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Original article

Variation of proteolytic activity in ewe during in vivo digestion of two pea-based diets differing by their fermentability characteristics

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Abstract — In order to study the effects of a small difference in starch and nitrogen availability on proteolysis, two different diets were supplied to four ewes fitted with rumen fistulae. They differed in the ratio of fermentable nitrogen over fermentable energy, with 144 g of fermentable nitrogen (FN) per kg of fermentable energy (FE) for diet I and 126 g FN·kg^{-1} FE for diet II. The diets were constituted of 700 g hay grass, 200 g ground pea and either 100 g ground wheat (diet I) or 100 g corn starch (diet II). After two weeks of an adapting period to the diets, rumen content was sampled after feeding over time. The rate of disappearance of soluble proteins was 2.5 times higher with diet II and ammonia concentrations were significantly lower (from –28 to –43%) with diet II. Total proteolytic activity, by considering all the bacterial compartments, was significantly higher with diet II (+40 EU/mL·h^{-1}): changes in the total proteolytic activity in the particulate and the liquid phases of the rumen could explain the difference observed between the two diets. Moreover, with diet II, exopeptidase activities increased more in the liquid phase, especially leucine aminopeptidase and Dipeptidyl peptidase I (DPP-I), and the diversity of endopeptidase activities increased in the particulate phase. These two facts could account for the higher total proteolytic activity in the rumen content with diet II.

sheep / rumen / proteolysis / bacteria

Résumé — Variation de l'activité protéolytique au cours de la digestion in vivo de 2 régimes à base de pois chez les brebis. Deux régimes différant par leur composition en azote fermentescible par kg d'énergie fermentescible (régime I : 144 g AF·kg^{-1} EF ; régime II : 126 g AF·kg^{-1} EF), ont été fournis à 4 brebis fistulées à l'entretien. Ils étaient constitués de 700 g de foin, 200 g de pois broyé et de soit 100 g de blé broyé (régime I) soit 100 g d'amidon de maïs (régime II). Après deux semaines d'adaptation à chacun des régimes, du contenu ruminal a été prélevé en cinétique après la prise du repas. La vitesse de disparition des protéines solubles a été 2,5 fois supérieure avec le régime II et les

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concentrations en ammoniac plus faibles (de –28 à –43 %). L'activité protéolytique totale, en considérant l'ensemble des compartiments bactériens, a été significativement supérieure avec le régime II (+40 UE/mL·h^{-1}) : l'évolution de l'activité protéolytique totale des phases particulaire et liquide du rumen pourrait expliquer les différences observées entre les deux régimes. De plus, avec le régime II, les activités exopeptidasiques ont davantage augmenté dans la phase liquide, et plus particulièrement la leucine aminopeptidase et la Dipeptidyl Peptidase I (DPP-I), et la diversité des activités endopeptidasiques a augmenté dans la phase particulaire. Ces deux événements pourraient expliquer l'augmentation de l'activité protéolytique totale du contenu ruminal avec le régime II.

ovin / rumen / protéolyse / bactérie

1. INTRODUCTION

The composition of the diet has repercussions on the proteolytic activity in the rumen [25, 30]. Fundamentally different diets have often been studied in vivo: fresh fodder results in ruminal proteolytic activity nine times higher than that resulting from dried fodder, due in particular to the higher soluble protein content of fresh fodder [14, 21, 32]. Cereal-based diets induce higher proteolytic activities than those obtained with fodder [25]. However, studies with neither of these types of diet provide insight into the enzymatic processes involved in the ruminant in vivo. Ruminal proteolysis involves a number of different enzymes (endopeptidases, exopeptidases) at various steps in protein degradation [6], and many in vitro studies on bacteria have focused on the characterisation of these enzymes [3, 31]. In addition, although 75 to 95% of total ruminal proteolytic activity is associated with the particulate fraction [4, 8, 19], several major proteolytic species, like *P. ruminicola* and *S. bovis* are associated with the liquid phase [31]. Since the nature of the protein has some effects on proteolytic activity we chose to constitute the diets with a source of protein rather resistant to bacterial enzymatic activities, that is peas (*Pisum sativum* t.) [22] in order to favour effects on proteolysis. Moreover, the use of peas in ruminant feed is currently limited by a lack of information concerning their nutritive value. The digestibility of pea is also often considered to be highly variable [12].

We investigated the differences in total proteolytic activity in sheep fed two different diets containing pea and only varying by their ratio of fermentable nitrogen over fermentable energy, and tried to identify the ruminal compartments involved in the observed differences to get more information than those previously published on the in vivo bacterial scale [16, 27].

2. MATERIALS AND METHODS

2.1. Animals, diets and sample preparation

Rumen contents were obtained from four ewes fitted with rumen fistulae. Two different diets were supplied in a two stage procedure when each diet was given to every animal simultaneously. They differed in the ratio of fermentable nitrogen over fermentable energy (FN·kg^{-1} FE), determined according to the French system based on the calculation of IDP (Intestinal Digestible Protein). Fermentable organic matter and degradable nitrogen were calculated using nutritive values of feeds given in tables indicating IDP [1]. The following diets were distributed twice daily (07 a.m. and 05 p.m.):

– diet I consisted of 700 g of grass hay, 200 g of ground peas and 100 g of coarsely ground wheat, giving 144 g FN·kg^{-1} FE;

Table I. Chemical composition of the diets.

	Dry matter (DM)	DM constituents (%)		
	(%)	ashes	crude protein	fiber (NDF)
Grass hay	90.8	6.4	11.6	60.3
Peas	86.1	3.3	23.7	15.5
Wheat	86.3	2.5	14.2	13.0
Corn starch	98.0	0	0	0

– diet II consisted of the same base diet, except that the wheat was replaced by 100 g of corn starch, giving 126 g FN·kg^{-1} FE.

The chemical composition of the two diets is presented in Table I.

Animals were allowed to adapt to the diet for two weeks before rumen content sampling. Rumen juice (150 mL) was removed in anaerobic conditions before feeding and 30 min, 1 h 30, 2 h 30, 4 h and 6 hours after feeding. Each sample was fractionated into three compartments:

– the liquid phase was obtained from 20 mL of rumen juice by centrifugation at high speed to harvest particles, protozoa and bacteria (6000 *g*, 20 min, 4 °C) [10];

– the large particles were removed from 100 mL of rumen juice by filtration with a sieve (0.2 mm pores) and low-speed centrifugation (500 *g*, 5 min, 4 °C) [28] was then used to separate small particles (at the top of the pellet), protozoa (at the bottom of the pellet) and free bacteria (supernatant). Protozoa were discarded and small particles were resuspended in NaCl (9 g·L^{-1}). The suspension was then subjected to a second low-speed centrifugation: the pellet, containing bacteria associated with small particles, was resuspended in 0.1 M phosphate buffer (1/10 of the initial volume). The supernatant was pooled with the supernatant from the first low-speed centrifugation. The pooled supernatant was subjected to high-speed centrifugation (6000 *g*, 20 min, 4 °C) to harvest the free bacteria present in the liquid phase of the rumen, which constituted the third compartment. The pellet was resuspended in 0.1 M phosphate buffer. The different steps of the fractionation are summarised in Figure 1.

2.2. Analysis

2.2.1. Fermentation parameters

pH was measured with an Orion pH meter, (420A model), directly on rumen content. The concentrations of volatile fatty acids (VFA) were determined by gas chromatography (Delsi Nermag), as previously described [9]. The concentration of lactate was determined with a lactic acid kit (Boehringer-Mannheim, Roche). These concentrations were all measured in the liquid phase.

2.2.2. Proteolytic activities

Total proteolytic activity, endopeptidase (serine, cysteine, metallo and aspartate proteinases) and exopeptidase (dipeptidylaminopeptidase DPP I, leucine and alanine aminopeptidases) activities, were all measured in the three compartments defined above.

To determine total proteolytic activity, we assessed azocasein (Sigma) degradation [4]. One enzymatic unit (EU) corresponds to a change in OD_{450} of 0.01·h^{-1} due to azocasein hydrolysis. DPP-I activity was determined using Gly-Arg-4-methoxy-2-naphthylamide (Gly-Arg MNA) as a fluorogening

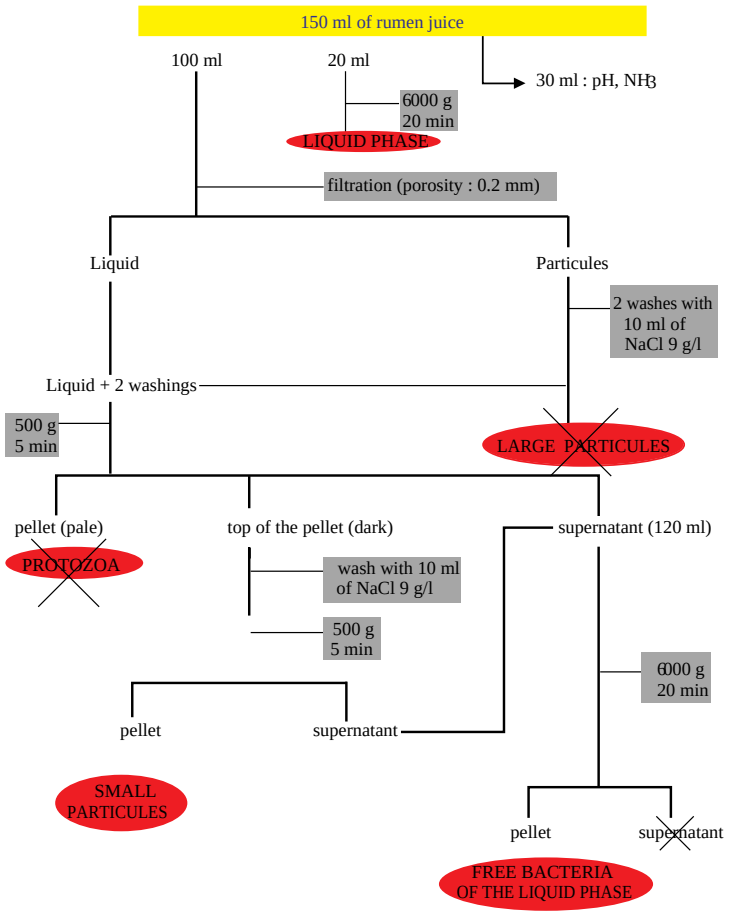


Figure 1. Fractionation of the rumen content.

substrate [33] with incubation for 6 h at 39 °C under anaerobic conditions. MNA was determined by its fluorescence (excitation: 350 nm, emission: 420 nm). Leucine and alanine aminopeptidase activities were measured using ala-p-nitroanilide (Ala-pNA) and leu-p-nitroanilide (Leu-pNA) as chromogenic substrates [33], with incubation for 4 h at 39 °C. The pNA released was assayed by diazotisation [2]. All substrates and standards were obtained from Sigma Chemicals (France). Proteinase inhibitors were used to determine the distribution of endopeptidases, as previously described [4]. Phenylmethylsulfonyl fluoride (PMSF), p-chloromercuribenzoate (pCMB), ethylenediamine tetracetic acid (EDTA) and pepstatin A were used to inhibit serine, cysteine, metallo and aspartate proteinases respectively. For each enzyme, inhibition is expressed relative to total cumulative inhibition.

2.2.3. Products of proteolysis

Residual quantities of proteins dissolved in the liquid phase were monitored. After protein precipitation by a method described

elsewhere [24], the protein content of the pellet was determined by the Bradford method (BioRad kit).

Derivatisation of free amino acids (AA) by the method of Husek [15], as modified by others [11], made it possible to determine the free AA in the supernatant by GPC (capillary column OV1701, Interchrom). Acid hydrolysis was performed to determine the AA originating from peptides, differentiating these AA from the free AA. Acid hydrolysis was performed by incubation with 12 N HCl for 22 h at 110 °C. After hydrolysis, samples were filtered (0.2 µm pores) and the acid was evaporated under vacuum at 70 °C. Samples were resuspended in 0.5 mL of 0.1 M HCl. The concentration of ammonia was determined with an ammonia-sensing electrode (Orion Research, mode 95–12).

2.2.4. Statistical treatment

The two diets were each fed to four sheep and the diets were then compared by analysis of variance with repeated measures, using the MIXED procedure of the SAS package (SAS 6.12 version, 1997). The variance structure between the various sampling times was classified as auto-regressive according to Alaike and Schwarz-Bayesian criteria [17]. The various treatments were compared using the least mean square method.

3. RESULTS

3.1. Fermentation parameters

No significant difference ($P > 0.05$) in pH was observed between the two diets. It varied between 6.7 to 6 for both diets. Similarly, changes in VFA concentration were not significantly different ($P > 0.05$) between the diets (Fig. 2). In contrast, lactic acid concentration was significantly higher for diet II ($P < 0.05$) and remained stable, at around 4 mM after the animal had finished eating (Fig. 2).

3.2. Catabolism of proteins

During digestion, the level of hydrolysis of soluble proteins was higher with diet II than with diet I (Fig. 3). The peak concentration observed 30 min after feeding corresponds to the time at which the sheep finished ingesting their food. The difference between the diets was significant ($P < 0.05$) for all time points and was greatest during the hour after the animal had finished eating: the rate of disappearance of soluble proteins was 2.5 times higher with diet II (diet I: 0.17 $g{\cdot}L^{-1}{\cdot}h^{-1}$; diet II: 0.43 $g{\cdot}L^{-1}{\cdot}h^{-1}$).

No free AA or peptide AA were detected during the 6 h of the time course study for either of the diets tested.

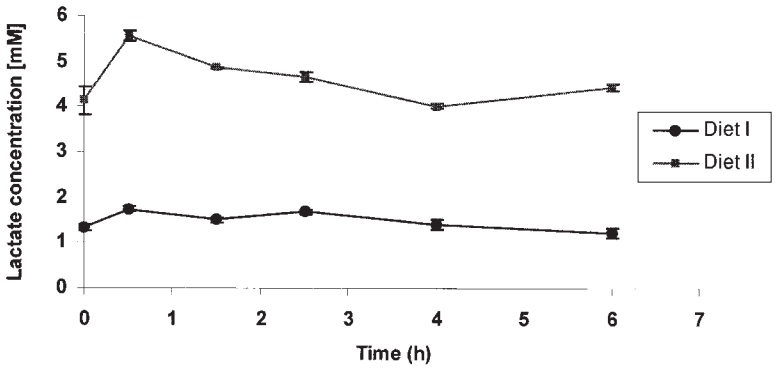


Figure 2. Changes in lactate concentrations (mM) in sheep fed two different diets ($n = 4$ sheep).

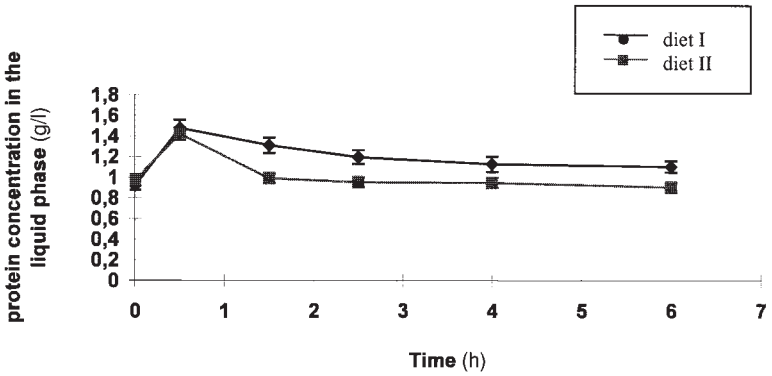


Figure 3 . Changes in soluble protein concentration in the rumen liquid phase (n = 4 sheep).

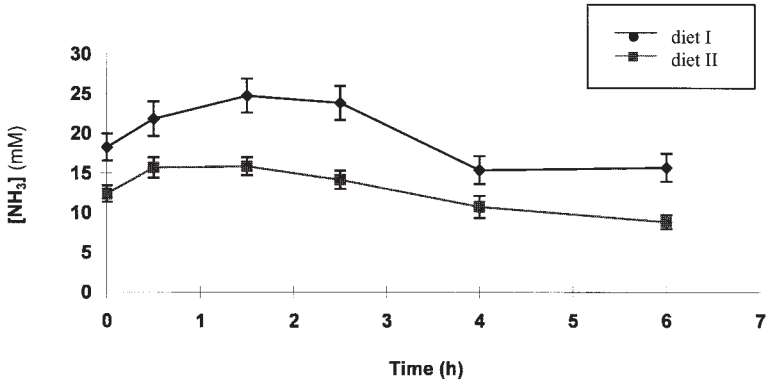


Figure 4. Changes in ammonia concentration in the liquid phase of the rumen (n = 4 sheep).

Ammonia concentration (Fig. 4) increased and reached a maximum around 1 h 30 to 2 h after the animals had finished eating for both diets. A difference ($P < 0.05$) was observed between diets at all points in the time course and this difference varied significantly, from 28 to 43%, according to the time point considered: ammonia concentrations were significantly higher with diet I.

3.3. Proteolytic activities

3.3.1. Total proteolytic activity

Total proteolytic activity was mainly associated with particles, with 70 to 75% of proteolytic activity associated with particles for both diets (Figs. 5a and 5b). The level of proteolytic activity in the liquid phase was lower, from 16 to 26%. Free bacteria in the liquid phase were responsible for only low levels of activity, accounting for 5 to 7% of total proteolytic activity.

Very few changes over time in proteolytic activity associated with free bacteria were detected since a major time × treatment interaction was observed at almost all time points ($P < 0.05$). In contrast, the changes in proteolytic activities over time in the particulate and liquid phases differed significantly between diets ($P < 0.05$). With diet I, the proteolytic activity of the

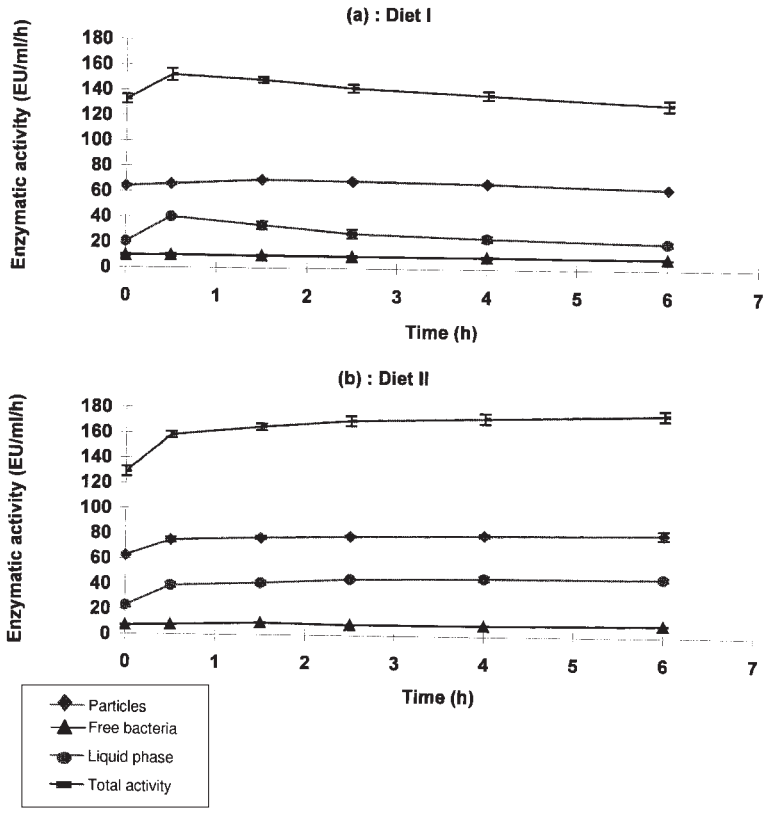


Figure 5. Changes in total proteolytic activity in various rumen compartments (n = 4 sheep).

particulate compartment varied little over time (Fig. 6a), whereas it clearly increased after feeding with diet II (Fig. 5b). The proteolytic activity of the liquid phase was stimulated with both diets after feeding, but decreased thereafter with diet I (Fig. 5a) whereas it remained at a constant level with diet II (Fig. 6b). Total proteolytic activity, calculated by summing the activities in the three compartments, was significantly higher ($P < 0.05$) with diet II (+40 EU·mL^{-1}·h^{-1}).

3.3.2. Exopeptidases

With diet I, we observed a decrease in the activity of the three kinds of exopeptidase tested in the liquid phase, from 30 min or 1 h 30 after feeding to 6 h (Figs. 6a, 7a, 8a). Focusing on the exopeptidases of the particulate phase, we noted an increase in leu aminopeptidase activity, with less clear-cut changes for the other two exopeptidases. With diet II, we observed stimulation of DPP I in the liquid phase after 30 min (Fig. 6b) and the other two exopeptidases displayed a large peak in activity, followed by a decrease to the initial state (Figs. 7b, 8b). In the particulate compartment, the three activities decreased immediately after feeding and then gradually increased.

The difference between diets in the pattern of DPP I activity in the liquid phase was statistically significant ($P < 0.05$), whereas there was a time × treatment interaction for the ala and the leu aminopeptidases. The pattern of activity associated with

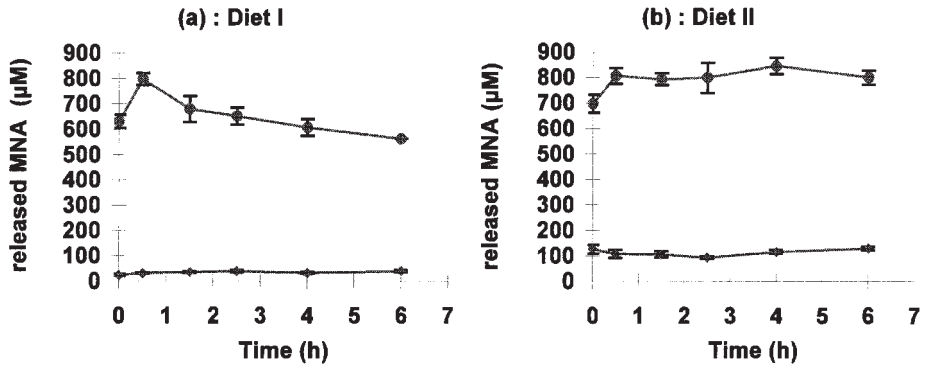


Figure 6. Changes in DPP I activity in the liquid and particulate phases of the rumen (n = 4 sheep).

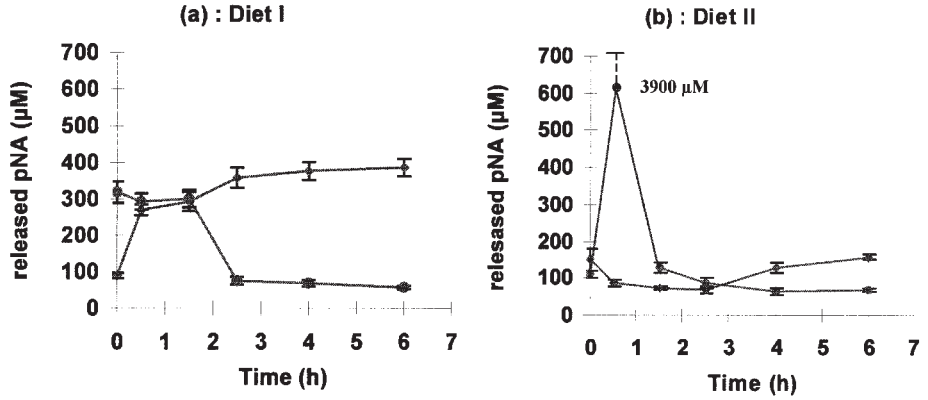


Figure 7. Changes in Leucine Aminopeptidase activity in the liquid and particulate phases of the rumen (n = 4 sheep).

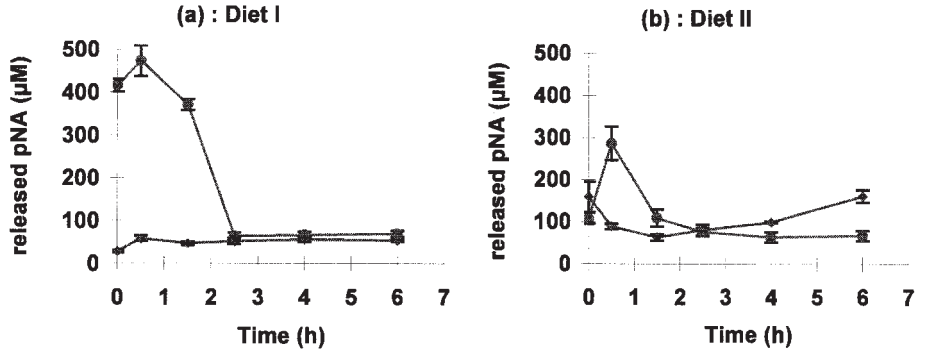


Figure 8. Changes in Alanine Aminopeptidase activity in the liquid and particulate phases of the rumen (n = 4 sheep).

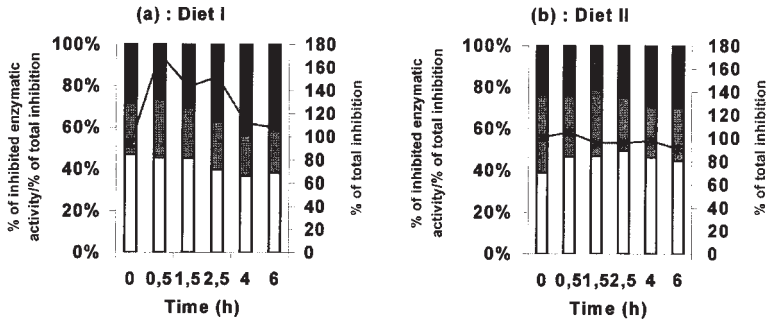


Figure 9. Changes in endopeptidase activities in the liquid phase of the rumen (n = 4 sheep).

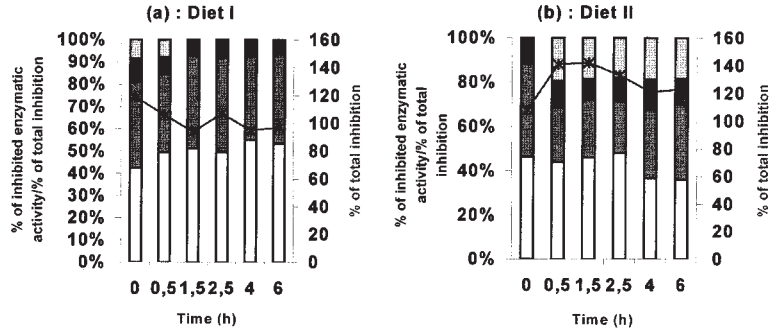
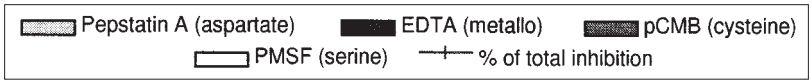


Figure 10. Changes in endopeptidase activities in the particulate phase of the rumen (n = 4 sheep).



particles differed significantly ($P < 0.05$) between diets for DPP I and leu aminopeptidase, and there was a time × treatment interaction for the ala aminopeptidase.

3.3.3. Endopeptidases

With diet I, the proportion of metalloproteinase activity in the liquid phase increased (+15 points) from 1 h 30 after feeding whereas the proportions of cysteine and serine proteinase activity decreased (–5 and –10 points respectively) (Fig. 9a). In the particulate compartment, aspartate proteinase activity disappeared (–7 points) and the proportion of serine proteinase activity increased (+9 points) (Fig. 10a). With diet II, the proportions of serine and metallo proteinase activity increased (+6 and +4 points respectively) whereas the proportion of cysteine proteinase activity decreased (–8 points) (Fig. 9b). Concerning the endopeptidases associated with particles, aspartate proteinase activity became detectable (+19 points) whereas the proportion of serine proteinase activity decreased (–16 points) (Fig. 10b).

There was a significant difference ($P < 0.05$) between diets in the pattern of metallo and aspartate proteinase activity in the liquid phase. The pattern of serine and cysteine proteinase activity also differed significantly between diets but only until t_{4h}. Thereafter, there was a time × treatment interaction. Statistical analysis gave similar results for the particulate phase, except for changes in the activity of cysteine proteinases, which did not differ significantly between diets.

4. DISCUSSION

The modification of diets that consisted in replacing wheat by starch simultaneously induced a decrease in ammonia concentration and an increase in proteolytic activity. The discussion focuses on these phenomena and tries to explain how the decrease of the ratio of fermentable nitrogen over fermentable energy induced the stimulation of the proteolysis.

The difference in ammonia concentration between diets is probably due to the replacement of wheat by starch in diet II. This led to a slight decrease in protein intake ($-8\ g \cdot d^{-1}$) but the impact of this factor was probably of minor importance since the difference in protein content was low. It also led to an increase in the level of starch intake ($+35\ g \cdot d^{-1}$). Some authors [26] have shown that the addition of starch to a cellulose-based diet causes an increased nitrogen utilisation in the rumen. This efficiency of starch valorisation was linked by the authors to the energetic yield of microbial fermentation. Starch is indeed the carbohydrate that provides the most energy for rumen bacteria [18]. Moreover, as the availability of fermentable carbohydrate increases, so ammonia concentration decreases [23]: this phenomenon is due to an increase in the amount of amino nitrogen directly incorporated into bacterial proteins. These data have been confirmed by others [29], who suggest that the catabolism of proteins would cause excessive ammonia production when energy is no longer available. The hydrolysis of nitrogen compounds would then provide energy for the maintenance of micro-organisms and would not be used as a nitrogen source for microbial growth.

The faster disappearance of dissolved proteins with diet II was sustained by stimulation of the total proteolytic activity of the rumen contents as a whole. There are two possible reasons for this:

– the first is based on the works of Wallace and Brammall [31], who suggested that the effects of diet on proteolytic activity are probably not due to the inhibition/stimulation of enzymatic activity, but instead, to differences in the size and composition of microbial populations. The proteins of these two diets are not easily fermentable: pea proteins consist of 60 to 90% globulins [13] and wheat proteins of 30 to 40% glutelins plus 40 to 50% prolamins, with the association of these two types of protein giving rise to gluten, which has a highly branched structure. This may explain why peptides and amino acids did not accumulate with either diet: Since dissolved proteins seemed to be more extensively used with diet II, we suggest that this diet had a positive effect on bacterial synthesis. Moreover, by replacing wheat by starch, amylolytic bacteria were probably stimulated. The large increase in leucine aminopeptidase activity in the liquid phase was specific to the exopeptidase activity of *Streptococcus bovis*, which is one of the most amylolytic rumen species [5]. Further evidence supporting this notion is provided by the concentration of lactic acid, which was higher with diet II. The increase of the degradable starch supply probably induced a stimulation of the amylolytic activity associated with the production of lactic acid. Since amylolytic bacteria are known to be highly proteolytic [7], this lactate increase has to be linked to a higher proteolytic activity. Thus, the higher level of proteolytic activity in sheep fed diet II may be due to an increase in the amylolytic population;

– a second possibility is that ammonia directly affects proteolytic activity as it has already been shown with pure cultures [24]. Protein catabolism is high at low concentrations of ammonia [20]. Other studies have shown inhibition of proteolytic activity when protein concentration is high and have focused on the possible effect of the products of protein breakdown, such as ammonia, on proteolysis. Authors [29] have shown a decrease in casein degradation by bacteria from the liquid phase when ammonia concentration is increased from 0–14 mM to

19–47 mM. This may explain why proteolytic activities were the lowest with diet I, in both the liquid and particulate phases, when the concentration of ammonia in the rumen was high. Changes in the enzymatic apparatus may be indicators of an effect of ammonia. With diet I, the decrease in proteolytic activity in the liquid phase was at least partly due to a decrease in exopeptidase activities. The decrease in the diversity of endopeptidase activities may account for the decrease in proteolytic activity in the particulate phase. With diet II, the stimulation of the proteolytic activity observed in the liquid phase may be connected to an increase of the activity of the three exopeptidases. The increase in total proteolytic activity in the particulate phase may be due to an increase in the diversity of endopeptidase activities.

5. CONCLUSION

Thus, the effect of ammonia is complex and differs according to the type of the proteolytic enzyme considered. However, this effect cannot be separated with the increase of the fermentable energy availability since it is known that its increase allows a better use of ammonia by bacteria. Different targets can be distinguished: exopeptidases in the liquid phase and endopeptidases in the particulate phase. Further studies are required, involving the stabilisation of energy provision and variation of the amount of ammonia.

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