OPPORTUNITIES TO INCREASE THE CONTRIBUTION OF PEPTIDES TO METABOLIZABLE PROTEIN

Douglas B. Bates
Animal Science Department, IFAS,
University of Florida, Gainesville 32611

Introduction

As a consequence of the rumen fermentation, protein digested in the small intestine of the ruminant is comprised of microbial protein and undegraded dietary protein that escapes microbial digestion in the rumen (Broderick et al., 1991). Taken together, these two sources contribute to metabolizable protein that is absorbed by the ruminant and available to meet the ruminant's protein requirements. Protein requirements of young growing ruminants and productive cows often exceed the ruminal synthesis of microbial protein. Therefore, undegraded intake protein (UIP) or "escape" protein must compliment microbial protein in order for optimal animal performance to be realized (Orskov, 1982). Recognition of this requirement resulted in the development of Metabolizable Protein Systems in the United States (NRC, 1985), United Kingdom (ARC, 1984) and France (INRA, 1988). These systems distinguish between dietary protein that is degraded in the rumen and dietary protein that escapes ruminal degradation. Use of the Metabolizable Protein Systems proposed by the NRC (1989, 1996) to predict protein requirements of cattle often concludes that some dietary UIP requirement exists for high producing dairy cows and for the following scenarios relevant to cow-calf production in Florida: 1) managment of replacement heifers to achieve adequate rates of gain on pasture to breed at two years of age, and 2) management of pregnant cows in negative energy balance during late gestation.

Under conditions when the animal's need for UIP is increased, supplementation to increase UIP supplied by the diet is complicated by the extensive breakdown of protein within the rumen (Broderick et al., 1991). Ruminal degradation of dietary protein to ammonia can result in inefficient nitrogen utilization by the ruminant because ammonia often is produced in excess of microbial requirements (Leng and Nolan, 1982). Peptides that arise from the action of rumen microbial proteinase may be transported into microbial cells or further hydrolyzed to amino acids. Accumulation of peptides in rumen fluid indicate that these processes may be rate limiting *in vivo* (Chen et al., 1987a); peptides can accumulate in rumen contents to concentrations exceeding 20 mg dl^{-1} (Annison, 1956; Broderick and Wallace, 1988; Broderick and Craig, 1989; Chen et al., 1987b). A number of authors have recognized the importance of ruminal peptide metabolism in determining overall degradation of a dietary protein and the contribution of peptides to meeting requirements of both ruminal microbes and host animal (Broderick and Craig, 1989; Chen et al., 1987a,b; Williams and Cockburn, 1991). Transient accumulation of peptides in the rumen of cattle and sheep fed different protein supplements has been reported (Annison, 1956; Broderick and Wallace, 1988; Broderick and Craig, 1989; Chen et al.; 1987b;

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Williams and Cockburn, 1991), and peptides that arise from the partial hydrolysis of dietary protein can make an appreciable contribution to metabolizable protein reaching the small intestine of the host animal. Chen et al. (1987b) measured the flow of peptides from the rumen of cattle supplemented with soybean meal and estimated that peptides leaving the rumen could account for more than 25% of the milk protein synthesized by lactating dairy cows. Inhibition of peptide hydrolysis may be one strategy to enhance the quantity and/or quality of amino acids escaping deamination in the rumen and, thus, available to meet the host ruminant's protein requirements (Morrison and Mackie, 1996).

Breakdown of Protein in the Rumen

Dietary protein ingested by ruminants is degraded to varying degrees in the rumen to ammonia and volatile fatty acids. This process represents the sum of a large number of microbial activities including protein hydrolysis, peptide degradation, amino acid deamination, and fermentation of resulting carbon skeletons (Cotta and Hespell, 1986). Ruminal digestion of protein to peptides and amino acids is carried out by microbial proteinases and peptidases (Cotta and Hespell, 1986), and the rate at which protein and peptides are hydrolyzed usually determines the extent to which protein degrades before passing out of the rumen (Lewis and Emory, 1962; Russell et al., 1983; Chen et al., 1987a).

The first step of proteolysis in the rumen involves extracellular or cell associated endoproteinases that degrade protein into large oligopeptides (Wallace and Cotta, 1988). Both ruminal bacteria and protozoa produce proteolytic enzymes (Tamminga, 1979); however, the predominant proteolytic organisms in the rumen are bacteria (Brock et al., 1982; Wallace et al., 1987). Proteolytic bacteria comprise between 12 and 38% of the total bacterial population in the rumen (Bryant and Burkey, 1953; Fulghum and Moore, 1963). Although the nature of the proteolytic population varies with diet (Wallace and McPherson, 1987), predominant proteolytic ruminal bacteria include *Prevotella ruminicola*, *Ruminobacter amylophilus*, *Butyrivibrio fibrisolvens*, *Selenomonas ruminantium*, *Eubacterium ruminantium*, *Lachnospira multiparus* and *Streptococcus bovis* (Wallace and Brammel, 1985). On the basis of cellular location, activity against various substrates, and sensitivity to inhibitors, Wallace and Brammel (1985) concluded that the proteolytic activity of *P. ruminicola* was most similar to the activity of rumen contents. Recently, Avgustin et al. (1997) proposed the redefinition of *P. ruminicola* based upon phenotypic diversity among ruminal isolates of this species. Strains related to *P. ruminicola* subspecies brevis type strain GA33 were assigned to a new species, *P. brevis*, whereas strains related to type strain B14 were assigned to *P. bryantii*. Most of the isolates formerly classified as *P. ruminicola* subspecies ruminicola were placed in a redefined species called *P. ruminocola*. A small group of isolates with G:C ratios lower than the other species were reclassified as *P. albensis*. Proteinase activities varied widely among the new species with *P. brevis* exhibiting the highest proteolytic activity. Other microbial species, however, play important roles in ruminal proteolysis because they act cooperatively to degrade protein (Schwingel and Bates, 1996; Wallace, 1985). For instance, synergistic interaction between *S. bovis* and *S. ruminantium* enabled rapid growth of a co-culture of the two organisms on a

medium containing casein as the sole nitrogen source, even though neither species was capable of growth on this medium as a pure culture (Wallace, 1985).

The principal type of endoproteinase activity found in rumen contents is a cysteine - protease type which is sensitive to ρ - chloromercurobenzoate (Attwood and Reilly, 1996). Other types of protease activities are present, but more variable (Brock et al., 1982; Kopecny and Wallace, 1982). These include serine - protease activity (sensitive to inhibition by phenylmethylsulphonyl fluoride), metalo.-protease (sensitive to EDTA and 1, 10 - phenanthroline) and aspartic-protease (sensitive to pepstatin-A; Wallace and Cotta, 1988).

Oligopeptides produced by the action of ruminal proteinases are broken down in turn to smaller peptides and amino acids. Peptide hydrolysis in the rumen follows a distinctive pattern characteristic of dipeptidyl aminopeptidease activity (Wallace and McKain, 1989; Wallace et al., 1990a,b). Although ciliate protozoa have more activity against dipeptides (Wallace et al., 1990a), the ruminal hydrolytic activity most active against oligopeptides is bacterial in origin. The hydrolysis of alanine containing peptides of different lengths (Broderick et al., 1988; Wallace and McKain, 1991; Wallace et al., 1990a, b) and arylamide substrates (Wallace and McKain, 1991) suggested that the principal bacterial peptidase activity in the rumen of sheep and cattle is dipeptidyl aminopeptidase activity type I (DAP-I). Peptidase activities of pure cultures (Wallace and McKain, 1991) and selective isolates from rumen fluid (McKain et al., 1992) indicated that the only predominant ruminal bacteria to possess DAP-I activity is *P. ruminicola*. Wallace et al. (1993b) reported that the hydrolysis of high molecular weight oligopeptides followed a similar pattern when *P. ruminicola* was compared to rumen fluid. All strains of the redefined *P. ruminicola* produce DAP-I, but the relative activities against different peptide substrates is species specific (Avgustin et al., 1997). Other species of ruminal bacteria occupy different niches in the microbial ecosystem responsible for peptide breakdown in the rumen; ie., *Streptococcus bovis* is the only rumen bacterial species with appreciable leucine aminopeptidase activity (Wallace and McKain, 1991).

Schwingel and Bates (1996) studied the degradation of soluble soybean meal proteins by *P. brevis* GA33 and mixed ruminal microorganisms *in vitro* using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This technique facilitated the quantification of peptides (molecular weight ≥ 14 kDa) released during the partial hydrolysis of soybean proteins. Several peptides derived from digested soybean proteins appeared during incubations with these microorganisms; peptides with molecular weights of 23, 28, 59 and 64 kDa accumulated in 48 hr incubations of *P. brevis*, whereas the accumulation of the 23 and 29 kDa peptides was only transient (appearing at 4 and 2 hr incubation, respectively) in incubations of mixed ruminal bacteria. The early stages of protein degradation by mixed ruminal microbes were similar to those seen in the degradation of soybean protein by pure cultures of *P. brevis* GA33; however, interactions between *P. brevis* and other microorganisms appeared to be responsible for the rapid and complete degradation of soybean proteins observed with cultures of mixed ruminal microorganisms. N-terminal amino acid sequencing and amino acid analyses were

conducted on peptides accumulating in the cultures with *P. brevis* to establish the origin of the peptides, and to provide information about peptides that resisted rapid proteolytic digestion. A peptide of 23 kDa was of particular interest because it could be easily isolated as a pure peptide, and its N-terminus was identical to the N-terminus of the β subunit of β-conglycinin. The N-terminus of this peptide was NH_2-leucine-lysine-valine-arginine-glutamine-aspartic acid-glutamine-asparagine-proline-phenylalanine-tyrosine-leucine-arginine-serine. Wallace and McKain (1991) reported that peptides with N-termini containing leucine were resistant to peptidolytic attack by *P. ruminicola* M384. In addition, The N-termini of the 23-, 59- and 64- kDa products were enriched with basic amino acids. The authors proposed that this characteristic may be related to the resistance of these peptides to degradation by *P. brevis*.

Strategies to Limit Peptide Degradation in the Rumen

A number of authors have recently identified aspects of ruminal peptide metabolism that may be exploited to increase the contribution of peptides to metabolizable protein. Suggested strategies to limit ruminal peptide degradation have focussed on the 1) effect of ionophores on ruminal peptide metabolism (Yang and Russell, 1993), 2) blocking the N-termini of peptides thus taking advantage of low ruminal carboxypeptidase activity (Wallace, 1992), 3) relative resistance of hydrophobic peptides to ruminal peptidases (Wallace et al., 1993a; Wallace, 1996), 4) use of 1, 10-phenanthroline to inhibit the breakdown of dipeptides produced through the action of DAP-I (Wallace et al., 1996), and 5) reducing the involvement of *Prevotella* species in the ecology of the rumen (Madeira and Morrison, 1997).

The effect of ionophores on ruminal peptide metabolism is not well understood (Wallace, 1996). Although Wessels et al. (1996) did not observe an effect of lasalocid on ruminal peptide concentrations, Whetstone et al. (1981) reported an increase in the concentration of peptides in a rumen-simulating continuous culture upon prolonged exposure to ionophores. Similar results were reported with sheep rumen *in vivo* (Wallace et al., 1990c). Adaptation of the rumen microbial ecosystem is required because acute addition of ionophores to ruminal fluid *in vitro* had no effect on the rate of peptide breakdown (Wallace et al., 1990c). Chen and Russell (1991) concluded that the principal effect of ionophores on "N sparing" in the rumen is inhibition of gram-positive, amino acid fermenting bacteria from the genera *Peptostreptococcus* and *Clostridium*; however, use of an rRNA approach to assess the role of these bacteria indicated that monensin can not completely counteract the activity of these microorganisms (Krause and Russell, 1996).

An implication of the low carboxypeptidase activity in the rumen is that peptides can be blocked effectively from degradation by compounds which react with their N-termini; i.e., blocking the N-terminal of the peptide Ala_3 reduced its rate of degradation by 89% (Wallace and McKain, 1989). This approach represents a second strategy to limit peptide degradation in the rumen. Although the effectiveness of N-terminal protection of peptides would be expected to decrease as the length of the peptide increases, hydrolysis of oligopeptides derived from a trypsin digestion of casein (Trypticase) also was inhibited by

N-terminal modifications (Wallace et al., 1993). Sheep fed an N-acetylated peptide mixture showed no signs of increasing ruminal carboxypeptidase activity over time (Wallace, 1996).

The resistance of a peptide to hydrolysis also may be affected by its primary structure (amino acid sequence) as well as secondary and tertiary structures (folding, solubility and disulfide bridges; Broderick et al., 1991). Hydrophobic peptides are more resistant to degradation than hydrophilic ones (Chen et al., 1987c; Depardon et al., 1996). When Chen et al. (1987c) fractionated Trypticase with 90% (wt/vol) isopropyl alcohol, mixed ruminal bacteria hydrolyzed the alcohol-soluble fraction (which contained a high concentration of proline) at a much slower rate than the alcohol-insoluble peptides. Also, peptides from Trypticase and gelatin hydrolyzate which resisted 96 hr incubation in rumen fluid had an abundance of proline. The experiments of Yang and Russell (1992) indicated that lysine and methionine dipeptides containing proline were degraded slowly by mixed ruminal bacteria.

A fourth strategy employs a chelator of divalent metal cations (1, 10-phenanthroline) which inhibits ruminal dipeptidase activity, thus decreasing degradation of dipeptides created through the action of DAP-I (Wallace et al., 1995; Wallace et al., 1996). The main rumen bacterial species with high dipeptidase activity (*Fibrobacter succinogenes*, *P. ruminicola*, *L. multiparus*, and *M. elsdinii*) are affected. This inhibitor dramatically decreased production of ammonia during an incubation of a pancreatic digest of casein with rumen microbes.

A fifth strategy involves methods to decrease the concentration of *Prevotella* species in the rumen and their associated effects on ruminal peptide breakdown. The "smugglin concept" has been proposed as one means to selectively lower *Prevotella* numbers in the rumen (Morrison and Mackie, 1996). This approach utilizes the lack of specificity shown by most bacterial peptide permeases to promote cellular uptake of an amino acid mimetic: a toxic compound which is transported by peptide permeases, but not by more specific amino acid permeases (Ringrose, 1980). While most ruminal bacteria transport amino acids more selectively than peptides, species of *Prevotella* transport peptides in preference to amino acids (Pittman et al., 1967; Ling and Armstead, 1995). Protamine, a polycationic, low molecular weight protein, has a bacteriocidal effect against strains B14 (reclassified as *P. bryantii*) and D31d (reclassified as *P. ruminicola*), but the authors concluded that the effects of this compound were too broad to permit its successful use in manipulating ruminal peptidolysis (Madeira and Morrison, 1997). Species of *Prevotella* are some of the most abundant ruminal microorganisms and carry out many important functions besides protein digestion. Broderick et al. (1991) concluded that elimination of *P. ruminicola* almost certainly would be counterproductive to overall rumen function.

Summary

Recent research indicates that peptides may make an important contribution to

metabolizable protein. As our understanding of ruminal proteolysis increases, there will be opportunities to increase the contribution of peptides, either synthetic in nature or derived from the partial hydrolysis of feed proteins, to protein that is absorbed by the ruminant. Strategies that currently are being researched include design of specific peptides that are resistant to microbial peptidase activities, and alterations in the microbial population responsible for peptide breakdown.

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