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Manipulation of Microbial Protein Degradation in the Rumen: Development of the "Smugglin Concept" to Control Protein Digestion

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Manipulation of Microbial Protein Degradation in the Rumen: Development of the "Smugglin Concept" to Control Protein Digestion

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Summary

One approach that might slow down ruminal protein degradation is the "smugglin concept", which involves incorporation of growth-inhibitory compounds into larger molecules (peptides) that are normally transported by protein-degrading rumen microorganisms. The effects of peptides containing toxic amino acid analogs, as well as the polycationic peptide salmine, on the growth of ***Prevotella ruminicola*** *were assessed. Results obtained indicate that the smugglin concept may be applicable for the study and manipulation of peptide metabolism by P. ruminicola and, probably, other peptide-fermenting bacteria in the rumen. Such manipulation could be used to control protein digestion in the rumen.*

Introduction

It has been estimated that as much as 25% of the protein fed to grazing and forage-fed animals is wasted due to its rapid degradation by the rumen microorganisms. Therefore, from an economic and environmental perspective, the nitrogen cycle of intensive livestock systems could be managed more effectively. Compounds such as ionophores can reduce ruminal ammonia production, but their anti-bacterial effects are too broad for its widespread use with grazing and forage-fed livestock. Another strategy to manipulate the activities of rumen bacteria responsible for protein degradation is the "smugglin concept". The "smugglin concept" involves the selective inhibition of microorganisms by the incorporation of inhibitory compounds into the normally transported peptides. We report here the effects of several synthetic peptides, as well as the polycationic peptide salmine, on the growth of *Prevotella ruminicola,* an important proteolytic rumen bacterium.

Procedure

Determination of mode of action of salmine

We have previously reported that salmine is bacteriocidal to *P. ruminicola* (1995 Nebraska Beef Report, p. 13). The minimal inhibitory concentration (MIC) is between 10 and 15 μg ml^{-1}. Although salmine can interfere with DNA replication, polycationic peptides like salmine can also permeabilize the outer membrane of Gram negative bacteria such as *E. coli*. Therefore, it was necessary to assess whether the smugglin concept would be involved with the inhibition of *P. ruminicola*, by ruling out other modes of action such as permeabilization of the cell. Permeabilization of the outer membrane increases the sensitivity of Gram negative bacteria to hydrophobic antibiotics. *P. ruminicola* strains were tested for increased sensitivity to monensin and novobiocin, either in the presence or absence of a sub-MIC of salmine (~5 μg ml^{-1}). Cultures were incubated at 37°C in a defined, anaerobic medium containing ammonia and glucose as nitrogen and energy sources, respectively, for up to 48 hours, and growth was assessed by the final optical density (OD_{600}) of the cultures. Levels of novobiocin and monensin ranged from 0.1 to 40 μg/ml and 0.7 to 5 μg/ml, respectively.

To conclusively demonstrate that salmine disrupted the outer membrane of *P. ruminicola*, we tested for such an effect by measuring the release of alkaline phosphatase, a periplasmic enzyme, following treatment of mid-log phase cells with salmine. Cells grown to mid-log phase were harvested, washed and treated with either sucrose followed by cold water (osmotic shock; positive control) or salmine, and release of alkaline phosphatase into the menstruum was measured and compared with cell-free supernatant from washed cells (negative control).

Effects of ethionine- or oxalysine-peptides upon ***P. ruminicola***

Ethionine acts as an inhibitor of methyltransferases because of its structural similarity with methionine. Oxalysine appears to affect ribosomal RNA and probably, protein biosynthesis in a variety of prokaryote and eukaryote microorganisms. Pentapeptides containing either ethionine or oxalysine were kindly provided by Dr. L. Zhang and Dr. F. Naider, Department of Chemistry, CUNY, Staten Island, NY. These peptides were used in disk diffusion assays with *P. ruminicola* B_14 and D31d, grown on basal agar media, in the presence or absence of 0.2% (w/v) Trypticase (BBL). Sterile paper-filter disks were placed on the surface of the plates and saturated with the solutions of peptides. The bacterial strains were tested for susceptibility (indicated by the appearance of a zone of clearance around the disks) to increasing amounts of either triornithine, Tyr-Asp-Ala-Orn-Orn-Orn-Ala ($YDAO_3A$), Tyr-Asp-Chloroalanine-Asn-Ser-Chloroalanine-Ala (YDCNSCA), Oxalysine-Leu-Leu-Leu-Gly (OL_3G), Lys-Leu-Leu-Ala-Ethionine (KL_2AEth) or Lys-Leu-Leu-Leu-Ethionine (KL_3Eth).

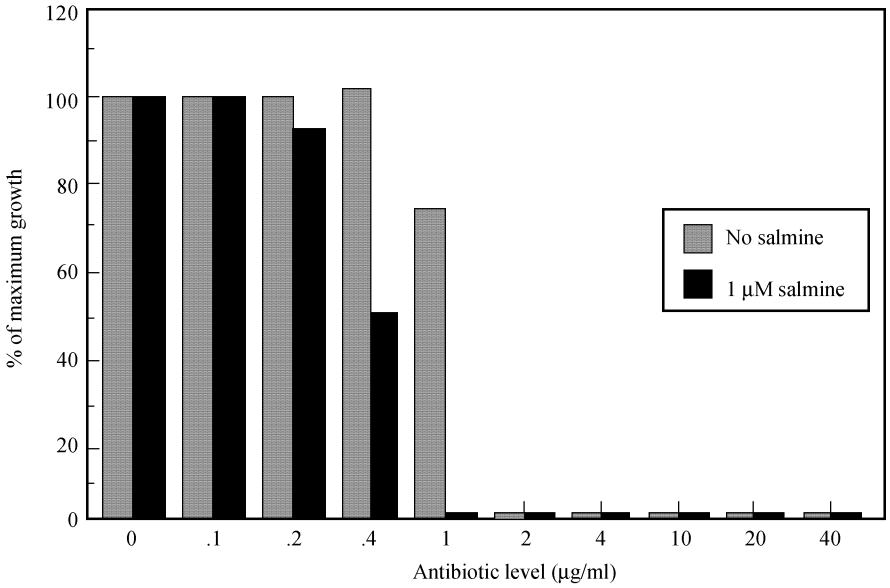

Figure 1. Effect of sub-MIC of salmine on the sensitivity of *P. ruminicola* to the hydrophobic antibiotic novobiocin.

Results

In the presence of a sub-MIC of salmine, there was a four-fold increase in the sensitivity of *P. ruminicola* to novobiocin (Figure 1), and a ten-fold increase in sensitivity to monensin (data not shown). Because the mode of action of these two antibiotics is very different, it seems likely that salmine is exerting its effect by making the membrane more accessible to these hydrophobic antibiotics. The release of alkaline phosphatase activity following treatment with salmine (Table 1) indicates that the outer membrane of *P. ruminicola* is sufficiently damaged by polycationic peptides to permit the release of periplasmic proteins and result in cell death.

Levels as high as 1.5 mg of tri-ornithine did not inhibit growth of either *P. ruminicola* strain, but 75 µg of $YDAO_3A$ was inhibitory to *P. ruminicola* B_14. The peptide YDCNSCA did not inhibit growth of *P. ruminicola* within the range tested (0-250 µg). OL_3G (50 µg) and KL_3Eth (100 µg) were inhibitory to strain B_14 growing on ammonia, causing zones of clearing of 1.8 cm (Figure 2). Inhibition by these last two oligopeptides was prevented by including peptone (0.2 % w/v) in the growth medium. We consider these findings evidence that the same transport system was being used for the uptake of both the synthetic and the peptone peptides. Moreover, the findings are consistent with earlier studies that showed that only oligopeptides ranging from four amino acid residues in length up to 2,000 Da are utilized for growth, whereas free amino acids and a range of di- and tri-peptides are either poorly or not used.

Table 1. Distribution of alkaline phosphatase activity in cell fractions of *P. ruminicola,* and total recovery of enzyme activity in treated cells as compared with control cultures.[1]

Fraction	% of total activity[1] Osmotic shock	Salmine (20 µM)
Sucrose supernatant	2.5 (±0.35)	——
Cold water - periplasmic fraction	53.0 (±5.65)	——
Supernatant after salmine treatment	——	14.2(±1.1)
Periplasm-less cells after osmotic shock	25.0(±4.9)	——
Periplasm-less cells after salmine treatment	——	67.0(±2.8)
% Recovery[2]	84	84

[1]Average of 2 experiments; ± SD.
[2]Relative to untreated cells harvested from the same culture used to provide cells for osmotic shock and salmine treatment.
Less than 3.5% of total alkaline phosphatase activity was present in cell-free culture fluid and wash fractions.

Conclusions

Results obtained with oxalysine- and ethionine-containing peptides indicate that the smugglin concept may be applicable for the study and manipulation of peptide metabolism by *P. ruminicola* and, probably, other peptide-fermenting bacteria in the rumen. Such manipulations are warranted to reduce nitrogen excretion in animal waste, as well as production costs. Previous studies have suggested that peptides that are hydrophobic in nature and/or possess proline residues are less susceptible to hydrolysis in the rumen. Salmine has a bacteriocidal effect on *P. ruminicola*, probably due to the polycationic nature of the protein disrupting the outer membrane of the bacterium. Although micromolar concentrations of polycationic peptides like salmine may not inhibit growth, the sensitivity of *P. ruminicola* to monensin could be dramatically increased. Considering that some cereal grains possess polycationic proteins, perhaps some of the variability in the ruminal digestion of grain proteins can be attributed to their deleterious effect on bacteria such as *P. ruminicola.* Therefore, unlike the other peptides tested, salmine does not appear to possess great potential for use as a smugglin agent, because of its action of disrupting the outer membrane of *P. ruminicola.* However, it may still prove useful in other studies of ruminal protein digestion.

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I. With Peptides

II. Without Peptides

Figure 2. The effect of chloroalanine, ethionone, or oxalysine-containing peptides on growth of *P. ruminicola* in the presence (I) or absence (II) of 0.2% (w/v) Trypticase. Disks are saturated with either anaerobic water (a); 10 mM acetic acid (b); 100 µg KL_3G (c); 50 µg oxalysine-L_3 (d); 100 µg KL_3-ethionine (e); 100 µg KL_2-A-ethionine (f); and 250 µg YD-chloroalanine-NS-Chloroalanine-A (g).