

January 1997

Biochemical Characterization of a Peptidase Enzyme from the Ruminal Bacterium *Prevotella ruminicola*

Humberto Madeira
University of Nebraska-Lincoln

Mark Morrison
University of Nebraska-Lincoln

Follow this and additional works at: <http://digitalcommons.unl.edu/animalscinbcr>



Part of the [Animal Sciences Commons](#)

Madeira, Humberto and Morrison, Mark, "Biochemical Characterization of a Peptidase Enzyme from the Ruminal Bacterium *Prevotella ruminicola*" (1997). *Nebraska Beef Cattle Reports*. 448.
<http://digitalcommons.unl.edu/animalscinbcr/448>

This Article is brought to you for free and open access by the Animal Science Department at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Nebraska Beef Cattle Reports by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Biochemical Characterization of a Peptidase Enzyme from the Ruminal Bacterium *Prevotella ruminicola*

Humberto Madeira
Mark Morrison¹

The characterization by molecular biology techniques of a bacterial enzyme involved with ruminal protein digestion brings new insights and strategies which seek to optimize protein nutrition of beef cattle.

Summary

*The dipeptidyl aminopeptidase activity of the rumen bacteria **Prevotella ruminicola** is localized in the cell periplasm/cytoplasm, possesses pH optimum of 7.5, is inhibited by cysteine protease inhibitors, and is calcium-dependant. The production of this enzyme is not affected by different nitrogen sources or stage of growth. These results provide relevant information on how this enzyme is affected by ruminal environment (pH) and on possible use and design of specific inhibitors. Additionally, the studies show how the techniques of molecular biology provide understanding of the structure function and expression of enzymes, and how this information is the first step in developing new approaches to optimize protein nutrition of beef cattle.*

Introduction

An important step in the ruminal degradation of feed proteins is the conversion of peptides, generated by the proteolytic activity of the microflora, into amino acids. *Prevotellaruminicola*, besides being a predominant proteolytic species, is also considered to be one of the most active bacteria involved with the degradation of peptides. Recent attention has been directed towards a dipeptidyl aminopeptidase enzyme characterized by its cleavage of the

diagnostic substrate Glycyl-Arginyl-4-methoxy- β -naphthylamide (Gly-Arg-MNA) to Gly-Arg, and free-MNA. This enzyme is thought to be the predominant peptidase in the rumen and we have shown that its inactivation could reduce ammonia production by as much as 25 percent (see article by Madeira, Peng, and Morrison, in this Beef Report). Although such findings offer the potential for productive alterations in protein nutrition of beef cattle, such potential is unlikely to be achieved unless the methods of controlling enzyme activity are highly selective, with minimal negative effects upon other enzymes, microorganisms, and the beef animal. For these reasons it is critical to understand the structure and function of this enzyme, and molecular biology techniques provide the tools necessary to obtain such knowledge. We describe here some of the knowledge we have obtained about this peptidase enzyme and explain how this knowledge provides new insights for our goal of improving protein nutrition.

Procedure

Effect of pH, rumen fluid, nitrogen sources, and stage of growth on peptidase activity

Overnight cultures of *P. ruminicola* strain B₁4 were grown on a defined medium (1995 Beef Report, p. 13). Peptidase activity was determined anaerobically by incubating cells with the diagnostic substrate Gly-Arg-MNA. Upon the action of the peptidase on the substrate, the fluorescent compound MNA is released and is quantified using a fluorescence spectrophotometer. Specific activity is expressed as nmols of MNA released/min/mg protein. To test the effect of pH on enzyme activity, cells were resuspended in buffers with

pH values of 6.0, 6.5, 7.0, 7.5, and 8.0 before peptidase assays. To test the effects of rumen fluid and nitrogen sources, cultures receiving either 5 percent (v/v) rumen fluid or no rumen fluid were incubated in the presence of two levels of ammonia (ammonium chloride, 1 mM and 10 mM), gelatin (porcine skin gelatin) and peptides (Trypticase) to a final concentration of 10 mM as nitrogen equivalents. Peptidase activity of cultures harvested at different stages of growth was also tested; the same nitrogen sources described above were used, with cultures harvested at either mid-log phase (5 h), or stationary phase of growth (14 h).

Effect of Inhibitors on Peptidase activity

Much can be learned about how a peptidase enzyme cleaves its substrate by first treating the enzyme with specific chemicals. If enzyme activity is lost, then the chemical is diagnostic for the presence of a certain structure, critical to protein digestion. Treatments comprised of additions of 1 mM of either the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), the cysteine protease inhibitors parahydroxy-mercuribenzoic acid (pCMB) and iodoacetate (IAA), or the metal-binding compounds ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol-bis-tetraacetic acid (EGTA) to the assay mixture. For the EDTA and EGTA treatments, the requirement for either Ca⁺⁺ or Mg⁺⁺ ions was assessed by adding either 5 mM calcium chloride or magnesium chloride to the assay buffer. The effect of reduced sulfhydryl groups was tested by the addition of the reducing agent dithiothreitol (DTT) at 5 mM. The effect of oxygen on enzyme activity was also assessed by harvesting the cells and conducting the enzyme assays aerobically.

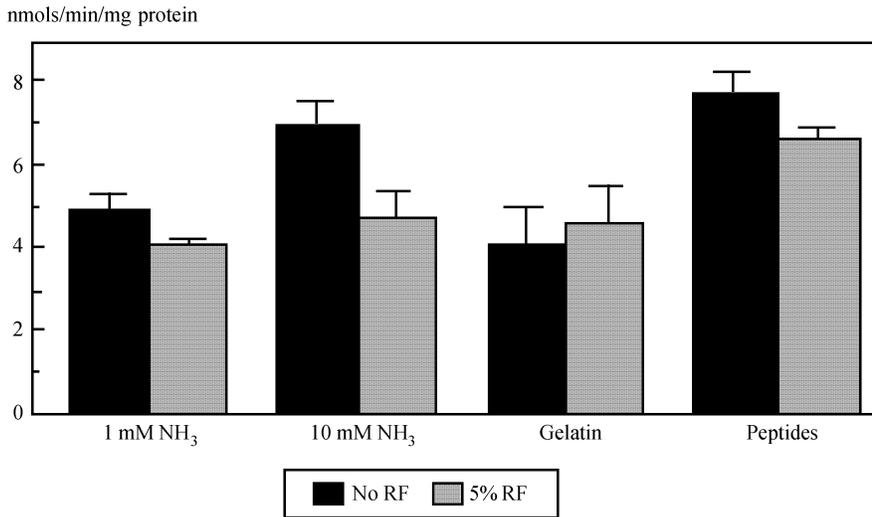


Figure 1. Effect of nitrogen sources and rumen fluid (RF) on peptidase activity of *P. rumenicola* (n=4, error bars represent standard deviations).

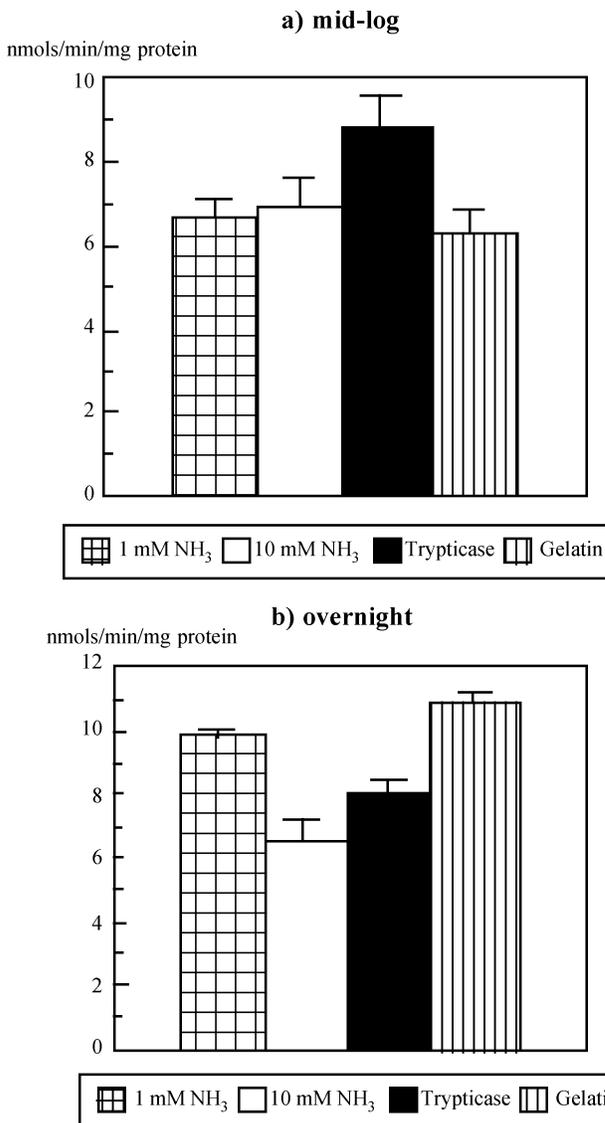


Figure 2. Effectiveness of stage of growth on peptidase activity of *P. rumenicola* cultures growing on different nitrogen sources (n=4, error bars represent standard deviations).

Localization of Peptidase Activity

Localization of enzyme activity - whether it is extracellular, membrane associated or intracellular - needs to be determined, so that inhibitory compounds "reach" their target. To determine the location of the peptidase enzyme, fifty ml cultures were harvested and resuspended in one-tenth of the original volume in phosphate buffer, pH 7.5. These resuspended cells were broken using a French pressure cell under a flux of nitrogen gas because oxygen was found to inhibit the enzyme (see Figure 3). Following cell disruption, a low spin centrifugation was applied to remove any unbroken cells, and the remaining suspension was then recentrifuged at 80,000 rpm for 30 min. The supernatant liquid contains any intracellular proteins while the pellet contains membrane-bound proteins. After resuspending the membrane proteins in phosphate buffer, the cell fractions were stored in liquid nitrogen (-80°C) until analyzed for peptidase activity using Gly-Arg-MNA as a substrate.

Results

Effect of pH, rumen fluid, nitrogen sources, and stage of growth on peptidase activity

Enzyme activity was maximal at pH 7.5 and therefore, all subsequent assays were conducted at this pH. Enzyme activity decreased markedly (~40%) at a pH of 6.0. *P. rumenicola* is recognized as being one of the more acid-tolerant ruminal bacteria. However, buffer pHs typical of those seen in animals receiving high-concentrate diets decreased Gly-Arg-MNase (peptidase) specific activity. Therefore, it seems possible that the peptidase activity may be affected by diet and(or) ruminal pH.

Addition of rumen fluid had little impact on peptidase activity (Figure 1), suggesting that there are no requirements for nutrients or co-factors that could be present in ruminal fluid. Similarly, enzyme activity did not appear to be modulated in response to nitrogen source (Figure 1) or stage of growth

(Continued on next page)

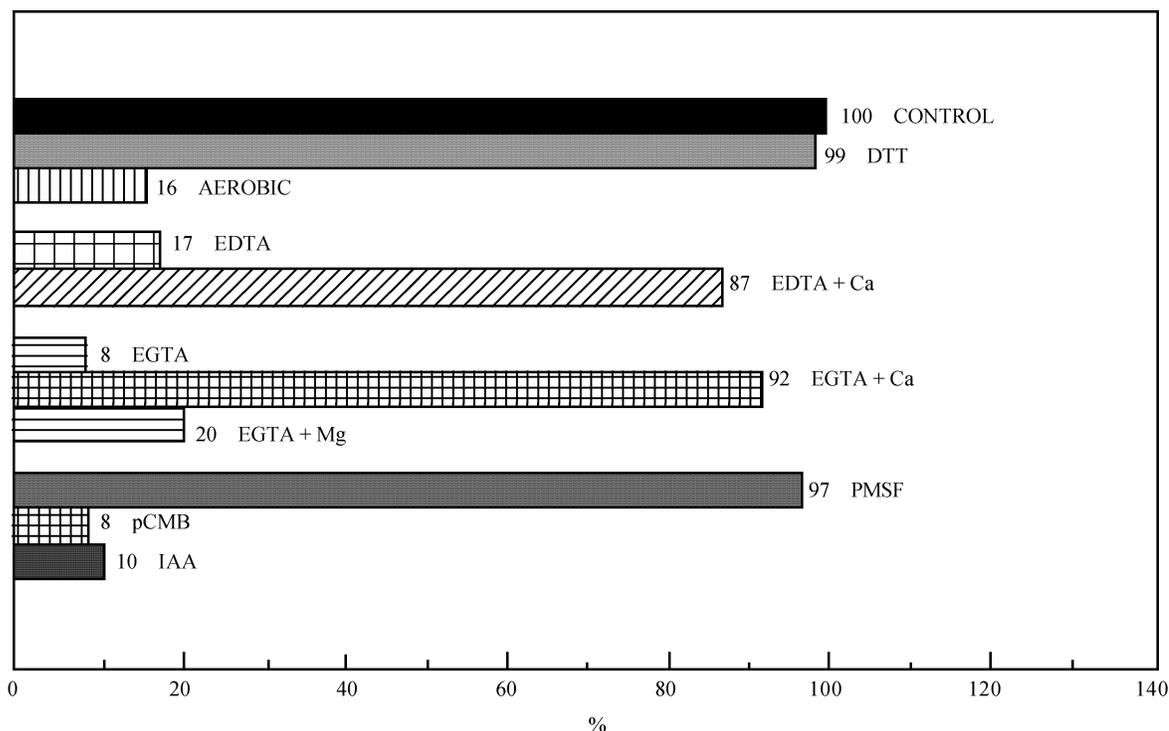


Figure 3. Effect of inhibitors on peptidase activity of *P. ruminicola*. Results are presented as percentage of control, and are average of two experiments, with four replicates per treatment.

(Figure 2), suggesting that this enzyme activity is always produced by *P. ruminicola*.

Effect of Inhibitors on Peptidase activity

Although the serine protease inhibitor, PMSF, as well as the reducing agent, DTT, had no effect on enzyme activity (Figure 3), cysteine protease inhibitors (pCMB and IAA) reduced activity drastically. The enzyme activity is also very sensitive to oxygen because enzyme activity was decreased by approximately 80 percent when the incubation was conducted aerobically. The metal-binding compounds EDTA and EGTA also caused a decrease in enzyme activity, which indicates the enzyme must contain divalent cations for maximal enzyme activity. The more pronounced effect of EGTA suggested a requirement for calcium ions, and this was confirmed by the addition of excess calcium ions (Figure 3), that reverted the inhibition caused by EGTA and EDTA. However, magnesium could not replace calcium in restoring enzyme

activity, suggesting that the divalent cation requirement for enzyme activity is quite specific for calcium. Cell-free assays conducted with the cytoplasmic/periplasmic fraction resulted in a similar inhibition profile (data not shown).

Localization of Peptidase Activity

More than 90 percent of the peptidase activity under investigation is present in the intracellular fraction. Little activity was found in the cell-free supernatant (3%), showing that it is not extracellular. These findings determine that an inhibitor specific for this enzyme will only be effective if it is capable of crossing the bacterial cell wall. Therefore, the design of any inhibitory compound must ensure it is compatible with those structures involved with the transport of nutrients into the bacterium.

Conclusions

The dipeptidyl aminopeptidase produced by *P. ruminicola*, which degrades the diagnostic substrate Gly-Arg-MNA

is produced in similar amounts irrespective of the nitrogen source used for growth, other nutritional factors, or stage of growth. Therefore, manipulation of the diet is unlikely to result in measurable changes in enzyme activity. Enzyme activity however is decreased by low pH, removal of calcium ions, and by chemicals which bind to a cysteine residue which is critical to the cleavage of the dietary protein. Because the enzyme appears to be located inside the bacterium, the peptides present in ruminal fluid must first be transported across the bacterial cell wall before they can be degraded to amino acids, ammonia, and VFA. These molecular details now provide at least two new strategies which may control ruminal ammonia production: inhibitors which irreversibly bind to the peptidase enzyme, or compounds which irreversibly bind to the bacterium's peptide transporter.

¹Humberto Madeira is a graduate student; and Mark Morrison is Assistant Professor, Animal Science.