Estimation of Arginine Metabolism in Putrefactive Bacteria Using Liquid Chromatography

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(Received September 24, 1993)

Liquid chromatography (LC) using on-column derivatization coupled with a column-switching technique was developed to study the metabolism of amino acids in bacteria. Simultaneous analysis of 12 kinds of polyamines and their precursor amino acids was performed using this system.

Degradation products of amino acids due to *Photobacterium phosphoreum*, a putrefactive bacterium, was analyzed by the LC method. The arginine (Arg) metabolism in the bacterium was found to be such that Arg was converted to agmatine by the Arg-decarboxylase system, and agmatine was subsequently converted to putrescine by agmatinase.

The method was applicable to the determination of Arg, ornithine and lysine decarboxylase activities, Arg-dihydrolase activity and other enzyme activities in putrefactive bacteria.

Keywords — liquid chromatography; on-column derivatization; polyamine; arginine decarboxylase; arginine dihydrolase; *Photobacterium phosphoreum*

Introduction

Investigation of the metabolism of amino acids in bacteria is valuable in studies of bacteriological identification and classification. Decarboxylation is an enzymatic reaction carried out by a specific decarboxylase in bacteria. Arginine (Arg) decarboxylase, ornithine (Orn) decarboxylase and lysine (Lys) decarboxylase are the three principal enzymes¹⁾ involved in polyamine biosynthesis; they are also known to be inductive enzymes and to produce agmatine (Agm), putrescine (Put) and cadaverine (Cad) as a metabolite, respectively. Study of the decarboxylase activity has, to date, been done by the Møller²⁾ or Falkow³⁾ method as the conventional mi-

crobiological technique. However, these methods require 4 or more days, and the results are often difficult to interpret when the enzymatic activity is weak, because the decarboxylase activity is determined by the change in color of a pH indicator in a medium alkalized by decarboxylation. In addition, the pathway of Arg metabolism cannot always be determined by these methods, because Arg has two degradation pathways²⁾: the Arg-decarboxylase system and the Arg-dihydrolase system (Fig. 1).

Instead of the conventional method for evaluating decarboxylase activity, Gerlach *et al.*⁴⁾ reported a semi-micro test based on an enzymatic reaction between a bacterium and an amino acid in a buffer solution; the results

Fig. 1. Degradation Pathways of L-Arginine by (A) Decarboxylation and (B) Dihydrolase

from their method were in good agreement with those using the Møller or Falkow method.^{2,3)} However, since Gerlach's method also employed a pH indicator as an index of the determination, a problem of sensitivity and reliability similar to that with the Møller method remained. A thin layer chromatographic method⁵⁾ and a gas chromatographic method⁶⁾ were also reported for determining the Arg-dihydrolase and Orn or Lys decarboxylase activity, respectively. These methods, however, identified only the single enzyme activity that participated in an enzymatic reaction on an individual substrate; furthermore, both methods were time-consuming.

We report here a simultaneous means of ascertaining polyamines and precursor amino acids using liquid chromatography (LC) in order to determine the decarboxylase activities and Arg-dihydrolase activity. The method was used to analyze enzymatic degradation products in *Photobacterium phosphoreum* (*P. phosphoreum*), a putrefactive aerobic bacteria, isolated from spoiled foods. The pathway of polyamine production in the bacterium was also elucidated.

Materials and Methods

1. Reagents — All standards of polyamines and amino acids except monocarbamylputrescine (MCP) were purchased from Wako Pure Chemicals. MCP was synthesized according to the method reported by Akamatsu and Unemoto.⁷⁾ The obtained MCP was shown to be contaminated sodium acetate by the results of elemental analysis, and we verified the MCP by mass spectrometric (MS) analysis; a molecular ion (M^+) at m/z 131 and a protonated molecular ion ($[M+H]^+$) at m/z 132 were obtained by electron impact ionization (EI) and by chemical ionization (CI), respectively. The MCP was further confirmed by an M^+ at m/z 135 by EI and an $[M+H]^+$ at m/z 136 by CI of a deuterated MCP; the four active protons of the terminal-N ($-NH_2$) and the carbamyl group ($-CO-NH_2$) of MCP molecule were substituted with deuterium.

2. Apparatus — The LC system coupled with an on-column derivatization method and a column-switching technique described previously⁸⁾ was used with slight modification, i.e., a stepwise elution system was carried out using a rotary valve (Model FCV-3AL; Shimadzu) (Fig. 2). The switching of the rotary valve was operated by a system-controller (Model SCL-6A; Shimadzu). Detailed LC conditions are

Fig. 2. Schematic Diagram of Proposed LC System

TABLE I. Operative Conditions for Liquid Chromatography

Analytical column	: Asahipak ODP-50 (150 mm×4.6 mm i. d.)
Mobile phase	(A) : 30 mM BBS (pH 10.6)-MeOH (80 : 20, v/v)
	(B) : 30 mM BBS (pH 10.6)-MeOH-MeCN (70 : 20 : 10, v/v/v)
	(B) : 30 mM BBS (pH 10.6)-MeCN (76 : 24, v/v)
Elution : stepwise	A : equilibration, B : 0.01—12 min, C : 12—35 min
Flow rate (eluent)	: 0.5 ml/min
Column temperature	: 40 °C
Reaction column	: Asahipak C4P-50 (4 mm×4 mm i. d.)
Reagent	: 50 mM borax-MeOH (96 : 4, v/v) containing 2 mM OPA & NAC
Flow rate (reagent)	: 0.1 ml/min
Reaction time	: 1 min
Reaction temperature	: ambient
Detection	: fluorescence ; λ_{ex} 330 nm. λ_{em} 430 nm
Injection volume	: 10 μ l

BBS : borate buffer solution.

OPA : *o*-phthalaldehyde, NAC : *N*-acetyl-L-cysteine.

presented in Table I.

3. Determination of Polyamines Produced in Arg Fortified Medium—*P. phosphoreum* previously isolated and identified by Itaya *et al.*⁹⁾ was inoculated in 5 ml of Møller decarboxylase base (Difco) medium with 3% added NaCl and 1% Arg, followed by incubation at 25°C for 1–4 d under aerobic and anaerobic conditions, respectively; the anaerobic condition was performed using a chamber filled with hydrogen gas. The

polyamines produced in the medium were determined by the proposed LC method using the extraction and cleanup procedure described.¹⁰⁾ A medium without Arg was also used as the control.

4. Preparation for Bacterial Suspension of *P. phosphoreum*—The organism of *P. phosphoreum* was cultivated at 25°C for 72 h in 60 ml of brain heart infusion broth with 3% added NaCl. The broth was centrifuged at $1800 \times g$ (3000 rpm) for 10 min. The precipitate was washed

twice with 20 ml of physiological saline, and the organism was suspended and adjusted to 10^8 cell/ml in the saline.

5. Measurement of Amino Acid Metabolites in *P. phosphoreum*—The method described by Gerlach *et al.*⁴⁾ was slightly modified. Each substrate solution was prepared as follows: NaCl each amino acid (Arg, citrulline (Cit), Orn and Lys) and pyridoxal phosphate were respectively made up as a 1%, 3% and 1 mg % in 4 mM potassium phthalate buffer with an adjusted pH of 5.7. To 1 ml of each amino acid solution, 0.5 ml of the cell suspension was added, followed by incubation at 25°C for 3 and 24 h under aerobic conditions. To 10 μ l of the incubated solution, 1 ml of 0.05 M HCl was added, followed by filtration with a membrane filter (0.45 μ m). A 10 μ l aliquot was then injected into the LC system.

Results and Discussion

1. LC Conditions

Various LC methods are currently widely used for the determination of polyamines or amino acids. However, few reports have been published to date on the simultaneous determination of polyamines and precursor amino acids. Although Carlucci and Karmas¹¹⁾ and Patchett *et al.*¹²⁾, respectively, reported the simultaneous determination of these compounds, the methods required a tedious derivatization procedure for LC detection, and the kinds of polyamines and amino acids analyzed were not sufficient to elucidate amino acid metabolism in bacteria.

We recently developed an on-column derivatization method which uses LC to determine histamine¹³⁾ and polyamines¹⁰⁾ in foods. We also reported that the on-column method coupled with a column-switching technique is applicable for the routine analysis of polyamines.⁸⁾ Most amino acids and polyamines were derivatized to each fluorophore by this on-column derivatization method. However, the simultaneous analysis of polyamines and their precursor amino acids listed in Fig. 1 could not be performed

by an isocratic elution because of the difference in the hydrophobicity of the compounds. Thus, the on-column derivatization method coupled with a column-switching technique reported previously⁸⁾ was upgraded by also coupling a stepwise elution system of the mobile phase with the LC system.

The mobile phase for the stepwise elution was first examined by adding acetonitrile or methanol to the base solution of 30 mM borate buffer (BBS, pH 10.6). A good chromatographic separation of Cit and Arg was accomplished with the eluent (A) 30 mM BBS-methanol (80:20); for MCP, histamine (Him), Agm and Orn was performed with the eluent (B) 30 mM BBS-methanol-acetonitrile (70:20:10); and for Lys, 1,3-diaminopropane (Dap), Put, spermidine (Spd), spermine (Spm) and Cad was performed with the eluent (C) 30 mM BBS-acetonitrile (76:24).

In the next step, a time program for the switching of the rotary valve was examined so that all 12 compounds described above could be separated from each other. As shown in Fig. 3, they were well separated and all of them were eluted within 32 min under the conditions shown in Table I.

The calibration graphs of the peak area versus polyamine or amino acid concentration showed good linearity over the range of 0.2–4 ng for Him; 0.5–10 ng for Cit, Arg, MCP, Lys, Put and Cad; 1–20 ng for Orn, Agm and Spd; and 2–40 ng for Dap and Spm.

2. Arg Degradation by *P. phosphoreum* in Møller Medium

We attempted to detect the Arg metabolites Agm and Put produced in the Møller medium using the proposed LC method in order to evaluate the conventional Møller method. *P. phosphoreum* was used as a test organism, because the bacterium is one of the putrefactive bacteria able to produce Him; moreover, Arg metabolism in the bacterium is currently unclear.

As described in the Experimental section, *P. phosphoreum* was incubated in the Møller

decarboxylase medium for a certain period, then polyamines were extracted from the medium followed by chromatography using the LC system. Agm was detected after one day incubation under both aerobic and anaerobic conditions (Fig. 4). The concentration of Agm in the medium varied little for 4 d. These results suggested the presence of Arg-decarboxylase.

Although Orn-decarboxylase in *P. phosphoreum* was said to be negative, Put was also detected in the medium. The concentration of Put was significantly lower than that of Agm, and was found to increase with the prolongation of incubation period. Put is also known to be produced from Agm by Agm-decarboxylase or Agm-dihydrolase (Fig. 1). However, we were not able to determine the pathway of Put production in these experiments.

Although the color change of the pH indicator was not observed in the control

Fig. 3. Typical Chromatogram of Standard Mixture

Peaks: 1=L-citrulline (10 ng), 2=L-arginine (10 ng), 3=monocarbamylputrescine (4 ng), 4=histamine (4 ng), 5=agmatine (20 ng), 6=L-ornithine (20 ng), 7=L-lysine (20 ng), 8=1,3-diaminopropane (40 ng), 9=putrescine (20 ng), 10=spermidine (20 ng), 11=spermine (40 ng), 12=cadaverine (20 ng).

medium without Arg, both Agm and Put were detected in the medium, similar to that fortified with Arg (Fig. 5). These polyamines were considered to be produced from the Arg in the catabolites of peptone or meat extract fortified in the Møller base medium. Thus, the Møller method is viewed as not very sensitive, and hence not always able to yield an accurate result in the detection of decarboxylase activity. The Møller method also required an anaerobic condition for the incubation in order to prevent alkalization caused by the oxidation and deamination of peptone on the surface of the medium.¹⁴⁾ Furthermore, the method required at least 4 d for incubation with observations every day for positive or negative results.

With the LC method, we were able to detect the polyamines produced in the medium after 24 h incubation with high sensitivity. Moreover, it was found that there is little difference in the polyamine content in the medium under either aerobic or anaerobic conditions. Thus, there was no need of an

Fig. 4. Time Course of Polyamine Content in Arginine Fortified Medium under Aerobic (—) and Anaerobic (---) Conditions

●,○: arginine; ▲,△: putrescine.

anaerobic condition, *i.e.*, an aerobic condition was sufficient for the incubation when the proposed method was used for the polyamine detection.

3. Arg Metabolic Pathway in *P. phosphoreum*

As shown in Fig. 3, the proposed LC method allowed determination of polyamines and the precursor amino acids involved in Arg, Orn and Lys metabolism. Thus, the presence of each enzymatic activity shown in Fig. 1 and a pathway of the polyamine production could be determined by the LC method. Hence, the estimation of enzymatic activity for Arg metabolism in the *P. phosphoreum* was examined. The test solution was prepared according to Gerlach's method.⁴⁾ The left column in Table II represents each amino acid added as the substrate, and each row represents polyamines and amino acids detected in the metabolites. When Arg was a substrate, Agm was detected, whereas Cit and Orn were not; they are produced by the Arg-dihydrolase system as intermediate products, as shown in Fig. 1. Since Cit and Orn were not in the least decomposed when either was used as the substrate, the Arg-dihydrolase system including Orn-carbamoyltransferase and Orn-decarboxylase shown in Fig. 1 was found not to work, *i.e.*, these enzyme activities were negative.

Put was also detected after 24 h incubation when Arg was used as the substrate. This Put was thought to be produced from Agm by agmatinase, because Orn-decarboxylase was

Fig. 5. Time Course of Polyamine Content in Control Medium without Arginine under Aerobic (—) and Anaerobic (---) Conditions

●, ○: arginine; ▲, △: putrescine.

negative and no MCP as an intermediate metabolite of Agm by Agm-dihydrolase was detected. Therefore, we concluded that Arg was converted to Agm by Arg-decarboxylase, and the Agm formed was sequentially converted to Put by agmatinase in *P. phosphoreum*. Incidentally, since Cad was detected when Lys was used as the substrate, the presence of Lys-decarboxylase was verified.

On the basis of the results of these experi-

TABLE II. Degradation of L-Arginine (Arg), L-Citrulline (Cit), L-Ornithine (Orn) and L-Lysine (Lys) in *P. phosphoreum*

Substrate	Amino acids or polyamines detected (%)							
	Arg	Cit	Orn	Lys	MCP	Agm	Put	Cad
Arg	0.44	—	—	—	—	0.22	0.02	—
Cit	—	0.67	—	—	—	—	—	—
Orn	—	—	0.67	—	—	—	—	—
Lys	—	—	—	0.44	—	—	—	0.23

Each value is the average of 3 determinations in each incubated solution.

—: not detected.

ments, we have demonstrated that the proposed LC method is highly accurate, sensitive and useful for the estimation of enzymatic activity of amino acid metabolism,

such as the decarboxylase system and dihydrolase system for Arg in bacteria. In particular, the Arg degradation pathway in *P. phosphoreum* was clarified in this study.

References

- 1) J.F. MacFaddin, "Biochemical Tests for Identification of Medical Bacteria," 2nd ed., translated by Y. Takeda, E. Yabuuchi and T. Miwatani, 1st ed., Igakushoin, Tokyo, 1985, pp.67—80.
- 2) V. Møller, *Acta Pathol. Microbiol. Scand.*, **36**, 158 (1955).
- 3) S. Falkow, *Am. J. Clin. Pathol.*, **29**, 598 (1958).
- 4) E.H. Gerlach, S. Steiner and R.H. Weaver, *Am. J. Med. Technol.*, **33**, 372 (1967).
- 5) G.A. Williams, D.J. Blazevic and G.M. Ederer, *Appl. Microbiol.*, **22**, 1135 (1971).
- 6) M.A.S. Lambert and C.W. Moss, *Appl. Microbiol.*, **26**, 517 (1973).
- 7) N. Akamatsu and T. Unemoto, *Annu. Rep. Inst. Food Microbiol.*, **15**, 77 (1963).
- 8) K. Saito, M. Horie and H. Nakazawa, *Anal. Chem.*, **66**, 134 (1994).
- 9) T. Itaya, M. Iijima, K. Saito, H. Masaki, A. Aoki, A. Saito, K. Ando, Y. Tokumaru and M. Bando, *Jpn. J. Food Microbiol.*, **8**, 203 (1992).
- 10) K. Saito, M. Horie, N. Nose, K. Nakagomi and H. Nakazawa, *Anal. Sci.*, **8**, 675 (1992).
- 11) F.V. Carlucci and E. Karmas, *J. Assoc. Off. Anal. Chem.*, **71**, 564 (1988).
- 12) M.L. Patchett, C.R. Monk, R.M. Daniel and H.W. Morgan, *J. Chromatogr.*, **425**, 269 (1988).
- 13) K. Saito, M. Horie, N. Nose, K. Nakagomi and H. Nakazawa, *J. Chromatogr.*, **595**, 163 (1992).
- 14) D.J. Blazevic and G.M. Ederer, "Principles of Biochemical Tests in Diagnostic Microbiology," New York: John Wiley and Sons, 1975, pp.29—35.