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Effects of Methionine or Lysine Served as Sole Nitrogen and Carbon Sources on Level of Free Amino Acids and Activity of Transaminases at *in vitro* Incubation of Rumen Microorganisms

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Abstract: This study aimed at investigating the effect of Met or Lys as sole Nitrogen (N) and Carbon (C) sources on Glutamic Oxaloacetic Transaminase (GOT), Glutamic Pyruvic Transaminase (GPT) and Amino Acid (AA) metabolism of rumen microorganisms *in vitro*. Three fistulated goats were used as a source of ruminal microorganisms. Microbial suspensions with or without Met or Lys as substrate were anaerobically incubated at 39°C for 16 h. Free AA and transaminases in the supernatants of the incubation were analyzed by HPLC and an automatic biochemistry analyzer, respectively. When Met was used as a unique source of N and C, the content of free Val and His were significantly ($p<0.01$ and $p<0.05$) reduced but Gly was increased ($p<0.01$). No significant differences in the other free AA were found as well as for GOT and GPT activities. When Lys was used as a sole source of N and C, the content of free His and activities of GOT and GPT were significantly lower ($p<0.01$) than that of controls however Tyr was significantly increased ($p<0.05$). The results indicated that Val and Cys are probably the most important AA of rumen microorganisms when Met serves as the only source of N and C. But when Lys serves as a sole source of N and C, His becomes the most important AA. At the same time, GPT plays a weaker role when Met serves as a sole source of N and C.

Key words: Rumen microorganisms, methionine, lysine, free amino acids, GOT, Glutamic-Pyruvic Transaminase (GPT)

INTRODUCTION

Lysine (Lys), as Methionine (Met) is limiting amino acid for ruminants (National Research Council, 2001). However, both of them can not be added directly to the diets of ruminants to overcome nutritional deficiencies because free amino acids are rapidly degraded by rumen bacteria so inhibiting degradation of Met and Lys in rumen is one of fundamental objectives for ruminant nutrition research.

Therefore, it is very important to study the metabolic regulation of microbial Met and Lys degradation to screen the ruminant microorganisms which are active in degrading Met and Lys. It was demonstrated before that some ionophores were able to inhibit ruminal deaminase (Moloney *et al*., 1996) that some metal ions influenced branch chain amino acid transaminase of ruminal protozoa (Wakita and Hoshino, 1975) and that cadmium could stimulate Glutamic Oxaloacetic Transaminase (GOT) and Glutamic Pyruvic Transaminase (GPT) (Faixova and Faix, 2002). This study was aimed to detect effects on the activities of GOT and GPT and the metabolism of amino acids of ruminant microorganisms with Met or Lys as the sole nitrogen and carbon source *in vitro*. The purpose of this study was also to provide evidence for the isolation and cultivation of Met and Lys degrading ruminal microorganisms and the regulation of amino acid metabolism.

MATERIALS AND METHODS

Experimental animals and collection of rumen fluid: Ruminal microorganisms were obtained for experiment from three mature China Salon goats (body weight of 29±4 kg) which fitted with permanent rumen fistulae. The animals were fed twice daily (09:00 and 17:00). Alfalfa hay (1.35 kg) and a concentrate mixture (450 g, consisting of corn 80%, soybean meal 12%, wheat bran 6.3%, salt 1% and $CaHPO_4$ 0.3%, compound premix of the trace elements and vitamins 0.4%) were given daily. Fresh water was available at all times. After 6 months of feeding, rumen fluid of the three goats was collected before morning

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feeding by an aspirator bottle and strained through four layers of surgical gauze. Feed particles were removed according to method of Lee *et al.* (2000). Fresh rumen fluid free feed particle was bottled, filled with nitrogen, sealed by rubber stoppers for experimental use.

Preparation of medium: Media was prepared by Susane's method (Lowe *et al*., 1985) and some improvements were made.

Mineral-salts solution: Mineral-salts solution contained, per liter: $MgCl_2 \cdot 6H_2O$, 14 g; KH_2PO_4, 2 g; NH_4Cl, 3 g; KCl, 5 g; $CaCl_2 \cdot 2H_2O$, 0.6 g.

Haemin solution: Haemin solution was prepared by dissolving haemin 0.1 g in 10 mL ethanol. This was then adjusted to a volume of 1000 mL with 0.05 m NaOH.

Vitamin solution: Each liter of HEPES buffer contained: 1, 4 naphthoquinone, 0.25 g; calcium D-pantothenate, 0.2 g; nicotinamid, 0.2 g; riboflavin, 0.2 g; thiamin· HCl, 0.2 g; pyridoxine·HCl 0.2 g; biotin, 0.025 g; folic acid, 0.025 g; cyanocobalamin, 0.025 g and p-aminobenzoic acid, 0.025 g. Then the prepared solution was filtered through a membrane (0.22 μm pore diameter).

Trace elements solution: The trace elements contained per liter of 0.2 mol L^{-1}-HCl: $MnCl_2 \cdot 4H_2O$, 0.25; $NiCl_2 \cdot 6H_2O$, 0.25; $NaMoO_2 \cdot 6H_2O$, 0.25; H_3BO_3, 0.25 g; $FeSO_4 \cdot 7H_2O$, 0.20 g; $CoCl_2 \cdot 6H_2O$, 0.05 g; SeO_2, 0.05 g; $NaVO_2 \cdot 6H_2O$, 0.05 g; $CuCl_2 \cdot 2H_2O$, 0.025 g.

Basal solution: The basal solution contained: mineral-salts solution, 75 mL; trace elements solution, 10 mL; haemin solution, 10 mL; resazurin solution (1 g L^{-1}), 1 mL; K_2HPO_4, 0.45 g. The final volume was made up to 830 mL with de-ionized water and the pH was adjusted to 7.4 with 1 m KOH.

Confection of medium: About 830 mL basal solution and 4 g Na_2CO_3 were diluted to 1000 mL with de-ionized water. The solution was then put into a big flask with three jaws and boiled on an electric stove for 5 min to expel the oxygen. Finally, N_2 was poured over the medium for 5 min. When the solution temperature cooled to room temperature, 0.25 g $Na_2S \cdot 9H_2O$ and 0.25 g L-cysteine·HCl were added. Then the mixed solution was removed to serum bottles (before removing the solution, the empty bottle was filled with high purity nitrogen gas). Each bottle was filled with 100 mL, closed with butyl rubber stoppers and then sealed with aluminum lids under high purity nitrogen. Lastly, the media were autoclaved at 121°C for 20 min. Into each bottle 1 mL vitamin solution was injected before the media was used.

Inoculation of rumen microorganism and experiment design: About 9 bottles (bottle volume: 120 mL) of media were taken and were randomly divided into three groups with 3 bottles in each group. One group was injected with 10 and 0.1 mL Met (Beijing Jing Ke Hong Da biotech company, China) per bottle as trial group 1. About 3 bottles of another group were injected with 10 and 0.1 mL Lys (Shanghai Chemical Reagent Company, China) per bottle as trial group 2. About 3 bottles were not injected with any amino acid and served a control group. Ruminal fluid (5 mL) was injected into each bottle. Each goat was a replicate. Lastly all nine bottles were incubated at 39°C.

Sampling and determining: About 2 mL of suspension were withdrawn from the reaction vessel at 0, 8 and 16 h during the fermentation and divided them into two parts. One part was immediately added to equal volumes of 4% (w/v) sulfosalicylic acid (Or-Rashid *et al*., 2001) in 2 mL Eppendorf tubes. The deproteinized samples were centrifuged at 9901 g and 4°C for 30 min and the supernatant was carefully collected and stored at -20°C for determining of free amino acids. The other part was used to determine the GOT and GPT. The free amino acids were analyzed by Agilent 1100 (Chromtech, Idstein, Germany) high performance liquid chromatography. GOP and GPT were determined through the use of a Hitachi 7600-020 (Hitachi Corp., Minato-ku, Japan) automatic biochemistry analyzer by rate method. A 0 was assigned if the amino acid was undetectable.

Statistical analysis: All data from the experiment were analyzed using the AVONA procedure of SAS (1990) for completely randomized designs. Statistical significances of differences among treatments were assessed using the Duncan's multiple-range test. Significant differences were described as significant or highly significant if p value were <0.05 or <0.01, respectively.

RESULTS AND DISCUSSION

Control group: There were no significant differences for all amino acids and GPT among 0, 8 and 16 h fermentations. There was a significant difference ($p<0.05$) between 0 and 8 h of incubation only for GOT. But after 16 h of incubation, GOT increased to 5.0 IU L^{-1} and the differences between 0 and 16 h no longer became significant (Table 1). That indicated that some metabolic products or substrates inhibited GOT before 8 h of incubation. Further research is needed to determine identity of the inhibitor of GOT. However, in total, metabolic activities of microorganisms stopped as the sources for nitrogen and carbon were depleted.

Table 1: Effect of incubation time and resources of carbon and nitrogen on GOT, GPT (IU L^{-1}) and free amino acids concentration (μmol L^{-1}) of incubation fluid*

	Control			Trial 1 (Met)			Trial 2 (Lys)		
Acids	0 (h)	8 (h)	16 (h)	0 (h)	8 (h)	16 (h)	0 (h)	8 (h)	16 (h)
GOT	6.0±1.0^{a}	4.00±1.000^{c}	5.0±1.0^{abc}	5.7±0.600^{ab}	5.0±0.000^{abc}	4.7±0.600^{bc}	4.30±0.50^{c}	2.70±0.60^{d}	2.3±0.60^{d}
GPT	6.7±0.6^{ab}	5.30±0.600^{b}	5.0±0.0^{b}	6.3±0.600^{a}	5.0±0.000^{b}	5.3±0.600^{b}	5.00±00.0^{b}	3.70±0.60^{c}	3.3±0.60^{c}
Met	1.1±0.4^{A}	1.90±1.400^{A}	2.5±0.8^{A}	981.6±265.3^{B}	1113.8±126.9^{B}	894.5±277.5^{B}	6.00±8.40^{A}	1.22±0.30^{A}	6.1±8.60^{A}
Lys	1.6±0.2^{A}	2.00±0.500^{A}	1.2±0.9^{A}	3.2±1.400^{A}	2.6±1.600^{A}	1.2±0.500^{A}	977.50±10.5^{B}	921.54±85.0^{B}	877.0±83.1^{B}
Cys	45.1±9.9^{a}	35.90±16.60^{ab}	29.9±5.8^{ab}	47.7±14.50^{a}	41.0±7.600^{ab}	22.5±7.000^{bB}	49.10±13.1^{aA}	43.50±7.70^{a}	44.7±13.6^{a}
Glu	10.6±2.1^{a}	8.40±0.600^{ab}	8.0±0.7^{ab}	9.6±2.400^{ab}	8.3±1.700^{ab}	5.9±5.400^{b}	8.60±1.80^{ab}	7.70±0.50^{ab}	6.7±4.00^{ab}
Ser	1.6±0.7^{ab}	0.90±0.200^{ab}	5.7±8.2^{a}	1.7±0.900^{ab}	1.0±0.400^{ab}	0.2±0.100^{b}	1.30±0.50^{ab}	0.00^{b}	1.3±1.20^{ab}
His	2.3±0.7^{bcd}	2.20±0.400^{cd}	2.8±1.8^{abc}	2.6±1.000^{abc}	1.7±0.400^{cdA}	3.6±0.300^{abB}	3.80±0.80^{aB}	1.00±0.20^{dA}	1.9±0.30^{cd}
Gly	3.2±1.0^{bAC}	2.00±0.500^{bAC}	5.2±6.0^{bAC}	14.0±3.100^{aB}	17.1±3.600^{aB}	13.9±4.100^{aB}	2.00±0.90^{bAC}	2.50±0.30^{bAC}	3.2±0.20^{bAC}
Thr	13.4±2.7^{a}	11.60±3.200^{ab}	11.8±1.5^{ab}	13.4±3.000^{a}	14.3±2.500^{aA}	10.5±3.000^{ab}	11.20±4.20^{ab}	5.70±5.80^{bB}	11.7±3.90^{ab}
Arg	5.0±1.4^{abA}	4.20±1.200^{ab}	4.4±1.2^{ab}	4.8±1.200^{ab}	5.3±1.200^{aA}	2.4±2.400^{bc}	4.30±2.60^{ab}	1.30±0.50^{cB}	4.6±1.30^{ab}
Ala	1.4±0.6^{ab}	0.80±0.500^{b}	0.7±0.7^{b}	2.2±1.600^{a}	1.4±0.900^{ab}	1.2±1.100^{ab}	0.60±0.60^{b}	1.00±0.40^{ab}	1.5±0.30^{ab}
Tyr	6.6±2.2^{c}	8.14±2.180^{bc}	11.7±5.5^{abc}	8.6±1.200^{bc}	10.7±1.400^{abc}	10.0±4.800^{abc}	5.70±1.00^{c}	14.10±6.10^{ab}	15.0±3.60^{a}
Trp	0.7±0.0	0.70±0.100	4.5±0.6	5.2±8.200	1.0±0.900	3.1±4.900	1.30±0.20	1.10±0.30	8.8±13.8
Phe	4.6±2.5^{ab}	1.80±0.200^{b}	1.0±1.1^{b}	7.9±6.200^{ab}	1.8±1.400^{b}	4.9±4.000^{ab}	4.10±1.40^{ab}	0.30±0.10^{b}	19.4±26.0^{a}
Ile	2.0±0.7^{b}	2.10±0.200^{b}	1.9±0.4^{b}	5.6±6.700^{ab}	3.3±1.100^{ab}	3.6±1.500^{ab}	3.80±1.80^{ab}	2.10±0.20^{b}	12.2±14.5^{a}
Leu	3.3±1.2	2.60±0.200	2.7±0.8	11.8±14.10	4.5±1.100	2.5±1.800	5.40±2.70	5.80±1.00	10.0±9.60
Pro	8.6±3.8	7.10±9.100	2.4±1.1	2.9±1.500	6.3±1.600	5.8±3.200	5.10±6.60	19.60±31.4	3.5±0.50
Val	2.1±1.0^{aA}	1.60±0.400^{a}	1.4±0.5^{abc}	0.0^{B}	0.0^{B}	0.0^{B}	1.80±2.10^{aA}	0.20±00.0^{bc}	1.0±1.00^{abc}

*Values within a row with different small superscripts, different capital superscripts represent significant difference ($p<0.05$) and highly significant difference ($p<0.01$), respectively the values are expressed as mean±standard deviation, n = 3

Trial group 1: When Met was used as source of N and C for microorganisms (Trial group 1), every amino acid concentration had different changes when compared with the control group. Furthermore, there were also significantly differences ($p<0.05$) among Cys, His and Val as fermentation time become longer. Cys was significantly decreased ($p<0.05$) with incubation time (Table 1). Val was especially depleted by 0 h of incubation after adding of Met. This could indicate that the addition of Met speeded up the metabolisms of Cys and Val. Their metabolic pathways are probably via the pyruvic acid pathway (Wang *et al*., 2002). This also indicated that Val and His became limiting amino acids when microorganism growth depended solely on Met as the unique source of N and C. It could be very useful to confect medium for the screening the microorganism that degrade Met. Content of His as 1.7 μmol L^{-1} at 8 h of fermentation which was lower than that at 0 h. But by 16 h of fermentation, its concentration increased to 3.6 μmol L^{-1} which was significantly higher than that at 8 h of incubation (Table 1). This means that the synthetic metabolism of His played a leading role after 8 h of incubation and that the catabolic metabolism of His played a leading role before 8 h of incubation. However, it is not clear what factor plays a key role in the control of synthesis or degradation. GOT did not change significantly among the different fermentation times. But GPT at 8 and 16 h was significantly lower ($p<0.05$) than that at 0 h (Table 1). This indicated that transamination via the Glu-Ala pathway was inhibited when Met was the only source of C and N in rumen microorganisms.

Trial group 2: Activities of GOT and GPT at 8 h of incubation were all lower ($p<0.05$) than that at 0 h (Table 1). This indicated that the action of GOT and GPT became weaker with increasing time of incubation when Lys served as the unique source of C and N in rumen microorganisms. It may have been the result of a lack of transamination when Lys was added (Wang *et al*., 2002). At the same time, many kinds of amino acids in suspension were significantly or highly significantly changed when fermentation time changed (Table 1). Content of his at 8 h and 16 h was highly significantly ($p<0.01$) and significantly ($p<0.05$) lower than that at 0 h. On the other hand, Tyr content significantly increased as incubation time got longer. The results showed that adding Lys speeded up the degradation of his and the synthesis of Tyr. It also indicated that His was import for the growth of rumen microorganism when Lys served as the sole source of N and C.

The concentrations of Arg, Phe and Ile in suspension were also lowest at 8 h (Table 1). This means that the synthesis and degradation of these amino acids occurred at the same time during the 16 h incubation period. Degradation was greatest during the earlier stage whereas synthesis was the greatest during the later stage. Nevertheless, their metabolic mechanism was not clear. Amin *et al*. (2001) (Amin and Onodera, 1997) reported in a study of the amino acid metabolism of rumen microorganism using mixture of C and N sources that the synthesis of Phe occur via the aminotransfer on phenylpyruvic by various rumen bacteria and protozoa. However, in present research, Phe content was not

influenced ($p>0.05$) by source of amino acids and fermentation time. The results showed that this metabolic path did apparently not play a leading role when Lys or Met served as unique sources of C and N.

CONCLUSION

There were differing effects of Met and Lys used as sole source of C and N on GOT, GPT and amino acid metabolism of rumen microorganisms. When Met was used, the rates of Val and Cys degradation were increased and the metabolism of His was influenced. When Lys was used, the degradation of His and the synthesis of Tyr were increased. These data suggested that Val and Cys were probably important amino acids of rumen microorganisms when Met was the sole source of N and C; however His may be an important amino acid when Lys is the sole source of N and C. The effects of Met and Lys on GPT and GOT were not the same.

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