Amino Acids (2003) 24: 135–139
DOI 10.1007/s00726-002-0326-4

Amino
Acids
Printed in Austria

Studies on the utilization of methionine sulfoxide and methionine sulfone by rumen microorganisms *in vitro*

M. M. Or-Rashid, R. Onodera, and **S. Wadud**

Laboratory of Animal Nutrition and Biochemistry, Division of Animal Science, Miyazaki University, Miyazaki, Japan

Received December 28, 2001
Accepted May 21, 2002
Published online October 14, 2002;

Summary. An *in vitro* experiment was conducted to test the ability of mixed rumen bacteria (B), protozoa (P), and their mixture (BP) to utilize the oxidized forms of methionine (Met) *e.g.*, methionine sulfoxide (MSO), methionine sulfone (MSO_2). Rumen contents were collected from fistulated goats to prepare the microbial suspensions and were anaerobically incubated at 39°C for 12 h with or without MSO (1 mM) or MSO_2 (1 mM) as a substrate. Met and other related compounds produced in both the supernatants and hydrolyzates of the incubation were analyzed by HPLC. During 6- and 12-h incubation periods, MSO disappeared by 28.3 and 42.0%, 0.0 and 0.0%, and 40.6 and 62.4% in B, P, and BP suspensions, respectively. Rumen bacteria and the mixture of rumen bacteria and protozoa were capable to reduce MSO to Met, and the production of Met from MSO in BP (156.6 and 196.1 μmol/g MN) was about 17.3 and 14.1% higher than that in B alone (133.5 and 171.9 μmol/g MN) during 6- and 12-h incubations, respectively. On the other hand, mixed rumen protozoa were unable to utilize MSO. Other metabolites produced from MSO were found to be MSO_2 and 2-aminobutyric acid (2AB) in B and BP. MSO_2 as a substrate remained without diminution in all-microbial suspensions. It was concluded that B, P, and BP cannot utilize MSO_2; but MSO can be utilized by B and BP for producing Met.

Keywords: Methionine – Methionine sulfoxide – Methionine sulfone – Rumen microorganisms

Introduction

The importance of rumen microorganisms in the protein nutrition of ruminant animal is out of question. Rumen microorganisms synthesize protein as their cell protein and usually accounts for the largest proportion (60–85%) of the total amino acid nitrogen entering the small intestine of ruminants (Buttery and Foulds, 1985). The unique role of methionine (Met) as the initiator of protein synthesis in both prokaryotes and eukaryotes is well documented (cited by Ejiri et al., 1979). Inadequate supply of Met can retard the normal function of the bodies of the ruminant animals as well as other monogastric animals.

In many food/feed proteins, Met is the most or one of the most limiting of the essential amino acids. Methionine sulfoxide (MSO) and methionine sulfone (MSO_2) are the two main products formed by oxidation of Met. MSO is readily formed under mild oxidative conditions while the formation of MSO_2 requires relatively severe oxidative conditions (Ganapathy and Leibach, 1982). The loss of available Met from fishmeal in the form of MSO during feed processing has been reported (Miller et al., 1965). Oxidation of Met can also occur during the conservation and storage of animal feedstuffs.

MSO can be reduced to Met by animal tissues (Nagamine et al., 1991; Ganapathy and Leibach, 1982) and can support the growth of rat in absence of Met (Anderson et al., 1976; Njaa, 1962). Although very extensive works about the utilization of MSO and MSO_2 in animals (Anderson et al., 1976; Njaa, 1962; Ganapathy and Leibach, 1982; Bennett, 1941; Miller and Samuel, 1970) have been conducted. But very few reports related to microorganisms are available (Ejiri et al., 1979, 1980) without measuring the rate of reduction capability of MSO and MSO_2, while no report has practically been made by using rumen microorganisms. It is worthwhile to observe from the standpoint of the host nutrition whether rumen microorganisms utilize the oxidized products of Met *e.g.*, MSO and MSO_2.

The present study concerns the utilization of MSO and MSO_2 by mixed rumen bacteria (B), protozoa (P), and their mixture (BP).

Materials and methods

Animals and their management

Three mature rumen-fistulated goats (Japanese native breed, castrated, live weight of 35 ± 5 kg) fed on a daily ration consisting of lucerne (*Medicago sativa*) cubes (23 g DM/kg $BW^{0.75}$) (DM, dry matter; $BW^{0.75}$, metabolic body weight) and concentrate mixture (8 g DM/kg $BW^{0.75}$) in two equal portions given at 0900 and 1700 were used for the experiments. The goats were housed in individual pens under approximately constant environmental conditions with a good ventilation system. The animals had free access to water.

Preparation of rumen microbial suspensions

Rumen contents obtained from the fistulated goats before the morning feed were strained through four layers of surgical gauze into a separator funnel which was gassed with a mixture of 95% N_2 and 5% CO_2. The strained contents were then incubated in a prewarmed water bath at 39°C for about 60 min to allow feed debris to float. The suspensions of mixed bacteria (B) and mixed protozoa (P) and B plus P (BP) were prepared according to 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.

Incubation and sample treatments

Microbial suspensions (20 ml) were anaerobically incubated with and without 1 mM MSO or MSO_2 as a substrate in 30 ml Erlenmeyer flasks in a prewarmed shaking water bath at 39°C for 12 h. All incubations contained 0.5 mg/ml of rice starch. Anaerobiosis was maintained by gassing with a mixture of 95% N_2 and 5% CO_2 that was passed through a column of copper turnings heated at 350°C. Samples (1 ml) were collected at 0, 6, and 12 h into 2 ml Eppendorf tubes each of which contained 1 ml of 4% (w/v) sulfosalicylic acid (including 10 mM EDTA-3K) for deproteinization and kept overnight at 4°C. The deproteinized samples were centrifuged at 27,000 × g for 35 min at 4°C. The supernatant fluids were filtered through a membrane filter (0.45 μm porosity) and stored at 4°C until HPLC analyses. 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) and stored at 4°C.

Methods of analyses

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-formation 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 (Helrich, 1990) from 1 ml (triplicate of each) of microbial suspensions.

Experimental design

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. 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. The statistical significance of the results was analyzed by using Student's t-test (Snedecor and Cochran, 1980). Differences at $P < 0.05$ were considered to be significant.

Results

Disappearance of MSO

Rate of disappearance of MSO from the suspensions of *in vitro* cultures is shown in Table 1. The disappearance of MSO (1 mM) in B during 6- and 12-h incubation periods were 28.3 and 42.0%, respectively; while in BP, these values were 40.6 and 62.4%, respectively. On the other hand, MSO in P suspension remained unchanged throughout the incubation period. When the disappearance rate was expressed in the form of "per g microbial nitrogen (MN)", the disappearance rate in B (41.9 μmol/g MN/h) was 22.6% higher compared with BP during the 12-h incubation period. And the difference was statistically significant ($P < 0.05$).

Production of Met from MSO

Table 1 shows the production of Met from MSO by rumen microbial fractions during a 12-h incubation period. In B suspensions, Met production from MSO was found to be 61.4 and 76.7 μM in the supernatant of the incubation medium, and 50.1 and 66.8 μM in the hydrolyzates of bacterial cells during 6- and 12-h incubations, respectively. As a result, 39.5 and 34.2% of the disappeared MSO remained as Met after 6- and 12-h incubations, respectively. In P suspensions, Met was not produced during the experimental period.

In BP suspensions, the production of Met was 193.9 and 240.2 μM in the supernatants of the medium, and 44.3 and 58.1 μM in the hydrolyzates of BP cells during 6- and 12-h incubations, respectively. It was observed in BP that Met equivalent to 58.7 and 47.8% of the disappeared MSO was produced during 6- and 12-h

Table 1. Conversion of methionine sulfoxide (MSO) to Met, methionine sulfone (MSO_2) and 2-aminobutyric acid (2AB) in the suspensions of mixed rumen bacteria (B), protozoa (P), and B plus P (BP) during a 12-h incubation period*

Compounds	B		P		BP	
	6 h	12 h	6 h	12 h	6 h	12 h
MSO						
S	717.5 ± 48.3	580.1 ± 37.3	0.00	0.00	594.3 ± 44.7	376.1 ± 29.6
Met						
S	61.4 ± 5.76	76.7 ± 8.24	0.00	0.00	193.9 ± 16.5	240.2 ± 20.5
H	50.1 ± 6.29	66.8 ± 4.21	0.00	0.00	44.3 ± 6.23	58.1 ± 5.72
T	111.5 ± 10.87	143.5 ± 11.43	0.00	0.00	238.2 ± 21.4	298.3 ± 24.6
(μmol/g MN)	133.5 ± 14.6	171.9 ± 17.2	0.00	0.00	156.6 ± 17.3	196.1 ± 16.3
MSO_2						
S	18.7 ± 2.71	32.4 ± 4.13	0.00	0.00	25.7 ± 3.76	46.2 ± 5.73
(μmol/g MN)	22.4 ± 3.2	38.8 ± 4.8	0.00	0.00	16.9 ± 2.9	30.4± 4.1
2AB						
S	6.3 ± 2.4	15.7 ± 3.62	0.00	0.00	17.2 ± 3.12	33.4 ± 4.54
(μmol/g MN)	7.5 ± 2.1	18.8 ± 3.4	0.00	0.00	11.4 ± 2.7	22.0 ± 3.1

* Values are shown as concentrations of the products (μM) (Mean values of nine determinations ± standard deviation) remained in the incubations

S, supernatant; *H*, hydrolyzate; *T*, S + H; *MN*, microbial nitrogen. Average microbial nitrogen measurements (mg N/ml) in B, P, and BP suspensions were 0.835, 0.782, and 1.521, respectively

incubations, respectively. About 80.5–81.4% of the total synthesized Met was accumulated as free form in the supernatant of BP. When the production abilities of different microbial fractions were calculated on the basis of "per g MN", the production of Met from MSO in BP was higher than that in B ($P < 0.05$, both in 6- and 12-h incubations).

Production of MSO_2 from MSO

Table 1 presents the production of MSO_2 in the supernatant of the medium from MSO by B, P, and BP suspensions during a 12-h incubation period. In B suspension, 18.7 and 32.4 μM of MSO_2 was produced in the supernatant of the medium during 6- and 12-h incubations, respectively. It was observed that 6.6 and 7.7% of the disappeared MSO was converted to and remained as MSO_2 during 6- and 12-h incubations, respectively. MSO_2 was not found to produce by P.

In BP suspensions, the amount of MSO_2 increased in the supernatant of the incubation medium. As a whole, MSO_2 remained by 6.3 and 7.4% of molar amounts of the disappeared MSO during 6- and 12-h incubations, respectively. When expressed as "per g MN", the amount of MSO_2 remained was higher in B than that in BP ($P < 0.05$, both in 6- and 12-h incubations).

Production of 2-aminobutyric acid (2AB) from MSO

The production of 2AB from MSO by B, P, and BP is shown in Table 1. In B suspension, 2.2 and 3.7% of the disappeared MSO remained as 2AB during 6- and 12-h incubations, respectively. On the other hand, no formation of 2AB appeared in P.

In BP suspensions, 4.2 and 5.4% of the MSO that disappeared was converted to and remained as 2AB at the same incubations period as mentioned above. The production of 2AB in BP (per g MN) was 1.2–1.5 folds higher than that in B.

Discussion

Our report is the first to deal with the quantitative study on the metabolism of MSO by the three different fractions of rumen microorganisms *i.e.*, mixed rumen bacteria, mixed rumen protozoa, and their mixture, so far as we are aware. In the present experiment a significant amount of Met was found to be produced from MSO by the mixed rumen bacteria and a small portion of the synthesized Met was found in the hydrolyzates of bacterial cells (Table 1) which indicated that rumen bacteria efficiently utilized Met produced from MSO for their cell protein synthesis (Armstead and Ling, 1993; Or-Rashid et al., 2001; Wright and Hungate, 1967).

On the contrary, mixed rumen protozoa were not capable to synthesize Met from MSO because it could not degrade MSO at all. Therefore, it seemed that methionine sulfoxide reductase, the enzyme that catalyzes the reduction of MSO to Met might not be present in rumen protozoa.

The rumen microbial ecosystem mainly consists of bacteria and protozoa. In this experiment, BP suspension was also employed for incubation to speculate the real activities of the rumen microbial ecosystem and to investigate interactions between bacteria and protozoa. To our surprise, the rumen protozoa alone could not produce any Met from MSO, but when they were mixed with rumen bacteria, Met production (μmol/g MN) capability of BP suspensions increased by 17.3 and 14.1% than that of B alone during 6- and 12-h incubations, respectively. It seems that the association of P with B in the suspension may accelerate the activities of the relevant enzyme [*i.e.*, methionine sulfoxide reductase] and can be regarded as a positive interaction between the bacteria and the protozoa, although the reason of the higher value of Met production in BP in our observation is exactly not known. Thus it is suggested that co-existence of P in B suspension may have beneficial effect in the conversion of MSO to Met in the natural rumen microbial ecosystem. Although Ejiri et al. (1979) have reported that *Escherichia coli* could reduce MSO to Met; the evidence of the reduction of MSO to Met by mixed rumen bacteria and the mixture of mixed bacteria and protozoa, and that incapability of mixed protozoa shown in the present research was a new demonstration in the field of rumen microbiology.

In the present study, B and BP also produced MSO_2 from MSO. Rumen protozoa were not able to form MSO_2 from MSO as it could not degrade MSO. Present experiment also revealed that MSO_2 production ability of BP was 24.6% lower than that of B (22.4 μmol/g MN). It is known that protozoa consume oxygen and may have a significant role in ruminal oxygen scavenging (Williams and Coleman, 1992). Thus association of P with B in the suspension (*i.e.*, in BP suspension) markedly decrease the oxidation of MSO to MSO_2 compared with B suspension and the MSO that not oxidized to MSO_2 in BP, may be used further to produce Met.

The present observation also revealed that 2AB was formed from MSO by B and BP, while P failed to do the same. Incubation of Met in mixed protozoa (Onodera and Migita, 1985) and in *E. coli* (Meister, 1965) has been reported to produce 2AB via 2-oxobutyric acid. Considering the aforementioned fact, it seemed that 2AB was produced from Met being formed at first from MSO.

It was evident from the present study that the different rumen microbial fractions, *i.e.*, B, P, and BP, were neither capable of producing Met from MSO_2 nor able to degrade MSO_2 during the experimental period. It was documented from a study conducted by Ejiri et al. (1979) that *E. coli* had no ability to synthesize Met from MSO_2 via MSO.

From this *in vitro* study it is concluded that MSO can be utilized by the rumen bacteria and the mixture of rumen bacteria and protozoa because of their ability to reduce this compound to Met, by which, ultimately host animal might be benefited. On the other hand, rumen microorganisms, *i.e.*, B, P, and BP cannot utilize MSO_2 from producing Met.

Acknowledgements

The authors are extremely grateful to Professor H. Ogawa, the University of Tokyo, Japan and Dr. Takashi Hasegawa, Miyazaki University, Japan for inserting permanent rumen fistulae in goats. We would like to thank MONBUSHO for the award of a research scholarship to Mamun M. Or-Rashid since 1996–2001.

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Authors' address: Shaila Wadud, Laboratory of Animal Nutrition and Biochemistry, Division of Animal Science, Miyazaki University, Miyazaki 889-2192, Japan, Fax. +81-985-58-7201, E-mail: rafatkun@hotmail.com