Occurrence of δ -Aminovaleric Acid in the Culture Medium of Rumen Ciliate Protozoa

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An unknown ninhydrin positive spot on the paper chromatogram of the endogenous metabolites of mixed rumen ciliates was isolated by using 'Dowex' 50-X8 column and silica gel column and identified as δ -aminovaleric acid by the data of elementary analysis, specific optical rotation, mass spectrum, paper chromatogram and infrared spectrum.

Identification of unknown substances in the metabolites of rumen ciliates is naturally essential to elucidate the metabolism of rumen ciliates. Six unknown ninhydrin positive spots (US-1~US-6) have been detected in a paper chromatogram of endogenous metabolites of rumen ciliates.¹⁾ One of them (US-1) has already been identified as L-(—)-pipecolic acid²⁾ and shown to be produced from lysine as an end product by rumen ciliates.³⁾

Present paper describes on isolation and identification of an another one (US-2).

MATERIALS AND METHODS

1. Cultural method of rumen ciliate protozoa. Rumen ciliates were collected as follows: rumen contents collected through fistula of a goat maintained on daily ration containing 3~5 kg of fresh grass, 300 g of starch pulp and 200 g of mixed concentrate feed and filtered through four layers of gauze. After the filtrate was incubated in the separating funnel at 39°C for about 60 min, sediment containing ciliates at the bottom of the separating funnel were collected and washed five times with warmed B-9 buffer solution⁴⁾ by centrifugation at $120 \times g$ for 30 sec. A 30 ml sample of wet living ciliates was incubated in 1,000 ml of B-9 buffer solution containing 50 mg of dihydrostreptomycin sulphate to suppress bacterial action⁵⁾ at 39°C for 24 hr. In this case, an another 50 mg of dihydrostreptomycin sulphate was supplemented into the medium at the time of 12 hr.

2. Separation of endogenous amino acids. After incubation, the medium was deproteinized by adding 100 g of trichloroacetic acid (TCA), leaving it in the cold room over night and filtrating it through Toyo Filter Paper No. 2. The filtrate was evaporated *in vacuo* to about 300 ml and washed with an equal volume of ethyl ether to remove TCA. The water soluble fraction was evaporated *in vacuo* to about 10 ml and desalted according to the method of Block *et al.*⁶⁾ using 'Amberlite' CG-120 (H form) and 'Amberlite' CG-400 (OH form). The desalted solution containing amino acids mixture was evaporated to dryness and a part of the amino acids mixture was paper chromatographed.

3. Isolation of US-2 from amino acids mixture

a) Fractionation with 'Dowex' 50-X8. 'Dowex' 50-X8 (200~400 mesh) was washed with 4 N HCl, water, 4 N NaOH and water successively. A column (3 $\times$ 55 cm) was prepared from this resin suspending in a small amount of 1.5 N HCl. After the amino acids mixture desalted was dissolved in 5 ml of 1.5 N HCl, poured into the column and percolated, elution with 1.5 N HCl and 2.5 N HCl was carried out according to the method of Onodera and Kandatsu.²⁾

b) Removal of proline contaminating US-2 fraction with silica gel column. Silica gel (Wakogel C-200, 120~200 mesh) was suspended in water and unprecipitated particles within 20 min removed five times by decantation. A column (2.5 $\times$ 25 cm) was prepared with this silica gel after suspending it in a small amount of 85% EtOH and washed with 500 ml of 85% EtOH. The US-2 fraction containing a small amount of proline was dissolved in 3 ml of 85% EtOH, poured into the column and eluted with 85% EtOH. The eluates were collected every 10 ml by fraction collector. Each fraction was thin-layer chromatographed.

c) Crystallization of US-2. US-2 isolated was

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desalted through 'Amberlite' CG-120 and 'Amberlite' CG-400, evaporated to dryness and crystallized by adding 0.4 ml of water, 1.2 ml of EtOH and more than 6 ml of ethyl ether to the dried residue and allowing the mixture to stand at room temperature for 30~60 min. The crystals were washed three times with the gradient solution of EtOH and ethyl ether and finally washed three times with ethyl ether alone. The crystals obtained were dried at 55°C for an hour.

d) *Esterification of US-2.* About 3 mg of the crystal of US-2 was dissolved in ethanolic hydrochloric acid and left at room temperature for 2 hr with refluxing. The mixture was dried in a desiccator containing KOH under reduced pressure.

4. Analytical methods

a) Paper chromatography was carried out by using (1) *n*-BuOH: AcOH: H₂O (4: 1: 2), (2) Phenol: H₂O (4: 1) as solvents for two-dimensional development, and by using (1), (2) and (3) 77% EtOH for one-dimensional development.

b) Thin-layer chromatography was carried out by using Phenol: H₂O (4: 1) as solvent for one-dimensional development.

c) The specific optical rotation was measured by Automatic Polari Meter (Model DIP-SL, Japan Spectroscopic Co., Ltd.).

d) Infrared spectrometry was carried out by using Infrared Spectro Photometer (Type IR-G, Japan Spectroscopic Co., Ltd.).

RESULTS

Paper chromatogram of endogenous amino acids was shown in Fig. 1. The spot of US-2 was detected under the spots of proline and US-1 (pipecolic acid).

In the case of the fractionation by 'Dowex' 50-X8 column, US-2 was detected in the two-dimensional paper chromatogram of the eluate of the 300 ml of 2.5 N HCl followed the eluates of 1,500 ml of 1.5 N HCl and 100 ml of 2.5 N HCl together with a small amount of proline. Since US-2 appeared not to be completely isolated from proline by 'Dowex' 50-X8 column, a silica gel column was used to separate these two amino acids. As shown in Fig. 2, US-2 was completely isolated by the silica gel column by repeating two times because proline was not removed completely by the first fractionation alone. From this US-2 fraction, 20 mg of US-2 crystal was gained.

FIG. 1. Paper Chromatogram of Endogenous Amino Acid of Rumen Ciliates Incubated in B-9 Medium.

The results of elementary analysis of the US-2 crystal was as follows:

Found: C; 40.01, H; 7.61, N; 9.56, Cl; 23.42%.

Calcd. for C₆H₁₁O₂N·HCl: C; 39.09, H; 7.81, N; 9.11, Cl; 23.08%.

The molecular weight of US-2 was determined as ethyl ester by mass spectrometry according to the method of Biemann *et al.*⁷⁾ The mass spectrum was shown in Fig. 3. A peak found at *m/e* 145 seemed to be a parent peak. The molecular weight of US-2 was therefore estimated as 117. The most intense peak appeared at mass 30 which could be thought as an amine peak characteristic to amino esters. But there was no peak at *m/e* 102 as an ester peak. US-2, therefore, seemed to have an amino group at a terminal carbon atom.

The specific optical rotation of US-2 was found to be zero, which allowed us to presume the lack of asymmetric carbon atom. This agreed with the result of mass spectrum of US-2. From these results US-2 was estimated as δ -aminovaleric acid (DAV).

In one-dimensional paper chromatograms of US-2 alone, the mixture of US-2 and authentic DAV·HCl (Sigma), and authentic DAV·HCl alone, the spot of the mixture of US-2 and

FIG. 2. Removal of Proline Contaminating US-2 Fraction with Cilica Gel Column.

FIG. 3. Mass Spectrum of Ethyl Ester of US-2.

authentic DAV·HCl did not separate and the *R_f* values of three spots were the same in each solvent, *i. e.* 0.47 with solvent (1), 0.81 with (2) and 0.58 with (3).

Melting points were as follows: US-2; 157°C, authentic DAV·HCl; 94.5°C (the value from the literature⁸⁾; 91°C), treated authentic DAV·HCl (recrystallized after treating by silica gel column); 157°C, lactam of DAV·HCl; 182°~183°C (cited from the literature⁸⁾). Since the value of US-2 agreed not with that of authentic DAV·HCl but with that of treated authentic DAV·HCl and was approximate to

the value of lactam of DAV·HCl rather than authentic DAV·HCl, the crystal of US-2 obtained seemed to be the mixture of DAV·HCl and its lactam which might be formed during isolation and crystallization.

The infrared spectrum of US-2 in KBr disc did not correspond to that of authentic DAV·HCl (probably because of the production of lactam as described above), but almost agreed with that of treated authentic DAV·HCl recrystallized after treating by silica gel column (Fig. 4). In these spectrum, a strong absorption band at 1730 cm⁻¹ let us certainly presume the formation of lactam. The infrared spectra of the ethyl esters of the crystal of US-2 and authentic DAV·HCl showed the same absorption bands in chroloform (Fig. 5).

All these results led us to the conclusion that ninhydrin positive unknown spot (US-2) in the culture medium of rumen ciliate protozoa was δ -aminovaleric acid.

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FIG. 4. Infrared Spectra of US-2 and Treated Authentic δ -Aminovaleric Acid HCl (DAV·HCl) (see text) in KBr Disc.

FIG. 5. Infrared Spectra of Ethyl Esters of US-2 and Authentic δ -Aminovaleric Acid HCl (DAV·HCl) in CHCl_3 .

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