Enzyme Activities Related to Protein Catabolism and Michaelis Constant Values of the Glutamate Dehydrogenase System in Sheep Rumen Bacteria and Protozoa: A Postprint

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Abstract

This study aimed to investigate the enzyme activities related to protein catabolism and the Michaelis constant (Km) values of the glutamate dehydrogenase system in sheep rumen bacteria and protozoa, to provide enzymological evidence for elucidating the characteristics of protein catabolism in these microorganisms. Six Chinese Merino (Xinjiang type) sheep approximately one year old [average body weight
(32.00$±1.36)kg], fittedwithpermanentrumenfistulas, werefedadietwithaconcentrate−
to − forageratioof30 : 70.Rumenfluidwascollectedatsixtimepoints :
beforefeeding(0h)andat1.5, 3.0, 6.0, 9.0, and12.0hafterfeeding, withthreereplicates.Bacterialandprotozoally
1)Theactivitiesofprotease, alanineaminotransferase, aspartateaminotransferase, andglutamatedehydroger
feeding; glutamateandammoniaconcentrationsalsodisplayedsimilarpatterns.Theactivitiesofthesefourenzy
0.01).2)Glutamateconcentrationinprotozoallysateswasextremelysignificantlyhigherthanthatinbacteriallysa
0.01); ammoniaconcentrationinprotozoallysateswassignificantlyorextremelysignificantlyhigherthanthatin
0.05orP < 0.01).3)TheKmvaluesofglutamatedehydrogenasefromsheeprumenbacteriaandprotozoafornicoti
7and1.48×10−7mol/L, respectively; theKmvaluesforglutamatewere8.41×10−
6and4.91×10−6mol/L, respectively; theKmvaluesforreducednicotinamideadeninedinucleotide(NADH)wer
8and2.70×$10-8 mol/L, respectively; the Km values for α-ketoglutarate were
1.16$×10−6and2.07×10−6mol/L, respectively; theKmvaluesforammoniawere2.97×10−
5and1.40×$10-5 mol/L, respectively. These results suggest that, overall, the activities of protease, glutamate dehydrogenase, alanine aminotransferase, and aspartate aminotransferase in sheep rumen bacteria and protozoa peaked at 1.5 h post-feeding and then gradually decreased; the activities of protease, alanine aminotransferase, aspartate aminotransferase, and glutamate dehydrogenase in sheep rumen protozoa were all extremely significantly higher than those in bacteria, indicating more vigorous protein catabolism in protozoa; glutamate

transamination mechanisms exist in rumen protozoa, and there may also be a mechanism for resynthesizing amino acids utilizing ammonia.

Full Text

Enzyme Activities Related to Protein Catabolism and Michaelis Constant Values of Glutamate Dehydrogenase System in Rumen Bacteria and Protozoa of Sheep

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Abstract

This study was conducted to determine enzyme activities related to protein catabolism and the Michaelis constant (Km) values of the glutamate dehydrogenase (GDH) system in rumen bacteria and protozoa of sheep, providing enzymological evidence to explain protein catabolism characteristics in these microorganisms. Six healthy Chinese Merino sheep (Xinjiang type) approximately one year old [average body weight (32.00±1.36) kg] were fitted with permanent rumen fistulas and fed a diet with a concentrate-to-forage ratio of 30:70. Rumen fluid was collected sequentially before feeding (0 h) and at 1.5, 3.0, 6.0, 9.0, and 12
(1) The activities of protease, glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT), and GDH in both bacterial and protozoal crushing liquids exhibited a dynamic pa
feeding. Glutamate and ammonia contents showed similar trends. The activities of these four enzymes involved in
0.01). (2) Glutamate content in protozoal crushing liquid was significantly higher than in bacteria ($P <$ 0.01). Ammonia content in protozoal crushing liquid was significantly or extremely significantly higher than in ba
0.05 or $P < 0.01$). (3) The Km values of GDH for NAD in bacterial and protozoal crushing liquids were 2.60×10^{-7} and 1.48×10^{-7} mol/L, respectively; for glutamate were 8.41×10^{-6} and 4.91×10^{-6} mol/L; for NADH were 3.80×10^{-8} and 2.70×10^{-8} mol/L; for α-ketoglutarate were 1.16×10^{-6} and 2.07×10^{-6} mol/L; and for ammonia were 2.97×10^{-5} and 1.40×10^{-5} mol/L, respectively. These results indicate that the activities of protease, GPT, GOT, and GDH in rumen bacteria and protozoa of sheep generally peak at 1.5 h after feeding and then gradually decrease. Protozoal activities of these enzymes are significantly higher than bacterial activities, suggesting more vigorous protein catabolism in protozoa. Additionally, rumen protozoa possess not only a glutamate transamination mechanism but also potentially a mechanism for reusing ammonia to synthesize amino acids.

Keywords: rumen; bacteria; protozoa; protein catabolism; enzyme activity; Km value

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Introduction

Understanding the mechanisms of feed protein degradation and utilization by rumen microorganisms is fundamental to regulating rumen nitrogen metabolism in ruminants. Elucidating the mechanisms of protein degradation and deamination in rumen protozoa and bacteria is a prerequisite for achieving rumen protein metabolism regulation. Protein is the primary nitrogen source in ruminant diets, and low nitrogen utilization efficiency in ruminants is largely attributed to significant nitrogen wastage during extensive dietary protein degradation in the rumen. Rumen microorganisms (mainly bacteria and protozoa) secrete enzymes that hydrolyze proteins into polypeptides, dipeptides, or tripeptides, which are further hydrolyzed into amino acids and ultimately fermented into ammonia nitrogen. Rumen protozoa have long been considered active participants in deamination and a major cause of inefficient rumen nitrogen metabolism. Studies have reported that rumen ciliates synthesize proteins depending on the bacteria they ingest, without utilizing ammonia (NH_3) as a nitrogen source. Research indicates that ammonia nitrogen concentration in the rumen with protozoa is twice that in defaunated rumen. Amino acid deaminase activity in protozoal cell extracts is at least three times higher than in bacteria. However, the reasons for higher ammonia production efficiency in rumen protozoa compared to bacteria remain unclear. Enzyme activities related to protein catabolism in rumen microorganisms are important factors affecting dietary protein degradation in ruminant livestock. This study investigated the dynamic changes and kinetic characteristics of four enzymes involved in protein catabolism within rumen bacterial and protozoal cells: protease (hydrolyzing proteins to peptides or amino acids), glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase (catalyzing amino transfer and amino acid formation), and glutamate dehydrogenase (GDH) [catalyzing amino acid deamination and ammonia (NH_4^+) utilization for amino acid synthesis], providing enzymological evidence for understanding protein catabolism characteristics in rumen bacteria and protozoa of sheep.

1.1 Experimental Animals

Six Chinese Merino sheep (Xinjiang type) fine-wool wethers approximately one year old, with an average body weight of (32.00$±$1.36) kg, were selected and surgically fitted with permanent rumen fistulas before the experiment. The sheep were fed a diet with a concentrate-to-forage ratio of 30:70, offered in two equal portions at 09:00 and 21:00 daily, with concentrate and roughage (corn stalks) mixed before feeding. Fresh water was available ad libitum. Diet composition and nutrient levels are presented in .

Composition and nutrient levels of the diet (DM basis) %

1.2 Sample Collection and Processing

Each sheep was sampled once per week for three weeks. Sampling time points were before feeding (0 h) and at 1.5, 3.0, 6.0, 9.0, and 12.0 h after feeding. Rumen fluid was collected using a 20-mesh nylon filter, with 120 mL collected per sheep at each time point. From this, 100 mL of rumen fluid was measured and pooled across all sheep, and 480 mL of the mixed fluid was used for separating rumen protozoa and bacteria and preparing cell-free extracts for enzyme activity determination.

1.3 Separation and Disruption of Rumen Bacteria and Protozoa

The pooled rumen fluid (480 mL) was centrifuged at 150×g for 10 min, the pellet was discarded, and the supernatant was centrifuged at 650×g for 20 min to separate bacteria and protozoa, yielding a protozoal pellet and bacterial supernatant.

The protozoal pellet was resuspended in 25 mL of ice-cold phosphate buffer and centrifuged at 4 °C, 650×g for 20 min (repeated twice). The bacterial supernatant was centrifuged at 10,000×g for 20 min, the supernatant was discarded, and the pellet was collected and resuspended in 50 mL of ice-cold phosphate buffer, then centrifuged at 4 °C, 10,000×g for 20 min (repeated twice). The final pellets were collected, dissolved in ice-cold physiological saline, adjusted to 40 mL, and homogenized. Disruption was performed using an ultrasonic disruptor (400 W, 15 min total, 3 s on/5 s off, 60 cycles, repeated three times). The crushing liquid was centrifuged at 4 °C, 10,000×g for 20 min, and the supernatant was collected and stored at -20 °C for determination of enzyme activities, Km values of the GDH system, and glutamate, ammonia, and protein contents.

1.4 Determination of Protein Content and Enzyme Activities

Protein content in bacterial and protozoal crushing liquids was determined using the method of Zhang et al. with bovine serum albumin as the standard. Enzyme activities of protease, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase, and glutamate dehydrogenase were determined using the method of Palmquist et al. A UV-Vis spectrophotometer (754PC) with quartz cuvettes (1.0 cm path length) and an SSW microcomputer-controlled thermostatic water bath (Shanghai Boxun Industrial Co., Ltd.) were used. NADH and NAD were Sigma products, and all other reagents were analytical grade.

1.5 Determination of Km Values of Glutamate Dehydrogenase in Rumen Bacteria and Protozoa

The deamination reaction catalyzed by GDH is: Glutamate + NAD → α-ketoglutarate + NADH + Ammonia. This study determined the Km values for five substrates in the reaction.

For Km determination, substrate concentrations were: glutamate at 2.5, 5.0, 10.0, 20.0, and 40.0 mmol/L; NAD at 0.05, 0.10, 0.50, 1.00, 2.00, and 4.00 mmol/L; NADH at 0.157, 0.313, 0.625, 0.250, and 0.500 mmol/L; α-ketoglutarate at 1.25, 2.50, 5.00, 10.00, and 20.00 mmol/L; and ammonia at 10, 20, 40, 80, and 160 mmol/L. Each substrate concentration was assayed following the same procedure as for GDH activity determination. Initial reaction velocities were measured, and Lineweaver-Burk plots were constructed by taking the double reciprocal of the Michaelis-Menten equation: $1/v = Km/vmax \cdot 1/[S] + 1/vmax$, where v is initial reaction velocity (mol/min), [S] is substrate concentration (mol/L), and vmax is maximum reaction velocity (mol/min). The x-intercept equals -1/Km and the y-intercept equals 1/vmax, allowing graphical determination of vmax and Km values.

1.6 Determination of Glutamate and Ammonia Contents in Rumen Bacteria and Protozoa

Glutamate and ammonia contents were determined enzymatically using the method of Guilbault. For glutamate determination, standard solutions with final concentrations of 0, 10, 25, 50, 100, and 200 mmol/L were prepared with NAD at 2 mmol/L final concentration and 100 U GDH in a total reaction volume of 3 mL. After mixing and incubating at 37 °C for 40 min, absorbance at 340 nm was measured using a UV-Vis spectrophotometer (754PC) to establish a standard curve. Samples were adjusted to fall within the standard concentration range and analyzed similarly. For ammonia determination, standard solutions with final concentrations of 0, 1, 2, 4, 7, and 10 mmol/L were prepared with NADH at 4 mmol/L final concentration and 100 U GDH in a total reaction volume of 3 mL, followed by the same procedure.

1.7 Data Processing

Experimental data are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS 16.0 software for one-way ANOVA, with Duncan' s multiple comparison for means and independent samples t-test for bacteria versus protozoa comparisons.

2.1 Dynamic Changes of Key Enzyme Activities Related to Protein Catabolism in Rumen Bacteria and Protozoa of Sheep

As shown in , the activities of protease, GPT, GOT, and GDH in both bacterial and protozoal crushing liquids exhibited a "low-high-low" dynamic pattern over feeding time, peaking at 1.5 h post-feeding (except for bacterial GPT) and returning to pre-feeding levels by 12.0 h. Protozoal protease activity was nearly 50% higher than bacterial activity at each time point ($P<0.01$), while protozoal GPT, GOT, and GDH activities were approximately double those of bacteria ($P<0.01$).

Activities of enzymes related to protein catabolism in rumen bacteria and protozoa crushing liquid of sheep

2.2 Dynamic Changes of Glutamate and Ammonia Contents in Rumen Bacterial and Protozoal Crushing Liquids

As shown in , glutamate contents in both bacterial and protozoal crushing liquids increased initially then decreased over feeding time, reaching maximum values at 1.5 h post-feeding. Protozoal glutamate content was significantly higher than bacterial content at all time points ($P<0.01$). Ammonia contents in both bacterial and protozoal crushing liquids showed similar dynamic patterns, peaking at 1.5 h post-feeding. Protozoal ammonia content was significantly higher than bacterial content at 1.5 h ($P<0.01$) and significantly higher at 6.0, 9.0, and 12.0 h ($P<0.05$).

Contents of glutamate and NH_4^+ in rumen bacteria and protozoa crushing liquid of sheep

2.3 Comparison of Km Values of Glutamate Dehydrogenase in Rumen Bacteria and Protozoa of Sheep

Lineweaver-Burk plots are presented in [Figure 1: see original paper] and [Figure 2: see original paper]. Calculated Km and vmax values are shown in . For GDH with NAD^+, bacterial Km was 2.60$×10^{-7}$ mol/L with vmax of 2.17$×10^{-4}$ mol/(L·min), while protozoal Km was 1.48$×10^{-7}$ mol/L with vmax of 6.70$×10^{-5}$ mol/(L·min), indicating higher protozoal affinity. For glutamate, bacterial Km was 8.41$×10^{-6}$ mol/L (vmax 1.35$×10^{-4}$ mol/(L·min)) and protozoal Km was 4.91$×10^{-6}$ mol/L (vmax 1.79$×10^{-4}$ mol/(L·min)). For NADH, bacterial Km was 3.80$×10^{-8}$ mol/L (vmax 1.89$×10^{-4}$ mol/(L·min)) and protozoal Km was 2.70$×10^{-8}$ mol/L (vmax 2.73$×10^{-4}$ mol/(L·min)). For α-ketoglutarate, bacterial Km was 1.16$×10^{-6}$ mol/L (vmax 4.00$×10^{-4}$ mol/(L·min)) and protozoal Km was 2.07$×10^{-6}$ mol/L (vmax 6.67$×10^{-4}$ mol/(L·min)). For ammonia, bacterial Km was 2.97$×10^{-5}$ mol/L (vmax 3.57$×10^{-4}$ mol/(L·min)) and protozoal Km was 1.40$×10^{-5}$ mol/L (vmax 3.33$×10^{-4}$ mol/(L·min)).

[Figure 1: see original paper] Km value of GDH to NAD in rumen bacteria crushing liquid

[Figure 2: see original paper] Km value of GDH to NAD in rumen protozoa crushing liquid

Km values of GDH to NAD in rumen bacteria and protozoa crushing liquid

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3.1 Dynamic Changes of Key Enzyme Activities Related to Protein Catabolism in Rumen Bacteria and Protozoa of Sheep

This study determined changes in key enzyme activities related to protein catabolism in rumen bacterial and protozoal crushing liquids at various time points before and after feeding with a 30:70 concentrate-to-forage ratio. The activities of protease, GDH, GPT, and GOT in both rumen bacteria and protozoa showed a pattern of initial increase followed by decrease, generally reaching maximum values at 1.5 h post-feeding and then declining gradually. Chen et al. reported that peptide concentrations in sheep rumen fluid increased initially then decreased slowly, peaking at 1.5 h. Rapid rumen protein degradation reaches its most active stage approximately 1.5 h after feeding, which may lead to rapid accumulation of small peptides and amino acids. Studies by Wang and Zhai indicated that rumen ammonia nitrogen concentration showed similar trends across different diets, being higher at 1.5-3.0 h post-feeding and then gradually decreasing. This pattern suggests that deamination reactions are most active at 1.5 h post-feeding. Rumen ammonia nitrogen concentration is directly affected by dietary protein content and is also related to protozoal quantity and species. Higher dietary protein increases available protein substrates for rumen bacteria, enhancing protease, GPT, GOT, and GDH activities and consequently increasing rumen ammonia nitrogen concentration. Since these four enzyme activities are significantly higher in rumen protozoa than in bacteria, defaunation significantly reduces rumen ammonia nitrogen concentration. This study provides enzymological theoretical support for the dynamic patterns of peptide and ammonia nitrogen concentrations in rumen fluid.

3.2 Comparison of Km Values of Glutamate Dehydrogenase Reaction Systems in Rumen Bacteria and Protozoa

No previous reports have documented Km values for GDH reaction systems in rumen bacteria and protozoa. Shino et al. reported that GDH from *Brevibacterium flavum* had Km values of 3.08 mmol/L for ammonia, 100.00 mmol/L for glutamate, and 5.72 mmol/L for α-ketoglutarate. Kujo et al. found that GDH from anaerobic hyperthermophiles had Km values of 0.025 mmol/L for NAD, 0.170 mmol/L for glutamate, 0.005 mmol/L for NADH, 0.066 mmol/L for α-ketoglutarate, and 9.700 mmol/L for ammonia. Newbold et al. demonstrated that *E. coli* GDH had strong affinity for ammonia and α-ketoglutarate (Km values of 2.33 and 0.71 mmol/L, respectively) but weak affinity for glutamate

(Km 98.00 mmol/L). The Km value patterns for GDH substrates in rumen bacterial crushing liquid observed in this study are similar to those reported for these bacterial species. Comprehensive comparison of GDH activities and Km values reveals: (1) Rumen protozoal GDH activity is higher than bacterial activity, with 42-43% lower Km values for glutamate and NAD$^+$, indicating 42-43% higher affinity and faster catalytic rates, which partially explains why protozoal GDH activity and ammonia production rates exceed those of bacteria. The lower bacterial Km for α-ketoglutarate suggests higher affinity, favoring the reverse reaction for glutamate synthesis and potentially resulting in lower bacterial ammonia production efficiency. (2) Regarding ammonia utilization, the Km for α-ketoglutarate (1.16$\times10^{-6}$ mol/L) is lower than for ammonia (2.97$\times10^{-5}$ mol/L) in bacteria, with similar trends in protozoa. Therefore, α-ketoglutarate availability is the primary limiting step for ammonia assimilation and amino acid resynthesis in both bacteria and protozoa, warranting further investigation into cellular α-ketoglutarate content and its effects on amino acid resynthesis.

3.3 Potential Mechanism for Ammonia Reutilization in Amino Acid Synthesis in Rumen Protozoa

Protozoa have long been considered net ammonia producers in the rumen, unable to utilize ammonia and obtaining nitrogen by ingesting small feed particles and bacteria. However, Williams et al. found that ciliates could synthesize lysine from diaminopimelic acid in bacterial cell walls and suggested that holotrichs could utilize ammonia for amino acid resynthesis. This study determined ammonia Km values of 2.97$\times10^{-5}$ mol/L in bacteria and 1.40$\times10^{-5}$ mol/L in protozoa, indicating higher protozoal GDH affinity for ammonia that favors the reverse reaction for amino acid synthesis, providing substrates for subsequent transamination by GPT and GOT. Regarding ammonia utilization, the lower Km for α-ketoglutarate compared to ammonia in both bacteria and protozoa suggests that α-ketoglutarate availability is the main limiting factor for ammonia assimilation and amino acid resynthesis. Further research is needed to investigate cellular α-ketoglutarate content and its influence on amino acid resynthesis in both microorganisms.

Conclusions

1. The activities of protease, GDH, GPT, and GOT in rumen bacteria and protozoa of sheep generally peak at 1.5 h post-feeding and then gradually decrease.
2. The activities of protease, GPT, GOT, and GDH in rumen protozoa are significantly higher than in bacteria, indicating more vigorous protein catabolism in protozoa.
3. Rumen protozoa possess not only a glutamate transamination mechanism but also potentially a mechanism for utilizing ammonia to resynthesize

amino acids.

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