Analysis of Peptide Metabolism by Ruminal Microorganisms

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Methods were developed for the determination of oligoalanine and other short-chain peptides and peptide analogs in ruminal fluid by using reverse-phase high-pressure liquid chromatography. Chromatographic analysis of the breakdown of $(Ala)_3$ and $(Ala)_4$ in ruminal fluid in vitro revealed that the predominant mechanism of hydrolysis was a dipeptidyl peptidase-like activity. Hydrolysis proceeded from the N terminal of the peptide chain; N-acetyl–$(Ala)_3$ was broken down at 11% of the rate of breakdown of $(Ala)_3$ or $(Ala)_3$–p-nitroanilide. $(Ala)_2$–p-nitroanilide was hydrolyzed most rapidly of the arylamide substrates tested, but fluorogenic 4-methoxy-2-naphthylamide (MNA) compounds were more convenient and potentially more versatile substrates than p-nitroanilides. Gly-Arg-MNA was the most rapidly hydrolyzed dipeptidyl peptidase substrate, suggesting that ruminal peptidase activity was predominantly of a type I specificity.

The conversion of dietary protein to ammonia in the rumen effectively deprives the ruminant animal of some of the nutritive value of its consumed amino acids. As a consequence, there is considerable interest in suppressing microbial catabolism of proteins, peptides, and amino acids in the rumen (15). Peptides seem to be particularly significant in this process and accumulate in ruminal fluid after feeding (4, 6).

Methods employed in the study of peptide metabolism in bacteria usually involve the use of radioactively or chemically labeled substrates or fluorescence techniques for detecting intact peptides, followed by separation and identification of products by thin-layer chromatography (19, 20). Almost all experiments done with ruminal microorganisms have dealt with the metabolism of mixed peptides derived from protein hydrolysates and have used general analytical procedures that did not identify individual peptides (6–8, 22–25, 29; P. B. Cooper and J. R. Ling, Proc. Nutr. Soc. 44:144A, 1985). We have previously used a fluorescamine procedure based on methods described by Perrett et al. (21) and Nisbet and Payne (18) to compare the metabolism of individual oligopeptides of various composition (5), but this method measured the disappearance of total peptide and so did not distinguish peptide substrates from intermediates that might accumulate during hydrolysis.

In the present study we investigated methods for measuring peptide metabolism by ruminal microorganisms. A hydrophobic ion-pairing technique was developed for the isocratic separation of peptides by high-pressure liquid chromatography (HPLC), permitting the assay of individual alanine oligopeptides and other similar molecules in ruminal contents. The information derived with this technique enabled us to identify suitable peptide-arylamide substrates for measuring ruminal peptidase activity.

MATERIALS AND METHODS

Animals. Two mature wether sheep, fitted with permanent ruminal fistulae, were fed 450 g of a mixed diet at 0800 and 1600 h. The diet consisted of 67% grass hay and 33% concentrate (27). Strained ruminal fluid was prepared about 2 h after the morning feeding by straining ruminal contents through four layers of muslin.

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Incubation procedure. Strained ruminal fluid (3 volumes) was mixed with 1 volume of anaerobic 100 mM potassium phosphate (pH 7.0), as described previously (5). A portion (0.6 ml) of this mixture was added to 0.2 ml of buffer containing the peptide substrate (5 mM) and incubated at 39°C. Peptides were dissolved in 25 mM potassium phosphate buffer. Peptide–p-nitroanilides and 4-methoxy-2-naphthylamides were dissolved in ethanol and then mixed with 25 mM phosphate buffer or 0.15 M sodium acetate buffer (pH 5.5), respectively, to final concentrations of 25% (vol/vol) ethanol and 5 mM peptide-arylamide. Parallel incubations were done with p-nitroaniline (pNA) and 4-methoxy-2-naphthylamine (MNA). Reactions were terminated by adding 0.2 ml of 30% (wt/vol) perchloric acid (PCA). Acid-treated samples were stored at −20°C for later analysis.

Analysis of peptide substrates and products. Frozen samples were thawed and centrifuged (12,000 × g for 5 min). The supernatant was analyzed for total peptides by the fluorescamine procedure described previously (5), except that the sample size was 100 μl and the acidic PCA-containing solution was used in the fluorescamine procedure rather than the neutral cell-free supernatant used before. This was done to avoid the necessity for immediate analysis. Although a loss of sensitivity of 55 to 60% was incurred, the fluorescence was still easily measured in the fluorimeter and the assay was still linear within the concentration range used. The revised method had the important advantage that samples could be reanalyzed as necessary.

The supernatant from the PCA extract was analyzed for individual substrates and products by HPLC. The HPLC apparatus was an LDC/Milton Roy system (Riviera Beach, Fla.) fitted with a Spherisorb S5 ODS (5 μm, C18; LDC/Milton Roy) column (250 by 4.6 mm). The pump rate was 1.5 ml/min, and the detector was set at 206 nm. PCA-treated samples were thawed, centrifuged at 12,000 × g for 5 min, and filtered through 0.45-μm-pore-size membrane filters or glass fiber filters before being injected (20-μl loop). The eluant used for the analysis of alanine oligopeptides was a mixture comprising 90% 30 mM orthophosphoric acid plus 5 mM heptane sulfonic acid (HSA) and 10% methanol. Alanine–p-nitroanilides were analyzed for disappearance of substrate by HPLC, using 80% 30 mM phosphoric acid–20% methanol as the eluant. Gly–Pro–p-nitroanilide was determined with a 70% 30 mM phosphoric acid–30% methanol eluant, and N-acetyl–$(Ala)_3$ [N-Ac$(Ala)_3$] was determined
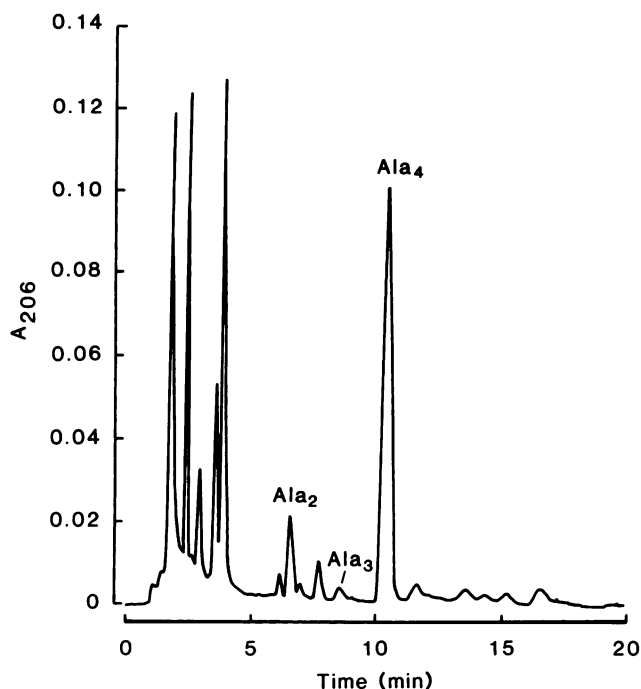
FIG. 1. HPLC analysis of metabolism of $(Ala)_4$ in ruminal fluid. $(Ala)_4$ was mixed with ruminal fluid and incubated at 39°C as described in the text. The chromatogram shows the $(Ala)_4$ remaining after 10 min of incubation, along with an emerging $(Ala)_2$ peak, but little accumulation of $(Ala)_3$.

with 100% 30 mM phosphoric acid. Run times of 35 min were used to clear some residual peaks derived from the digesta.

pNA release was determined by the diazotization procedure of Appel (1). MNA was determined by its fluorescence (excitation wavelength = 350 nm, emission wavelength = 425 nm) in 40-fold dilutions of PCA supernatants in 0.15 M sodium acetate buffer (pH 5.5). Protein was determined with the Folin reagent (13) with bovine serum albumin as the standard.

Chemicals. HPLC-grade methanol and HSA were obtained from FSA Laboratory Supplies, Loughborough, England. Orthophosphoric acid was analytical grade from BDH Chemicals Ltd, Poole, England. Peptides, peptide–p-nitroanilides, Lys-Ala-MNA, Arg-Arg-MNA, and Leu-MNA were from Sigma Chemical Co., Poole, England. MNA, Gly-Arg-MNA, Leu-Val-MNA, and N-Ac(Ala)$_3$ were from Bachem Ltd, Bubendorf, Switzerland.

RESULTS

HPLC analysis of peptides in ruminal fluid. Reverse-phase HPLC with HSA as the ion-pairing agent in a dilute phosphoric acid-methanol mobile phase was used to separate and quantitate alanine oligopeptides in ruminal fluid. Retention times (minutes) for alanine oligomers were as follows: $(Ala)_2$, 6.4; $(Ala)_3$, 8.3; $(Ala)_4$, 10.0; $(Ala)_5$, 13.1. There was remarkably little interference from other compounds found in ruminal fluid (Fig. 1). PCA extracts of ruminal contents could therefore be injected directly onto the column. So long as a thorough methanol wash was done daily, deterioration of the column was minimal over several hundred injections. Trichloracetic acid was investigated as an alternative to PCA, but its absorbance prevented quantitation of the ala-

nine peptides on the resulting chromatogram. When HSA was omitted from the eluant, retention times (minutes) for the different peptides were as follows: $(Ala)_2$, 3.0; $(Ala)_3$, 4.5; $(Ala)_4$, 6.9; $(Ala)_5$, 12.5. Thus, the smaller oligomers were not well resolved when HSA was not present, particularly in the presence of ruminal fluid in which numerous early peaks occurred.

The method was used to analyze for several other peptides in ruminal fluid. Molecules with two basic groups were retained for a longer time than those with a single positive charge. Their retention times (minutes) with 90% 30 mM phosphoric acid plus 5 mM HSA and 10% methanol were as follows: Gly-His, 23.6; His-Gly, 25.1; Lys-Asp, 28.6; Glu-Lys, 32.3; Asp-Lys, 21.1. In the absence of HSA and methanol, the retention times of the same compounds were 2.7, 2.6, 2.8, 3.3, and 2.7 min, respectively. Increasing the methanol content of the eluant in the presence of HSA would probably give more satisfactory intermediate retention times, although this aspect was not investigated.

Peptides with one or no positive charge were generally retained for shorter times with the 90% 30 mM phosphoric acid–5 mM HSA–10% methanol eluant. Examples of retention times (minutes) include the following: Ala-Gly, 5.0; Gly-Ala, 6.1; $(Gly)_2$-Ala, 6.5; Ala-$(Gly)_2$, 5.0; Pro-$(Gly)_2$, 7.9; Gly-Asp, 4.4; Gly-Ser, 3.9; Asp-Glu, 5.4. Considerable variation occurred depending on the nature of the amino acid side chains. Glycine oligomers did not separate well with the same eluant. Retention times (minutes) were as follows: $(Gly)_2$, 4.24; $(Gly)_3$, 4.41; $(Gly)_4$, 4.56; $(Gly)_5$, 4.71; $(Gly)_6$, 4.86. However, if the methanol was omitted from the eluant, separations were improved, with retention times (minutes) as follows: $(Gly)_2$, 5.9; $(Gly)_3$, 6.9; $(Gly)_4$, 7.7; $(Gly)_5$, 8.8; $(Gly)_6$, 10.3. Peptides containing aromatic amino acid residues were retained on the column for more than 20 min with the 90% 30 mM phosphoric acid–5 mM HSA–10% methanol eluant, as were those with longer aliphatic side chains, such as $(Leu)_3$. However, elution could be accelerated by increasing the proportion of methanol. For example, phenylalanine oligomers, which were effectively absorbed by the column under the conditions used for separating alanine peptides, eluted at the following times (minutes) with 70% methanol–30% 30 mM phosphoric acid–5 mM HSA: $(Phe)_2$, 6.3; $(Phe)_3$, 9.5; $(Phe)_4$, 14.1.

Analysis of intermediates formed during hydrolysis of alanine peptides. The chromatogram shown in Fig. 1 is the profile of a sample taken during an experiment to study the metabolism of $(Ala)_3$ and $(Ala)_4$ in ruminal fluid. The rate of hydrolysis of $(Ala)_3$ determined with fluorescamine was underestimated because $(Ala)_2$ accumulated (Fig. 2). $(Ala)_2$ accumulated once more as a consequence of $(Ala)_4$ hydrolysis, again with the fluorescamine procedure giving a poor indication of its rate of breakdown (Fig. 3). Results are expressed as relative fluorescence because different peptides have different fluorescence responses, which are not related directly to their amino acid contents (4). Relative molar fluorescence responses for pure alanine peptides are as follows: $(Ala)_2$, 1.0; $(Ala)_3$ and $(Ala)_4$, 1.29 (4). HPLC analysis revealed additionally that little $(Ala)_3$ was present as an intermediate.

Metabolism of synthetic peptides. $(Ala)_2$-pNA was more rapidly hydrolyzed than either $(Ala)_3$-pNA or Ala-pNA, and N-Ac(Ala)$_3$ was degraded at 11% of the rate of $(Ala)_3$-pNA (Table 1). The rate of breakdown of $(Ala)_3$-pNA (7.2 nmol/mg of protein per min) compares with rates of 7.3 and 5.8 nmol/mg of protein per min for $(Ala)_3$ and $(Ala)_4$ calculated from the 20-min samples in Fig. 2 and 3. Gly-Pro-pNA was
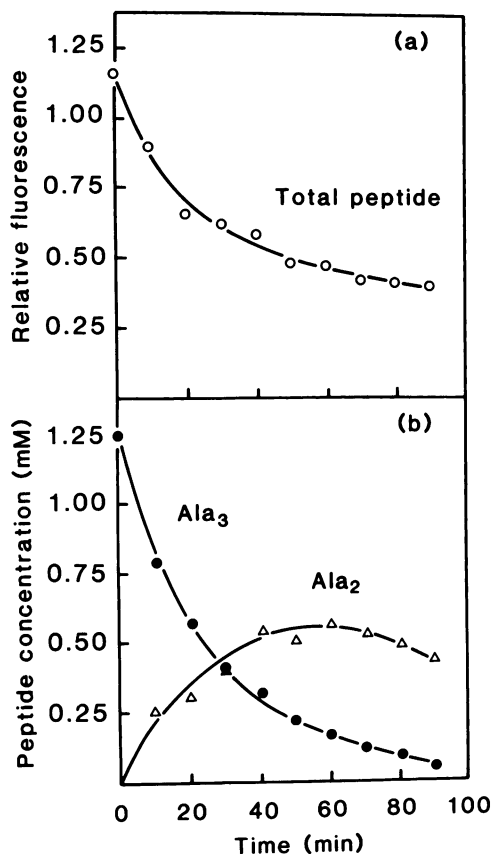
FIG. 2. Hydrolysis of (Ala)$_3$ added to mixed ruminal microorganisms. (a) Total peptides determined by fluorimetry, relative to 1.25 mM (Ala)$_3$. (b) HPLC analysis of (Ala)$_2$ ($\triangle$) and (Ala)$_3$ ($\bullet$) in the same samples. The protein content of the mixture was 4.71 mg/ml.

FIG. 3. Hydrolysis of (Ala)$_4$ by mixed ruminal microorganisms. (a) Total peptides determined by fluorimetry, relative to 1.25 mM (Ala)$_4$. (b) HPLC analysis of (Ala)$_2$ ($\triangle$), (Ala)$_3$ ($\bullet$), and (Ala)$_4$ ($\blacktriangle$) in the same samples. The protein content of the mixture was 3.79 mg/ml.

broken down at less than a quarter the rate of breakdown of (Ala)$_2$-pNA. The measurements given in Table 1 were done by HPLC analysis of the substrate remaining in the incubation mixture. pNA, measured by diazotization, was found to be metabolized by the mixed population, which for example gave the appearance that pNA was released from Ala-pNA at only 55% of the rate of disappearance of the substrate during a 30-min incubation.

No significant metabolism of MNA was detected during 1-h incubations with ruminal microorganisms. Peptide-MNA substrates released MNA in a linear manner, except for Leu-Val-MNA, but at rates slower than pNA derivatives (Fig. 4). Gly-Arg-MNA was most rapidly broken down, at a rate corresponding to 5.06 nmol/mg of protein per min, compared with 3.64, 1.89, 1.40, and 1.52 nmol/mg of protein per min for Lys-Ala-MNA, Arg-Arg-MNA, Leu-Val-MNA, and Leu-MNA, respectively.

DISCUSSION

The results presented here illustrate some of the pitfalls that can occur in the measurement of peptide metabolism. The general fluorescamine technique, although frequently used for the purpose (18–20), is inappropriate for determining how rapidly individual peptides are metabolized, unless it is established first that accumulation of intermediates does not occur. Thus, previous estimates of alanine peptide metabolism (5) were too low due to the accumulation
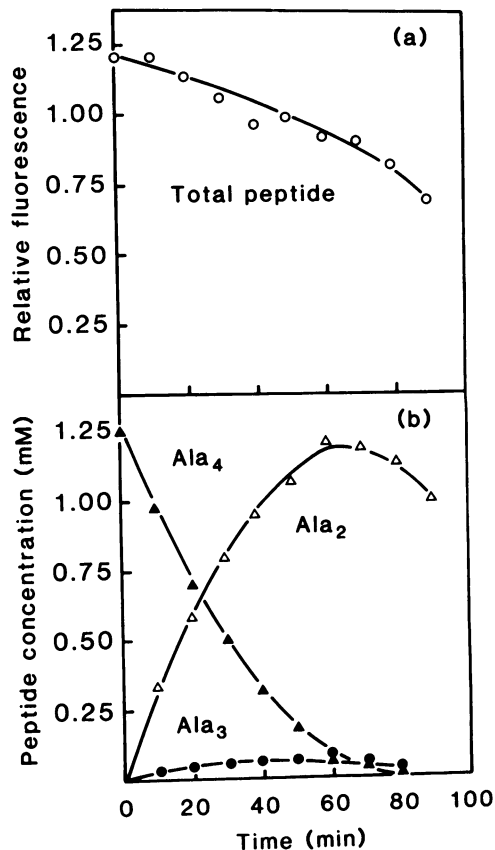
of (Ala)$_2$. HPLC analysis also indicated that (Ala)$_4$ is cleaved to (Ala)$_2$ rather than (Ala)$_3$, another characteristic that was not evident from fluorescamine analysis.

The principle of the chromatography was that the low pH effectively rendered carboxylic groups uncharged, and the HSA paired with the positively charged free amino groups, making the whole complex much more hydrophobic (11). Hence, the hydrophobicities and retention times of different peptides depend to a great extent on the positive charge present on the molecule. Clearly, the conditions for optimum HPLC depend on the peptide of interest. Options that are available for improving separations include changing the pH

TABLE 1. Hydrolysis of peptide–p-nitroanilides and N-Ac(Ala)$_3$ by mixed ruminal microorganisms

Substrate	Rate of hydrolysis[a] (nmol/mg of protein per min) (mean [SE])
(Ala)$_3$-pNA	7.2 (0.84)
(Ala)$_2$-pNA	20.7 (4.36)
Ala-pNA	11.4 (1.00)
N-Ac(Ala)$_3$	0.8 (0.56)
Gly-Pro-pNA	4.9 (1.45)

[a] Results are means and standard errors for four observations. The average protein content was 2.31 mg/ml of the assay mixture. The incubation time was 20 min, except for (Ala)$_2$-pNA, for which it was 10 min.
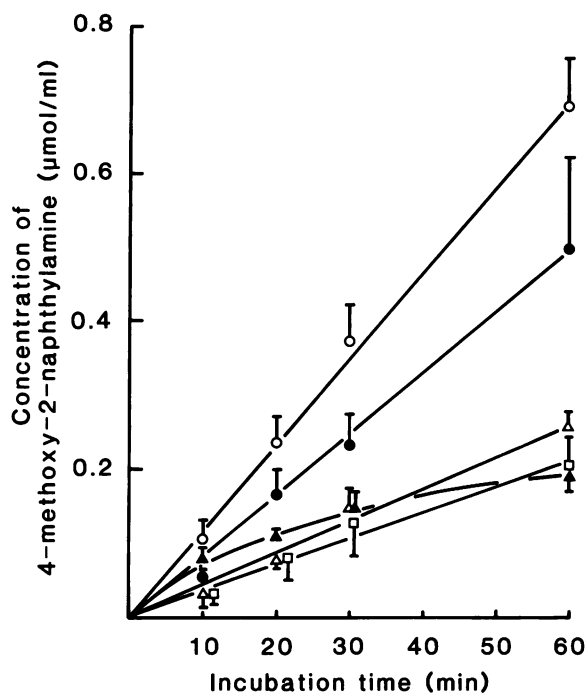
FIG. 4. Release of MNA from Gly-Arg-MNA (O), Lys-Ala-MNA (●), Arg-Arg-MNA (△), Leu-Val-MNA (▲), and Leu-MNA (□) by mixed ruminal microorganisms in vitro. Results are means and standard errors for four incubations with diluted ruminal fluid, as described in the text. The average protein content of the incubation mixture was 2.31 mg/ml.

and the organic solvent concentration, as well as the addition of a counterion, such as the hydrophobic HSA used here. Alternatively, a hydrophilic anion might be used as an ion-pairing agent to reduce the retention times of nonpolar peptides (10). It should be noted that the detection wavelength used here (206 nm) is not specific for peptide bonds. Indeed, methanol, one of the eluants used in the present procedures, absorbs at 206 nm. This was not a problem with the back-off facility available in our apparatus, but other nonpolar eluants such as acetonitrile might be used to replace methanol under different circumstances. It should also be noted, therefore, that the early peaks found in ruminal fluid (Fig. 1) need not be peptides, in agreement with previous observations (7, 25).

Chromogenic and fluorogenic substrates are often used to measure peptidase activities. pNA is the most frequently used chromophore, and *p*-nitroanilides have been used to measure proteinase and peptidase activities of ruminal microorganisms (3, 26). Because of the large background absorbance by ruminal fluid at wavelengths similar to those at which pNA absorbs, diazotization was necessary to quantify the pNA released. However, in addition to this inconvenience, it transpired that pNA was a poor indicator of substrate breakdown because it was metabolized in ruminal fluid. 4-Methoxy-2-naphthylamides release MNA, which fluoresces at wavelengths that are not subject to interference by ruminal fluid, thus providing a convenient and sensitive assay method. MNA was not metabolized in ruminal fluid. MNA and pNA derivatives were not compared directly in the present study, but it would appear that MNA substrates are broken down more slowly. The rate of hydrolysis of Leu-MNA, 1.52 nmol/mg of protein per min, was consider-

ably less than was observed previously with Leu-pNA (2.81 nmol/mg of protein per min [5]).

The fact that no (Ala)$_3$ was detected during (Ala)$_4$ hydrolysis, that *N*-Ac(Ala)$_3$ was slowly metabolized compared with (Ala)$_3$ and (Ala)$_3$-pNA, and that (Ala)$_2$-pNA was broken down more rapidly than other alanine-pNAs suggests that the main peptidase activity in ruminal fluid is an exopeptidase that cleaves dipeptides from the N-terminal end of the peptide chain. It has been proposed that these enzymes, previously classified as dipeptidyl aminopeptidases (17), should now be named simply dipeptidyl peptidases (16). Dipeptidyl peptidases of both aminopeptidase and carboxypeptidase types occur in several mammalian tissues (16, 17). Cathepsin C, an aminopeptidase, is the best known example. Reports of similar enzymes in microorganisms are much less common (2, 12, 28), although dipeptidyl carboxypeptidase activities have been found in *Escherichia coli* (30) and *Corynebacterium equi* (14) and a dipeptidyl peptidase type IV occurs in *Streptococcus mitis* (9).

The diagnostic peptide-arylamide substrates for types I to IV recognized in mammalian systems are Gly-Arg-X, Lys-Ala-X, Arg-Arg-X, and Gly-Pro-X, respectively (16, 17). Thus, the results with MNA derivatives (Fig. 4) suggest that the activity is more similar to the type I and II enzymes than the type III enzyme. Arg-Arg-MNA was hydrolyzed at a rate similar to Leu-MNA and was therefore probably hydrolyzed by a leucine aminopeptidase activity present at an activity lower than that of dipeptidyl peptidase in ruminal contents. The type IV substrate Gly-Pro-pNA (16, 17) was hydrolyzed more slowly than Ala-pNA (Table 1) or Leu-pNA (5) and was probably broken down by the same enzyme. Type I enzymes have a much higher affinity for (Ala)$_2$-X compounds than do type II enzymes, so it would appear that the predominant ruminal peptidase was most similar to mammalian type I enzymes. More detailed classification will await identification of the peptidase-producing organisms and purification of the relevant enzyme(s).

Our studies of peptide metabolism in the rumen are continuing. The HPLC techniques described here will enable the enzyme mechanism to be characterized further, and the use of peptide-MNA substrates will facilitate the continuous assay of the relevant peptidase activities. MNA-linked compounds are also useful as histochemical substrates (16), and it is hoped that combinations of these techniques will enable us to identify the peptidolytic organisms of greatest importance in the ruminal ecosystem.

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