A survey of peptidase activity in rumen bacteria

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(Received 21 February 1991; revised 22 May 1991; accepted 29 May 1991)

Twenty-nine strains of 14 species of rumen bacteria were screened for their ability to hydrolyse Ala₂, Ala₅, GlyArg-4-methoxy-2-naphthylamide (GlyArg-MNA) and Leu-MNA. Several species, notably *Megasphaera elsdenii*, were active against Ala₂, and a smaller number, including *Bacteroides ruminicola*, *Butyrivibrio fibrisolvens*, *Ruminococcus flavefaciens*, *Lachnospira multipara* and *Ruminobacter amylophilus*, broke down Ala₅. *Streptococcus bovis* had an exceptionally high leucine arylamidase activity. However, only *Ba. ruminicola* hydrolysed GlyArg-MNA. Further investigation revealed that only *Ba. ruminicola* and *Bu. fibrisolvens* hydrolysed Ala₅ to Ala₃ and Ala₂, with little Ala₄ being produced, in a manner similar to rumen fluid. The activity of *Ba. ruminicola* against synthetic peptidase substrates, including GlyArg-MNA, LysAla-MNA, ArgArg-MNA, GlyPro-MNA, LeuVal-MNA, and Ala₃-*p*-nitroanilide, was similar to that of rumen fluid, whereas the activity of *Bu. fibrisolvens* was quite different. Since the main mechanism by which peptides are broken down in the rumen is similar to dipeptidyl aminopeptidase type I, for which GlyArg-MNA is a diagnostic substrate, it was concluded that *Ba. ruminicola* was the most important single species in peptide breakdown in the rumen.

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.*, 1990*a, 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*, *Megasphaera 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.*, 1990*a, 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.*, 1990*b*).

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.

Abbreviations: HSA, heptane sulphonic acid; MNA, 4-methoxy-2-naphthylamide; pNA, *p*-nitroanilide.

Table 1. *Peptidase activities of different species of rumen bacteria*

Bacterial suspensions were incubated with substrates as described in Methods for periods up to 60 min. The values given below are means of duplicate determinations on each of two cultures on separate days.

Species	Strain	Peptidase activity [nmol min ⁻¹ (mg protein) ⁻¹]				Source/reference
		Ala ₂	Ala ₅	GlyArg-MNA	Leu-MNA	
<i>Anaerovibrio lipolytica</i>	5S	0	0	0	0	ATCC 33276
<i>Bacteroides multiacidus</i>	46/5	2.2	0.5	0	0	Flint & Stewart (1987)
<i>Bacteroides ruminicola</i> subsp. <i>ruminicola</i>	M384	3.3	2.3	10.1	0	Wallace & Brammall (1985)
	223/M2/7	12.1	11.1	5.4	0.3	Flint & Stewart (1987)
	2202	3.2	2.1	2.5	0	NCFB 2202
subsp. <i>brevis</i>	F115	4.5	7.5	3.9	0	Flint <i>et al.</i> (1988)
	118B	4.0	5.5	2.5	0	NCFB 1935
<i>Butyrivibrio fibrisolvens</i>	JW11	0	0.6	0	0.1	Wallace & Brammall (1985)
	SH13	1.6	4.3	0	0	Wallace & Brammall (1985)
<i>Eubacterium ruminantium</i>	GA 195	1.6	1.0	0	0.2	ATCC 17233
	C2/A164	1.1	1.2	0	0	Rowett Research Institute
<i>Fibrobacter succinogenes</i>	S85	2.4	0.2	0	0	ATCC 19169
	BL2	12.7	1.4	0	0	NCFB 2576
<i>Lachnospira multipara</i>	D15d	3.9	2.9	0	0	B.A. Dehority, Wooster, Ohio, USA.
	D32	3.9	2.2	0	0	ATCC 19207
<i>Megasphaera elsdenii</i>	LC1	16.5	0.2	0	0	NCFB 2264
	J1	14.4	0.1	0	0	Rowett Research Institute
<i>Ruminobacter amylophilus</i>	WP91	3.9	3.3	0.3	1.1	NCFB 2234
	WP225	1.2	3.4	0.4	1.2	Rowett Research Institute
<i>Ruminococcus albus</i>	J6	0.2	0.2	0	0	Varel <i>et al.</i> (1989)
	SY3	0	0.1	0	0	Varel <i>et al.</i> (1989)
<i>Ruminococcus flavefaciens</i>	FD1	1.1	6.6	0	0	NCFB 2215
	007	2.2	1.5	0	0	Stewart <i>et al.</i> (1990)
<i>Selenomonas ruminantium</i>	Z108	2.9	0.1	0	0	Flint & Bisset (1990)
	JW2	4.2	0	0	0	Wallace & Brammall (1985)
<i>Streptococcus bovis</i>	C277	2.5	0.9	0.1	4.3	Wallace & Brammall (1985)
	ES1	1.7	0.2	0.3	4.1	Rowett Research Institute
<i>Veillonella parvula</i>	L59	0	0.1	0	0	Rowett Research Institute
	692	0	0	0.1	0	Rowett Research Institute

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 peptides were dissolved in 25 mM-potassium phosphate buffer. *N*-Ac-Ala₃-pNA was dissolved in 100% dimethyl sulphoxide. The reaction was stopped by the addition of 0.2 ml 1.25 M-orthophosphoric acid. Peptide-4-methoxy-2-naphthylamides (peptide-MNAs) were dissolved in ethanol and then mixed with 0.15 M-sodium acetate buffer, pH 5.5, to final concentrations of between 1 and 4% (v/v) ethanol and 1 mM-peptide-MNA. Parallel incubations were done with 4-methoxy-2-naphthylamine and ethanol. Reactions were stopped by the addition of 0.2 ml 25% (w/v) trichloroacetic acid. Acid-treated samples were stored at -20 °C for later analysis.

In experiments to determine the influence of PMSF, the initial concentration of Ala₅ was 0.313 mM and the incubation time was 60 min, except for strained rumen fluid for which 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.*, 1983). 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 incubations had no influence on the rate of breakdown of Ala₅.

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 (5 µm, C18; FSA Chromatography) column (250 by 4.6 mm) and a 20 µl loop. The pump rate was 1.5 ml min⁻¹ and 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 mM-heptane sulphonic acid (HSA) and 20% (v/v) methanol. The alanine-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 mM-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 0.25 M-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.

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 Leu-MNA were obtained from Bachem.

Results

Twenty-nine strains of 14 species of predominant rumen bacteria were screened for their ability to hydrolyse Ala₂, Ala₅, GlyArg-MNA and Leu-MNA (Table 1). Several species were active against Ala₂, with very high activities being shown by both strains of *M. elsdenii*, one strain of *Ba. ruminicola* (223/M2/7) and one strain of *Fibrobacter succinogenes* (BL2). Fewer species were active against Ala₅, 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 Ala₅ disappearance from the culture medium also revealed which alanine peptides were formed as products of the hydrolysis. Ala₄ was the major product from *L. multipara* D15d, *Rc. flavefaciens* FD1 and *Rb. amylophilus* WP91. The *Ba. ruminicola* strains and *Bu. fibrisolvens* SH13 hydrolysed Ala₅ to Ala₃ and Ala₂, with little Ala₄ being produced. PMSF had little influence on Ala₅ 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, Ala₃, Ala₄ and Ala₅ were hydrolysed at similar rates by *Ba. ruminicola* (Fig. 1) and Ala₄ was more slowly hydrolysed relative to Ala₃ and Ala₅ by *Bu. fibrisolvens* (Fig. 2). Concentrations of products from Ala₄ were small with *Bu. fibrisolvens*, but otherwise the patterns of products appeared similar for *Ba. ruminicola* and *Bu. fibrisolvens*.

Table 2. Influence of PMSF on Ala₅ hydrolysis

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

Species	Ala ₅ hydrolysis [nmol min ⁻¹ (mg protein) ⁻¹]	
	- PMSF	+ PMSF
<i>Ba. ruminicola</i> M384	2.9	3.3
<i>Bu. fibrisolvens</i> SH13	5.4	4.2
<i>L. multipara</i> D15d	1.7	1.7
<i>Rc. flavefaciens</i> FD1	3.3	3.0
Strained rumen fluid	4.2	4.0

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.

Peptide substrate	Rate of hydrolysis [nmol min ⁻¹ (mg protein) ⁻¹]			
	<i>Ba. ruminicola</i> M384		<i>Bu. fibrisolvens</i> SH13	
	Mean	SE	Mean	SE
Ala ₂	4.34	0.29	4.20	0.48
Ala ₃	4.40	0.64	5.18	0.27
Ala ₄	4.70	1.36	2.11	0.32
Ala ₅	3.74	0.85	3.96	0.37
Ala-pNA	7.48	0.50	0.73	0.40
Ala ₂ -pNA	11.26	0.18	3.70	0.36
Ala ₃ -pNA	21.57	1.99	1.28	0.11
<i>N</i> -Ac-Ala ₃	0.79	0.11	0.17	0.15
<i>N</i> -Ac-Ala ₄	1.54	0.26	0.46	0.17
<i>N</i> -Ac-Ala ₃ -pNA	0.70	0.12	0.04	0.04

Ba. ruminicola hydrolysed Ala₂-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 Ala₃-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).

Fig. 1. Hydrolysis of (a) Ala₃, (b) Ala₄ and (c) Ala₅ by *Ba. rumincola* M384. Concentrations of Ala₂ (○), Ala₃ (●), Ala₄ (□) and Ala₅ (■) were measured by HPLC.

Fig. 2. Hydrolysis of (a) Ala₃, (b) Ala₄ and (c) Ala₅ 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. rumincola* M384. Concentrations of Ala-pNA (○), Ala₂-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 Ala-pNA (○), Ala₂-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.

Substrate	Rate of MNA release [nmol min ⁻¹ (mg protein) ⁻¹]	
	Mean	SE
GlyArg-MNA	19.82	1.94
LysAla-MNA	11.28	0.67
ArgArg-MNA	15.08	1.32
LeuVal-MNA	2.05	0.28
GlyPro-MNA	5.10	0.91

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 Ala₂ 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 Ala₅, 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 *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 Ala₅ hydrolysis, and since N-Ac-Ala₃-pNA was not hydrolysed, it seems unlikely that endopeptidase activity was responsible for Ala₅ 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, *Bacteroides fragilis* has cell-associated arylamidase 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 *Ba. ruminicola* in peptide hydrolysis *in vivo* must depend largely on its high population size. Van Gylswyk (1990) estimated that *Ba. ruminicola* comprised 60.1% of the bacterial population

in dairy cows fed grass silage. While such a high proportion may not be typical, *Ba. 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 *Ba. 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.

We thank Nicola Watt and Sylvia Duncan for supplying bacterial cultures, and Dr H. J. Flint for discussion and constructive criticism of the manuscript.

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