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Determination of Total and Separate Stereoisomers of Diaminopimelic Acid in Five Species and Mixed Rumen Bacteria Using High-Performance Liquid Chromatography

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Abstract: Total (with one peak) and separate three stereoisomers of 2,6-diaminopimelic acid (DAP) in hydrolysed bacterial species and mixed rumen bacteria were determined. The contents of the total DAP of the hydrolysed mixed rumen bacteria collected from sheep and cattle on the average were 30.1 and 12.3 µmol g bacterial DM $^{-1}$. The contents of *meso*- and LL- DAP in the hydrolysed mixed rumen bacteria collected from sheep on the average were 26.2 and 3.8 and those collected from cattle were 8.0 and 4.1 µmol g bacterial DM^{-1} , respectively. DD-DAP was not detected in the mixed rumen bacteria of the sheep and cattle. The contents of total and separate stereoisomers of DAP in *Prevotella ruminicola B* $_1$ *4*, *Streptococcus bovis JB1*, *Selenomonas ruminantium Z108*, *Fibrobacter succinogenes S85* and *Anaerovibrio lipolytica 5S* ranged from 5.68 to 32.54, and from 5.63 to 32.12 µmol g bacterial DM $^{-1}$ for total DAP and *meso*-DAP, respectively. LL-DAP was absent from pure cultures.

Key words: Rumen bacteria, diaminopimelic acid, HPLC, stereoisomers of DAP

INTRODUCTION

2,6-Diaminopimelic acid (DAP) is known to be a constituent amino acid of peptidoglycan in various bacterial cell walls. DAP has been studied in the rumen ecosystem both as a marker of bacterial biomass[12] and as a precursor of lysine production by rumen protozoa[16,17]. These were confirmed by Masson and Ling[14], El-Waziry *et al* [6] and El-Waziry and Onodera[7]. DAP is chiefly synthesized by bacteria and partially used for lysine synthesis and partially incorporated in the peptidoglycan of bacterial cell walls[10]. Bacteria[24,1] and plants[2] synthesize lysine from *meso*-DAP, and LL-DAP can be a precursor of lysine via *meso*-DAP[23].

There have been some studies concerning the determination of the stereoisomers of DAP using HPLC in *Streptomyces* and *Nocardia* [20], rumen bacteria[2,5], rumen digesta[21,5,18] and feed samples[5,18]. However, they obtained only two peaks, one was for the mixture of DD- and LL-DAP and another was for *meso*-DAP. Zanol and Gastaldo[25] reported a method for the determination of the three stereoisomers of DAP. However, they did not actually apply the method for the analysis of any natural DAP-containing substances like bacterial cell walls. Then, we established quantitative and sensitive determination methods for the total DAP with one peak and the separate three stereoisomers of DAP and applied the methods to the analysis of mixed rumen bacterial hydrolysates from goats[8].

In the present study, we planned to determine the contents of total and stereoisomers of DAP in five species of ruminal bacteria and mixed culture from Welsh sheep and mixed culture from Japanese cattle.

MATERIALS AND METHODS

Apparatus: A liquid-chromatography pump (UP-980), variable wavelength ultraviolet detector (875-UV), and column oven (860-CO) were obtained from Japan Spectroscopic (Tokyo, Japan); the injector (CC-EKE 005) was from Senate Science (Tokyo, Japan); the data analyzer (Chromatopac C-R6A) was from Shimadzu (Kyoto, Japan), and the LiChrospher 100 RP-18 column particle size was from Kanto Chemical (Tokyo, Japan) under the licence of E. Merck (Darmstadt, Germany).

Chemicals: 2,6-Diaminopimelic acid (DAP) (a mixture of LL-, DD- and *meso*-form) and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) were purchased from Sigma

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(St. Louis, MO, U.S.A.). Triethylamine phosphate was obtained from Tokyo Organic Chemicals (Tokyo, Japan); Acetone and acetonitrile were from Wako Pure Chemical Industries (Osaka, Japan). Ultra pure water made with Milli-Q Labo (Nihon Millipore, Tokyo, Japan) was used to prepare the mobile phases and other solutions according to the previous method[8].

Chromatography: The mobile phases used for isocratic elution were as follows: a mixture of 0.05 M triethylamine phosphate (pH 3.0) and acetonitrile with a ratio of 72:28 was used for the determination of total DAP with one peak and a mixture of those solutions with a ratio of 78.5:21.5 was used for the determination of the separate three stereoisomers of DAP according to El-Waziry *et al*[8]. Before use, the mobile phases were filtered through membrane filter (HV 0.45-µm, Nihon Millipore Kogyo K.K., Tokyo, Japan) and degassed *in vacuo* with ultrasonication. The flow-rate was 1 ml min $^{-1}$; column temperature, 40°C; monitoring wave-length at 325 nm with a UV detector; column, LiChrospher 100 RP-18 (250 x 4 mm I.D.) of 5 µm particle size[8].

Sample preparation:

Preparation of freeze-dried rumen bacteria: Rumen bacteria were collected from the rumen contents of ruminally fistulated cattle (Japanese native breed) fed on a daily ration consisting of dry Rhodes grass (*Chloris gayana* Kunth) and concentrate mixture. The rumen samples were collected before the morning feed and strained through four layers of surgical gauze. The strained rumen fluid was incubated in a separatory funnel at 39°C for about 60 min. Then approximately 200 ml of the lower liquid phase was transferred to centrifuge tubes and centrifuged at 390 x g for 2 min to remove protozoa after cooling in ice-cold water. The supernatant was centrifuged at 27,000 x g for 30 min and sediments (mainly rumen bacteria) were taken and kept at -20°C over-night and then freeze-dried.

Freeze-dried mixed and five species of rumen bacteria of the Welsh sheep fed on a daily ration consisting of grain-based concentrate and chopped hay were prepared by Ling[13].

Hydrolysis of rumen bacteria: Four mg of freeze-dried and powdered mixed and five species of rumen bacteria were hydrolyzed in 4 ml of 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 pure water. The filtrate was evaporated to dryness *in vacuo*, washed three times with pure 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. The filtrate was used for the derivatisation and injection using HPLC according to El-Waziry *et al* [8].

RESULTS AND DISCUSSION

Total DAP: The content of the total DAP of the hydrolysed mixed rumen bacteria from Welsh sheep on the average was 30.1 µmol g bacterial dry matter $^{-1}$ (DM) and from cattle on the average was 12.3 µmol g bacterial DM^{-1} (Table 1). The average nitrogen contents of the bacteria collected from sheep and cattle were 0.077 and 0.050 g N g bacterial DM $^{-1}$, respectivley (Table 1). The amounts of DAP detected, therefore, could be expressed on the average as 391.3 and 244.6 µmol g bacterial N $^{-1}$ in mixed rumen bacteria from the Welsh sheep and the Japanese cattle, respectively. The present value of mixed rumen bacteria collected from the Welsh sheep are slightly higher than the value of mixed rumen bacteria collected from the Japanese goats (26.6 µmol total DAP g bacterial DM $^{-1}$) reported by El-Waziry *et al* [8], but both values were higher than that collected from the Japanese cattle (12.3 µmol total DAP g bacterial DM $^{-1}$). If the diet of the ruminant animal changes, the quality and the quantity of the bacteria will also change[9,11]. Hungate[11] stated that when cattle are fed with only forage diets, the gram negative bacteria will be predominant in the rumen. However, if they consume more concentrate, the proportion of gram positive bacteria will increase. The different bacteria have different peptidoglycans in the cell wall, which would lead to higher or lower DAP concentration. If the forage is changed, this may cause a change of bacterial species, and hence peptidoglycan and DAP contents[3]. The diets of cattle, goats and sheep in the present study were different, therefore, the contents of DAP in rumen bacteria of three species of animals were different as mentioned above. Dufva *et al* [4] reported that cattle rumen contents contained 7.8-50.4 µmol total DAP g bacterial DM $^{-1}$. The value of mixed rumen bacteria collected from the Welsh sheep and from Japanese cattle are within that ranged values of that authors. Russell and Robinson[19] reported lower DAP content of strains *Streptococcus bovis* (1.95-8.41 µmol total DAP g bacterial DM $^{-1}$). Webster *et al* [21] found 17.4 µmol total DAP g bacterial DM $^{-1}$ in mixed rumen bacteria from sheep and this value lower than the value of the Welsh sheep, but higher than that of the Japanese cattle. When the present values expressed per N, the value of mixed rumen bacteria from the Welsh sheep (391.3 µmol g bacterial N $^{-1}$) seems slightly higher than those in mixed rumen bacteria from the Japanese goats (320.5 µmol g bacterial N $^{-1}$,[8]) , and those from goats reported by Onodera *et al* [17]

Table 1: Nitrogen content per g of bacterial DM and total and single content of stereoisomers of diaminopimelic acid in hydrolysed mixed rumen bacteria collected from sheep, cattle and goats[a]

		Content of stereoisomers and total DAP (µmol/g bacterial DM)				
Organism	Nitrogen[b]	*meso*-	LL-	DD-	Sum of three DAP-isomers	Total DAP[c]
Mixed rumen bacteria (Welsh sheep)	0.077±0.002	26.24±5.94	3.79±0.21	0	30.03	30.13±0.79
Mixed rumen bacteria (Japanese cattle)	0.0502±0.001	7.96±0.96	4.13±0.36	0	12.09	12.28±0.94
Mixed rumen bacteria (Japanese goats)	0.083±0.003	21.24±1.37	5.10±0.11	0	26.34	26.60±5.36

a. Values are shown as mean ± S.D. (n =3)
b. g/g bacterial DM
c. Determined by one-peak method

Table 2: Nitrogen content per g of bacterial DM and total and single content of stereoisomers of diaminopimelic acid in hydrolysed five species rumen bacteria collected from sheep[a]

		Content of stereoisomers and total DAP (µmol/g bacterial DM)				
Organism	Nitrogen[b]	*meso*-	LL-	DD-	Sum of three DAP-isomers	Total DAP[c]
Prevotella ruminicola $B_1$4	0.090±0.006	5.63±0.05	0	0	05.63	5.68±0.09
Streptococcus bovis JB1	0.092±0.003	5.68±0.42	0	0	05.68	5.73±0.53
Selenomonas ruminantium Z108	0.091± 0.004	18.30±3.47	0	0	18.30	18.19±1.42
Fibrobacter succinogenes S85	0.105±0.002	22.19±0.58	0	0	22.19	21.77±2.94
Anaerovibrio lipolytica 5S	0.102±0.005	32.12± 0.42	0	0	32.12	32.54±3.21

a. Values are shown as mean ± S.D. (n =3)
b . g/g bacterial DM
c. Determined by one-peak method

(326.5 µmol g bacterial N^{-1} . The present values of mixed rumen bacteria collected from the Japanese cattle and those reported by Weller *et al* [22] (range 203.5-228.7 µmol g bacterial N^{-1} from sheep) were lower than the present value of mixed rumen bacteria collected from the sheep and those collected from the goats[8]. In the present study, the contents of total DAP in *Streptococcus bovis JB1*, *Prevotella ruminicola B 1 4*, *Selenomonas ruminantium Z108*, *Fibrobacter succinogenes S85* and *Anaerovibrio lipolytica 5S* on the average were 5.63, 5.68, 18.19, 21.77 and 32.54 µmol g bacterial DM^{-1} , respectively (Table 2).

The present values of *Streptococcus bovis JB1*, *Prevotella ruminicola B 1 4* are within the range of 1.95-8.41 µmol total DAP g bacterial DM^{-1} of those reported by Russell and Robinson[19] in *Streptococcus bovis* strains. While the present value of *Fibrobacter succinogenes* S85 was similar to the value of *Seleomonas lactilytica PC18* (20.0 µmol total DAP g bacterial DM^{-1})[4].

The value of *Selenomonas ruminantium Z108* was similar to the value of mixed rumen bacteria (17.4 µmol total DAP g bacterial DM^{-1}) reported by Webster *et al* [21]. These discrepancies may be due to the bacterial composition in the rumen and hence the diet fed.

Three stereoisomers of DAP

The content of each stereoisomer of DAP was also shown in Table 1. The contents of *meso*- and LL-DAP in the hydrolysed mixed rumen bacteria collected from the Welsh sheep on the average were 26.24 and 3.79 µmol g bacterial DM^{-1} (340.78 and 49.22 µmol g bacterial N^{-1} for *meso*- and LL-DAP, respectively), and those in the hydrolysed mixed rumen bacteria collected from the Japanese cattle on the average were 7.96 and 4.13 µmol g bacterial DM^{-1} (158.66 and 82.27 µmol g bacterial N^{-1} for *meso*- and LL-DAP, respectively). DD-DAP was not detected in these samples (Tables 1). The difference of DAP contents (especially *meso*-DAP) between the Welsh sheep and the Japanese cattle may due to the difference between the diets. *Prevotella ruminicola B 1 4*, *Streptococcus bovis JB1*, *Selenomonas ruminantium Z108*, *Fibrobacter succinogenes S85* and *Anaerovibrio lipolytica 5S* possessed the values of *meso*-DAP on the average ranged from 5.6 to 32.1 µmol g bacterial DM^{-1} (Table 2). LL-DAP was detected in only mixed rumen bacteria collected from the Welsh sheep and the Japanese cattle and goats, but we did not identify the bacterium which contains LL-DAP.

Dugan *et al* [5] showed only two peaks of the three stereoisomers of DAP in mixed rumen bacteria of the dairy cow, *meso*-DAP (7.6 µmol g bacterial DM^{-1}) and the mixture of DD- and LL-DAP (1.02 µmol g bacterial DM^{-1}). These values were much lower than those of the Welsh sheep and the value of *meso*-DAP almost similar to the value of *meso*-DAP of the Japanese cattle. The value of LL-DAP of the Japanese cattle was similar to the values of LL-DAP of the Japanese goats and the Welsh sheep, but *meso*-DAP was much lower. The reason may be due to

some species of bacteria collected from the Japanese cattle which have a much content of LL-DAP.

The present study confirmed that *meso*-DAP was the major and the most common form of DAP occurring in the rumen bacteria of the sheep, cattle and goats used, and that although LL-DAP was found in mixed rumen bacterial populations, it was absent from pure cultures of *Streptococcus bovis JB1*, *Prevotella ruminicola B* $_1$ *4*, *Selenomonas ruminantium Z108*, *Fibrobacter succinogenes S85* and *Anaerovibrio lipolytica 5S*. DD-isomer was not detectable in bacteria used in the present experiment.

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