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Original article

In vitro study of molecular weight, hydrophobicity and amino acid composition of peptides during breakdown of a casein hydrolysate by two strains of *Prevotella ruminicola*

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Abstract — The molecular weight, amino acid composition and hydrophobicity of the peptide residue produced by hydrolysis of protein by two strains of *Prevotella ruminicola* (23 and S17/3) were determined. These last two characteristics could play a role in the control of proteolysis. Both strains produced dipeptidyl peptidases (DAP-1) but only *P. ruminicola* 23 synthesised alanine aminopeptidase. The area of 3–5 kDa peptides decreased, while the peptides directly assimilable by bacteria (0.5–1 kDa and < 0.5 kDa) increased with strain S17/3, but decreased with *P. ruminicola* 23. The amino acid compositions showed that the proportions of these compounds changed little with time and there was no proline enrichment. Similarly, reverse phase HPLC showed no evidence of enrichment of the culture medium by hydrophobic peptides during the growth phase of *P. ruminicola*. These experiments show that the changes in the various peptide classes resulting from the hydrolysis and uptake of peptides by *P. ruminicola* differed with time and depended on the strain used. The nature of the enzyme activity and the use of other nitrogen sources may explain the difference between the two strains. © Inra/Elsevier, Paris.

rumen bacteria / proteolysis / aminopeptidases / peptides / hydrophobicity / molecular weight / amino acids

Résumé — Étude in vitro du poids moléculaire, de l'hydrophobicité et de la composition en acides aminés au cours de la dégradation d'un hydrolysat de caséine par deux souches de *Prevotella ruminicola*. Au cours de l'hydrolyse de peptides par deux souches de *Prevotella ruminicola* (23 et S17/3), nous avons étudié le poids moléculaire, la composition en acides aminés et l'hydrophobicité du résidu peptidique. Ces deux dernières caractéristiques pourraient jouer une rôle dans le contrôle

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de la protéolyse. Les deux souches produisent des dipeptidyl-peptidases (DAP-1) mais seule, *P. ruminicola* 23 synthétise de l'alanine aminopeptidase. L'analyse du poids moléculaire des peptides montre que la part de la fraction 2 à 3 kDa augmente au cours du temps alors que celle de la fraction 3 à 5 kDa diminue. Les fractions peptidiques directement assimilables par les bactéries (0.5 à 1 kDa et < à 0.5 kDa) augmentent avec la souche S17/3 et diminuent en présence de *P. ruminicola* 23. L'étude de la composition en acides aminés montre que la proportion de ces composés évolue peu au cours du temps et ne fait apparaître aucun enrichissement en proline. De même, l'analyse par HPLC en phase inverse des peptides ne met pas en évidence un enrichissement du milieu de culture en peptides hydrophobes pendant la phase de croissance de *P. ruminicola*. Ces résultats mettent en évidence que les évolutions des différentes classes de peptides, qui sont la résultante de l'hydrolyse et du transport des peptides par *P. ruminicola*, diffèrent au cours du temps et dépendent de la souche utilisée. La nature des activités enzymatiques et l'utilisation d'autes sources azotées pourraient expliquer les différences constatées entre les deux souches. © Inra/Elsevier, Paris.

bactéries du rumen / protéolyse / aminopeptidases / peptides / hydrophobicité / poids moléculaire / acides aminés

1. INTRODUCTION

Much of the protein in the diet of ruminants (30–90 %) is broken down in the rumen to give amino acids, ammonia and volatile fatty acids. This process is the result of a variety of microbial activities, including hydrolysis of proteins and peptides, the transport of hydrolysis products, deamination of amino acids and the fermentation of aliphatic chains [8]. Nitrogen metabolism leads to the transformation of food proteins into microbial nitrogen-containing compounds (proteins, DNA, RNA, etc.) which can greatly reduce the supply of nitrogen to the host ruminant [17]. However, the factors that control the breakdown of proteins by rumen micro-organisms are not yet fully understood. For example, it has been suggested that peptides that are resistant to breakdown may be hydrophobic [6]. Whereas Wallace et al. [29] showed that hydrophobic tetrapeptides are more rapidly hydrolysed than are hydrophilic peptides. According to Yang and Russell [35], the presence of proline in synthetic peptides leads to slower hydrolysis, whereas other work has shown that the residual mixture of unhydrolysed peptides does not become enriched in proline during hydrolysis by an inoculum of rumen bacteria [10, 12]. Some studies indicate that high molecular weight peptides are incorporated into bacterial cells faster than small peptides or amino acids [7], whereas others have shown that only small peptides can be transported by bacterial permeases [20]. The substrates used, the bacterial profile (that can vary with the food) and the analytical problems inherent in in vivo studies could give rise to these conflicting conclusions.

In order to minimise these problems, *P. ruminicola*, which plays a key roles in protein hydrolysis in the rumen [11, 12, 19, 28, 29], was used in this study. The present study was therefore carried out to determine the changes in molecular weight, amino acid composition and hydrophobicity of peptides resulting from hydrolysis by this bacterium of a casein hydrolysate (tryptone) which is a sustrate commonly used in the literature.

2. MATERIALS AND METHODS

2.1. Organisms

P. ruminicola 23 was kindly provided by Dr Fonty (Inra, Theix). *P. ruminicola* S17/3 was isolated in our laboratory [9]. It was identified using the morphological and biochemical criteria described by Buchanan and Gibbons [4] and Holdeman et al. [14]. The strains were maintained in glucose-cellobiose-soluble starch medium [13].

2.2. Incubation with peptides

The anaerobic culture techniques were similar to those described by Hungate [15]. The composition (per litre medium) was 225 mg K_2HPO_4, 450 mg NaCl, 450 mg $(NH_4)_2SO_4$, 225 mg KH_2PO_4, 45 mg $CaCl_2$, 95 mg $MgSO_4$, 1 mg Hemin, 1 700 mg acetate, 600 mg propionate, 300 mg butyrate and 100 mg each of isobutyrate, isovalerate, 2-methyl-butyrate, 1 mL vitamin solution [24], 2 500 mg glucose, 2 500 mg cellobiose and 500 mg soluble starch. The medium was brought to the boil under CO_2 and cooled. Na_2CO_3 (0.4 % w/v) and resazurin were added and the solution was bubbled with CO_2 until the Na_2CO_3 dissolved. Cysteine (0.05 % w/v) was then added and the medium was sterilised (120 °C, 20 min.). Pfennig's micromineral solution [25] (final concentration 1 % v/v) was sterilised by filtration. The nitrogen source was a pancreatic digest of casein, the tryptone (Difco, France). The tryptone (final concentration 2 mg·mL^{-1}) was sterilised by filtration.

Bacterial growth was estimated by measuring the optical density (OD) at 660 nm. The samples that were used to determine the enzyme activities, physico-chemical properties of the peptides and the amino acid composition were collected at 0, 4, 8, 12 and 16 h and were stored at –20 °C. All operations were conducted in three replicates.

2.3. Enzymatic activities

Total proteolytic activity was measured using azocasein as a substrate. An enzyme unit corresponds to an OD variation of 0.01 h^{-1} [11]. Dipeptidyl peptidases type I like activity (DAP-1) (EC 3.4.14.1) was determined using Gly-Arg-4-methoxy-2-naphthylamide (MNA) as fluorogenic substrate [27]. The incubation time was 6 h under anaerobic condition at 39 °C. MNA was assayed by fluorescence (excitation wavelength = 350 nm, emission wavelength = 420 nm). Aminopeptidase activities were measured on Ala-pNA and Leu-pNA as chromogenic substrates [27]. Incubation was for 4 h at 39 °C under anaerobic conditions. The pNA released was measured by diazotisation [1]. Gly-Arg-MNA, MNA, Leu-pNA, Ala-pNA, pNA and other peptides were from Sigma.

2.4. Chemical analysis

Ammonia concentration was determined with an ammonia gas sensing electrode (ORION, mode 95–12).

2.4.1. Peptide analysis

The molecular weight profiles of hydrolysates were determined by high performance size exclusion chromatography (HPSEC) and reverse-phase HPLC (RP-HPLC) was used to separate peptides according to their hydrophobicity [11, 12]. Changes in the peptide molecular weight distribution during incubation was monitored by integration the areas under the peaks of chromatograms obtained by HPSEC at various times (0, 4, 8 and 16 h). The peptides used to calibrate the column were (molecular weights in brackets) α-lactalbumin (14 000), aprotinin (6 512), streptomycin (1 457), Leu-enkephalin (601) and Phe-Gly-Phe-Gly (426). The areas under the peaks were integrated using Gold software (Beckman Instruments Inc., USA). Peaks were separated into five ranges (3–5, 2–3, 1–2, 0.5–1 and < 0.5 kDa). The proportions, at t = 0 h, of the fractions 3–5, 2–3, 1–2, 0.5–1 and < 0.5 kDa were 34.2, 27.6, 18.2, 6.3 and 13.8 % during the incubation with strain 23 of *P. ruminicola*, and 42.5, 34.6, 13.0, 6.4 and 3.5 % in the study with strain S17/3. Absorbance was measured at 280 nm in order to avoid the interferences occurring at 220 nm due to VFA and to other compounds present in the medium [11]. The various ingredients in the media analysed by HPSEC and RP-HPLC showed no absorption at t = 0 h.

2.4.2. Amino acid analysis

Amino acids were analysed by GLC by the method of Husek [16] modified by Depardon et al. [12]. No release of amino acids (analysed without hydrolysing the sample) was detected by the methods used. The results of the analyses therefore refer to amino acids in peptide residues.

The HPLC and GC analyses were conducted after filtering though 0.2 µm. The peptides analysed were the residue of the substrate and were the products of hydrolysis.

2.5. Statistical analysis

The time effect on amino acid composition was tested using the Kruskal-Wallis test.

3. RESULTS

3.1. Growth and enzyme activities

Both strains produced endopeptidases during their exponential growth stage. At t = 16 h, the endopeptidase activities of *P. ruminicola* 23 was 24.7 and that of S17/3 was 21.1 U·mL^{-1} (*figure 1*). Both strains studied had DAP-1 activity. However, this activity changed little in strain 23 during incubation, whereas the S17/3 strain greatly increased MNA production from 3.9 at t = 0 h to 40.1 µM at the end of growth. Only strain 23 hydrolysed alapNa. The alanine aminopeptidase of this strain increased steadily from t = 4 h to t = 16 h (*figure 2*).

3.2. Ammonia

The ammonia concentration was changed little by the 23 strain, so production was equal to consumption. In contrast, *P. ruminicola* S17/3 decreased the ammonia concentration from 8 h to 2.19 mM at t = 16 h (*figure 3*).

3.3. Changes in peptide molecular weight

Both strains decreased the area of 3–5 kDa peptides, whereas the 2–3 kDa fraction increased (*figure 4*). *P. ruminicola* 23 decreased the area of < 0.5 kDa peptides from 37.2 to 18.9 at t = 16 h. The 0.5–1 kDa peptide fraction also decreased during the first 8 h to reach 6.2, but then increased to the end of bacterial growth. The S17/3 strain tended to increase the area of small peptides (< 0.5 kDa and 0.5–1 kDa) during the 16 h incubation: the 0.5–1 kDa and < 0.5 kDa fractions increased from 12.8 and 6.7 at t = 0 h to 20.3 and 13.6 at t = 16 h.

3.4. Analysis of the amino acid composition and hydrophobicity

Quantitative analysis of amino acids was used to calculate the percentage of the peptides that were broken down at the end of the exponential phase. The percentage degradation by *P. ruminicola* 23 was 25.9 (± 1.7) and that by S17/3 was 10.4 (± 3.4).

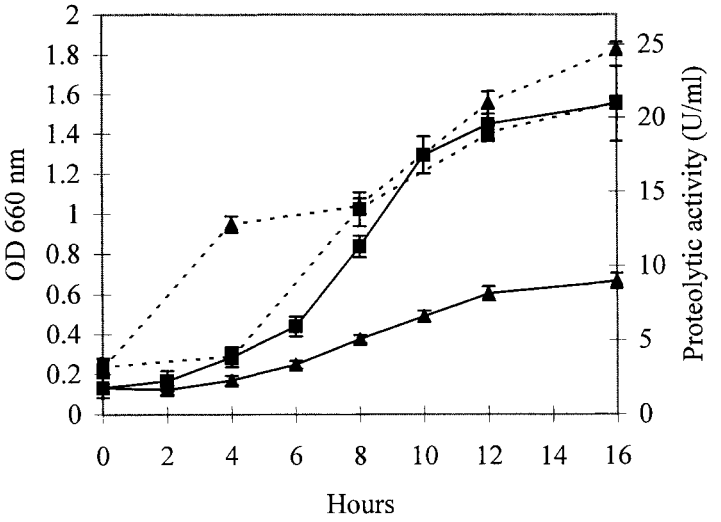

Figure 1. Growth (OD) of *P. ruminicola* 23 (-▲-) and S17/3 (-■-) and the endopeptidase activities (U·mL^{-1}) of *P. Ruminicola* 23 (-▲-) and S17/3 (-■-).

There was no significant change in the proportions of the various amino acids after incubation with strain 23 (*table I*). Incubation with the S17/3 strain changed the proportions of one hydrophobic amino acid, leucine, and of one hydrophilic amino acid, glutamic acid ($P < 0.1$). As this change in amino acid composition could have altered the hydrophobicity of the peptides, we examined reverse-phase HPLC during the growth stage of *P. ruminicola* S17/3 (*figure 5*). The zone corresponding to the most hydrophilic peptides changed. The areas under the peaks eluting at 8.9 and 11.7 min increased, whereas the areas under the peaks at 10.7 and 15.9 min decreased. These changes were small and there was no overall change in the hydrophilic or hydrophobic nature of the peptides during incubation.

4. DISCUSSION

4.1. Changes in the molecular weights of peptides and in endopeptidase and exopeptidase activities

The changes in the various peptide classes resulting from the hydrolysis and uptake of peptides by *P. ruminicola* differed with time and depended on the strain used. Compounds such as peptides and amino acids must be transported across the lipid bilayer by specialised proteins [23]. This transport partly depends on the molecular weight of the substrate. Westlake and Mackie [33] showed that the pentapeptide (LeuTryMetArgPhe) is transported intact into the intracellular medium, indicating that *Streptococcus bovis* has a mechanism for transporting 750 Da peptides. Others have demonstrated that *P. ruminicola* preferentially assimilates peptides in the range 250–1 650 Da [22] or 200–1 000 Da [18] and that peptides larger than four amino acids are used more efficiently than di- and tri-peptides or amino acids [21]. High molecular weight peptides can only be incorporated into bacterial cells after hydrolysis by enzymes. Consequently, the observed increase in the proportion of 2–3 kDa peptides was probably due to the hydrolysis of larger peptides (3–5 kDa). Unlike studies using radiolabelled peptides, HPSEC did not directly measure the uptake of peptides, but the results obtained do not seem to support the findings of Cooper and Ling [7], that high molecular weight peptides (45 amino acids) are incorporated into microbial cells faster than small peptides (seven amino

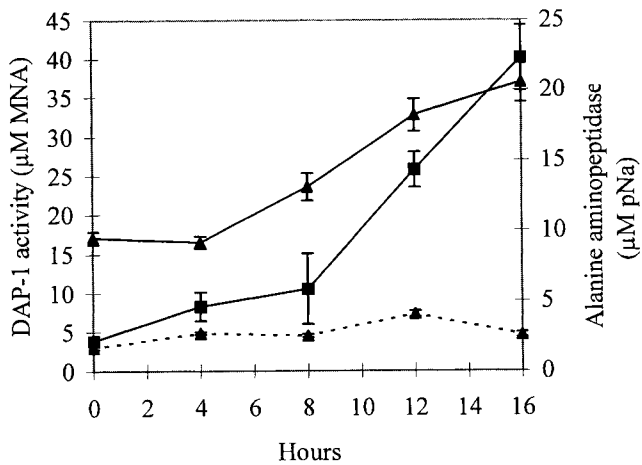

Figure 2. DAP-1 activities (µm MNA) of *P. ruminicola* 23 (-▲-) and S17/3 (-■-) and alanine aminopeptidase (µm pNa) activity of *P. ruminicola* 23 (-▲-).

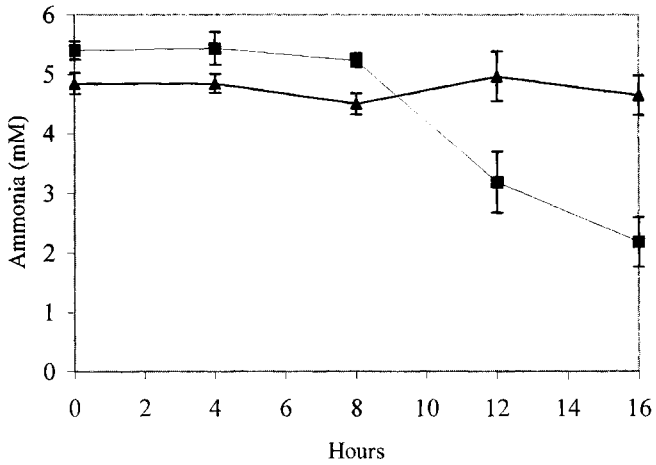

Figure 3. Changes in ammonia concentration (mM) with *P. ruminicola* 23 (-▲-) and S17/3 (-■-).

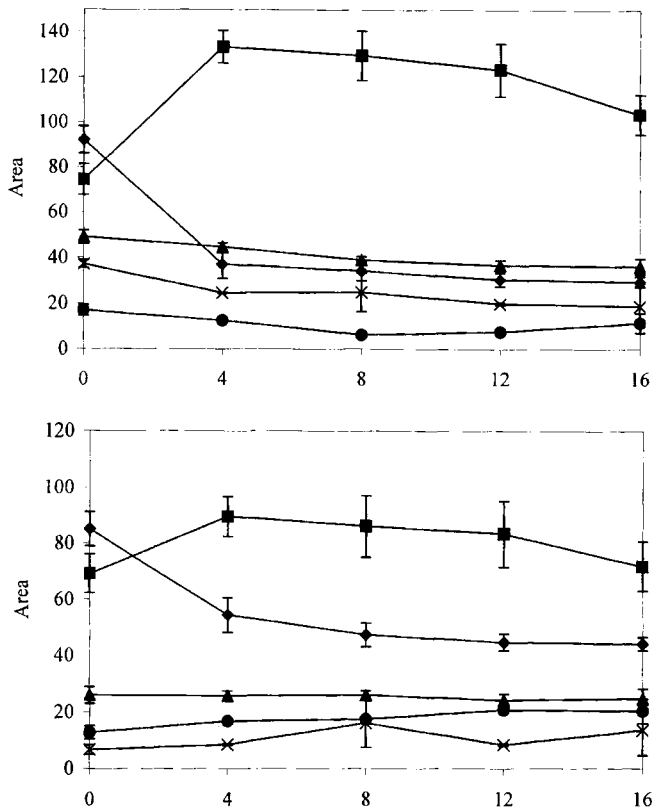

Figure 4. Changes in the areas of the peptide peaks (HPSEC) during incubation with *P. ruminicola* 23 (top) and S17/3 (bottom); -◆- : 3–5 kDa, -■- : 2–3 kDa, -▲-: 1–2 kDa, -●-: 0.5 kDa, -✕- < 0.5 kDa.

Table I. Amino acid composition (%) of degradation-resistant peptides.

		P. ruminicola 23		*P. ruminicola* S17/3.	
	0 h	8 h	16 h	8 h	16 h
ALA	4.0	4.2	4.2	4.0	4.2
GLY	2.7	2.8	2.8	2.7	2.3
VAL	6.0	6.4	6.3	6.1	5.7
LEU	8.7	9.1	7.6	8.8	7.2*
ILE	5.5	5.9	5.8	5.6	4.7
MET	3.9	3.9	3.8	4.0	3.4
PHE	6.8	7. 1	6.9	6.9	6.8
PRO	10.0	10.0	10.6	10.2	10.6
THR	4.7	4.6	4.7	4.8	5.0
SER	6.7	6.4	6.8	6.8	6.9
GLU	18.0	15.8	17.0	17.6	19.0
ASP	6.8	6.7	7.2	6.9	7.2
LYS	13.3	14.2	13.2	13.0	14.1
TYR	2.9	3.1	3.0	2.9	3.1

* $P < 0.10$ determined by the Kruskal-Wallis test.

acids), or those of Armstead and Ling [2], that four fractions of ^{14}C-peptides (1 000–2 000, 500–1 000, 200–500, < 200) were all assimilated similarly by an inoculum of rumen bacteria.

The temporal changes in the peptide fractions assimilable by bacteria (0.5–1 kDa and < 0.5 kDa) showed that they depended on the bacterial strain. The increase of these peptide fractions when incubated with strain S17/3 agrees with the results of Wright [34], who showed that peptides from a trypsin digest of *Chlorella* proteins labelled with ^{14}C were transformed into lower molecular weight compounds. Depardon et al. [11, 12] also showed that bacterial growth depended on the proportions of soya peptides and tryptone that were directly assimilable by the bacteria. However, incubation with *P. ruminicola* 23 produced no increase in small peptides.

The nature of the enzyme activities and the use of other nitrogen sources may explain the differences between the two strains. *P. ruminicola* can use both ammonia and peptides as a nitrogen source [21]. The great decrease in the ammonia concentration with *P. ruminicola* S17/3 shows that this compound provided much of the nitrogen required by this strain, leading to an accumulation of small peptides. In contrast, there was no peptide accumulation with strain 23. The enzyme activities could also provide an explanation. Both strains produced DAP-1 and neither had any leucine aminopeptidase activity, in agreement with McKain et al. [19]. But strain 23 had less DAP-1 activity than strain S17/3, so that more dipeptides could have been produced, leading to the accumulation of small peptides < 0.5 kDa. Other exopeptidases could also have hydrolysed the tryptone. Wallace et al. [32] recently showed that *P. ruminicola* produces four distinct dipeptidyl peptidases.

4.2. Hydrophobicity and amino acid composition

RP-HPLC analysis of the peptides not assimilated by *P. ruminicola* S17/3 showed

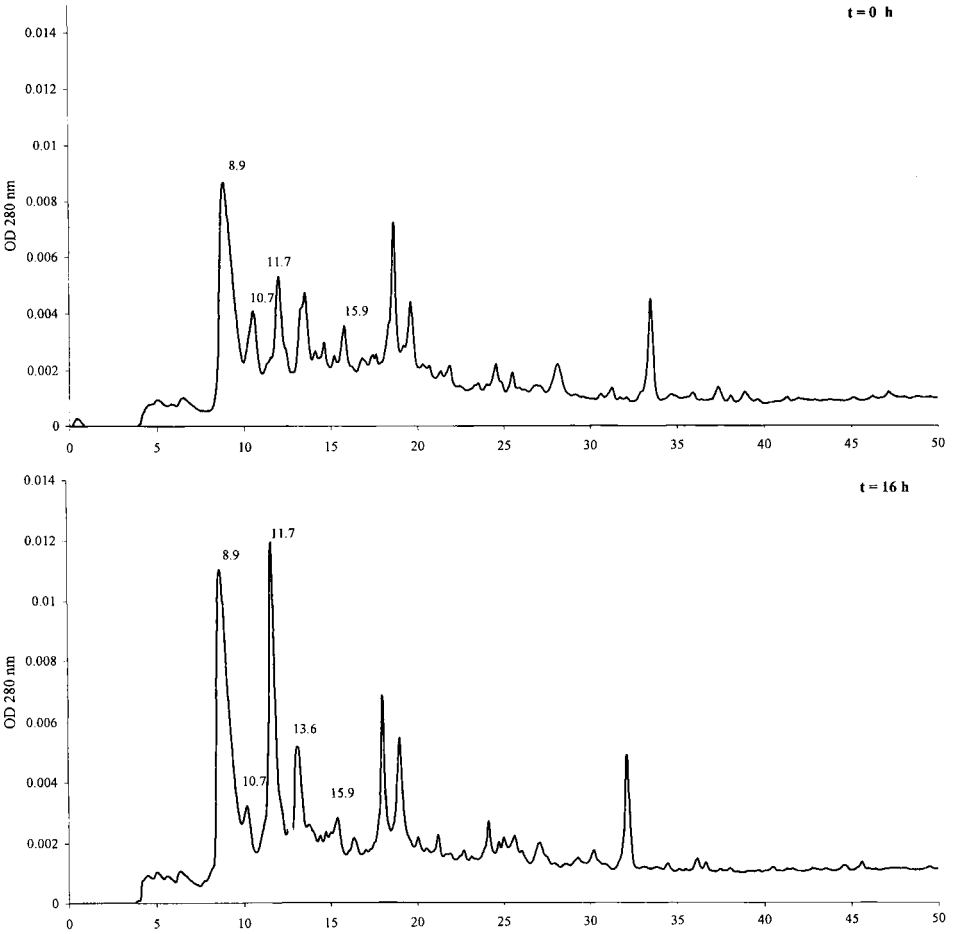

Figure 5. Reverse-phase HPLC of peptides in early (0 h) and late (16 h) incubation with *P. ruminicola* S17/3.

no evidence of an increase in hydrophobic peptides in the medium. This contradicts the results of Chen et al. [5], who found evidence for a faster breakdown of hydrophilic peptides by *P. ruminicola* ($B_1$4). HPLC analyses have also shown that hydrophilic peptides are preferentially assimilated by rumen micro-organisms [6], but some hydrophobic peptides were also found hydrolysed. Depardon et al. [11, 12] found no evidence for faster breakdown of hydrophilic peptides than of hydrophobic ones, regardless of the peptide substrate used (tryptone or soya peptides). These contradictory results could be partly due to methodological differences. As Wallace [26] has shown, the inorganic substances and volatile fatty acids in the culture medium absorb strongly at wavelengths below 240 nm. The chromatograms obtained at 215 nm could therefore include non-peptide compounds. Depardon et al. [11, 12] detected peptides by their absorption at 280 nm, so that only peptides containing aromatic amino acids were detected. RP-HPLC may therefore be insufficient for determining the hydrophobicity of peptides,

which can also be determined from the amino acid composition of the peptides.

The amino acid composition data agreed with those obtained by reverse phase HPLC. The ratio between hydrophilic and hydrophobic amino acids changed very little with time. But the decrease in the peptide concentration during the growth phase of *P. ruminicola* may have made it impossible to detect any significant change in the hydrophobicity of peptides or in the amino acid composition. However, Wallace et al. [29] stated that hydrophobicity alone cannot explain the resistance of a peptide to breakdown. For example, tetrapeptides containing phenylalanine are hydrolysed faster than neutral or hydrophilic peptides.

The enrichment in glutamic acid of peptides hydrolysed by *P. ruminicola* S17/3 corroborates the results of Yang and Russell, and those of Wallace et al. [29], who showed that peptides containing amino acids with acid groups are broken down more slowly than basic or neutral amino acids. Similarly, the decrease in the proportion of Leu agrees with observations made in the rumen [31].

This study and our previous findings [10, 12] indicate that the peptide residue is not enriched in proline. In this respect they disagree with the results of Wallace et al. [30] and Broderick et al. [3], who found that proline (in C-terminal position) decreased the assimilation of dipeptides by rumen bacteria. Nevertheless, a peptide of any given amino acid composition is readily hydrolysed if the proline is in the N-terminal position [35]. For example, the percentage hydrolysis of ValPro is $0.050 \cdot h^{-1}$ while that of ProVal is $0.114 \cdot h^{-1}$. Thus, the resistance of a dipeptide to degradation seems to depend on the presence of proline and also on the amino acid sequence. Wallace et al. [30] showed that the tetrapeptide GlyHisArgPro was more readily hydrolysed than tetrapeptides which did not contain proline. Thus, the inhibiting effect no longer seems to operate for peptides containing more than two amino acids or when the proline is C-terminal. The inhibiting effect of proline therefore depends on structure and size of the peptide.

We used peptides of various sizes which were hydrolysed by various proteolytic enzymes acting together. The fact that a bacterium can transport peptides containing up to five amino acids across its cell wall [20, 33], combined with the random nature of enzyme action, makes the production of dipeptides with proline in the C-terminal position unlikely. This may partly explain the differences from the other studies cited, which found that this amino acid was inhibitory.

These results show that the amino acid composition of peptides hydrolysed by *P. ruminicola* varied little with time. This is in contrast to the in vivo findings in the rumen [31] or in vitro experiments [35] carried out under very different conditions. However, the hydrolysis of the same substrate under similar conditions by a mixed rumen inoculum indicated an enrichment in Val and a decrease in the proportions of Leu, Phe and Glu [12]. Although *P. ruminicola* is considered to be one of the most important proteolytic bacteria in the rumen its activity cannot alone explain the breakdown of peptides in the rumen. A better understanding of the processes at work could be obtained by using mixed cultures, combining *P. ruminicola* with other proteolytic bacteria.

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