Can a natural protease inhibitor decrease protein degradation of perennial ryegrass in the rumen?

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Abstract

Recently breeders identified two populations of perennial ryegrass (*Lolium perenne* L.) that were thought to be different in protease activity. One population was bred to contain an increased concentration of the protease inhibitor (cystatin) and one population was bred to have a normal protease inhibitor concentration (control). The two populations were tested in three experiments. In the enzyme assay no significant difference could be found between the cystatin and the control population. It was surmised that the protease inhibitor was inactivated by the freeze drying process. The gas production run was done with fresh material. A small difference ($P<0.01$) between the cystatin and the control population was found in the final ammonia concentration in the rumen fluid. Therefore it was concluded that the natural protease inhibitor in the cystatin population might decrease proteolysis in ruminants. The *in vivo* experiment showed no reducing effect on the ammonia concentration in the rumen of sheep grazing the cystatin population. It is concluded that either populations were not different, or the effect of the examined protease inhibitor on proteolysis of perennial ryegrass protein in the rumen is limited.

Keywords: Plant proteases, cystatin, rumen kinetics, sheep.

Introduction

Although large efforts have been made in the last two decades, losses of nitrogen to the environment are still a major problem in livestock production. The efficiency of nitrogen utilization by ruminants is low (16%). The rate of protein breakdown in the rumen frequently exceeds the level at which the released amino acids can be incorporated by the microorganisms. This is especially a problem when ruminants are fed on fresh grass, because grass consists of a large amount of rapidly degradable nitrogen. One possibility to improve nitrogen utilization by ruminants is to reduce the rate of protein degradation in the rumen. Very recently breeders identified, with genetic markers, the gene for cystatin (a protease inhibitor). Two different grass populations of perennial ryegrass (*Lolium perenne* L.) were created. One population was bred to contain an increased concentration of the protease inhibitor (cystatin) and was thought to slowdown the protein degradation another population was bred to have a normal protease inhibitor concentration (control) and was thought to have a faster protein degradation. These two populations were used in an enzyme assay test (exp. 1), a gas production run (exp. 2) and an *in vivo* (exp. 3) experiment to test the effect on the extent of protein degradation of perennial ryegrass in the rumen.

Materials and Methods

Experiment 1*:* The two populations were sown in pots and grown in the greenhouse. After two harvests, and 21 growing days the grass was harvested using scissors. The samples were immediately freeze dried, and milled through a 1mm sieve. An enzyme assay test for protein degradation as described by Mathis (2001) was performed. Triplicate forage samples that contained 15 mg N (≈1 g dry material) were weighed in 80 mL centrifuge tubes, and incubated in 60 mL of a borate-phosphate buffer solution (pH 8) for 1 hour at 39 ºC. Subsequently, 15 mL of separate enzyme solution (*Streptomyces griseus*) was added to the borate-phosphate buffer plus forage and incubated for 0, 4, 24 and 48 hours. Following the incubation, the samples were vacuum filtered through filter flocks. The residue was washed with

distilled water. Flocks and residue were quantitatively brought into a Kjehldahl receiver and analyzed for total nitrogen.
Experiment 2: Grass samples were taken from two established pastures, which were sown with seed from the two contrasting populations, at 28 days of regrowth. The grass was cut fresh at 4 cm, and cut into pieces of 4 cm lengths using a paper cutter. Approximately 4 g of fresh grass were weighed into 140 ml bottles and incubated in triplicate with 81 ml of N-free medium, 5 ml of rumen fluid and 1 ml of reducing agent. Rumen fluid was obtained from three Holstein-Friesian bullocks offered a standard diet, consisting of 80% good quality hay and 20% concentrates. Samples were incubated in the solution at 39 °C for 0, 1, 2, 3, 4, 6, 8, 10, 12 and 24 hours. Two bottles at each time point containing no substrate were included as a blank. Following the incubation, samples (5 mL) were taken and 5 mL of trichloroacitic (TCA) was added. NH_3 was analyzed by the indophenol method (Searle, 1984).
Experiment 3: Four grass fed sheep were equipped with rumen fistulas. Two grazed the cystatin and two grazed the control population. An experimental period lasted for 3 days. The first day was used for adaptation. On day 2 and 3 in the experimental period rumen liquid samples were taken during the photoactive period, at 7:00, 9:00, 12:00, 15:00, 18:00, 20:00 h. and 5 ml of TCA was added. After three days, sheep changed to the other population. In a third period the sheep changed back to the initial population. The rumen liquid samples were analyzed for NH_3.
Statistical analysis were done on all experiments, using the repeated measures design, with time as the within subjects factor and the population (cystatin or control) as between subjects factor.

Results and discussion

Experiment 1: During the incubation period, protein concentration was already decreased rapidly (from 17.0 g kg^{-1} DM to 10.0 g kg^{-1} DM). Due to grinding of the samples (1mm), the soluble protein could easily be released. The residual protein was efficiently degradated within the first 4 hours of incubation in a buffer solution with an enzyme assay. After 4 hours of incubation no further degradation took place. No significant differences could be found between the two grass populations. It was concluded that natural breakdown inside the plant cells was not inhibited by the cystatin. But it might be that the protease inhibitor was inactivated by the drying process. Therefore, an experiment with fresh grass might give different results.
Experiment 2*:* Figure 1 shows the NH_3 concentration (mg L^{-1}) in the bottles in the period after incubation of the grass samples. The mean NH_3 concentration was lower (P<0.01) in the cystatin population than in the control population, especially at 8, 10 and 24 hours after incubation when the difference was largest. This could be related to the reduced protease activity due to the protease inhibitor, but could also be due to the DM content and CP concentration, which were lower in the cystatin compared to the control population. When the curves were expressed as ammonia concentration per mg N_{input}, no significant differences were found between the two populations. However, the NH_3 concentration at the end of the gas production run was lower (P<0.05) in the cystatin population. This might be related to a slightly higher WSC concentration in this population, but could also be an effect of the cystatin protease inhibitor.

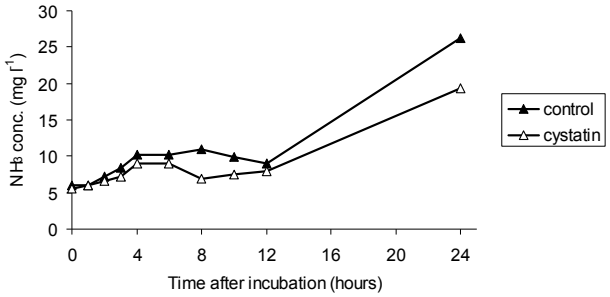

Figure 1. NH_3 concentration (mg L^{-1}) of the buffer solution with rumen liquids incubated either with control (closed symbols) or cystatin population (open symbols).

Experiment 3: Figure 2 shows the NH_3 concentration in the rumen liquid of four sheep grazing either the control or the cystatin population during the day. The NH_3 concentration in the rumen was at most time points lower (P<0.05) in the control than in the cystatin population, which was against expectation. It was only at the end of the day, at 20:00 h, when the NH_3 concentration was increasing that the NH_3 concentration of sheep grazing the cystatin population was higher than those grazing the control; however this was not significant (P=0.06). The animal variation was high and more animals should be used in further experiments.

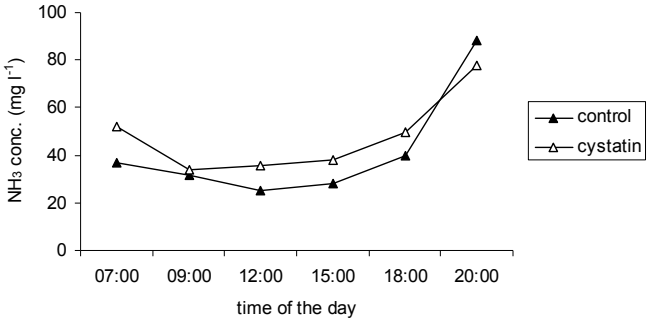

Figure 2. NH_3 concentration (mg L^{-1}) of rumen liquid samples of sheep grazing either the control (closed symbols) or the cystatin population (open symbols).

Conclusions

It is concluded that either the populations were not different, or the effect of the examined protease inhibitor (cystatin) on proteolysis of perennial ryegrass protein in the rumen is limited. In experiments with protease inhibitors, fresh material should be used, because the protease inhibitor could be deactivated by the drying process.

References

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