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# The proteolytic systems of ruminal microorganisms

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Protein breakdown in the rumen is generally regarded as detrimental to the efficiency of ruminant nutrition, certainly for animals on a relatively high plane of nutrition. Peptides and amino acids arising from proteolysis are potential nutrients for the growth of rumen microorganisms, but they are also liable to be degraded to ammonia and lost from the rumen. Proteolytic activity occurs in all three main categories of rumen microorganisms. Bacteria are mainly responsible for dietary protein breakdown, while ciliate protozoa break down particulate feed protein of appropriate size and also bacterial protein. The key features of rumen proteolytic activity are that it varies greatly from animal to animal and from feed to feed. The predominant mechanism of peptide degradation is biphasic, via dipeptidyl aminopeptidases which cleave dipeptides from larger peptides followed by dipeptidase. Dipeptidyl aminopeptidase activity occurs only in *Prevotella ruminicola* among the common rumen microbial species. In contrast, dipeptidase, which cleaves the dipeptide products from dipeptidyl aminopeptidase, is present in many species, including *P. ruminicola*, and is particularly high in rumen protozoa. Deamination of amino acids is carried out by a combination of numerous low-activity bacteria and protozoa and a much smaller number of high-activity species. Most ammonia production is probably carried out by the low-activity species, which again include *P. ruminicola*, but proliferation of the high-activity species may be a problem on certain diets.

The microbiology of protein breakdown in the rumen is of interest because it deals with one of the major inefficiencies of ruminant nutrition, namely the too-rapid conversion of protein to ammonia in the rumen and the subsequent loss of that ammonia by absorption across the rumen wall and excretion as urea (Leng and Nolan, 1984; Wallace and Cotta, 1988; Wallace, 1994). This review describes the microorganisms involved at different stages of the breakdown process and assesses the relative importance of different species in the light of population densities and the properties of the mixed rumen population.

## Proteolytic ruminal microorganisms

The mixed rumen microbial population has a proteolytic activity that is only moderate compared with other proteolytic micro-organisms and the host's own gastric and pancreatic secretions, but the length of time that feed material is retained in the rumen means that this activity is able to break down a substantial proportion of most dietary proteins (Ørskov and McDonald, 1979; Broderick et al, 1991). Many strains and species of rumen ciliate protozoa, bacteria and anaerobic fungi have been found to be proteolytic, and they contain a variety of different types of proteolytic enzymes (table I) (Wallace and Cotta, 1988; Wallace, 1994).

The predominant species of proteolytic bacterium found in the rumen of most animals is *Prevotella* (formerly *Bacteroides*) *ruminicola*, which has been identified as proteolytic in many studies (Blackburn and Hobson, 1962; Fulghum and Moore, 1963; Hazlewood and Nugent, 1978; Wallace and Brammall, 1985) and which can comprise more than 60% of the flora under some circumstances (Van Gylswyk, 1990). Its cell-associated, mainly cysteine protease activity, is fairly typical of the rumen bacterial population as a whole. Some animals possess *Butyrivibrio fibrisolvens* as the most prevalent proteolytic isolate (Blackburn and Hobson, 1962; Fulghum and Moore, 1963; Hazlewood et al, 1983; Wallace and Brammall 1985). *B. fibrisolvens*, which has a higher specific activity than most other species (Wallace and Brammall, 1985), appeared to be present when the dietary protein was more resistant to degradation (Wallace et al, 1987). Its extracellular serine protease activity

**Table I .** Properties of microbial proteases.

| Microorganism | Location of enzyme | Active site category | Enzyme type | References |
|---|---|---|---|---|
| Mixed ciliate protozoa | Intracellular, presumably in lyososome-like vesicles. (exc. extracellular) | Cysteine and aspartic proteases | Mixed, significant trypsin-like activity and high leucine aminopeptidase | Shinchi et al, 1986<br>Williams et al, 1961 |
| *Entodinium caudatum* | Intracellular | Cysteine and aspartic proteases | Trypsin-like, pH optimum 3,2 or 6.5-7.0 | Coleman, 1983<br>Williams et al, 1961 |
| *Entodinium simplex* | do. | Cysteine protease | Trypsin-like, pH optimum 4.5 | Lockwood et al, 1988 |
| *Ophryoscolex caudatus* | do. | Cysteine and metalloprotease | Trypsin and chymotrypsin-like, pH optimum 4.5 | Lockwood et al, 1988 |
| *Polyplastron multivesiculatum* | do. | Cysteine and lower metalloprotease | Trypsin and chymotrypsin-like specificities, pH optimum 4.5 | Lockwood et al, 1988 |
| *Epidinium caudatum ecaudatum* | do. | Mainly cysteine protease | No clear pattern | Lockwood et al, 1988 |
| *Isotricha* spp. | do. | Cysteine protease | Trypsin- and chymotrypsin-like specificities | Lockwood et al, 1988 |
| *Dasytricha ruminantium* | do. | Cysteine protease | Trypsin-like, pH optimum 4.5 | Lockwood et al, 1988 |
| Mixed bacteria | Loosely associated with cell envelope | Cysteine protease mainly | Mixture of specificity | Brock et al, 1982<br>Prins et al, 1983 |
| *Prevotella (Bacteroides) ruminicola* | Cell-associated and extracellular | Cysteine and aspartic proteases mainly | Mixed | Hazlewood and Edwards, 1981<br>Wallace and Brammall, 1985 |
| *Ruminobacter amylophilus* | Mainly cell-associated | Serine protease | Trypsin-like | Blackburn, 1968<br>Wallace and Brammall, 1985 |
| *Butyrivibrio fibrisolvens* | Extracellular | Serine protease | Not trypsin- or chymotrypsin-like | Cotta and Hespell, 1986<br>Strydom et al,1986<br>Wallace and Brammall, 1985 |
| *Streptococcus bovis* | Cell-associated | Mainly serine protease | Leucine aminopeptidase | Russell et al, 1981<br>Wallace and Brammall, 1985 |
| *Neocallimastix frontalis* | Extracellular | Metalloprotease | Endopeptidase, trypsin-like | Wallace and Joblin, 1985 |

(Wallace and Brammall, 1985; Cotta and Hespell, 1986; Strydom et al, 1986) is not typical of in vivo activity.

Rumen ciliate protozoa exhibit a variety of protease activities, the most important of which are cysteine and aspartic proteases (Forsberg et al, 1984) that have a mixture of specificities with a significant trypsin-like activity (Prins et al, 1983; Forsberg et al, 1984). An *Isotricha* sp. had a protease profile on gel electrophoresis that was quite different to the profile of *Dasytricha ruminantium,* and representatives of four different entodiniomorphid genera also had different protease patterns (Lockwood et al, 1988). Extracellular extracts of rumen ciliates were reported to have a higher activity than intracellular extracts (Shinchi et al, 1986), but although these enzymes have been characterised biochemically (Shinchi and Kandatsu, 1983; Shinchi et al, 1986), their relevance to the mixed ecosystem has not been established.

Protozoa do not hydrolyse soluble protein as readily as do the bacteria. In the studies of Ushida et al (1986, 1991), defaunation resulted in a higher activity of rumen fluid towards soluble protein, which was believed to be caused by increased numbers of more active bacteria. A similar trend was seen with sheep which had been ciliate-free from birth (Wallace et al, 1987). However, protozoa ingest protein particles, either in the form of feed particles or bacteria, and they may be of great significance in diets containing particles of the correct dimensions.

Reports of proteolytic activity associated with rumen anaerobic fungi are conflicting. Whereas *Neocallimastix frontalis* had a high specific activity metalloprotease (Wallace and Joblin, 1985), most other fungal isolates had little proteolytic activity, although aminopeptidase was present in all species (Michel et al, 1993). Experiments with gnotobiotic lambs indicate that fungi play a minor role in proteolysis in vivo (Bonnemoy et al, 1993).

Proteolytic activity and the microbial species responsible for that activity are heavily diet-dependent (Nugent and Mangan, 1981; Siddons and Paradine, 1981; Hazlewood et al, 1983). However, perhaps the most remarkable feature of proteolysis is its inherent variability : different animals on the same or similar diets and housed together had completely different patterns of proteolytic enzymes in polyacrylamide gels (Wallace and Cotta, 1988). Rational manipulation of ruminal proteolysis by altering the proteolytic population therefore appears only a distant possibility.

## Peptidolytic rumen microorganisms

Peptide breakdown to amino acids must occur for the amino acids to be incorporated into microbial protein, and when there is sufficient energy available to fuel biosynthesis, amino acids will be incorporated and peptide breakdown would not be considered to be a major inefficiency in fermentation. However, when energy is unavailable, or when the rate of peptide breakdown exceeds the rate at which it can be assimilated, peptide catabolism leads to excessive ammonia production and poor N retention.

The great majority of peptidase activity in rumen fluid is aminopeptidase (Wallace et al, 1990a). It is characterised by dipeptides rather than single amino acids being cleaved from the peptide chain (table II) (Wallace et al, 1990a, 1993). Enzymes of this nature are classified as dipeptidyl aminopeptidases (Webb, 1992). The main mechanism of hydrolysis in intact rumen microorganisms appeared, from the hydrolysis of diagnostic synthetic substrates, to be dipeptidyl aminopeptidase type I (DAP-1) (Wallace and McKain, 1989), although other activities were also apparent. This pattern differs from that obtained with sonicated bacteria, which indicated among others a strong X-Ala-p-nitroanilide arylamidase (Ala-DAP) activity (table II). The dipeptides released as a result of DAP activity are then broken down by separate dipeptidase activity. Ruminal peptide breakdown is therefore a two-stage process.

It was established by comparing the specific activities of mixed rumen protozoa and mixed bacteria prepared from rumen fluid that bacteria were mainly responsible for the breakdown of larger peptides (Newbold et al, 1989; Wallace et al, 1990c). When pure cultures of the most common rumen bacteria were screened for dipeptidyl aminopeptidase activity, remarkably the only common bacterial species that possessed DAP-1 and had high Ala-DAP activity was *P. ruminicola* (table III) (Wallace and McKain, 1991). Selective isolation, using fluorogenic DAP-1 substrate confirmed this unusual finding. *P. ruminicola*,

**Table II.** Aminopeptidase activities of sonicated rumen bacteria[1].

| Substrate | Rate of *p*-nitroaniline release (μmol $ml^{-1}$ $h^{-1}$) |
|---|---|
| Ala2-pNA | 0.85 |
| ValAla-pNA | 0.50 |
| GlyPro-pNA | 0.38 |
| GlyArg-pNA | 0.13 |
| Pro-pNA | 0.12 |
| Lys-pNA | 0.02 |
| Ala-, Arg-, Asp-, Gly-, Leu-, Met-, Tyr-, Val-pNA | < 0.01 |

[1]Author's unpublished results, expressed per ml of original rumen fluid.

**Table III.** Dipeptidylaminopeptidase and dipeptidase activities in rumen microorganisms.

| | Rate of peptide hydrolysis by whole cells (nmol $min^{-1}$ mg $protein^{-1}$) | | |
|---|---|---|---|
| Species | GlyArg- MNA[1] | Ala2-pNA[2] | Ala2[3] |
| Mixed rumen microorganisms | 5.0 | 1.9 | 0.7 - 2.8 |
| Mixed protozoa | ND | ND | 7.5 |
| *Dasytricha ruminantium* | ND | ND | 35.7 |
| *Entodinium* spp. | ND | ND | 46.4 |
| *Isotricha* spp. | ND | ND | 15.1 |
| Mixed bacteria | ND | 3.9 | 2.8 |
| *Anaerovibrio lipolytica* | 0 | 0 | 0 |
| *Butyrivibrio fibrisolvens* | 0 | 3.7 | 1.6 |
| *Eubacterium ruminantium* | 0.1 | 0.7 | 1.3 |
| *Fibrobacter succinogenes* | 0 | 0.3 | 12.7 |
| *Lachnospira multipara* | 0 | 1.1 | 3.9 |
| *Megasphaera elsdenii* | 0 | 0 | 16.5 |
| *Mitsuokella multiacidus* | 0 | 0 | 2.2 |
| *Peptostreptococcus anaerobius* | 0 | ND | ND |
| *Prevotella ruminicola* | 2.5 - 36.2 | 11.3 - 31.5 | 3.2 - 25.6 |
| *Ruminobacter amylophilus* | 0.3 | 0 | 3.9 |
| *Ruminococcus albus* | 0 | 0 | 0.1 |
| *Ruminococcus flavefaciens* | 0 | 0 | 2.2 |
| *Selenomonas ruminantium* | 0 | ND | 4.2 |
| *Streptococcus bovis* | 0 | 4.1 | 2.5 |
| *Veillonella parvula* | 0 | 0 | 0 |

ND: Not determined; [1]From Wallace and McKain (1989, 1991) and McKain et al (1992). MNA-4-methoxynaphthylamide; [2]Wallace and McKain (1991) and author's unpublished results; [3] From Newbold et al (1989, 1990), Wallace and McKain (1991), Wallace et al (1990b), Wallace et al (1993), Wallace et al (1995) and author's unpublished results.

like the mixed rumen population, had low aminopeptidase activity against amino acyl-p-nitroanilide substrates but high activity against dipeptidyl-p-nitroanilide substrates; it also cleaved dipeptides from longer peptides as the first step in peptide hydrolysis (Wallace et al, 1993). Once again, the pattern of peptide breakdown would be expected to change with diet and its influence on numbers of *P. ruminicola*. If organisms such as *S. bovis* were to prevail, their leucine aminopeptidase activity would predominate and amino acids might be cleaved singly from the peptide chain rather in pairs (Russell and Robinson, 1984; Wallace and Brammall, 1985). However, in the sheep that have been examined at the Rowett Research Institute, there is no doubt that, in total contrast to proteolysis being carried out by a large and variable number of species, oligopeptides are cleaved predominantly by only one bacterial species, namely *P. ruminicola*.

In contrast to the limited occurrence of dipeptidyl aminopeptidase, many species of protozoa and bacteria have dipeptidase activity (table III). Among the bacteria, *P. ruminicola* had activity against a wide range of dipeptides, while *M. elsdenii* also had a high activity (Wallace and McKain, 1991). The *P. ruminicola* dipeptidase is a Mn-metalloprotease (Wallace et al, 1995).

## Rumen microorganisms forming ammonia from amino acids

Many experiments were done in the 1950s, 60s and 70s to determine the metabolism and fate of amino acids in mixed rumen contents (Blackburn, 1965; Allison, 1970; Chalupa, 1976; Broderick and Balthrop, 1979). As well as being nutritionally wasteful, the products of amino acid breakdown may be toxic to the animal (Carlson et al, 1972; Onodera, 1993). Most recently the main issue has been the nature of the microbial population that is primarily responsible for ammonia production in vivo. For many years, it had been assumed that deamination was carried out by a large number of the main species of rumen bacteria that had been identified to produce ammonia from protein or protein hydrolysates (Bladen et al, 1961).

However, Russell and his colleagues at Cornell (Chen and Russell, 1988, 1989; Russell et al, 1988, 1991) calculated that these bacteria did not have sufficient activity to account for observed rates of ammonia production by the mixed population in their cattle, and isolated a group of bacteria that were much less numerous than the others, but which possessed a specific activity of ammonia production which was an order of magnitude greater than that of the other species. Moreover, these bacteria, unlike the others, were highly sensitive to monensin, and since ammonia concentrations are lower when ruminants receive this dietary ionophore, it was deduced that they must be significant ammonia producers in vivo. The species isolated, *Peptostreptococcus anaerobius, Clostridium sticklandii* and *Clostridium aminophilum* (Paster et al, 1993), were atypical of the main ruminal species, although a large number of clostridia have been isolated from the rumen over the years (Stewart and Bryant, 1988). These bacteria, and also the mimosine degrader, *Synergistes jonesii* (Allison et al, 1992; McSweeney et al, 1993), did not ferment sugars but used amino acids as their main source of carbon and energy as well as as a nitrogen source. The dichotomy is, therefore, that amino acid deamination could be carried out predominantly numerically abundant bacteria each having low activity, or by relatively few species each with exceptionally high deaminative activity (Figure 1).

Until now, there have been no reports of high-activity bacteria similar to those isolated at Cornell having been isolated elsewhere. Recently, however, cattle and sheep at the Rowett Research Institute were sampled and the numbers of monensin-sensitive Trypticase degraders enumerated (Eschenlauer, 1994). Their viable count indicated that bacteria capable of growth on Trypticase alone were present at 0.7% of the total bacterial population, numbers similar to those reported by Yang and Russell (1993), and like the Cornell bacteria they were monensin-sensitive. However, most of these bacteria, unlike the Cornell isolates, fermented sugars. Furthermore, ammonia production in the rumen fluid from which they were derived was much lower than in the Cornell studies and therefore it was not necessary to invoke the intervention of high-activity bacteria. This may be true in other studies too where the rates of ammonia production are relatively low.

Ammonia production in the rumen fluid of

| High numbers<br>Low activity | Low numbers<br>High activity |
|---|---|
| *Butyrivibrio fibrisolvens* | *Clostridium aminophilum* |
| *Megasphaera elsdenii* | *Clostridium sticklandii* |
| *Prevotella ruminicola* | *Peptostreptococcus anaerobius* |
| *Selenomonas ruminantium* | |
| *Streptococcus bovis* | |
| > $10^9$ ml$^{-1}$ | $10^9$ ml$^{-1}$ |
| 10-20 nmol $NH_3$ min$^{-1}$ (mg protein)$^{-1}$ | 300 nmol $NH_3$ min$^{-1}$ (mg protein)$^{-1}$ |
| Mainly monensin-resistant | Monensin-sensitive |

**Figure 1.** A summary of the properties of ammonia-producing bacteria from the rumen.

these same animals was inhibited less than half by monensin, similar to the inhibitions observed in similar sheep with monensin and tetronasin (Wallace et al, 1990b). Yang and Russell (1993) also found that the rate of ammonia production from casein was inhibited less than half by extremely high concentrations of monensin (5 mM cf. a likely concentration in vivo of 4 mM) (Wallace et al, 1981), although the effect was greater for lower rates of ammonia production. Decreased ammonia production with ionophores may indeed be due partly to elimination of high-activity bacteria, but it should also be recognised that the influence of ionophores extends beyond those species whose growth is inhibited and are generally recognised to be 'monensin-sensitive'. The deaminative activity of *P. ruminicola* and *Ruminobacter amylophilus*, bacteria that can grow when ionophores are present, was greatly diminished when they were grown with ionophores in the medium (Newbold et al, 1990). Furthermore, the deamination of reduced amino acids will be affected indirectly by monensin, via decreased hydrogenase activity (Russell and Martin, 1984; Hino and Russell, 1985). The long-term effects of ionophores on ammonia production in vivo are therefore due partly to an effect on the residual, apparently ionophore-insensitive species as well as to the suppression of the high-activity species.

It may be concluded that amino acid deamination is carried out by two distinct bacterial populations of either low-activity/high numbers or high-activity/low numbers characteristics. The former population is probably of greater significance under most circumstances. However, it is extremely important to suppress the proliferation of the high-activity species since the presence of only a small population of these organisms could have a major impact on the efficiency of N retention by the animal.

## Conclusions

The pattern of conversion of protein to ammonia is rather like a funnel. Many species participate in the initial proteolytic cleavage. The resultant oligopeptides then funnel down to to be hydrolysed largely by one species, *P. ruminicola*. Thereafter, the products diverge as many species of bacteria and protozoa break down dipeptides and amino acids. Regulation of the process has thus far concentrated on the first or last steps. Since the constriction in the flow is at *P. ruminicola*, it seems that the peptidases of this organism deserve detailed study.

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