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Synthesis of Diaminopimelic Acid, Lysine and Pipecolic Acid from Spartic Acid by Rumen Microorganisms

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Abstract: The current study was examined the formation of the three Stereoisomers (SI) of 2, 6-Diaminopimelic Acid (DAP), lysine and pipecolic acid from aspartic acid by mixed rumen bacteria and protozoa. Mixed rumen bacteria and protozoa were isolated from the rumen of sheep given concentrate mixture and hay and incubated with and without aspartic acid (5 mM). The microbial suspensions were anaerobically incubated at 39°C for 12 h. The results showed that mixed rumen bacteria produced meso-DAP by 0.221 mM, LL-DAP by 0.013 mM, lysine by 0.211 mM during the 12 h incubation periods. This is the first finding to show the synthesis of DAP-SI and lysine from aspartic acid by rumen bacteria. Mixed rumen protozoa produced meso-DAP by 0.231 mM, lysine by 0.443 mM and pipecolic acid by 0.042 mM during the 12 h incubation periods. DD-DAP was not detected during the incubation periods of mixed rumen bacteria and protozoa and also LL-DAP was not detected by mixed rumen protozoa. The synthesis meso-DAP from aspartic acid by rumen protozoa was demonstrated for the first time. The results indicated 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 produced meso-DAP, lysine and pipecolic acid from aspartic acid.

Key words: Stereoisomers of diaminopimelic acid, lysine, pipecolic acid, metabolism, mixed rumen bacteria, mixed rumen protozoa

INTRODUCTION

Lysine is the final products of the meso-DAP pathway in ruminal bacteria and protozoa (El-Waziry *et al*., 2000; El-Waziry, 2003). Many researchers demonstrated that 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). Aspartate kinase which catalyzed the first reaction of the conversion of aspartate to lysine was regulated by feed-back inhibition (Shewry and Miflin, 1977). In contrast, fungi included yeast allow the α-aminoadipic acid pathway for biosynthesis of lysine (Broquist, 1971; Strassman and Weinhouse, 1953). Onodera and Kandatsu (1974) established that mixed rumen protozoa have the ability to synthesize 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). Masson and Ling (1986) reported that mixed rumen bacteria were also shown to synthesize lysine from DAP. In general, 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). Later, lysine has been synthesized from each type of stereoisomers of DAP (DAP-SI) by mixed rumen bacteria and protozoa (El-Waziry *et al*., 1995a, b; El-Waziry and Onodera, 1996a, b; Onodera *et al*., 1997a, b; El-Waziry, 2003). 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 aimed to investigate the types of DAP-SI that can be synthesized from aspartic acid by mixed rumen bacteria and protozoa and to investigate whether these types were converted to lysine and pipecolic acid.

MATERIALS AND METHODS

Animals: A total of 3 mature rumen fistulated sheep with a mean live weight of 45 (SD 5 kg) kg, fed on a daily ration consisting of 900 hay and 250 g day^{-1} concentrate mixture in two equal portions given at 0900 and 1700 h 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 contents 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^{-1} 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 tools 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^{-1} 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 overnight and centrifuged at 27,000 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 analysed by HPLC according to El-Waziry *et al.* (1996a). Pipecolic acid was determined according to 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.

RESULTS AND DISCUSSION

Lysine is essential of amino acid for the growth of mammalian nutrition and must be present in the diet. Ruminant animals such as cattle, sheep and goats can live on a lysine-deficient diet because the amino acid is synthesized by microorganisms in the rumen (Edwards and Darroch, 1956).

In nature, lysine synthesize via different pathways. In bacteria, lysine is synthesized by the DAP and in yeast and other higher fungi lysine is produced via α-aminoadipic acid. Furthermore, mixed rumen protozoa and bacteria as well can produce lysine by the pathway of diaminopimelate decarboxylase (Onodera and Kandatsu, 1972; El-Waziry *et al.*, 1995a, b; El-Waziry and Onodera, 1996a, b; Onodera *et al.*, 1997a, b; El-Waziry, 2003). Onodera found that by an *in vitro* trial, lysine production by faunated goat rumen contents is 23% higher than that from defaunated goats.

Synthesis of stereoisomers of diaminopimelic acid, lysine and volatile fatty acids by mixed rumen bacteria suspensions: In the case of bacteria suspension, both supernatant of the medium and hydrolysate of bacteria were analyzed because bacteria can grow in this incubation and accumulate lysine in cell protein (El-Waziry and Onodera, 1996a). The conversion of aspartic acid to DAP-SI and lysine in bacteria suspensions (supernatants and hydrolysates) during the 12 h incubation period are shown in Table 1. The meso-DAP and LL-DAP produced from aspartic acid but DD-DAP was not detected in the media of bacteria suspensions. Average bacterial nitrogen in B suspension in the current experiment was 0.445±0.007 mg mL^{-1}. The values of DAP-SI were 0.091 mM (204.49 nmol mg^{-1} bacterial N) and 0.006 mM, (0.135 nmol mg^{-1} bacterial N) for meso-DAP and LL-DAP during 6 h incubation, respectively. However, the values of meso-DAP and LL-DAP during the 12 h incubation times were 0.221 mM (496.63 nmol mg^{-1} bacterial N) and 0.013 mM (0.292 nmol mg^{-1} bacterial N), respectively.

Table 1 also shows lysine production from aspartic acid in supernatant and hydrolysed of the media of mixed bacteria during the 12 h incubation periods. The amounts of lysine were 0.116 mM (260.67 nmol mg^{-1} bacterial N)

Table 1: The three stereoisomers of Diaminopimelic Acid (DAP) and lysine produced from aspartic acid by mixed rumen bacteria[a] (Mean±SD)

	Incubation time (h)	
Stereoisomers	6	12
meso-DAP (mM)	0.091±0.002[b]	0.221±0.001
meso-DAP (nmol mg^{-1} bacterial N)	204.49±4.49	496.63±2.25
LL-DAP (mM)	0.006±0.0001	0.013±0.0001
LL-DAP (nmol mg^{-1} bacterial N)	0.135±0.225	0.292±0.222
DD-DAP (mM)	ND[c]	ND
Lysine (mM)	0.116±0.001	0.211±0.002
Lysine (nmol mg^{-1} bacterial N)	260.67±2.25	474.16±4.49

[a]Aspartic acid was added to the bacterial suspensions as substrate (5 mM); [b]Values are shown as the differences between those with and without substrate after incubation at 39°C. [c]ND: Not Detected

and 0.211 mM (474.16 nmol mg^{-1} bacterial N) for 6 and 12 h, respectively. Patte *et al.* (1967) established that the production of DAP from aspartic acid but using *E. coli*. Many researchers demonstrated the characterizations of the enzymes catalyzing the reactions of the conversion of L-aspartate to L-lysine via DAP pathway (Cohen *et al*., 1965; Dewey and Work, 1952, Stadman *et al*., 1961; White *et al*., 1964). They also 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. This is the first finding to show the synthesis of DAP-SI and lysine from aspartic acid by rumen bacteria.

The amount of individual Volatile Fatty Acids (VFA) produced from aspartic acid in supernatant and hydrolysed of the media of mixed rumen bacteria during the 12 h incubation period as end products of lysine are shown in Table 2. The values were 0.031, 0.0012 and 0.0253 mM for acetic, propionic and butyric acids, respectively. Dohner and Cardon (1954) showed the first metabolic pathway of lysine in rumen microorganisms, they reported that 1 mole each of acetate and butyrate and 2 mols of ammonia were produced from lysine by *E. coli* isolated from bovine rumen. Onodera and Kandatsu (1975) found that that the main products from lysine were acetate butyrate and small amounts of propionate by mixed rumen bacteria. Therefore, the production of individual VFA from aspartic acid in the current study may be via lysine according to Dohner and Cardon (1954) and Onodera and Kandatsu (1975). Thus, the production of individual VFA from aspartic acid via DAP and lysine by mixed rumen bacteria was evidenced for the first time.

Synthesis of stereoisomers of diaminopimelic acid, lysine and pipecolic acid by mixed rumen protozoa suspensions: 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 (El-Waziry and Onodera, 1996a). Table 3 shows the conversion of spartic acid to DAP-SI, lysine and pipecolic acid in supernatant of the media of mixed rumen protozoa during the 12 h incubation period. The meso-DAP apparently was only produced from aspartic acid during incubation period but LL- and DD-DAP were not detected (Table 3). Average protozoal nitrogen in P suspension in the present experiment was 0.776±0.007 mg mL^{-1}. Aspartic acid partly was converted to meso-DAP by 0.025 mM (32.22 nmol mg^{-1} protozoal N) during a 6 h incubation and 0.231 mM (0.443 nmol mg^{-1} protozoal N) during the 12 h incubation. A negligible amount of DAP produce from aspartic acid during 12 h incubation was reported by Onodera *et al*. (1974). The differences between the current study and the finding of Onodera and Kandatsu (1974) may due 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 and by aspartate-β-semialdehyde dehydrogenase are common steps in *E. coli* of the biosynthesis of DAP, lysine, methionine, threonine and isoleucine. In this regards, 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). The synthesis meso-DAP from aspartic acid by rumen protozoa was demonstrated for the first time in this study.

The production of lysine from aspartic acid in supernatant of the media of mixed rumen protozoa during the 12 h incubation periods is shown in Table 3. The values were 0.243 mM (313.14 nmol mg^{-1} protozoal N) during a 6 h incubation and 0.443 mM (570.88 nmol mg^{-1} protozoal N) during the 12 h incubation period. Rumen ciliate protozoa synthesized a negligible amount of lysine from aspartic acid as reported by Onodera and Kandatsu (1974). The discrepancy between their finding and the present study may be also due to the differences in activity of the protozoa in the incubations. Onodera and

Table 2: Volatile fatty acids produced from aspartic acid by mixed rumen bacteria[a] (Mean±SD)

	Incubation time (h)	
Volatile fatty acids	6	12
Acetic acid (mM)	0.0085±0.001[b]	0.0310±0.0010
Propionic acid (mM)	0.0003±0.000	0.0012±0.0001
Butyric acid (mM)	0.0071±0.0001	0.0253±0.0010

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

Table 3: The three stereoisomers of Diaminopimelic Acid (DAP), lysine and pipecolic acid produced from aspartic acid by mixed rumen protozoa[a] (Mean±SD)

	Incubation time (h)	
Stereoisomers	6	12
meso-DAP (mM)	0.025±0.001[b]	0.231±0.03
meso-DAP (nmol mg^{-1} protozoal N)	32.22±1.28	297.68±38.12
LL-DAP (mM)	ND[c]	ND
DD-DAP (mM)	ND	ND
Lysine (mM)	0.243±0.093	0.443±0.025
Lysine (nmol mg^{-1} protozoal N)	313.14±22.00	570.88±32.00
Pipecolic acid (mM)	0.007±0.001	0.042±0.003
Pipecolic acid (nmol mg^{-1} protozoal N)	9.02±1.27	54.12±3.8

[a]Aspartic acid was added to the bacterial suspensions as substrate (5 mM); [b]Values are shown as the differences between those with and without substrate after incubation at 39°C; [c]ND: Not Detected

Kandatsu (1973, 1974) reported the first demonstration of lysine synthesis by the decarboxylation of DAP by rumen protozoa. 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 pipecolic acid production from aspartic acid in supernatant of the media of mixed rumen protozoa during the 12 h incubation period. The amount of pipecolic acid was 007 mM (9.02 nmol mg^{-1} protozoal N) during a 6 h incubation and 0.42 mM (54.12 nmol mg^{-1} protozoal N) during the 12 h incubation period. Rumen protozoa could produce pipecolate from lysine and it was eventually shown to be an end produce in the metabolism of lysine by mixed rumen protozoa (Onodera and Kandatsu, 1972). In this regard, Hussain-Yusuf *et al*. (2002) reported that mixed rumen protozoa and different species of protozoa as *Polyplastron* sp. and *Diploplasron* sp. Produced pipecolic acid when lysine was added in the media as a substrate.

CONCLUSION

The main substance produced from aspartic acid is lysine via DAP decarboxylase and then from lysine in the rumen are thought as present to be acetate, propionate butyrate, 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 microorganisms. The fate of pipecolic acid in the rumen is as possible physiological function in the host animal. 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, propionic and butyric acids and pipecolic acid from aspartic acid.

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