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METABOLISM OF ASPARTIC ACID BY MIXED RUMEN BACTERIA AND PROTOZOA, *IN VITRO*

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SUMMARY

Formation of the three stereoisomers (SI) of 2,6-diaminopimelic acid (DAP), lysine and pipecolic acid from aspartic acid by mixed rumen bacteria and mixed rumen protozoa were examined. Mixed rumen bacteria and protozoa were isolated from the rumen of sheep given a concentrate and hay diet, and incubated with and without aspartic acid. The microbial suspensions were anaerobically incubated at 39°C for 12 h. When aspartic acid was added to the media, the results showed that mixed rumen bacteria produced *meso*-DAP by 4.42%, LL-DAP by 0.025%, lysine by 4.22% during the12 h incubation periods, but DD-DAP was not detected during the incubation periods. Mixed rumen protozoa produced *meso*-DAP by 4.62%, lysine by 8.86% and pipecolic acid by 0.84% during the 12 h incubation periods, but LL-DAP and DD-DAP were not detected during the incubation periods. This means that mixed rumen bacteria have the ability to synthesis *meso*-DAP, LL-DAP and lysine from aspartic acid, meanwhile, the mixed rumen protozoa have the ability to produce *meso*-DAP, lysine and pipecolic acid from aspartic acid.

Keywords: Aspartic acid, stereoisomers of diaminopimelic acid, lysine, pipecolic acid, metabolism, mixed rumen protozoa, mixed rumen bacteria

INTRODUCTION

L-Lysine is one of the final products of the aspartate biosynthetic pathway in bacteria. In general, bacteria synthesize lysine from aspartate by the diaminopimelic acid (DAP) pathway (Cohen *et al.*, 1965; Dewey and Work, 1952; Stadman *et al.*, 1961; White *et al.*, 1964). Shewry *et al.* (1977) demonstrated that aspartate kinase, which catalyzed the first reaction of the conversion of aspartate to lysine, was regulated by feed-back inhibition. In contrast, yeast and other higher fungi employ the α-aminoadipic acid pathway for the biosynthesis of lysine (Broquis, 1971; Strassman and Weinhouse, 1953). Onodera and Kandatsu (1974) demonstrated that rumen ciliate protozoa have the ability to synthesis negligible amounts of DAP and lysine from aspartate using paper chromatography. The first demonstration of the synthesis of lysine by decarboxylation of DAP by rumen protozoa was reported by Onodera and Kandatsu (1973, 1974).They also demonstrated the synthesis of lysine from DAP bound in the peptidoglycan of the cell walls of *Escherichia coli* isolated from the rumen (Onodera *et al.*, 1974). Sauer *et al.* (1975) reported that, in mixed rumen cultures, lysine was synthesized mainly via DAP pathway as shown by the position of labeled carbons in the lysine molecule synthesized from [1-^{14}C]acetate or $H^{14}CO_3^-$. Later, rumen bacteria were also shown to synthesize lysine from DAP as reported by Masson and Ling (1986).

Generally, bacteria (White and Kelly, 1965; Asada *et al.*, 1981a) and plants (Bryan, 1980) synthesize lysine from *meso*-DAP by *meso*-diaminopimelate decarboxylase (EC 4.1.1.20) (Kelland *et al.*, 1985; Asada *et al.*, 1981b). Recently, lysine has been synthesized from each type of the stereoisomers of DAP (DAP-SI) by mixed rumen bacteria and mixed rumen protozoa as well (El-Waziry *et al.*, 1995a,b; El-Waziry and Onodera, 1996a,b; Onodera *et al.*, 1996, 1997a,b). With regard to the rumen bacteria and protozoa, however, there have been no reports concerning the types of DAP-SI synthesized from aspartic acid, and then converted to lysine and pipecolic acid. Therefore, the objective of this study was to investigate the types of DAP-SI that can be synthesized from aspartic acid by mixed rumen bacteria and protozoa. It is also of interest to investigate whether these types were converted to lysine and pipecolic acid.

MATERIALS AND METHODS

Animals

Three mature rumen fistulated sheep with a mean live weight of 45 (S.D.± 5 kg) kg, fed on a daily ration consisting of 900 g/day hay and 250 g/day concentrate mixture in two equal portions given at 0900 and 1700 were used for the experiment. The sheep were housed in individual pens under approximately constant environmental conditions. They had free access to fresh water.

Preparation of rumen microbial suspensions

Rumen content obtained from the fistulated sheep before morning feed were strained through four layers of surgical gauze into a separatory funnel which was gassed with a mixture of 95% N_2 and 5% CO_2. The contents were incubated at 39°C for up to 60 min to allow feed debris to float. The suspensions of bacterial (B) and protozoal (P) 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 suppress the biochemical activities of contaminating bacteria.

Incubation and sample treatments

Buffer solution (MB9) (Onodera and Henderson, 1980) and all glassware used in the present study were previously autoclaved and MB9 buffer was gassed with N_2+CO_2 again under aseptic conditions. Aspartic acid (Sigma Chemical Co., St. Louis, MO, USA) solution as substrate was prepared by dissolving in MB9 buffer. The microbial suspensions (20 ml) prepared above were incubated with and without aspartic acid (5 mM) in 30 ml Erlenmeyer flasks for up to 12 h at 39°C. All incubations contained 0.5 mg/ml rice starch. Samples collected (1 ml) at 0, 6 and 12 h were mixed with an equal volume of 20% trichloroacetic acid (TCA) for deproteinization, left over night and centrifuged at 27,000 x g for 20 min. The supernatant was washed three times with diethyl ether to remove TCA and evaporated to remove diethyl ether. Sediment of bacteria was hydrolyzed with 6 M HCl at 110°C for 20 h in sealed tubes. After cooling, the contents were filtered through filter paper (Whatman No. 2) and washed three times with distilled water. The filtrate was evaporated to dryness, washed three times with distilled water to remove HCl, dissolved again in 1 ml of pure water, and then filtered again through a 0.45 μm membrane filter (Toyo Roshi Kaisha, Tokyo, Japan) before analysis. All samples were subjected to analyses for DAP-SI, lysine, and pipecolic acid.

Analytical methods

Analyses of DAP-SI and lysine were carried out by HPLC according to El-Waziry *et al.* (1996). Pipecolic acid was carried out by the method of Armstrong *et al.* (1993). Concentrations of these compounds were expressed by the differences between the figures of incubations with and without substrates. Volatile fatty acids (VFA) were determined by gas chromatography according to Supelco, Inc. (1975).

RESULTS AND DISCUSSION

In nature, the biosynthesis of lysine proceeds via two different pathways. In bacteria, lysine is synthesized by the DAP pathway , and α -aminoadipic acid is the pathway of lysine synthesis in yeast and other higher fungi. Higher plants synthesize lysine from aspartate, alanine and acetate as well as bacteria and green by higher fungi (*Candida utilis*) synthesize lysine from acetate only (Vogel, 1959). Recently, mixed ruminal bacteria and protozoa have been shown to epimerize DAP-SI and to lysine not only from *meso*-DAP, but also from LL-and DD-DAP (El-Waziry *et al.*, 1995a,b; El-Waziry and Onodera, 1996a,b; Onodera *et al.* 1996, 1997a,b). The lysine production from *meso*-DAP was the greatest and followed by LL-and DD-DAP. The amount of lysine produced from each type of DAP-SI in the previous reports seemed to be lower than the value produced from mixed DAP-SI (El-Waziry *et al.*, 1995a,b; El-Waziry and Onodera, 1996a,b; Onodera *et al.*, 1996, 1997a,b). Onodera and Kandatsu (1974) reported that mixed rumen protozoa have the ability to form a negligible amount of lysine from aspartate using a paper chromatography. Pipecolic acid was eventually shown to be an end product in the metabolism of lysine by mixed rumen protozoa (Onodera and Kandatsu, 1972) There are no reports concerning the types of DAP-SI that can be synthesized from aspartic acid and convertible to lysine and pipecolic acid by rumen bacteria and protozoa.

Metabolism of three stereoisomers of diaminopimelic acid by rumen bacterial suspension

In the case of B suspension, both supernatant of the medium and hydrolysate of the bacteria were analyzed, because bacteria can grow in this incubation and accumulate lysine in the cell protein (El-Waziry and Onodera, 1996a).

Table 1 shows the conversion of aspartic acid to DAP-SI and lysine in the supernatant and hydrolysates of the B suspensions during the 12 h incubation period. *meso*-DAP and LL-DAP were only produced from aspartic acid during the incubation period, but DD-DAP was not detected (Table 1). As the results, aspartic acid was converted to *meso*-DAP and LL-DAP by 0.091 mM (1.82%) and 0.0006 mM (0.01%) during a 6 h incubation and 0.221 mM (4.42%) and 0.0013 mM (0.03%) during the 12 h incubation, respectively. Average bacterial nitrogen in B suspension in the present experiment was 0.445 ± 0.007 mg/ml, and the amount of *meso*-DAP and LL-DAP produced could be expressed as

204 and 0.135 nmol/mg N during a 6 h incubation and 497 and 0.292 nmol/mg N during the 12 h incubation, respectively. The production of DAP from aspartic acid was demonstrated by Patte *et al.* (1967) using *E. coli*.

Table 1. Amounts of the three stereoisomers of 2,6-diaminopimelic acid (DAP) and lysine produced from aspartic acid by mixed rumen bacteria[a]

	Incubation time (h)	
	6	12
Meso-DAP (mM)	0.091 ± 0.002[b,c]	0.221 ± 0.001
Meso-DAP (%)	1.82	4.42
Meso-DAP (nmol/mg bacterial N)	204.49 ± 4.49	496.63 ± 2.25
LL-DAP (mM)	0.006 ± 0.0001	0.0013 ± 0.0001
LL-DAP (%)	0.01	0.03
LL-DAP (nmol/mg bacterial N)	0.135 ± 0.225	0.292 ± 0.222
DD-DAP (mM)	ND[d]	ND
Lysine (mM)	0.116 ± 0.001	0.211 ± 0.002
Lysine (%)	2.32	4.22
Lysine (nmol/mg bacterial N	260.67 ± 2.25	474.16 ± 4.49

[a] Aspartic acid was added to the bacterial suspensions as substrate (5mM).
[b] Values are shown as the differences between those with and without substrate after incubation at 39°C.
[c] Values are shown with mean ± (n=3).
[d] Not detected.

Table 1 also shows the conversion of aspartic acid to lysine in supernatant and hydrolysed of the media of mixed rumen bacteria during the 12 h incubation period. As the results, the amounts of lysine were 0.116 mM (2.32%, 261 nmol/mg N) and 0.211 mM (4.22%, 474 nmol/mg N) for 6 and 12 h, respectively. In bacteria, Cohen *et al.* (1965); Dewey *et al.* (1952); Stadman *et al.* (1961) and White *et al.* (1964) demonstrated that the characterizations of the enzymes catalyzing the reactions of the conversion of L-aspartate to L-lysine by the way of α:ε DAP. They further reported that L-aspartate-β-semialdehyde was not only the intermediate of L-lysine, but also the intermediate of L-threonine, L-isoleucine and L-methionine. Therefore, the present study was confirmed their suggestion for the production of lysine from aspartic acid via DAP using mixed rumen bacteria.

Table 2 shows the amounts of volatile fatty acids (VFA) from aspartic acid in supernatant and hydrolysed of the media of mixed rumen bacteria during the 12 h incubation period. As the results, the amounts of acetic acid, propionic acid and butyric acid were 0.031, 0.0012 and 0.025 mM during the 12 h incubation period, respectively. The first study concerning metabolic pathway of lysine in rumen microorganisms reported by Dohner and Cardon (1954). They showed that 1 mole each of acetate and butyrate and 2 moles of ammonia were produced from lysine after incubation of 2 strains of *E. coli* isolated from the bovine rumen. Onodera and Kandatsu, (1975) also, examined the metabolites of lysine (1mM) by mixed rumen bacteria collected from goat rumen using L-[U-^{14}C]lysine. They found that the main products from lysine were acetate, butyrate and small amounts of propionate. Therefore, the production of VFA of acetate, propionate and butyrate from aspartic acid in the present study may be via lysine according to Dohner and Cardon (1954), and Onodera and Kandatsu, (1975).

Table 2. Amounts of volatile fatty acids produced from aspartic acid by mixed rumen bacteria[a]

Acid	Incubation time (h)	
	6	12
Acetic acid (mM)	0.0085 + 0.001[bc]	0.0310 +0.001
Propionic acid (mM)	0.0003 +0.000	0.0012 + 0.0001
Butyric acid (mM)	0.0071 + 0.001	0.0253 + 0.001

[a] Aspartic acid was added to the bacterial suspensions as substrate (5mM).
[b] Values are shown as the differences between those with and without substrate after incubation at 39°C.
[c] Values are shown with mean ± (n=3).

Metabolism of three stereoisomers of diaminopimelic acid by rumen protozoal suspension

In the case of P suspension, only supernatant fluid of the incubation was used for the analyses of DAP-SI and lysine, because in this incubation, protozoa cannot grow and accumulate any lysine in their cell protein.

Table 3 shows the conversion of aspartic acid to DAP-SI, lysine and pipecolic acid in supernatant of the media of mixed rumen protozoa during the 12h incubation period. *meso*-DAP apparently was

only produced from aspartic acid during incubation period, but LL-DAP and DD-DAP were not detected (Table 3). As a result, aspartic acid partly was converted to *meso*-DAP by 0.025 mM (0.5%) during a 6 h incubation and 0.231 mM (4.62%) during the 12h incubation period. In this study, average protozoal nitrogen of the protozoal suspension was 0.776 ± 0.007 mg/ml, and the amounts of *meso*-DAP produced during the incubation period were 32.22 and 297.68 nmol/mg protozoal nitrogen at 6 and 12h, respectively. Onodera and Kandatsu (1974) reported that a negligible amount of DAP was produced from aspartic acid during 12h incubation. The differences reported in this study and the study of Onodera and Kandatsu (1974) may be attributed to the activity of the protozoa during the incubation periods. Patte *et al.* (1967 reported that the 4-carbon chain of aspartate is incorporated into methionine. The two first reactions, catalyzed by β-aspartokinase (EC 2.7.2.4) and by aspartate-β-semialdehyde dehydrogenase (EC 1.2.1.11) are common steps in *Escherichia coli* of the biosynthesis of DAP, lysine, methionine, threonine and isoleucine. In this regard, the present results suggest that the mixed rumen protozoa have the ability to synthesize *meso*-DAP from aspartic acid via two reactions, β-aspartokinase and by aspartate-β-semialdehyde dehydrogenase according to Patte *et al.* (1967). In this study, the synthesis *meso*-DAP from aspartic acid by rumen protozoa was demonstrated for the first time.

Table 3 also shows the production of lysine from aspartic acid in supernatant of the media of mixed rumen protozoa during the 12h incubation period. As indicated from Table 3, the amount of lysine was 0.243 mM (4.86%) during a 6 h incubation and 0.443 mM (8.86%) during the 12 h incubation period.

Table 3. Amounts of the three stereoisomers of 2,6-diaminopimelic acid (DAP), lysine and pipecolic acid produced from aspartic acid by mixed rumen protozoa[a]

	Incubation time (h)	
	6	12
Meso-DAP (mM)	0.025 ± 0.001[b,c]	0.231 ± 0.03
Meso-DAP (%)	0.5	4.62
Meso-DAP (nmol/mg protozoal N)	32.22 ± 1.28	297.68 ± 38.12
LL-DAP (mM)	ND[d]	ND
DD-DAP (mM)	ND	ND
Lysine (mM)	0.243 ± 0.093	0.443 ± 0.025
Lysine (%)	4.86	8.86
Lysine (nmol/mg protozoal N)	313.14 ± 22.00	570.88 ± 32.00
Pipecolic acid (mM)	0.007 ± 0.001	0.042 ± 0.003
Pipecolic acid (%)	0.14	0.84
Pipecolic acid (nmol/mg protozoal N)	9.02 ± 1.27	54.12 ± 3.8

[a] Aspartic acid was added to the protozoal suspensions as substrate (5mM).
[b] Values are shown as the differences between those with and without substrate after incubation at 39° C.
[c] Values are shown with mean ± (n=3). [d] Not detected.

The amounts of lysine produced during the incubation were 313.14 and 570.88 nmol/mg protozoal nitrogen for 6 and 12 h, respectively. Onodera and Kandatsu (1974) demonstrated that rumen ciliate protozoa synthesized a negligible amounts of lysine from aspartic acid. This discrepancy may be also due to the differences in activity of the protozoa in the incubations. The first demonstration of the synthesis of lysine by the decarboxylation of DAP by rumen protozoa was reported by Onodera and Kandatsu (1973, 1974). Therefore, the lysine production from aspartic acid in the present study was probably via DAP-decarboxylase (El-Waziry and Onodera, 1996a,b).

Table 3 also shows the production of pipecolic acid from aspartic acid in supernatant of the media of mixed rumen protozoa during the 12 h incubation period. The amount of pipecolic acid was 0.007 mM (0.14%) during a 6 h incubation and was 0.042 mM (0.84%) during the 12 h incubation period. The amounts of pipecolic acid produced during the incubation period were 9.02 and 54.12 nmol/mg protozoal nitrogen for 6 and 12 h, respectively. Rumen protozoa could produce pipecolate from lysine and it was eventually shown to be an end product in the metabolism of lysine by mixed rumen protozoa (Onodera and Kandatsu, 1972). In this regard, the present study suggest that the pipecolic acid may have been produced from aspartic acid via lysine as an end product of lysine metabolism in rumen protozoa (Onodera and Kandatsu, 1972).

It can be concluded that the main substance produced from aspartic acid is lysine via DAP decarboxylase and then the from lysine in the rumen are thought as present to be acetate, butyrate, propionate, pipecolate and undetermined compound like ammonia. The VFA are in the fate to be absorbed and utilized by the host animal and ammonia is utilized by bacteria and other organisms. The

fate of pipecolate in the rumen and its possible physiological function in the host animal (Onodera, 1993).

It is interesting to have shown in the present study that mixed rumen bacteria and protozoa may contribute to the nutrition of the host animal by producing LL-DAP, *meso*-DAP, lysine, VFA of acetic, butyric and propionic acids and pipecolic acid from aspartic acid.

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