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Predicting ruminal degradability of lucerne and grass forage protein from *in vitro* solubility with non-specific bacterial protease or pancreatin

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ABSTRACT

Samples of lucerne (25) from the primary growth and 2 regrowths and samples of grass (9) from the primary growth were harvested in successive stages of maturity within one vegetation season and were used to test the applicability of protein solubilization during incubation with a non-specific protease from *Streptomyces griseus* (Sigma type XIV) or porcine pancreatin to predict *in sacco* ruminal degradability of crude protein (CP) in dried forage. The effective degradability (ED) of protein in the forage, calculated at $k = 0.06 h^{-1}$, ranged from 63 to 88%. The conditions for protease XIV activity given by Krishnamoorthy et al. (1983) and Aufrere and Carthailler (1988), at constant enzyme concentration in the incubation medium and short incubation period, were not suitable for predicting variability in *in sacco* protein degradability of lucerne due to morphological changes or growth type ($R^2 = 0.183$, $P = 0.03$, RSD = 5.94). The results were better when a constant ratio of enzyme to protein in a sample was maintained (4 U of protease per 100 mg of protein) and duration of incubation was extended to 24 h ($R^2 = 0.713$, $P < 0.001$, RSD = 3.52). However, the best fit between enzymatic solubility and effective degradability of lucerne protein was obtained using pancreatin (ca. 5 U of trypsin per 0.5 g of dry forage): $R^2 = 0.830$, $P < 0.001$, RSD = 2.71.

Validation of regression equations with samples of grass forage indicated that solubility with pancreatin was superior to the action of protease from *S. griseus* in predicting ruminal degradability of forage determined *in situ* in cows (R^2 and RSD = 0.96 and 1.64% vs. 0.59 and 5.15%, respectively).

The regression equations between ED (Y, %) and enzymatic solubility of protein (X, %) for the combined sets of lucerne and grass samples (n = 34) were for pancreatin: $Y = 1.18 X - 10.97$, $R^2 = 0.882$, $P < 0.001$, RSD = 2.84; for protease: $Y = 1.00 X + 2.93$, $R^2 = 0.544$, $P < 0.001$, RSD = 5.97.

KEY WORDS: lucerne, grass, protein, rumen degradability, bacterial protease, pancreatin, enzymatic solubility

INTRODUCTION

Changes in composition occurring in lucerne plants during maturation (increase of fibre and decrease of crude protein content, Andrieu et al., 1989) also induce changes in the extent of herbage protein solubility (Tamminga, 1982) and degradability (Balde et al., 1993). Thus, forages harvested at a wide range of morphological stages (from early vegetative to late generative) present a good model for testing the suitability of laboratory methods to predict ruminal degradability of protein by using linear regression.

Protein degradation has been estimated from measurements of digesta flow in the small intestine and from protein disappearance during incubation of feed in polyester bags suspended in the rumen (Van Straalen and Tamminga, 1990). Both methods require cannulated animals and are not suitable for routine screening of feedstuffs. Therefore, various solubility tests and enzymatic procedures have been developed (Broderick, 1982; Miller, 1982; Lindberg, 1985).

Pichard and Van Soest (1977) proposed using a commercial protease from *Streptomyces griseus*, which has a broad specificity for cleavage of peptide bonds, to estimate ruminal protein degradation. Poos et al. (1980) reported that *in vitro* digestion of different feed proteins using a neutral fungal protease was more highly correlated with *in vivo* ruminal degradation than was *in vitro* digestion using several other commercial proteases, including that from *S. griseus*. The method based on the use of the latter protease was further developed by Krishnamoorthy et al. (1983), Poos-Floyd et al. (1985), Aufrere and Cartailler (1988) and Aufrere et al. (1991). However, the results of predicting protein degradation were not always satisfactory.

Aufrere et al. (1991) suggested a 1 h incubation time for routine analysis of concentrate ingredients, and this duration of incubation also gave the best correlation between enzymatic and *in vivo* degradation in the work of Poos-Floyd et al. (1985). However, our preliminary results (Antoniewicz and Kosmala, unpublished) indicated that during 2 h incubation, the protease from *S. griseus* (1 mg of enzyme per 500 mg of sample) was not able to differentiate grass forage of 18 and 9% crude protein (early vegetative and late bloom stage, respectively) according to their effective ruminal degradability (81 and 69% *in sacco*, 74 and 73% *in vitro*, respectively).

The present study conducted with lucerne and grass forage was undertaken to assess the predictive ability of *in vitro* incubations with the protease from *S. griseus* as compared to the results obtained by the *in sacco* procedure or *in vitro* incubation with pancreatin.

MATERIAL AND METHODS

Feed samples

Lucerne herbage (a monoculture of *Medicago sativa*) of primary growth and regrowths was cut at successive stages of maturity throughout the whole vegetation season to produce a set of 25 samples covering a fairly wide range of composition and quality. Grass herbage (*Dactylis glomerata* 0.72; *Poa pratensis* 0.20; *Festuca pratensis* and others 0.08) of primary growth was cut from early vegetative to dry stem stage. Fresh herbage samples were dried at 35^{0}C and ground to pass a 1 mm sieve using a Wiley mill.

Dry forage was analyzed *in sacco* for protein degradability (Antoniewicz et al., 1995). The assay was conducted on cannulated cows, essentially as described by Ørskov et al. (1980). Calculations of effective degradability (ED) were done using models based on McDonald (1981) assuming a small particle outflow rate k of 0.06 h^{-1}, with no correction for microbial contamination. Lucerne forage was used to make calibrations (regression equations) for predicting ED from enzymatic solubility, and grass samples were used to validate the obtained equations.

Enzymatic methods

Assay with bacterial protease

Protease from *Streptomyces griseus*, type XIV (5.1 units/mg, Sigma Chemical Co., St. Louis, Missouri, USA) was used. One unit represents the quantity of enzyme that will hydrolyse casein to produce colour with the Folin-Ciocalteu reagent equivalent to 1 mmol tyrosine min^{-1} at pH 7.5 and 37^{0}C.

Forage samples (0.5 g) were incubated in duplicate with enzyme in 40 ml of borate-phosphate buffer pH 8.0 ($NaH_2PO_4 \cdot 2H_2O$ 8.6 g and $Na_2B_4O_7 \cdot 10H_2O$ 13,17 g l^{-1}). The amount of enzyme was either constant, i.e. 4 units (procedure I) or equivalent to protein content in the incubated sample, 4 U per 100 mg of protein (procedure II and III). Samples were incubated for 2 h (procedure I and II) or 24 h (procedure III) by shaking in a water bath at 39^{0}C. Next, they were filtered through polyester fibre (40 μm square pore size) and the solid residues were thoroughly washed with distilled water. Feed residues on the filters were deep frozen and in this form quantitatively transferred to Kjeldahl flasks for total N determination.

Assay with pancreatin

The enzyme used was porcine pancreatin (Polfa, Warszawa, Poland, 59 U of trypsin g^{-1}), a solution of 2 g l^{-1} 0.1 M phosphate buffer pH 7.4 (80 ml

0.2 M NaH_2PO_4+420 ml 0.2 M Na_2HPO_4 made up to 1 l with distilled water). Samples (0.5 g) were incubated with 40 ml of enzyme solution for 24 h at 39°C and further treated the same way as in the procedure with the protease from *S. griseus.*

Chemical and statistical analysis

Nitrogen was determined by the Kjeldahl method using Kjeltec Auto 1030 (Tecator, Hoganas, Sweden). The results of *in vitro* assays were compared to the ED of protein using linear regression and the analysis of variance. Nonaccountable residual mean square variance was expressed as residual standard deviation (RSD).

RESULTS

Protein solubility of lucerne forage by the *S. griseus* protease depended strongly on the conditions of incubation. The relationship against ED values was poorest at a constant enzyme concentration and 2 h incubation time (procedure I, Table 1, Figure 1). It improved when the enzyme concentration was equivalent to protein content in forage: slightly at 2 h incubation (procedure II) and significantly when incubation was prolonged to 24 h (procedure III). As was expected, the closest relationship between ED values and enzymatic solubility was obtained using pancreatin (Table 1, Figure 2). Solubility with pancreatin allowed to account for 83% of variability in ED values due to maturity and growth type of lucerne forage, while the accountable variance at the best procedure (III) for protease XIV was 71%. There was a very close correlation between the protein solubility results obtained using pancreatin (Y, %) and the protease from *S. griseus* according to procedure III (X, %): $Y=0.83X + 14.27$; $n=25$, $R^2=0.92$, $P<0.001$, RSD $=1.60$.

The results of predicting protein ED values of primary growth of grass using the equations obtained for lucerne (Table 1) and protease (according to procedure III) or pancreatin are shown in Figure 3. The regression statistics are shown in Table 1. The correlation coefficients and RSD were much better for pancreatin. Accountable variance in the prediction of ED values reached 96% but only 59% when the equations for pancreatin and protease, respectively, were applied.

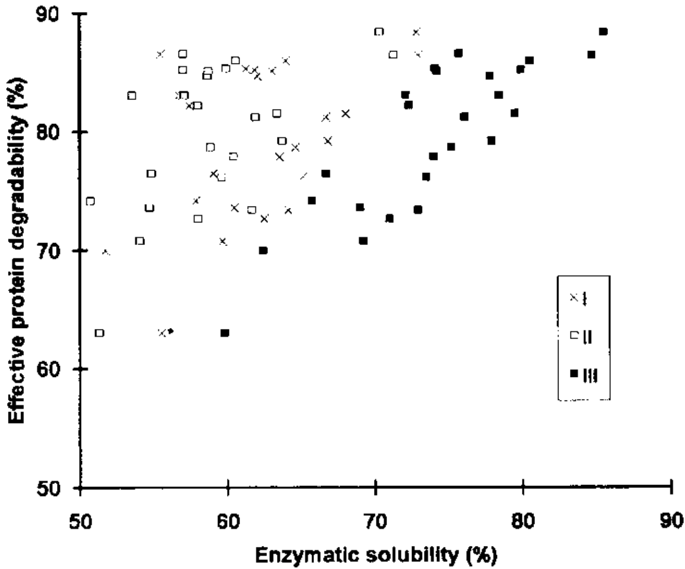

Figure 1. The relationship between ruminal effective degradability of protein of dried lucerne forage and protein solubility under action of protease from *Streptomyces griseus:*

I 4 units, 2 h

II 4 units per 100 mg of protein, 2 h

III 4 units per 100 mg of protein, 24 h

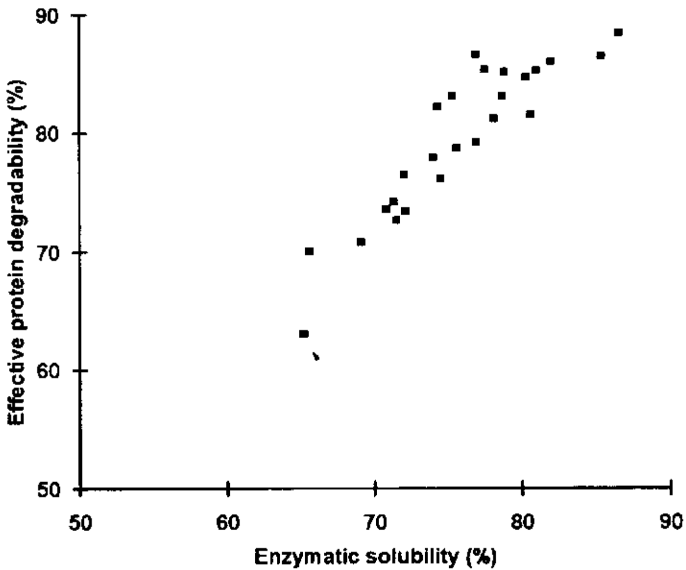

Figure 2. The relationship between ruminal effective degradability of protein of dried lucerne forage and protein solubility under action of pancreatin

TABLE 1

Statistical parameters of linear regression between enzymatic solubility (X,%) and *in sacco* effective degradability (Y,%) ($k=0{,}06\ h^{-1}$) of protein of dried lucerne forage (n = 25, model Y = a + bX)

	R^2	RSD	a	b	Significance of b
Calibration equations (lucerne forage)					
Pancreatin[1]	0.830	2.71	-2.43	1.08	P<0.001
Protase from *S. griseus*					
I	0.184	5.94	46.44	0.53	P=0.032
II	0.363	5.25	37.36	0.72	P=0.001
III	0.713	3.52	15.07	0.87	P<0.001
Validation equations (grass forage)					
Pancreatin	0.958	1.64	-1.08	0.96	P<0.001
Protease from *S. griseus*					
III	0.585	5.15	-25.28	1.22	P=0.014

[1] conditions of reaction (amount of enzyme per 0.5 g sample, time of incubation)
pancreatin 4.7 units of trypsin, 24 h
protease from *S. griseus*:
I 4 units, 2 h
II 4 units per 100 mg of protein, 2 h
III 4 units per 100 mg of protein, 24 h

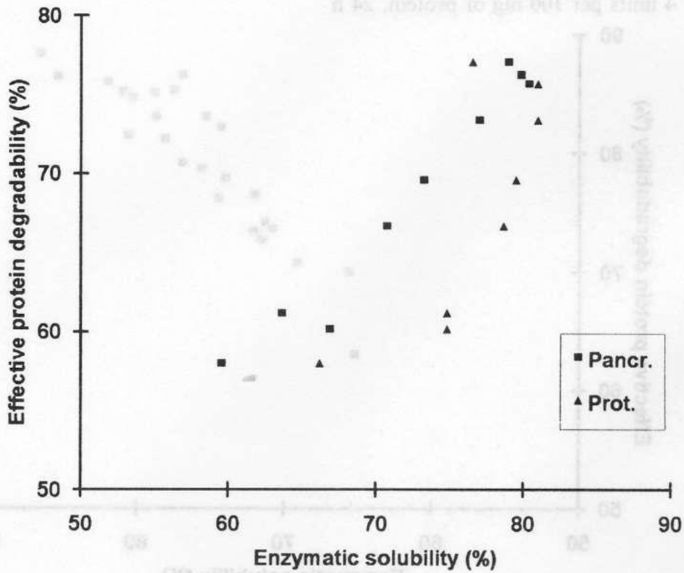

Figure 3. Prediction of the effective degradability of protein of dried grass forage using equations obtained for lucerne forage (Pancr. – pancreatin, Prot. – protease from *S. griseus* acc. to procedure III, compare Table 1).

DISCUSSION

The protease from *S. griseus* shows a higher rate of protein solubilization than is observed during *in sacco* incubation in the rumen, especially at a substrate-saturating enzyme concentration (Krishnamoorthy et al., 1983). This could explain the higher protein solubility than degradability from more mature forages of lower protein content when procedure I was used. The low correlation observed in this situation indicated that the enzyme to substrate (feed protein) ratio is of crucial importance. When the ratio is high, the reaction follows first-order kinetics and the rate depends strongly on substrate concentration. On the other hand, when substrate concentration is higher and the ratio of enzyme to substrate lower, the reaction follows zero-order kinetics, the rate of proteolysis is almost constant and not affected by substrate concentration. It is not quite clear which reaction order best describes proteolysis in the rumen. However, there is much to support the suggestion that the reaction deviates from first-order due to limiting enzyme activity (Van Soest et al., 1982). The experiments by Broderick (1978) support first-order kinetics, while those of Nugent and Mangan (1981) show that they could be zero-order. Our results indicate that excess enzyme should be avoided, and the results of *in vitro* solubilization correlate better with *in situ* measurements at a constant enzyme to substrate ratio. As protein solubilization under these conditions is less rapid, it was reasonable to increase the reaction time to 24 h.

The better correlation with *in situ* degradability found when lucerne protein was solubilized with pancreatin than with bacterial protease apparently seems unjustified. However, Craig and Broderick (1980) reported disproportionate release of lysine and, especially, arginine during *in vitro* degradation by mixed rumen microorganisms. Also artificial trypsin substrates inhibited *in vitro* degradation of casein by rumen organisms (Craig, unpublished, after Broderick, 1982). These data suggest that initial cleavage by trypsin-like proteases of rumen microbes may limit ruminal protein degradation. Production by microorganisms of trypsin-like proteases may be a good explanation of the satisfactory prediction of ED using porcine pancreatin. Solubility with this enzyme accounted for 83% of variance in the protein degradability results. Poorer results obtained with the protease from *S. griseus* can be a consequence of a very broad specificity of this enzyme that may obscure differences among feed protein in their susceptibility to microbial proteolysis in the rumen. Another reason may be a difference in the pH of the solution (7.4 for pancreatin and 8.0 for protease). It seems important to measure *in vitro* protein degradability under conditions similar to those in the rumen, because the pH affects solubility of different types of protein in feeds (Krishnamoorthy et al., 1983). However, it is worth noting that the values of

standard errors of estimate obtained in our study with *S. griseus* protease (Table 1) were much lower than those obtained by Assoumani et al. (1992) (8.1-16.5%).

With the use of *S. griseus* protease (according to procedure III), and also pancreatin, forages can be evaluated on a relative basis and protein solubilization results are in a reasonable agreement with those obtained by the nylon bag technique for ED estimation. The equation obtained for lucerne and applied to grass fits well in the case of pancreatin and relatively worse as far as protease is concerned.

It may be concluded that measuring solubility by protease from *S. griseus* at a constant enzyme to protein ratio or by porcine pancreatin provides a fairly accurate approach to predicting effective degradability of lucerne protein in the rumen. The equation obtained with pancreatin is more universal and may also be used satisfactorily for grass forage. This indicates that the extent of proteolysis of dry forage by this enzyme is not species-dependent.

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STRESZCZENIE

Szacowanie degradacji żwaczowej białka zielonki z lucerny i traw na podstawie rozpuszczalności *in vitro* pod działaniem nieswoistej proteazy bakteryjnej lub pankreatyny

Próbki zielonki z lucerny (25) z trzech pokosów i z traw (10) z dwóch pokosów pobierano w miarę postępujących stadiów dojrzałości w jednym sezonie wegetacyjnym i użyto do określenia przydatności oceny rozpuszczalności białka przy inkubacji z nieswoistą proteazą z *Streptomyces griseus* (Sigma, typ XIV) lub wieprzową pankreatyną do szacowania efektywnej degradacji (ED) białka ogólnego suszonych zielonek. ED białka w zielonkach obliczona dla $k=0,06$ h^{-1} wynosiła 63-88%.

Warunki działania proteazą XIV podane przez Krishnamoorthy'ego i in. (1983) i Aufrere i Carthailler (1988) (stałe stężenie enzymu w medium inkubacyjnym i krótki czas inkubacji) nie były odpowiednie do rozróżnienia zmienności w degradcji białka lucerny na skutek zmian w stadium

wegetacji ($R^2 = 0{,}183$, $P = 0{,}03$, $RSD = 5{,}94$). Lepsze wyniki uzyskano przy zastosowaniu stałego stosunku enzymu do białka w próbce i wydłużeniu czasu inkubacji do 24 godz ($R^2 = 0{,}713$, $P < 0{,}001$, $RSD = 3{,}52$).

Najlepszą zgodność między rozpuszczalnością białka lucerny pod działaniem enzymu a wartościami ED uzyskano stosując pankreatynę (ok. 5 jedn. trypsyny na 0,5 g suchej zielonki): $R^2 = 0{,}830$, $P < 0{,}001$, $RSD = 2{,}71$.

Sprawdzenie i potwierdzenie (walidacja) równań regresji przy użyciu prób zielonki z traw pokazało, że rozpuszczalność z pankreatyną pozwalała skuteczniej szacować degradację ocenianą *in situ* na krowach niż przy działaniu proteazą ze *S. griseus* (odpowiednio R^2 i RSD 96 i 0,11% vs. 59 i 5,15%).

Równania regresji do szacowania wartości ED (Y, %) na podstawie rozpuszczalności enzymatycznej białka (X, %) opracowane dla połączonego zbioru prób lucerny i traw były następujące:
dla pankreatyny $Y = 1{,}18\ X - 10{,}97$, $R^2 = 0{,}882$, $P < 0{,}001$, $RSD = 2{,}84$;
dla proteazy $Y = 1{,}00\ X + 2{,}93$, $R^2 = 0{,}544$, $P < 0{,}001$, $RSD = 5{,}97$.