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Article

Protein degradation rate as affected by plant proteases among fresh samples of perennial ryegrass cultivars (*Lolium perenne* L.)

Einfluss pflanzlicher Proteasen auf den Proteinabbau bei unterschiedlichen Englischen Raigrass Sorten (*Lolium perenne* L.)

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Summary

The objective of this study was to quantify the proteolytic activity of a set of 10 diploid early intermediate heading cultivars of *Lolium perenne* under rumenlike conditions. A field experiment was conducted in Northern Germany, where the perennial ryegrass cultivars were grown during two growing seasons. Leaves of the first and second cut were sampled in the field, sterilized with 800 ml·l^{-1} ethanol solution and incubated for 0, 6, and 24 h under rumenlike conditions (darkness, 39°C, pH 6.5) without the presence of rumen microbes. Results revealed that the leaf protein content declined with increasing incubation time, confirming the involvement of plant-mediated proteolysis in the degradation process. Gel electrophoresis illustrated that the decrease in protein content is probably mainly caused by the loss of the large subunit of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase), which was entirely degraded during the incubation time. Although differences among harvests and years were evident, genetic variation among the 10 diploid perennial grass samples concerning protein degradation rates and degradation characteristics was not detected.

Keywords: rumenlike conditions, Rubisco, plant protease, N-use efficiency

Zusammenfassung

Im vorliegenden Experiment wurden 10 diploide Sorten von Deutschem Weidelgras (*Lolium perenne*) des mittelfrühen Sortiments zum Zeitpunkt der früher Siloreife im ersten und zweiten Aufwuchs in Norddeutschland untersucht. Ziel war es in frischen Blättern die Auswirkungen pansenähnlicher Bedingungen auf die Aktivität pflanzlicher Proteasen im ersten und zweiten Aufwuchs zu bestimmen. Nach der Ernte erfolgte zeitnah die Aufbereitung des Pflanzenmaterials im Labor. Nach der Reinigung in destilliertem Wasser und 800 ml·l^{-1} Ethanol wurde jeweils 1 g des zerkleinerten Blattmaterials in dreifacher Wiederholung für 0, 6 und 24 h unter pansenähnlichen Bedingungen ohne Pansenmikroben inkubiert. Mit zunehmender Inkubationsdauer war eine Abnahme der Proteingehalte festzustellen, was die Beteiligung pflanzlicher Proteasen im Proteinabbau bestätigt. Die Gelelektrophorese unterstützt die Behauptung, dass die Minderung im Proteingehalt wahrscheinlich durch die Abnahme von Rubisco verursacht wird. Es traten deutliche Unterschiede zwischen den Erntezeitpunkten und Jahren auf, die genetische Variation zwischen den untersuchten Sorten zeigte jedoch keinen Einfluss hinsichtlich des Proteinabbaus.

Schlagworte: pansenähnliche Bedingungen, Rubisco, pflanzeneigene Proteasen, N-Nutzungseffizienz

1. Introduction

Forage feeding systems for ruminants are often characterized by inefficient N use, which may result in high N losses to the environment. The risk of losses is especially high under grazing conditions in combination with sandy soils (Trott et al., 2004). One main source is the inefficient conversion of plant protein to microbial protein in the rumen. It is crucial for the efficient use of ingested forage protein by ruminants that the availability of fermentable organic matter matches the availability of protein degradation products, with efficient conversion in microbial protein synthesis (e.g., Trevaskis et al., 2001). Such improvement in the efficient use may reduce the need of concentrates for high-yielding dairy cows on a herd basis. However, especially in forage-based diets, the protein degradation rate often exceeds the energy supply for microbial growth, and microbes use protein as energy source, releasing non-protein nitrogen (NPN) to the ruminal environment, mostly further converted to ammonia or nitrous oxides. To overcome this problem, one option at the plant level is to increase the availability of readily fermentable organic matter, as occurred by breeding high-sugar cultivars of perennial ryegrass (*Lolium perenne*) (Miller et al., 2001). The limitation of this approach is the content of water-soluble carbohydrates in sugar-rich cultivars, which is dependent on climatic conditions of the growing site. Another possibility to achieve the synchrony of protein and fermentable organic matter release in forage-based nutrition is to slow down the rate of protein degradation of the forage protein. Concerning protein degradation rate in fresh forages, as it is the case under grazing, up to now plant tissue has been regarded as passive victim of the degradation process of the rumen microbes. The contribution of plant-mediated proteolytic enzymes contained in ingested fresh forage and its contribution to protein degradation is ignored in current models for ruminant nutrition (Theodorou et al., 1996; Kingston-Smith et al., 2003). Assuming that half of ingested plant biomass at grazing consists of intact cells (Kingston-Smith and Theodorou, 2000), which stay viable and are metabolically active during the first hours after ingestion, the proteases in the intact cells may be involved in proteolysis. Conditions found in the rumen (elevated temperature, darkness, and anaerobic environment) may contribute to induce the plant cell death and increase plant-mediated protease activity (Beha et al., 2002; Atwood, 2005). Understanding the extent of protease activity under rumenlike conditions may contribute to reduce its activity and to improve the N use efficiency of the ruminant by decreasing the rate of protein degradation soon after feed ingestion as fresh forage. Whereas protease activity is variable between different plant species (Zhu et al., 1999), we hypothesize that variability of protein degradation mediated by plant proteases soon after ingestion among perennial ryegrass cultivars is high. The presence of variability among cultivars for the early postprandial protein degradation would allow the selection of new cultivars with improved protein quality. The objective of the present study was to quantify the plant-mediated proteolysis among a set of 10 diploid perennial ryegrass cultivars belonging to the intermediate early heading group.

2. Materials and Methods

2.1 Plant material, site and design of the experiment

The field plots with the different perennial ryegrass cultivars were located at Hohenschulen experimental station (53°18'N, 9°58'E, 32 m a.s.l.) of the Christian-Albrechts University, Kiel, Northern Germany, during the growing seasons of two years. The experimental site is characterized by its sandy loam soil with a long-term average annual rainfall of 755 mm and a daily mean temperature of 8.7°C (35 years average). During the two growing seasons, the average rainfall was 707 and 925 mm, respectively, and the daily mean temperature was 9.9 and 10.0°C, respectively. Ten diploid perennial ryegrass cultivars belonging to the intermediate early heading group were established in randomized complete block design with three replicates.

2.2 Management and sampling

The experimental plots were sown in the late summer and the plots were managed with four cuts in the first and second production year. Two out of the four cuts per year (the first and second cuts) were used in the experiment. N fertilizer was added in the form of ammonium calcium nitrate (26% N) at the rate of 300 kg $N \cdot ha^{-1}$ divided into four dressings of 100, 80, 80, and 40 kg $N \cdot ha^{-1}$ applied before the first, second, third, and fourth harvests, respectively. In addition, 80 kg P_2O_5 ha^{-1} were applied at one dose in spring each year. A potassium fertilizer (40% K_2O, 6% $MgSO_4$, 4% S, and 3% Na) was given at the rate of 360 kg K_2O ha^{-1} in two dressings, 200 and 160 kg K_2O ha^{-1}, before the first and third harvests, respectively. Folicur (1-(4-chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazol-1-ylme-

thyl) pentan-3-ol) was sprayed against crown rust at the rate of 0.7 $l \cdot ha^{-1}$ two weeks after each harvest beginning from the second one.

Plant material for the present study was sampled in the first and second cuts each year (June/July 2006 and May/June 2007) by hand clipping the plants at 5 cm above the soil surface at the early silage maturity stage. The development stage of plants was monitored at each harvesting date by cutting a representative sample of around 50 tillers randomly from each plot to ground level. The maturity stage of each tiller was determined according to Park (1980). The mean stage by count (MSC) was calculated as the average of the individual stage categories present in the herbage sample, weighed for the number of tillers at each stage (Moore et al., 1995), and used as covariable in the statistical model:

$$MSC = \frac{\sum_{i=1}^{17} Si \cdot N}{C}$$

where S_i is the stage number (1–17), N is the number of tillers in stage S_i, and C is the total number of tillers in herbage sample.

2.3 Sample preparation and incubation

The collected plant material of each of the 10 cultivars with three field replicates was immediately transported to the laboratory one plot after other under cooling conditions to minimize postharvest metabolic activity. In the laboratory, samples were rinsed several times with distilled water at room temperature. Microbial contamination was suppressed by soaking the intact plant material in 800 $ml \cdot l^{-1}$ ethanol solution and rinsed with water as proposed by Kingston-Smith et al. (2002). After this rinsing step, the leaves were cut into pieces of approximately 2 cm in length. The foliage of each cultivar was then separated into three subsamples each consisting of 1 g fresh matter (FM) in three replications. The first subsample was immediately frozen in liquid N and stored at –70°C for further analysis. The remaining subsamples were incubated in darkness under anaerobic conditions at 39°C for 6 and 24 h in Duran® bottles filled with 100-ml incubation buffer (50 mM Na_2HPO_4, 50 mM KH_2PO_4, 5 mM dithiothreitol [DTT], 20 $ml \cdot l^{-1}$ sodium azide, pH 6.8). At the end of the incubation, leaf residues were separated from buffer by filtration and immediately frozen in liquid N and stored at –70°C until required for protein content determination, using the method of Bradford (1976). The time span between sampling and first incubation took about 10–12 min for each sample and their laboratory replicates. Considering 30 field samples, the earliest new sampling of incubated foliage allowed a 6 h interval.

2.4 Analytical procedures

The plant material for residual protein content was prepared according to the method described by Kingston-Smith et al. (2003). Briefly, the frozen foliage was ground to a fine powder in liquid N with pestle and mortar. For each sample, 0.3–0.5 g were weighed in microtubes and thawed in 800 µl extraction buffer [50 mM Tris, 2mM DTT, 1 mM EDTA, 1 $g \cdot l^{-1}$ Triton X 100, pH 7.5]. The mixtures were homogenized and centrifuged for 10 min at 10.000×*g*. The extractable soluble protein content of the resulting supernatant was determined based on the method of Bradford (1976) using the Bio-Rad protein assay dye reagent concentrate (catalog number: 500-0006). The results were used to calculate the protein degradation rate of the samples and the supernatant was further used for gel electrophoresis.

For the gel electrophoresis, the polypeptides in the supernatant were separated with the Agilent 230 Protein Assay in the Agilent 2100 Bioanalyzer. The gels were loaded on equal protein basis to allow comparison of the polypeptide composition and relative abundance within and between the protein extracts of the cultivars. Sample preparation was performed according to the Agilent Protein 230 Assay Protocol. A control ladder varying from 14 to 230 kDa was used.

2.5 Calculations and statistical procedures

The protein contents ($\mu g \cdot g^{-1}$ FM) in leaf residues after incubation at 0, 6, and 24 h were transformed by natural logarithmic to achieve a linear slope representing the average degradation rates (h^{-1}) of the protein contents over the incubation period. The degradation rate was calculated for the times: 0–24, 0–6, and 6–24 h with the following equation:

$$h_{x-y}{}^{-1} = (lnB_x - lnB_y) / t_{y-x}$$

where $h_{x-y}{}^{-1}$ is the degradation rate from time *x* to *y*, B_x and B_y are the protein contents after *x* and *y* hours, respectively, and *t* is the respective incubation time.

The protein contents after 0, 6, and 24 h and the derived degradation rates were averaged over the two experimental years, as the sward age differed between years and subjected to a mixed model (proc mixed, SAS 9.1, SAS Institute Inc., Cary, NC, USA). For the protein content, the factors cultivar, incubation period, cut, and their interactions were included as fixed effects in the model. For the degradation rate, cultivar, cut, and the interaction "cultivar × cut" were included as fixed effects in the model. The MSC was used as covariable in both models to avoid differences between cultivars because of the development stage at sampling. Least square means with significant *F*-values were compared using student's t–test with previously defined contrasts. To avoid random significance between pair wise comparison, probabilities were corrected using the Bonferroni–Holm test (Holm, 1979).

3. Results

3.1 Protein content

Only the interaction between incubation period and cut influenced the protein content (Table 1). No cultivar effect was observed. Data presented in Table 1 point out that the protein content as means over cultivars decreased substantially with the incubation time in both cuts. The protein content showed higher values at incubation time of 0 h and decreased substantially within 24 h. The amount of protein loss from 0 to 6 h incubation time ranged between 63% and 74% for the first cut and 67% and 78% for second cut. After 24 h incubation time, only 11.1 and 9.6 $\mu g \cdot g^{-1}$ FM of the initial protein content was measured in the first and second cuts respectively.

3.2 Protein degradation characteristics

The gel electrophoresis did not show differences among the polypeptide profiles of the tested cultivars. Qualitative variations of the polypeptide profile among the incubation time treatment are exemplarily demonstrated for cultivar 9 in the first cut (Figure 1). After 0 h incubation time, several polypeptides were detectable, but the most abundant polypeptide was observed as a single band between 50 and 60 kDa. Another band was visible between 15 and 20 kDa. After 6 h incubation time, samples were characterized by a shift of the former most abundant polypeptide to the 40–50 kDa ranges. The protein degradation process was accompanied by an accumulation of polypeptides detectable between 15 and 20 kDa. Moreover, a band appeared in the region near 90 kDa. After 24 h incubation time, the band most abundant between 50 and 60 kDa at 0 h was no longer detectable. Additionally, shifts of polypeptides to the region between 40 and 50 kDa increased. Compared to the 6 h incubation time, a second band appeared near 40 kDa, and the band visible near 90 kDa was no longer detectable. Furthermore, the polypeptides below 20 kDa increased substantially after 24 h.

3.3 Protein degradation rates

Data on protein degradation rates as main effects are shown in Table 2. An impact of the tested factors was detected only for the first incubation period (0–6 h) where the cut influenced protein degradation rates, i.e. the second cut with 21% h^{-1} showed a higher protein degradation rate than the first cut with 18% h^{-1}. Although log transformed, the first incubation period (0–6 h) was characterized by

Table 1. The protein content in grass leaves as affected by incubation time and harvest as means over 10 genotypes
Tabelle 1. Einfluss von Inkubationszeit und Erntezeitpunkt auf den Proteingehalt in Blättern als Mittelwert über 10 Genotypen

	Incubation time			
	0 h	6 h	24 h	
Protein content, $\mu g\ g^{-1}$ FM				SEM
First cut	93.9 ^{aA}	31.2 ^{bA}	11.1 ^{cA}	3.12
Second cut	95.3 ^{aA}	26.9 ^{bB}	9.6 ^{cA}	

Different letters (a, b) among incubation times within each cut differ at $P < 0.001$.
Different letters (A, B) between cuts within incubation times differ at $P < 0.001$.

Table 2. Fractional degradation rate of the logarithmic-transformed protein contents of the tested cultivars per hour incubation
Tabelle 2. Proportionale Abbaurate des logarithmisch transformierten Proteingehaltes der geprüften Genotypen

	Cultivar											
Item	1	2	3	4	5	6	7	8	9	10	SEM	Sign.
Degradation rate												
0–6 h	0.20	0.22	0.19	0.18	0.20	0.22	0.19	0.22	0.21	0.19	0.012	n.s.
6–24 h	0.08	0.06	0.06	0.06	0.07	0.07	0.06	0.06	0.07	0.06	0.006	n.s.
0–24 h	0.10	0.09	0.09	0.08	0.10	0.10	0.08	0.09	0.10	0.09	0.005	n.s.

The mean stage by count was used as covariable in the statistical model; n.s., nonsignificant.

the highest degradation rates ranging from 18 to 22% h^{-1}. During the second incubation period (6–24 h), the degradation rate ranged from 5 to 8% h^{-1}. The degradation rate between 0 and 24 h varied from 8 to 10% h^{-1}. In all cases, differences between the degradation rates among the tested cultivars were not detected.

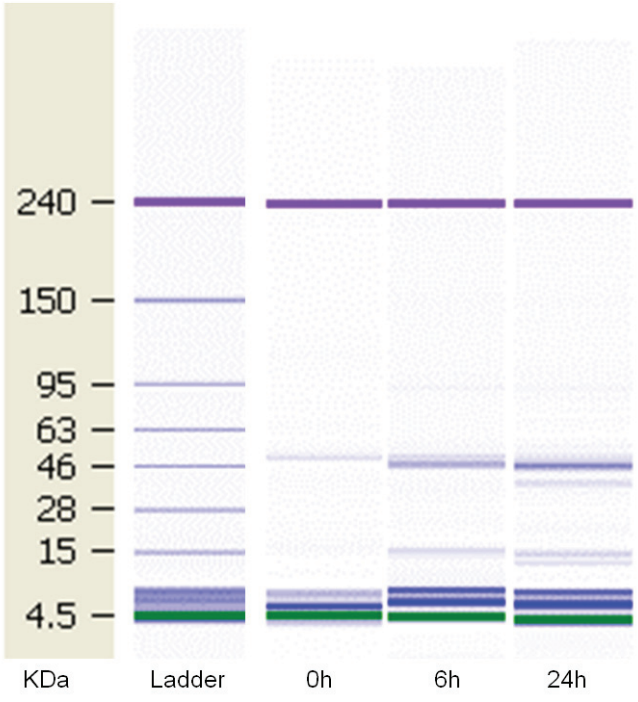

Figure 1. Qualitative changes of the polypeptide profile during incubation (Cultivar 9, first cut). The figure shows only one cultivar, as the differences among the tested cultivars were small.
Abbildung 1. Qualitative Änderungen im Polypeptidprofil im Laufe der Inkubation (Sorte 9, erster Schnitt). Die Abbildung zeigt eine Sorte, da die Differenzen zwischen Sorten eher gering ausfallen.

4. Discussion

The results presented here confirm the contribution of plant-mediated proteases in fresh forage on the degradation process of proteins, in accordance with others (Theodorou et al., 1996). There were apparently little effects among the 10 genotypes. The detected decline of the protein content during *in vitro* incubation under rumen similar conditions (elevated temperature, darkness, lack of O_2, and the absence of rumen microbes) was reported by various authors (Beha et al., 2002, Kingston-Smith et al., 2006). In the present study, the protein loss over 24 h incubation time accounted to 91% and 94% in the first and second cuts, respectively, with some variation accounted for the harvest time as well. These values agree with findings of Beha et al. (2002), who analyzed the protein degradation in perennial ryegrass leaves and detected protein losses of 82% under similar incubation conditions. The observed decrease in staining of the former most abundant Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) large subunit (LSU) (Figure 1) accompanied by an appearance of a possible breakdown product in an area between 40 and 50 kDa after 6 h incubation time is in accordance with the results of Beha et al. (2002) and Zhu et al. (1999).
The products of protein degradation mediated by plant proteases are used by rumen microbes. Zhu et al. (1999) pointed out that the patterns of protein degradation were similar in the presence and absence of rumen microbes for perennial ryegrass. However, a larger proportion of proteins below 20 kDa accumulated in the absence of rumen microbes in ryegrass but not in forage legumes. The results suggest that plant proteinases contribute substantially to plant protein breakdown in ryegrass, and small-sized proteins may be further degraded or incorporated by ru-

men microbes. In *in vivo* experiments, such accumulation of small-sized proteins may contribute to the amount in bypass protein (Gierus, 2010). In our study, the increased staining density in the region between 15 and 20 kDa confirmed the accumulation of low-molecular breakdown products in the absence of ruminal microbes.

Although the degradation rate did not differ between the tested cultivars, degradation rates of all cultivars showed a clear trend (Table 2). Even after linearization, the first incubation period (0–6 h) was characterized by the fast degradation rate, whereas during the second incubation period (6–24 h), the degradation rate decelerated. These results support the findings of Beha et al. (2002) who pointed out that one-third of the initial soluble protein content is already lost after four hours of incubation. The nature of soluble protein decides about their fate in the rumen environment, as not all soluble proteins are immediately available for the microbial activity (Gierus et al., 2005). Surprisingly, there were no differences among cultivars, which makes suppose that the genotypes were highly related.

Rubisco may have been involved in protein degradation mediated by plant proteases under rumenlike conditions (Figure 1). Feller et al. (2008) pointed out that not only the degradation extent but also the mechanisms involved in the degradation of Rubisco may depend on the biotic and abiotic environmental conditions, such as heat, drought, or water logging, some of them may occur in the rumen (Stoychev et al., 2013). Studies of Roulin and Feller (1998) demonstrated that the incubation of isolated pea chloroplast in darkness for several hours resulted in one major LSU fragment with a molecular weight of about 37 kDa. The authors concluded that this fragmentation was catalyzed by a metalloendopeptidase. Considering the Rubisco in ryegrass and its rapid degradation under rumenlike conditions, breeding programs should be targeted on the reduced degradation rate of Rubisco in the early hours after ingestion of the standing crop.

The initial step of the degradation process in the rumen is characterized by the attachment of the microbes to the feed particle. Assuming that up to half of the ingested fresh plant biomass at grazing consists of intact cells, the proteolysis mediated by microbes is initially limited by the structural compartmentation of cells (McAllister et al., 1994; Kingston-Smith and Theodorou, 2000). However, the time necessary for microbial attachment may be the time for plant protease activity to degrade protein. As the delay in time for microbial attachment and initial degradation, that is, the lag time, may be obtained with the nylon bag method, few studies measured the lag time for the protein content in fresh forages (Huws et al. 2012). In some studies, it depended mainly on plant species and sample preparation (Chaves et al., 2006; Nocek & Grant, 1987; Kim et al., 2005). In addition, the time necessary for microbial attachment before the degradation properly starts (also known as lag time) varied largely among *in situ* studies. In contrast, Edwards et al. (2007) measured a fast bacterial colonization of fresh forages, achieving stable population sizes after 15 min. Anyway, the protein loss observed in fresh samples as measured in the present study is probably related to stressors caused by the shift to the environment conditions as found in the rumen. Whereas sudden rise in temperature, darkness, and anaerobic environment compared to the growing environment conditions of the plant just before ingestion may have induced the protease activity (Kingston-Smith and Theodorou, 2000), differences accounted for genetic variation in the perennial grass cultivars tested were less relevant.

Extensive plant-mediated proteolysis during the lag phase may be a source of variation that may support the asynchrony theory between protein availability and fermentable organic matter release in the rumen in fresh forages used for grazing or ensiling. The improvement of the plant protein stability during the first hours after ingestion would noticeably improve N-use efficiency, as already suggested by Nugent and Mangan (1981). The improvement of protein quality of forage plants by means of breeding turned out difficult. An attempt to reduce the initial degradation rate obtained for alfalfa (*Medicago sativa* L.) resulted in the LIRD (low initial rate of degradation) cultivars, which is a breeding target using measurements very close to the protein degradation processes in the rumen. Although the LIRD cultivar was developed in association with fast degradation rate and bloat in cattle, the attempt is close to the target of synchronizing protein and fermentable organic matter availability in the rumen. Coulman et al. (1999) used a 4-hour nylon bag digestion *in situ* technique to determine the initial rate of dry matter disappearance in fresh leaf alfalfa material. The authors stated that the initial rate of degradation during the vegetative phase of the selected cultivars could be reduced by 15% approximately. However, these differences were only possible in the early vegetation stage and disappeared with increasing plant maturity. In addition,

in vivo trials of Berg et al. (1999) revealed that the LIRD character of the plants did not show reduction of the incidence of bloat in grazing experiments. The genetic improvement with LIRD cultivars may have been overlapped by the variation in plant maturity. These results indicate certain limitation in improving forage protein quality for ruminant nutrition, without negatively affecting the yield and the nutritive value of the target forage plant. In our study, the lack of differences in protein degradation rate between diploid cultivars of early intermediate maturity group of perennial ryegrass showed a low variation.

5. Conclusions

The genetic variation of early intermediate maturity group of perennial ryegrass for the activity of proteases in the first hours after defoliation is small. Although the tested cultivars represented a large range of cultivars from different origins and breeders, the narrow genetic background of the perennial ryegrass tested cannot be excluded as reason for the low variation. The most variation on protein degradation was caused by the cut (season). Management seems to be an important factor in forage production to minimize plant-mediated proteolysis potential.

The decline in protein content along the incubation period suggests that plant-mediated proteases were involved in the process of proteolysis under rumenlike conditions. Evaluation of the protein degradation characteristics showed that the large subunit of Rubisco, which was entirely degraded during the 24 hours of incubation, contributed to the major proportion of decline in protein content. Therefore, an important target of forage breeding programs should be the development of new cultivars characterized by their reduced plant-mediated proteolysis and by observing the degradation products.

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