A survey of peptidase activity in rumen bacteria

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Introduction

The breakdown of peptides to amino acids by rumen micro-organisms is an integral part of the conversion of protein to ammonia, a process that often leads to the inefficient use of dietary protein by ruminants (Leng & Nolan, 1984). Various species of protozoa, bacteria and anaerobic fungi are responsible for proteolysis in rumen fluid (Wallace, 1988; Wallace & Cotta, 1988), and the proteolytic population is highly variable (Wallace & Cotta, 1988). The role of different microbial species in the conversion of peptides to amino acids has received less attention. Ciliate protozoa have a high specific activity against dipeptides, but this falls as peptide size increases, and bacteria are most important in hydrolysing peptides with three or more amino acid residues (Newbold et al., 1989; Wallace et al., 1990a, b).

Bladen et al. (1961) screened rumen contents for bacteria which produced ammonia from Trypticase, and found significant activity in 28% of isolates in a 72 h incubation. Half of these were Bacteroides ruminicola, while some strains of Butyrivibrio fibrisolvens, Megaspheara elsdenii and Selenomonas ruminantium were positive. Ammonia production from Trypticase could arise from amino acids rather than peptides, however, as Trypticase contains some free amino acids. Furthermore, peptidolytic bacteria would also have to be able to deaminate amino acids in order to be identified from ammonia production. Thus, peptide hydrolysis was in some cases not necessary, and in others insufficient, for bacteria to have been picked out as peptidolytic in the study of Bladen et al. (1961).

Peptide hydrolysis in the rumen follows a distinctive pattern, characteristic of the peptides being broken down by a dipeptidyl aminopeptidase mechanism (Wallace & McKain, 1989; Wallace et al., 1990a, b). In this study, rumen bacteria which carry out a similar peptidolytic hydrolysis were identified by screening a laboratory culture collection of predominant rumen bacteria. A preliminary report of some of this work has been published (Wallace et al., 1990b).

Methods

**Rumen bacteria.** The sources of the different strains used in this survey are listed in Table 1. Bacteria were grown in Hungate tubes (Bellco Glass) under O₂-free CO₂ at 39°C in Hobson's medium 2 (Hobson, 1969). Overnight cultures were recovered by centrifugation (1100 g, 10 min), washed once with 25 mm-potassium phosphate buffer, pH 7·0, made anaerobic by boiling and bubbling with CO₂ and resuspended to half the original volume in the same buffer.
Incubation procedure. A portion (0.6 ml) of bacterial suspension was added to 0.2 ml peptide substrate (1 mM) and incubated at 39 °C under CO₂. Alanine peptides, alanine-p-nitroanilides (pNA) and N-Ac-alanine were dissolved in 25 mM-potassium phosphate buffer. N-Ac-Ala₃-pNA was dissolved in 100% dimethyl sulphoxide. Peptide-4-methoxy-2-naphthylamides (peptide-MNAs) were dissolved in ethanol and then mixed with 0.15 M-sodium acetate buffer, giving a final concentration of 2.5% in the assay volume of 0.8 ml. The reaction was stopped by the addition of 0.2 ml 1.25 M-orthophosphoric acid. Previous experiments had shown the relationship between peak area and peptide concentration to be linear.

In experiments to determine the influence of PMSF, the initial concentration of Ala₃ was 0.313 mM and the incubation time was 10 min. The strained rumen fluid was obtained from a sheep receiving 67% grass hay and 33% concentrate diet (Whitelaw et al., 1988). PMSF was added in a 20% (v/v) ethanol solution, giving a final ethanol concentration of 2.5% in the assay volume of 0.8 ml. The reaction was stopped by adding 0.2 ml 1.25 M-orthophosphoric acid.

The concentrations of dimethyl sulphoxide and ethanol used in these experiments were 40% (v/v) methanol. All samples analysed by HPLC were done in duplicate and the results were quantified by comparison with the peak area obtained when the peptide was added to rumen fluid containing 25 mM-phosphate buffer.

Analysis of peptide substrates and products. Supernatants from phosphoric-acid-treated samples were analysed for substrates and products by HPLC. The samples were thawed, centrifuged and filtered as described previously (Wallace & McKain, 1989). The HPLC apparatus was a Spectra-Physics system fitted with a Spherisorb ODS 2 column (5 µm, C18; FSA Chromatography) and a 20 µl loop. The detector was set at 206 nm. The eluant used for the analysis of the alanine oligopeptides was a mixture comprising 80% (v/v) 30 mM-orthophosphoric acid plus 5% (v/v) methanol. The alanyl-pNAs were eluted using 70% (v/v) 30 mM-orthophosphoric acid and 30% (v/v) methanol, the N-Ac-alanine peptides were eluted using 100% 30 mM-orthophosphoric acid plus 5% (v/v) HSA, and N-Ac-Ala₃-pNA was eluted with 60% (v/v) 30 mM-orthophosphoric acid and 40% (v/v) methanol. All samples analysed by HPLC were done in duplicate and the results were quantified by comparison with the peak area obtained when the peptide was added to rumen fluid containing 25 mM-orthophosphoric acid. Previous experiments had shown the relationship between peak area and peptide concentration to be linear. The release of MNA from the peptide-MNA substrates was measured by its fluorescence as described previously (Wallace & McKain, 1989). Protein was determined with the Folin reagent by the method of Herbert et al. (1971) with bovine serum albumin as the standard.
Peptidolytic rumen bacteria

Results

Twenty-nine strains of 14 species of predominant rumen bacteria were screened for their ability to hydrolyse Ala₂, Ala₃, GlyArg-MNA and Leu-MNA (Table 1). Several species were active against Ala₂, with very high activities being shown by both strains of M. elsdonii, one strain of Ba. ruminicola (223/M2/7) and one strain of Fibrobacter succinogenes (BL2). Fewer species were active against Ala₃, with the highest activities being shown by all strains of Ba. ruminicola, one strain of Bu. fibrisolvens (SH13), both strains of L. multipara, both strains of Rb. amylophilus and one strain of Rc. flavefaciens (FD1).

HPLC analysis of Ala₃ disappearance from the culture medium also revealed which alanine peptides were formed as products of the hydrolysis. Ala₁ was the major product from L. multipara D15d, Rc. flavefaciens FD1 and Rb. amylophilus WP91. The Ba. ruminicola strains and Bu. fibrisolvens SH13 hydrolysed Ala₃ to Ala₁ and Ala₂, with little Ala₄ being produced. PMSF had little influence on Ala₃ breakdown by rumen fluid or by individual bacterial species, except for slight inhibition (22%) with Bu. fibrisolvens (Table 2).

Exceptionally high leucine arylamidase activity was shown by Str. bovis, with lower activity being associated with Rb. amylophilus (Table 1). However, only Ba. ruminicola showed substantial activity against GlyArg-MNA.

Ba. ruminicola M384 and Bu. fibrisolvens SH13 were investigated further to determine their activities against a range of alanine peptide substrates (Table 3). The two species showed similar rates of hydrolysis with the alanine oligomers and similar low activity with the N-acetylated alanine peptides. However, Ba. ruminicola had a much higher activity against alanine-pNAs than Bu. fibrisolvens.

Further differences were seen when the disappearance of substrates and accumulation of intermediates from alanine peptides and alanine peptide-pNAs were measured during the incubations. As before, Ala₂, Ala₃ and Ala₄ were hydrolysed at similar rates by Ba. ruminicola (Fig. 1) and Ala₄ was more slowly hydrolysed relative to Ala₂ and Ala₃ by Bu. fibrisolvens (Fig. 2). Concentrations of products from Ala₂ were small with Bu. fibrisolvens, but otherwise the patterns of products appeared similar for Ba. ruminicola and Bu. fibrisolvens.

Ba. ruminicola hydrolysed Ala₃-pNA rapidly with almost no Ala-pNA formed (Fig. 3). In contrast, the breakdown of Ala₂-pNA by Bu. fibrisolvens was slower and Ala-pNA was formed (Fig. 4). When Ala₁-pNA was used as the substrate, Ba. ruminicola produced significant amounts of Ala-pNA with very little Ala₂-pNA, whereas Bu. fibrisolvens showed only low activity against Ala₃-pNA, with very small amounts of both intermediates formed.

When dipeptide-MNAs were used as the substrates Bu. fibrisolvens showed no activity, but Ba. ruminicola hydrolysed GlyArg-MNA, LysAla-MNA and ArgArg-MNA rapidly and had lower activity against LeuVal-MNA and GlyPro-MNA (Table 4). The release of MNA was linear over the first 10–20 min with GlyArg-MNA, LysAla-MNA and ArgArg-MNA, the first 30 min with GlyPro-MNA and 60 min with LeuVal-MNA (results not shown).

Table 2. Influence of PMSF on Ala₃ hydrolysis

The results are means of duplicate incubations in the absence or presence of 125 μg PMSF ml⁻¹.

<table>
<thead>
<tr>
<th>Species</th>
<th>Ala₃ hydrolysis [nmol min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-PMSF</td>
</tr>
<tr>
<td>Ba. ruminicola M384</td>
<td>2.9</td>
</tr>
<tr>
<td>Bu. fibrisolvens SH13</td>
<td>5.4</td>
</tr>
<tr>
<td>L. multipara D15d</td>
<td>1.7</td>
</tr>
<tr>
<td>Rc. flavefaciens FD1</td>
<td>3.3</td>
</tr>
<tr>
<td>Strained rumen fluid</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Table 3. Activity of Ba. ruminicola and Bu. fibrisolvens against a range of Ala peptides

The results are means and standard errors of the initial rate of peptide hydrolysis measured in duplicate from four samples.

<table>
<thead>
<tr>
<th>Peptide substrate</th>
<th>Rate of hydrolysis [nmol min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ba. ruminicola M384</td>
</tr>
<tr>
<td>Ala₁</td>
<td>Mean</td>
</tr>
<tr>
<td>Ala₂</td>
<td>4.34</td>
</tr>
<tr>
<td>Ala₃</td>
<td>4.40</td>
</tr>
<tr>
<td>Ala₄</td>
<td>4.70</td>
</tr>
<tr>
<td>Ala₅</td>
<td>3.74</td>
</tr>
<tr>
<td>Ala-pNA</td>
<td>7.48</td>
</tr>
<tr>
<td>Ala₁-pNA</td>
<td>11.26</td>
</tr>
<tr>
<td>Ala₂-pNA</td>
<td>21.57</td>
</tr>
<tr>
<td>N-Ac-Ala₃</td>
<td>0.79</td>
</tr>
<tr>
<td>N-Ac-Ala₄</td>
<td>1.54</td>
</tr>
<tr>
<td>N-Ac-Ala₃-pNA</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Chemicals. HPLC-grade methanol and HSA were obtained from FSA Laboratory Supplies. Analytical-grade orthophosphoric acid was obtained from BDH. Alanine peptides, alanine-pNAs, N-Ac-alanine peptides, N-Ac-Ala₃-pNA, LysAla-MNA ArgArg-MNA, GlyPro-MNA, MNA and PMSF were obtained from Sigma. GlyArg-MNA, LeuVal-MNA and MNA were obtained from Bachem.
Fig. 1. Hydrolysis of (a) Ala3, (b) Ala4 and (c) Ala5 by *Ba. ruminicola* M384. Concentrations of Ala3 (○), Ala4 (●), Ala5 (□) and Ala5 (■) were measured by HPLC.

Fig. 2. Hydrolysis of (a) Ala2, (b) Ala3 and (c) Ala4 by *Bu. fibrisolvens* SH13. Concentrations of Ala2 (○), Ala3 (●), Ala4 (□) and Ala5 (■) were measured by HPLC.

Fig. 3. Hydrolysis of (a) Ala2-pNA and (b) Ala3-pNA by *Ba. ruminicola* M384. Concentrations of Ala2-pNA (○), Ala3-pNA (●), Ala3-pNA (□), Ala2 (□) and p-nitroaniline (■) were measured by HPLC. Ala2 was not determined in (a). No free p-nitroaniline was detected in (b).

Fig. 4. Hydrolysis of (a) Ala2-pNA and (b) Ala3-pNA by *Bu. fibrisolvens* SH13. Concentrations of Ala2-pNA (○), Ala3-pNA (●) and Ala3-pNA (□) were measured by HPLC.
Table 4. Hydrolysis of dipeptide-MNA substrates by Ba. ruminicola M384

The results are means and standard errors of four observations.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate of MNA release [nmol min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>GlyArg-MNA</td>
<td>19.82</td>
</tr>
<tr>
<td>LysAla-MNA</td>
<td>11.28</td>
</tr>
<tr>
<td>ArgArg-MNA</td>
<td>15.08</td>
</tr>
<tr>
<td>LeuVal-MNA</td>
<td>2.05</td>
</tr>
<tr>
<td>GlyPro-MNA</td>
<td>5.10</td>
</tr>
</tbody>
</table>

Discussion

Previous experiments (Newbold et al., 1989; Wallace et al., 1990a, b) identified ciliate protozoa as having a higher dipeptidase activity than bacteria in the mixed rumen population, with protozoal specific activity against Ala₃ being more than twice that of mixed bacteria. From the present survey it is clear that several species of bacteria, notably M. elsdenii, individually possess high dipeptidase activity. Under normal circumstances where the rumen is colonized by ciliate protozoa, these bacteria may be suppressed. However, it is presumably these species which proliferated to replace the dipeptidase activity of protozoa when they were absent from the ecosystem (Wallace et al., 1987).

The main peptidase activity of the rumen is otherwise bacterial in origin (Newbold et al., 1989; Wallace et al., 1990a, b). Several of the species screened in this survey hydrolysed Ala₃, but only Bu. fibrisolvens and Ba. ruminicola did so in a manner similar to rumen fluid, in which the immediate products were the di- and tripeptides with little or no tetrapeptide formed (Wallace & McKain, 1989; Wallace et al., 1990a, b). Other species which have a high leucine aminopeptidase activity, particularly Rb. amylophilus and, as noted previously (Russell & Robinson, 1984; Wallace & Brammall, 1985), Str. bovis, must play a relatively minor role in peptide breakdown \textit{in vivo}. The hydrolysis of Ala₃, Ala₄ and Ala₃-pNA by these strains was, as with rumen fluid (Wallace et al., 1990a, b), effectively prevented by the presence of an acetyl group at the N-terminus. It should be noted here that the peptidase activities were measured in stationary phase cultures. Although the enzyme specificity is unlikely to be different in exponential phase cells, it is possible that marked changes in specific activity might occur.

When the activities of Ba. ruminicola and Bu. fibrisolvens against peptide arylamide substrates were compared, major differences between the two species and between the activity of Bu. fibrisolvens and that of rumen fluid became apparent. Ba. ruminicola and rumen fluid both hydrolysed GlyArg-MNA, LysAla-MNA, ArgArg-MNA and GlyPro-MNA (Table 4; Wallace & McKain, 1989), which are diagnostic substrates for the dipeptidyl peptidases types I to IV (McDonald & Barrett, 1986; McDonald & Schwabe, 1977). Type I activity was highest both in rumen fluid and in Ba. ruminicola. Bu. fibrisolvens had no activity against dipeptidyl peptidase substrates. Further, Ba. ruminicola hydrolysed Ala₂-pNA without Ala-pNA being formed, as would be expected from a dipeptidyl peptidase mechanism, whereas Ala-pNA was the main product with Bu. fibrisolvens.

The enzymic mechanism(s) responsible for the peptide hydrolysis by Bu. fibrisolvens remains unclear, therefore. As hydrolysis of Ala₃ and Ala₄ was blocked by an N-terminal acetyl group, an aminopeptidase activity is implied. However, blocking the C-terminus also decreased the rate of hydrolysis of alanine peptides. Since PMSF, which inhibits the main proteinase activities of Bu. fibrisolvens (Wallace & Brammall, 1985; Strydom et al., 1986), had little effect on Ala₃ hydrolysis, and since N-Ac-Ala₃-pNA was not hydrolysed, it seems unlikely that endopeptidase activity was responsible for Ala₃ breakdown.

As noted previously (Wallace & McKain, 1989), reports of dipeptidyl peptidases in bacteria are rare, and at present bacterial enzymes similar to type I dipeptidyl peptidases have not been identified. However, \textit{Bacteroides fragilis} has cell-associated aryiamide activities which are active against ValAla-pNA and GlyPro-pNA (Gibson & Macfarlane, 1988) and which could include a dipeptidyl peptidase. This can only be confirmed when the immediate products of hydrolysis have been analysed.

Ba. ruminicola is one of the principal ammonia-producing bacteria in the rumen (Bladen et al., 1961). We conclude that, unlike the other bacteria screened in this survey, it has a peptidase activity similar in specificity to the main activity present in rumen fluid. Its specific activities against Ala₃, Ala₄ and Ala₅ in pure culture [4-4, 4-7 and 2-1-11-1 nmol hydrolysed min⁻¹ (mg protein)⁻¹, respectively] compare with very similar specific activities in rumen fluid [7-3, 5-8 and up to 12-8 nmol min⁻¹ (mg protein)⁻¹; Wallace & McKain, 1989; Wallace et al., 1990a]. Similarly, the activity of Ba. ruminicola towards GlyArg-MNA was 2-5-10-1 nmol min⁻¹ (mg protein)⁻¹, compared to about 5 nmol min⁻¹ (mg protein)⁻¹ in rumen fluid (Wallace & McKain, 1989). Thus, the predominance of \textit{Ba. ruminicola} in peptide hydrolysis \textit{in vivo} must depend largely on its high population size. Van Gylswyk (1990) estimated that \textit{Ba. ruminicola} comprised 60-1% of the bacterial population.
in dairy cows fed grass silage. While such a high proportion may not be typical, *B. ruminicola* would normally be expected to be a significant member of the rumen bacterial population (Stewart & Bryant, 1988).

Much of the work that has been done on peptide metabolism in *B. ruminicola* has tended to emphasize the anabolic and growth-stimulatory role of peptides in this organism (Pittman & Bryant, 1964; Pittman *et al.*, 1967; Cotta & Russell, 1982; Russell, 1983). Experiments on its catabolic role, such as the chemostat studies of Russell (1983), should be extended to determine where (catabolic) peptidase activity is located, how it is regulated and if it can be inhibited. It may then be possible to devise ways of slowing peptide breakdown in the rumen, to the nutritional benefit of the host animal.

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**References**


