ficin, and neutral protease).

that remained undegraded after 24 h of incubation, most similar to the in situ ranking (Table 3). However, this result is of little importance because interactions between time of incubation, feed sample tested, and degradability method were significant by least squares means ($P < .0001$). Additionally, results of the contrast analysis confirmed that each of the in vitro methods studied resulted in estimates of feed protein degradability that conflicted with those obtained by the in situ method ($P < .0001$).

The results of the in vitro methods tested do not agree with those reported by Bartle et al. (3). The *S. griseus* method, with the alkaline buffer, did not consistently degrade soybean protein to a lesser extent than methods utilizing a buffer with pH 6.5.

TABLE 4. Regression of in situ on in vitro (by various methods) percentages of initial CP remaining undegraded at 0, .5, 1, 2, 4, 8, 12, 24, and 48 h of incubation of test feeds.

	R^2	SE[2]
Soybean meal		
In situ = −14.79 + 1.53 (*S. griseus*)[1]	.78	.29
In situ = 13.80 + 1.18 (ficin)[1]	.62	.33
In situ = −30.87 + 1.69 (neutral protease)[1]	.64	.45
Soy-PLUS		
In situ = 6.41 + 1.00 (*S. griseus*)	.94	.09
In situ = 46.50 + .66 (ficin)	.46	.26
In situ = −121.73 + 2.70 (neutral protease)	.75	.55
Light distillers grains		
In situ = −53.06 + 1.68 (*S. griseus*)	.96	.12
In situ = 8.47 + 1.03 (ficin)	.89	.13
In situ = −403.18 + 6.04 (neutral protease)	.72	1.33
Dark distillers grains		
In situ = 31.04 + .74 (*S. griseus*)	.95	.06
In situ = 57.74 + .51 (ficin)	.73	.11
In situ = −103.79 + 2.05 (neutral protease)	.11	2.05
Raw soybean		
In situ = −171.96 + 4.07 (*S. griseus*)	.58	1.22
In situ = −60.39 + 2.05 (ficin)	.83	.33
In situ = −24.77 + 1.69 (neutral protease)	.41	.71
Roasted soybean		
In situ = −106.72 + 2.27 (*S. griseus*)	.82	.37
In situ = −.87 + 1.20 (ficin)	.73	.26
In situ = −74.01 + 1.96 (neutral protease)	.61	.56

[1]*Streptomyces griseus* (13); ficin (21); neutral protease with amylase (2).

[2]SE of the estimate of the in vitro trait.

Correlations Between In Vitro and In Situ Estimates of Ruminal Protein Degradation

If an in vitro method is capable of accurately predicting in situ protein degradation, the regression of in vitro on in situ observation

TABLE 5. Regression of the percentage of initial CP remaining undegraded after in situ incubation of all test feeds on in vitro values at various time points throughout incubation.

	R^2	SE[2]
12-h In situ incubation, 12 h in vitro		
In situ = −7.13 + .90 (*S. griseus*)[1]	.21	.88
In situ = 46.45 − .20 (ficin)[1]	.01	1.15
In situ = −25.36 + 1.14 (neutral protease)[1]	.78	.30
18-h In situ incubation, 18 h in vitro		
In situ = .90 + .68 (*S. griseus*)	.10	1.03
In situ = 34.44 − .01 (ficin)	.00	1.27
In situ = −25.90 + 1.03 (neutral protease)	.76	.29
24-h In situ incubation, 24 h in vitro		
In situ = 13.75 + .30 (*S. griseus*)	.02	1.14
In situ = 22.11 + .23 (ficin)	.01	1.40
In situ = −26.88 + .93 (neutral protease)	.74	.28
18-h In situ incubation, 1 h in vitro		
In situ = −65.08 + 1.40 (*S. griseus*)	.57	.61
In situ = −23.01 + 1.06 (ficin)	.24	.94
In situ = −51.47 + 1.25 (neutral protease)	.78	.33
18-h In situ incubation, 2 h in vitro		
In situ = −64.43 + 1.43 (*S. griseus*)	.67	.50
In situ = −7.93 + .86 (ficin)	.15	1.02
In situ = −43.76 + 1.17 (neutral protease)	.80	.30
18-h In situ incubation, 4 h in vitro		
In situ = −68.37 + 1.62 (*S. griseus*)	.73	.50
In situ = 11.89 + .54 (ficin)	.07	.97
In situ = −29.47 + 1.01 (neutral protease)	.69	.34
18-h In situ incubation, 8 h in vitro		
In situ = −38.62 + 1.27 (*S. griseus*)	.49	.65
In situ = 28.82 + .18 (ficin)	.01	.98
In situ = −29.29 + 1.06 (neutral protease)	.81	.25
18-h In situ incubation, 12 h in vitro		
In situ = −13.56 + .89 (*S. griseus*)	.21	.86
In situ = 36.76 − .10 (ficin)	.00	1.13
In situ = −31.15 + 1.11 (neutral protease)	.78	.29

[1]*Streptomyces griseus* (13); ficin (21); neutral protease with amylase (2).

[2]SE of estimate of the in vitro trait.

points should yield a zero intercept with a slope of 1.0 and a high correlation coefficient (11). An in vitro method also may provide degradability estimates that simply relate, in a consistent manner, to those obtained by the in situ technique. None of the in vitro methods tested met these criteria, as shown in Table 4 by the lack of similarity between each of the regression equations developed with data obtained using the same in vitro degradability method but different feed samples and the marked differences in R^2 values.

Attempts have been made to develop regression equations that predict a static ruminal protein degradability value, i.e., extent of degradation assuming a dilution rate of .06/h, from the extent of protein degradation after an in vitro incubation (11, 21, 25). Although this technique fails to address the need for predicting protein degradation for individual cows with diverse rates of passage, it may be a viable alternative approach for commercial laboratory analysis. The regression equations developed with data from the neutral protease with amylase method explained 78, 76, and 74% of the variation in the in situ data obtained after 12, 18, and 24 h of incubation, respectively (Table 5). Additionally, prediction equations developed using data from the neutral protease with amylase method from shorter incubations explained much of the variation after 18 h *in situ*. The regression equation developed using the results from a 4-h *S. griseus* incubation explained 73% of the variation in the 18-h in situ results. However, relationships between other results of *S. griseus* incubation and in situ degradability were poor. Little relationship was found between the ficin and in situ results.

Some of our conclusions are not consistent with those of others. The reason is unknown. According to Krishnamoorthy (11), the residual N remaining after 18 h *incubation* with *S. griseus* closely agrees with in vivo degradability estimates (R^2 = .61). Poos-Floyd et al. (21) reported highly significant correlations between solubilization by the ficin enzyme after 1 or 4 h and in vivo protein degradation.

Assoumani et al. (2) reported that regression equations computed with sodium citrate buffer (pH 6.5) in vitro data from a much larger data base predicted nylon bag estimates quite accurately (R^2 = .91 and .92, respectively, for alkaline and neutral proteases). However, accuracy diminished when an alkaline buffer was utilized. Our findings are consistent with the results of their study.

CONCLUSIONS

Accurate estimation of ruminal protein degradability of feeds is essential for optimization of the rumen system. None of the in vitro methods evaluated resulted in feed protein degradation curves that had a consistent relationship with those generated by the in situ technique. However, relationships were found between protein degradability estimates obtained by the neutral protease with amylase method at specific time points and those measured in situ.

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