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Catabolism of methionine and threonine *in vitro* by mixed ruminal bacteria and protozoa

M. M. Or-Rashid[1], **R. Onodera**[1], **S. Wadud**[1], **S. Oshiro**[2], and **T. Okada**[3]

[1] Laboratory of Animal Nutrition and Biochemistry, Division of Animal Science, Faculty of Agriculture, Miyazaki University, Miyazaki shi, Japan
[2] Faculty of Agriculture, University of the Ryukyus, Senbaru, Nishihara-cho, Okinawa-Pre, Japan
[3] Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan

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Summary. *In vitro* studies were conducted to examine the metabolism of methionine (Met) and threonine (Thr) using mixed ruminal bacteria (B), mixed ruminal protozoa (P), and a combination of these two (BP). Rumen microorganisms were collected from fistulated goats fed with lucerne cubes (*Medicago sativa*) and a concentrate mixture twice a day. Microbial suspensions were anaerobically incubated with or without 1 mM each of the substrates at 39°C for 12 h. Met, Thr and their related amino compounds in both the supernatants and microbial hydrolyzates of the incubation were analyzed by HPLC. Met was degraded by 58.7, 22.1, and 67.3% as a whole in B, P, and BP suspensions, respectively, during 12 h incubation. In the case of Thr, these values were 67.3, 33.4, and 76.2% in B, P, and BP, respectively. Met was catabolized by all of the three microbial suspensions to methionine sulfoxide and 2-aminobutyric acid. Catabolism of Thr by B and BP resulted in the production of glycine and 2-aminobutyric acid, while P produced only 2-aminobutyric acid. From these results, the existence of diverse catabolic routes of Met and Thr in rumen microorganisms was indicated.

Keywords: Amino acids – Rumen bacteria – Rumen protozoa – Methionine – Threonine – 2-Aminobutyric acid

Introduction

It is generally accepted that free amino acids arise as intermediate products in the breakdown of proteins by rumen microorganisms. Furthermore, there is substantial indirect evidence that this intermediate of degradation is also an important substrate for biosynthesis by rumen microorganisms (Broderick et al., 1991). The metabolism of free amino acids in the rumen is still a concern of ruminant animal nutritionists as it is related to the N economy of the rumen

ecosystem, and hence the ruminant animals. In the mid and late 1970s, researchers published data on the apparent degradation rates of amino acids by mixed ruminal microorganisms (Chalupa, 1976; Prins et al., 1979) and five major genera of rumen bacteria (Scheifinger et al., 1976).

On the other hand, little information regarding the intermediate products formed in the catabolism of amino acids by rumen miocroorganisms is available (Lewis and Emery, 1962). In particular, information regarding the catabolism of methionine (Met) and threonine (Thr) is scant. Thr has been shown to be degraded by rumen bacteria, with the resultant production of 2-oxobutyric acid, ammonia, propionate, CO_2, and hydrogen gas (Lewis and Elsden, 1955; Van den Hende et al., 1963; Walker, 1958). Formation of glycine (Gly) from Thr through the action of threonine aldolase (EC 2.1.2.1) in *Trypanosoma brucei* (Linstead et al., 1977) has been reported. However, such information is not been available with regard to the catabolism of Thr by rumen bacteria, rumen protozoa and a combination of rumen bacteria and protozoa, a normal microbial ecosystem of the rumen.

Onodera and Migita (1985) identified 2-aminobutyric acid as a catabolite of both Met and Thr by mixed rumen protozoa, but it has not yet been demonstrated in the case of rumen bacteria. Not quantitatively, only qualitative data of methionine sulfoxide have been reported in rumen microorganisms (Salsbury et al., 1971).

In order to quantitatively examine the Met and Thr catabolism by rumen microorganisms considering the matters mentioned above in more detail, we have recently established a convenient selective HPLC method based on pre-column derivatization using 9-fluorenylmethyl chloroformate (FMOC-Cl) for the quantitative determination of Thr, Met and their related amino compounds (Or-Rashid et al., 2000). The present study was undertaken to quantitatively investigate the apparent and net degradation of Met and Thr and other metabolites produced *in vitro* during the metabolism of Met and Thr by mixed ruminal bacteria (B) and protozoa (P). A quantitative study on the catabolism of Met and Thr with a combination of rumen bacteria and protozoa (BP) was also carried out in order to know the fact in the rumen microbial ecosystem.

Materials and methods

Rumen microbial preparations

Samples of mixed ruminal microorganisms were obtained for experiments from three mature goats (Japanese native breed, body weight of 35 ± 5 kg); each was fitted with a permanent cannula in the rumen. The animals were fed 370–450 g of lucerne hay cubes and 115–140 g of concentrate feed (formula feed: Dairy Mix, Chubu-Shiryo, Japan), given in two equal portions at 9 a.m. and 5 p.m. Fresh water was available at all times.

Rumen digesta were removed before feeding in the morning and strained through several layers of surgical gauze. The suspensions of mixed bacteria (B), mixed protozoa (P), and B plus P (BP) were obtained by using the procedure of Onodera et al. (1992). The P suspensions always included 0.1 mg/ml each of chloramphenicol, streptomycin sulfate, and penicillin G potassium to stop the biochemical activities of the contaminating bacteria.

Incubations and sample preparations

Microbial suspensions (20 ml) were incubated in 30 ml Erlenmeyer flasks at 39°C for 12 h anaerobically with and without 1 mM each of Met, or Thr, or Gly as a substrate (Chalupa, 1976; Scheifinger et al., 1976) after being flushed briefly with a mixture of 95% N_2 and 5% CO_2. Rice starch was added (0.5 mg/ml) as an energy source in all incubation media. One milliliter of suspension was withdrawn from the reaction vessel at 0, 6, and 12 h during the fermentation and immediately added to equal volumes of 4% (w/v) sulfosalicylic acid (including 10 mM EDTA-3K) in 2 ml Eppendorf tubes. The deproteinized samples were centrifuged at 27,000 × g for 30 min at 4°C. The supernatant fluids were filtered through a membrane filter (0.45 μm porosity), and stored at 4°C. Microbial pellets were hydrolyzed with 4.0 M methanesulfonic acid at 160°C for 45 min (Chiou and Wang, 1988). Two ml of 3.5 M potassium hydroxide was added to the tube containing hydrolysate and vortex-mixed. Finally, the volume was adjusted up to 6 ml with distilled water and filtered through filter paper (Whatman, No. 2) followed by a membrane filter (0.45 μm porosity) and stored at 4°C.

Methods of analysis

All of the supernatant fluids of the incubations and hydrolysates of the pellets were subjected to analysis by HPLC according to Or-Rashid et al. (2000). Samples of P and BP were preserved by the addition of nine volumes of methylgreen-formalin salt solution (Onodera et al., 1977), kept at room temperature and then counted by direct light microscopy using a Fuchs-Rosenthal hemocytometer. The microbial nitrogen (MN) contents of B, P, and BP suspensions were determined by the Kjeldahl method (A O A C, 1990) from 1 ml (triplicate of each) of microbial suspensions.

In all experiments, incubations of microbial suspensions were carried out in triplicate, collected from one goat, followed by two other goats individually on different days. A control incubation was always run without the substrate to determine the endogenous products.

The following formula was used to calculate the net production of the products from the substrates.

$$\text{Net production of } X_i = (S_i - S_j) - (C_i - C_j),$$

where X = net amount of product, S = amount of product in the incubation of medium with substrate added, C = amount of product in the incubation of medium with substrate omitted, i = incubation period at 6 or 12 h, j = incubation period at 0 h.

Net production values of all the components found in the supernatants and hydrolysates were expressed as the means of nine observations with their standard deviations.

Results and discussion

Degradation of Met

Met (1 mM) was apparently degraded by 39.6 and 58.7% in B, 15.9 and 22.1% in P, and 50.7 and 67.3% in BP suspensions after 6 and 12 h incubations, respectively (Table 1). The apparent degradation was calculated by subtracting the Met concentration found in the supernatants after 6 and 12 h of incubation from the original concentration. When Met was incubated with B, or BP suspensions, a portion of Met was incorporated into the microbial cell protein (Broderick et al., 1991; Armstead and Ling, 1993; Ling and Armstead, 1995; Wright and Hungate, 1967) (Table 1). However, we have separately

Table 1. Net degradation of methionine (Met), and production of methionine sulfoxide and 2-aminobutyric acid by mixed ruminal bacteria (B), protozoa (P), and the combination of B plus P (BP) after 6 and 12 h incubation

Compounds		B		P		BP	
		6 h	12 h	6 h	12 h	6 h	12 h
		Concentration (μM)*					
Met	Supernatant	603.8 ±41.5	412.6 ±25.7	840.9 ±49.1	779.0 ±57.4	492.7 ±28.4	326.6 ±26.4
	Microbial pellet hydrolysate	60.8 ±12.7	67.1 ±13.4	−1.6 ±0.41	−1.9 ±0.35	56.1 ±5.5	65.7 ±8.9
	Net degradation	335.4	520.3	160.7	222.9	451.2	607.7
Methionine sulfoxide	Supernatant	27.5 ±5.9	25.1 ±4.8	18.9 ±3.9	15.1 ±4.4	36.3 ±7.3	24.7 ±4.1
2-aminobutyric acid	Supernatant	7.2 ±2.6	13.1 ±3.8	22.3 ±4.6	29.7 ±3.7	9.8 ±4.1	21.4 ±3.9

*Mean values ± standard deviation and obtained by using calculating formula as described in "Materials and methods" section. In control incubation (without substrate), average concentration (μM) at 0 h of Met, methionine sulfoxide and 2-aminobutyric acid are 301.4, 1.9 and 3.7 in B suspension; 286.3, 0.0 and 0.0 in P suspension; 547.7, 2.3 and 3.3 in BP suspension, respectively. In incubation with substrate, average contents (μM) at 0 h of Met, methionine sulfoxide and 2-aminobutyric acid are almost similar except Met (due to addition of substrate). Average microbial nitrogen (mg N/ml) in B, P, and BP suspensions were 0.821, 0.750, and 1.417, respectively. Protozoal compositions ($\times 10^4$ cells/ml) in P and BP were 131.3 and 127.1, 8.7 and 7.3, and 2.3 and 2.1 for *Entodinium, Diplodinium* and *Dasytricha*, respectively.

taken no account of Met synthesized *de novo* in their pellet fractions, *i.e.*, in cell protein. In P suspension, Met was released from the cell protein because the protozoal cells could not grow in this medium (Table 1). When protozoal suspensions are incubated *in vitro* in a buffer solution, they usually liberate endogenous amino acids into the medium (Onodera and Kandatsu, 1970; Morgavi et al., 1993). Therefore, the net degradation of Met was different from the apparent disappearance, and it was calculated by subtracting the sum of the values of Met content in the hydrolysates of microbial cells and Met found in the supernatants after 6 and 12 h of incubation from the original concentration. Thus, net degradation of Met were 33.5 and 52.0% in B, 16.1 and 22.3% in P, and 45.1 and 60.8% in BP after 6 and 12 h incubations, respectively. When the degradation rate was expressed with a unit of "per g microbial nitrogen (MN)", the net degradation rate of Met was more in B (52.8 μmol/g MN/h) followed by BP (35.7 μmol/g MN/h) and P (24.8 μmol/g MN/h) during a 12-h incubation period. The rate of net degradation in B was 2.1 fold higher than that in P which suggested that rumen bacteria have a higher Met degradation activity than protozoa. To our knowledge, this is the first report in which the net degradation ability of Met by rumen bacteria and protozoa have actually been demonstrated. The apparent degradation rates of some amino acids have been investigated by rumen microorganisms

(Chalupa, 1976; Prins et al., 1979) and by five species of rumen bacteria (Scheifinger et al., 1976). Apparent disappearance rate of Met (*in vitro*) was found to be 0.09 mM/h in rumen microbial suspensions of cattle (Chalupa, 1976) and this seems to be close to our reported values (0.07 and 0.08 mM/h in B and BP, respectively). On the other hand, apparent *in vitro* degradation of Met by mixed rumen microorganisms in rumen fluid of dairy cows was found to be 0.32 mM/h (Prins et al., 1979), and was inconsistent with our reported values. Prins et al. (1979) suggested that the rate of *in vitro* disappearance of amino acids depends on various factors: (a) the diet of the inoculum donor, (b) the form in which the amino acids were added and (c) the presence and absence of other energy sources during incubations.

Production of methionine sulfoxide from Met

On the basis of "per g MN", the formation of methionine sulfoxide from Met during a 6-h incubation period in P (25.2 μmol/g MN) and BP (25.6 μmol/g MN) were almost similar, but in the case of B (33.5 μmol/g MN), the production was about 32.0% higher than that of P and BP. Onodera and Migita (1985) by using rumen protozoa and Salsbury et al. (1971) by using mixed rumen microorganisms observed that Met was oxidized enzymatically to methionine sulfoxide. In this regard, Met is susceptible to oxidation, which in turn connects with the loss of protein activity (Teh et al., 1987). Our present experiment revealed that methionine sulfoxide production ability of BP was about 23.6–56.9% lower than that of B (33.5 μmol/g MN). Thus, the co-existence of P in B suspension decrease the oxidation of Met to methionine sulfoxide.

Production of 2-aminobutyric acid from Met

A considerable amount of 2-aminobutyric acid was found to be produced from Met in B, P, and BP (Table 1). The production of 2-aminobutyric acid in P (29.7 and 39.6 μmol/g MN in 6 and 12 h, respectively) was about 2.5–3.4 and 2.6–2.8 folds higher than that of B and BP during the experimental periods. Anaerobic incubation of Met in mixed protozoa (Onodera and Migita, 1985) and in *Escherichia coli* (Meister, 1965) have been reported to be produced 2-aminobutyric acid via 2-oxobutyric acid. The present study revealed that 2-aminobutyric acid was formed from Met by the different rumen microbial fractions and their relative efficiency in this respect.

Other undetectable compounds from Met

In B suspension, Met decreased apparently as a whole by 39.6%. And it was incorporated in cell protein as Met by 15.3%, and converted to methionine sulfoxide by 6.9% and 2-aminobutyric acid by 1.8% of the whole disappeared Met from the supernatant in 6 h incubation. About 30.1% of the added Met (1 mM) was not recovered in 6 h incubation and may have been converted to "other undetectable compounds" such as 2-oxobutyric acid, ammonia, methyl

mercaptan, and propionic acid (Meister, 1965; Onodera and Migita, 1985); methanethiol, S-methyl-L-cysteine, and S-adenosylmethionine (Zikakis and Salsbury, 1969); 2-oxo-γ-methiolbutyric acid (Ishimoto et al., 1971), which were not detectable owing to our HPLC method limitation. In the case of P and BP, these values were 11.9 and 40.1% and may have been converted to other undetectable compounds as mentioned above.

Degradation of Thr

Thr apparently disappeared by 45.5, 25.1 and 66.3% during 6 h incubation in B, P, and BP suspensions, respectively (Table 2). In the case of 12 h incubation, these values were 67.3, 33.4, and 76.2%, respectively. On the basis of "per g MN", Thr disappearance (net) was more in B (444.0 and 657.4 μmol in 6 and 12 h, respectively) followed by BP (402.6 and 454.9 μmol in 6 and 12 h, respectively) and P (323.7 and 431.3 μmol in 6 and 12 h, respectively).

A portion of apparently disappeared Thr was also incorporated in the microbial cell protein of B and BP (Broderick et al., 1991; Armstead and Ling, 1993; Ling and Armstead, 1995; Wright and Hungate, 1967) (Table 2) similar

Table 2. Net degradation of threonine (Thr), and production of glycine (Gly) and 2-aminobutyric acid by mixed ruminal bacteria (B), protozoa (P), and the combination of B plus P (BP) after 6 and 12 h incubation

Compounds		B		P		BP	
		6 h	12 h	6 h	12 h	6 h	12 h
		Concentration (μM)*					
Thr	Supernatant	545.0 ±36.5	327.0 ±28.7	749.0 ±39.9	666.1 ±27.4	337.0 ±18.4	238.0 ±20.7
	Microbial pellet hydrolysate	60.3 ±8.7	88.6 ±13.9	−1.5 ±0.35	−2.4 ±0.27	52.7 ±6.5	72.3 ±10.3
	Net degradation	394.7	584.4	252.5	336.4	610.3	689.7
Gly	Supernatant	151.0 ±32.9	98.4 ±15.1	0.0	0.0	195.6 ±29.6	136.7 ±17.0
	Microbial pellet hydrolysate	54.3 ±6.4	66.7 ±8.1	0.0	0.0	42.8 ±5.7	52.3 ±7.1
2-aminobutyric acid	Supernatant	45.7 ±6.6	65.3 ±7.8	116.3 ±15.8	139.8 ±22.7	28.2 ±4.6	34.1 ±5.1

*Mean values ± standard deviation and obtained by using calculating formula as described in "Materials and methods" section. In control incubation (without substrate), average concentration (μM) at 0 h of Thr, Gly and 2-aminobutyric acid are 795.6, 1025.3 and 4.5 in B suspension; 736.7, 927.4 and 0.0 in P suspension; 1505.1, 1885.3 and 3.2 in BP suspension, respectively. In incubation with substrate, average contents (μM) at 0 h of Thr, Gly and 2-aminobutyric acid are almost similar except Thr (due to addition of substrate). Average microbial nitrogen (mg N/ml) in B, P, and BP suspensions were 0.889, 0.780, and 1.516, respectively. Protozoal compositions ($\times 10^4$ cells/ml) in P and BP were 135.4 and 134.1, 9.8 and 8.3, and 2.7 and 2.9 for *Entodinium*, *Diplodinium* and *Dasytricha,* respectively.

to the case of Met as mentioned before. According to the report of Chalupa (1976) and Prins et al. (1979), apparent degradation of Thr by mixed rumen microorganisms were 0.50 and 0.39 mM/h, respectively. These values seem to be much higher than those obtained by our experiments (0.08 and 0.11 mM/h in B and BP, respectively). And probable causes were those as mentioned previously.

Production of Gly from Thr

A greater portion of Gly was accumulated as free form in the incubation medium, whereas a small amount of Gly produced by B and BP was incorporated into cell protein (Table 2). On the other hand, Gly was not found to be produced by P. Therefore, it seemed that threonine aldolase [EC 4.1.2.5], the enzyme responsible for the conversion of Thr to Gly, might not be present in rumen protozoa different from rumen bacteria. Emmanuel et al. (1974) reported that Gly might not be produced from Thr in mixed rumen microbes during *in vitro* experiment. In this respect, our observation was quite different from their observation. Thus, it is the first observation to show that rumen bacteria and the mixture of rumen bacteria and protozoa are able to produce Gly from Thr. Gly that was produced from Thr might be further converted to volatile fatty acids (*e.g.*, acetic acid, propionic acid, butyric acid), ammonia, CO_2, as reported in rumen microorganisms (Wright and Hungate, 1967). We also measured the disappearance rate of Gly in three different rumen microbial suspensions. It apparently disappeared from the supernatant by 50.6 and 62.3% in B, 7.1 and 10.3% in P, and 56.1 and 69.7% in BP after 6 and 12 h incubations, respectively. In B and BP, a portion of Gly that disappeared was found to be incorporated into microbial protein (data not shown). Incorporation of extracellular Gly directly without intervening deamination in mixed rumen bacterial protein was also reported by Wright and Hungate (1967).

Production of 2-aminobutyric acid from Thr

As shown in Table 2, 2-aminobutyric acid was formed from Thr in B, P and BP suspensions as in the case of Met metabolism. The production of 2-aminobutyric acid during a 6-h incubation period in P (149.1 μmol/g MN) was about 2.9 and 8.0 fold higher than those in B and BP, respectively. It was interesting to note that the production trend of 2-aminobutyric acid from Thr in different microbial suspensions was P > B > BP, while that from Met was P > BP > B (Table 1 and 2), suggesting again that there might be a complicated interaction between B and P, which was not clearly understood in this experiment. Onodera and Migita (1985) showed that deamination of Thr could yield 2AB via 2-oxobutyric acid in mixed rumen protozoa. Considering the aforementioned fact, it seemed that 2-aminobutyric acid was produced in mixed ruminal bacteria and protozoa from Thr via 2-oxobutyric acid in the present experiment.

Other undetectable compounds (in the case of Thr)

In B suspension, Thr decreased (apparently) as a whole by 45.5%. And it was incorporated in cell protein as Thr by 13.3%, and converted to Gly by 45.1% and 2-aminobutyric acid by 10.0% of the whole Thr disappeared from the supernatant in 6 h incubation. About 14.4% of the added Thr (1 mM) was not recovered and there might have been "other undetectable compounds" formed such as 2-oxobutyric acid, volatile fatty acids, CO_2, ammonia (Onodera and Migita, 1985; Wright and Hungate, 1967). In the case of P and BP, these values were 13.6 and 31.9% and may have been converted to other undetectable compounds as mentioned above.

Results of the present studies indicate that diverse catabolic routes of Met and Thr are present in rumen microorganisms. Data from the studies also indicate that the ability of rumen protozoa to degrade Met and Thr is much lower than that of rumen bacteria.

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Authors' address: Mamun M. Or-Rashid, Laboratory of Animal Nutrition and Biochemistry, Division of Animal Science, Faculty of Agriculture, Miyazaki University, Miyazaki shi 889-2192, Japan, Fax: +81-985-58-2884, E-mail: mamun76@hotmail.com

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