Creatinine Decomposing Enzymes in *Pseudomonas putida*

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Creatinine was found to be rapidly decomposed by several strains belonging to *Pseudomonas*, one of which was assigned to *P. putida*. Analyses of the metabolites indicated that creatinine was converted to sarcosine via creatine and further to glycine. The enzymes involving in a metabolic pathway of creatinine, i.e. creatinine amidohydrolase (EC 3.5.2), creatine amidinohydrolase (EC 3.5.3.3) and sarcosine dehydrogenase (EC 1.5.99.1), were found to be inducibly formed and accumulated in the bacterial cells. A novel assay method for sarcosine dehydrogenase activity was also described.

In 1937 Dubos and Miller¹⁾ have shown that creatinine is inducibly decomposed by some bacterial strains belonging to genus *Corynebacterium*,²⁾ but its metabolic pathway has remained unclear. The reversible transformation of creatinine to creatine and further decomposition to sarcosine have been deduced in *P. aeruginosa*,^{3~5)} *P. ovalis*,^{6,7)} *Arthrobacter ureafaciens*^{8,9)} and in an unidentified bacterium.^{10,11)} On the other hand, *P. stutzeri*¹²⁾ was shown to convert creatinine to methylguanidine and acetic acid, while *Clostridium paraputrificum*^{13,14)} converted it to 1-methylhydantoin and ammonia.

In order to obtain the enzyme preparations which are utilizable to the clinical assay of creatinine and creatine contents in human serum and urine, we attempted to isolate the microorganisms capable of producing creatinine decomposing enzyme system and found that several strains belonging to *Pseudomonas* could grow well aerobically in a medium containing creatinine as the sole source of carbon and nitrogen. One of the strains, C-83, was taxonomically characterized and found to be very close to *Pseudomonas putida*. The present paper deals with the inducible formation of creatinine decomposing enzymes by the strain

C-83. A novel assay method for sarcosine dehydrogenase activity was also shown in this report.

MATERIALS AND METHODS

Enzyme activity assay

Creatinine amidohydrolase (EC 3.5.2.: creatininase). Enzyme activity was assayed by following the decrease in creatinine caused by the enzyme reaction. To 0.5 ml of 50 mM potassium-sodium phosphate buffer (pH 7.5) containing one μ mole of creatinine was added 0.5 ml of enzyme solution (or diluted cell suspension) at 37°C and after incubation for 10 to 30 min, 2 ml of 1% picric acid, 0.5 ml of 7.5% NaOH and 10 ml of deionized water were added to the reaction mixture (Jaffe reaction). The absorbance at 520 nm was measured after 30 min with a Hitachi Spectrophotometer Type 101. From difference in absorbances before (O.D.b) and after (O.D.t) the enzyme reaction, activity units per ml of the original enzyme solution were calculated using the following equation.

$$\text{Creatininase units/ml} = (\text{O.D.b} - \text{O.D.t}) / \text{O.D.b} \times 1/T \times 2 \times \text{dilution factor} \quad (1)$$

where T was the reaction time in min and O.D.b was usually between 0.68~0.7. The values of 0.1~0.25 for (O.D.b—O.D.t) were most suitable to obtain the accurate enzyme activity.

*Creatine amidinohydrolase**¹ (EC 3.5.3.3: creatinase). Decrease in creatine by the enzyme reaction was

*¹ After the purified sarcosine dehydrogenase became available (T. Yoshimoto *et al.*, unpublished), another assay method of creatinase was developed, which measures the diformazane formed by coupling the creatinase reaction with sarcosine dehydrogenase, PMS and with NTB. This assay method will be reported elsewhere in details.

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Abbreviations used were: PMS, phenazine methosulfate; NTB, nitrotertrazolium blue; Creatininase, creatinine amidohydrolase; Creatinase, creatine amidinohydrolase; *P. Pseudomonas*.

followed by diacetyl- α -naphthol method.¹⁵⁾ To 0.5 ml of 0.1 M phosphate buffer (pH 8.0) containing 0.5 μ mole of creatine was added 0.5 ml of the enzyme solution (or diluted cell suspension) at 37°C, and after incubation for 10 to 30 min, 1.0 ml of diacetyl*² and 2.0 ml of α -naphthol*² and 6.0 ml of deionized water were added to the reaction mixture. After 30 min, the color developed was measured at 530 nm. The activity units per ml of the original enzyme solution was calculated by using the equation 2.

Creatinase units/ml = $(O.D.b - O.D.t) / O.D.b \times 1/T \times$ dilution factor (2)

where the value of O.D.b was that of color development due to 0.5 μ mole creatine under the conditions and was usually between 0.7 and 0.72.

Sarcosine dehydrogenase (EC 1.5.99.1). Diformazane¹⁶⁾ formed by the reduction of nitrotetrazolium blue (NTB) which was coupled with phenazine methosulfate (PMS) and the dehydrogenase reaction was measured. To 0.5 ml of 0.5 M sarcosine solution in 50 mM Tris-HCl buffer (pH 7.5) containing 0.1% Triton X-100, were added 0.5 ml of the enzyme and 0.1 ml of PMS-NTB mixture*³ at 37°C. After incubation for 15 min, 3 ml of 0.3 N HCl was added to the reaction mixture and the absorbance at 570 nm due to diformazane formation was measured. The activity units per ml of the original enzyme solution were calculated from the following equation.

Sarcosine dehydrogenase units/ml = $O.D.t / 0.165 \times 1/15 \times 2 \times$ dilution factor (3)

The value of 0.165 was that of the optical density at 570 nm caused by the reduction of one μ mole of sarcosine under the conditions. Linear relationship was observed between amounts of the enzyme added and the optical density until 0.8 (Fig. 1).

In equations 1, 2 and 3, one unit of the respective activities was defined as the enzyme quantities which catalyze decomposition of one μ mole of substrate per min under the conditions. These three enzymes were all located in the bacterial cells, but the activities of these enzymes were fully detectable by using the cell suspension system, by which no appreciable trouble due to the turbidity of the bacterial cells was observed.

Other assay methods and chemicals used

Metabolites from creatinine were identified by thin layer chromatography on Kiesel gel 60 (Merck) with the solvent system of phenol-ethanol-water (14:4:1

*² Stocked 1% diacetyl aqueous solution was diluted 20-fold with water just before use and its 1.0 ml was used. α -Naphthol, 1% in 1.5 N NaOH, was freshly prepared and used.¹⁶⁾

*³ One mg of PMS and 10 mg of NTB were dissolved in 10 ml of deionized water and stored in a refrigerator until use.

FIG. 1. Relationship between Amounts of Sarcosine Dehydrogenase Preparation Added and Absorbance at 570 nm Caused by Coupling the Dehydrogenase Reaction with Phenazine Methosulfate and Nitrotetrazolium Blue.

The enzyme preparation partially purified by salting out with 0.7 saturation of $(NH_4)_2SO_4$ was dialyzed against 10 mM Tris-HCl buffer (pH 7.4) and diluted as shown in abscissa, and 0.5 ml each was assayed as described in the text.

by vol). After 5 hr development at room temperature, the chromatograms were exposed in iodine vapour or sprayed successively with 1% picric acid and 1 N NaOH before or after being heated at 100°C for 50 min, and the metabolites were identified by comparison with parallel runs of authentic samples. Sarcosine*⁴ and glycine*⁴ were also identified using an automatic amino acid analyzer of Nihon Denshi, Type JLC-6AH.

Disruption of the bacterial cells was carried out using a Vibrogen Cell Mill of Edmund Bühler, Germany. A specific test medium for *Pseudomonas*, NAC agar medium "EIKEN,"¹⁷⁾ and a kit for the assay of urea by the urease-indophenol method¹⁸⁾ were supplied from Eiken Kagaku Co., Tokyo. Creatinine and the related compounds were purchased from Wako Pure Chemicals Co., Osaka. Phenazine methosulfate (PMS) and nitrotetrazolium blue (NTB) were products of Dojin Chemical Laboratories Co., Kumamoto. Other chemicals were of reagent grade and used without further purification.

RESULTS AND DISCUSSION

1. Screening of microorganisms capable of producing creatinine-decomposing enzymes.

About 300 soil samples were subjected to

*⁴ Both compounds were also detectable by the ninhydrin reaction on the plates of thin-layer chromatography.

the screening test for creatinine decomposing activity. Sixty mg of each soil sample was suspended in 10 ml of sterilized water and its 0.5 ml was spread over a solid medium composed of 0.5% creatinine, 0.5% K_2HPO_4 , 0.3% NaCl and 2% agar (pH 7.0). After incubation at 30°C for 48 hr, the colonies appeared were isolated and further subjected to shaking culture to test the ability to form the creatinine decomposing enzyme(s). The microorganisms were grown aerobically in a medium composed of 0.5% creatinine, 0.1% yeast extract, 0.025% malt extract and 0.05% NH_4Cl in 0.1 M potassium phosphate buffer, pH 7.4 (standard creatinine-enriched medium) at 30°C for 40 hr. Of 216 strains of bacteria, 12 strains of fungi and 82 strains of *Streptomyces* which were grown in the creatinine medium, six strains were found to grow well and decompose creatinine almost completely within 12 hr in the shaking culture.

2. Taxonomic characteristics

Six strains selected could grow well in the NAC agar medium. This indicates that all of these strains belong to genus *Pseudomonas*, since only this group of the bacteria can grow in the NAC medium.¹⁷⁾ A strain designated C-83 was used for further experiments on the production of creatinine decomposing enzyme(s) and the taxonomic characterization, because of its high ability to produce creatinine decomposing enzyme(s).

FIG. 2. Electron Microscopic Photograph of the Strain C-83. ($\times 10,000$)

TABLE I. CHARACTERIZATION OF STRAIN C-83

Gram staining	—	Acid formation from	
Shape	rod	glucose	+
Catalase	+	lactose	—
		maltose	—
Cytochrome oxidase	+	mannitol	—
O F test	oxidative	salicin	—
Motility	+	sucrose	—
Flagella	+	xylose	— ^{a)}
	(polar, mono ^{a)})	levulose	+
Growth on NAC agar	+	Starch hydrolysis	—
Fluorescent pigments	+	Gelatin hydrolysis	—
		Nitrate reduction	—
Pyocyanine	—	Arginine dihydrolase	+
Carotenoides	—	Ornithine	—
		decarboxylase	—
Citrate (Simmons')	+	Lysine decarboxylase	—
		Tween 80 hydrolysis	—

^{a)} Disagreed with the properties of typical *P. putida*.

Figure 2 shows an electron microscopic photograph of the strain C-83. In Table I the taxonomic characteristics of the strain are summarized. This experiment was carried out in cooperation with Messrs I. Ishizuka and M. Ikeda of Eiken Kagaku Co. From the comparison with the description in Bergey's Manual,¹⁸⁾ the strain C-83 seems to be very close to *Pseudomonas putida*. Thus, we assigned it to *P. putida* and decided to call *P. putida* var. *naraensis* C-83, since the strain was isolated from soil in Nara Prefecture.

3. Identification of metabolites from creatinine and the detection of enzymes related to creatinine metabolism in *P. putida* var. *naraensis* C-83.

Figure 3 shows the time course of cell growth, decrease in creatinine concentration, changes in pH and creatinine decomposing activity during the aerobic cultivation of the strain C-83 in the standard creatinine-enriched medium. The strain grew well in the medium and decomposed creatinine very rapidly. Increase of creatinine decomposing activity was slightly delayed from the cell growth.

FIG. 3. Time Course of Cell Growth, Changes in Creatinine Content, pH and Creatinine Decomposing Activity of Strain C-83 Grown in Creatinine Enriched Medium.

Strain C-83 was grown aerobically at 37°C in 70 ml of the standard creatinine enriched medium in a 500 ml Sakaguchi flask. Five ml aliquots of the culture broth were periodically withdrawn and used for the determinations of turbidity, creatinine content, pH and creatinine-decomposing activity.

In order to know the metabolic pathway of creatinine in the strain C-83, the metabolites were analyzed by thin-layer chromatography. The results are illustrated in Fig. 4, indicating that creatinine is converted to creatine and then to sarcosine which is further converted to glycine. Approximately stoichiometric conversion of sarcosine to glycine was confirmed by the incubation with the cell-free extract of bacterial cells grown in creatine or creatinine medium, as checked by using an amino acid analyzer. Urea formation was also proved by liberation of ammonia according to the method of urease-indophenol.¹⁹⁾ These results indicate that the strain C-83 metabolizes creatinine by the pathway shown below, as has previously been deduced in *P. aeruginosa*,^{3~5)} *P. ovalis*,^{6,7)} and *Arthrobacter ureafaciens*.^{8,9)} Creatinine

These results also suggest that the bacterial cells contain three enzymes involving in creatinine decomposition: (A), creatinine ami-

FIG. 4. Thin-layer Chromatograms of Metabolites by Strain C-83 from Creatinine and Creatine.

The 15 hr's culture broth in Fig. 3, 70 ml, was centrifuged at 8000 rpm for 15 min, and the bacterial cells precipitated were washed twice with 20 mM phosphate buffer (pH 8.0) and then suspended in 30 ml of the same buffer. The cell suspension was mixed with an equal volume of 30 mM creatinine or creatine solution dissolved in the same buffer at 37°C, and 2 ml aliquots were periodically removed, centrifuged at 8000 rpm for 15 min, and the supernatants were concentrated to 0.5 ml *in vacuo*. The concentrates were spotted on the plates and chromatographed as mentioned in the text.

dohydrolase (creatininase); (B), creatine amidohydrolase (creatinase); and (C), sarcosine dehydrogenase. In fact, these three enzymes were easily detectable in the culture broth of C-83 grown in creatinine containing media. In Table II are compared the activities of the three enzymes in the culture broths from various media, and Table III summarizes the effect of creatinine and the related compounds on the formations of these enzymes. The addition of creatinine induced the formations of these enzymes, and simultaneous supplement of glucose markedly inhibited the formations (Table II). Creatine was equally effective with creatinine on the formations of all the enzymes (Table III). This will be explainable from the fact that equilibrium of reversible creatinine-creatine reaction exists near the mid point, as has been indicated by Akamatsu *et al.*^{10,11)} and confirmed by us (T. Yoshimoto *et al.*, un-

TABLE II. COMPARISON OF ACTIVITIES OF CREATININASE, CREATINASE AND SARCOSINE DEHYDROGENASE OF STRAIN C-83 CELLS GROWN IN VARIOUS MEDIA

The strain C-83 was aerobically grown in the media shown below, and 20 hr after cultivation, aliquots of the culture broths were removed to check the activities of these three enzymes.

Standard medium: 0.5% creatinine, 0.1% yeast extract, 0.025% malt extract, 0.05% NH_4Cl , 0.1 M potassium phosphate buffer (pH 7.4) in tap water.

Bouillon medium: 1% polypepton, 1% meat extract, 0.5% NaCl (pH 7.2).

Medium used	Enzyme activities, (units/ml of culture broth) $\times 10^2$		
	Creatininase	Creatinase	Sarcosine dehydrogenase
A. Standard	2.3	1.4	28
B. A-creatinine	0	0	0
C. Bouillon+0.5% creatinine	1.0	/	21
D. A+0.5% sucrose	1.8	1.2	/
E. A+0.5% glucose	0.1	0.2	0.5

TABLE III. EFFECT OF CREATININE AND THE RELATED COMPOUNDS ON PRODUCTIONS OF THREE ENZYMES BY STRAIN C-83

The compounds were dissolved, respectively, in the medium composed of 0.1% yeast extract, 0.025% malt extract and 0.05% NH_4Cl , 0.1 M phosphate buffer, pH 7.4 in tap water. Cultivations were carried out at 37°C for 15 hr.

Chemicals added	Conc. (%)	Activities, (units/ml) $\times 10^2$		
		Creatininase	Creatinase	Sarcosine dehydrogenase
None	/	0	0	0
Creatinine	0.5	3.01	1.53	22.2
Creatine	0.5	2.80	1.58	16.0
Sarcosine	0.5	0.14	0.08	4.0
Dimethylglycine	0.5	0.05	0.04	21.8
Betaine	0.5	0.02	0.05	56.0
Urea	0.5	0	0.05	0
Glycosylamine	0.5	0	0.05	0
Guanidine	0.5	0	0.10	0
Hydantoin	0.5	0	0.10	0
1-Methylhydantoin	0.5	0	0	0

TABLE IV. FORMATION OF CREATININE DECOMPOSING ENZYMES BY RESTING CELL SUSPENSION SYSTEM

The 20 hr's culture broth in bouillon medium, 100 ml, was centrifuged at 8000 rpm for 15 min and the bacterial cells precipitated were washed with 50 mM phosphate buffer (pH 7.4) and suspended in 50 ml of the same buffer. This cell suspension, 5 ml, was mixed with an equal volume of the buffer containing creatinine, creatine or yeast extract and shaken at 37°C for 6 hr.

Supplemented with	Enzyme activities, (units/ml) $\times 10^2$		
	Creatininase	Creatinase	Sarcosine dehydrogenase
A. None	0	0	0.4
B. 0.5% creatinine	1.5	0.3	8.0
C. B+0.1% yeast extract	1.5	0.4	8.5
D. 1.0% creatinine	1.3	0.5	/
E. 0.5% creatine	1.2	0.3	6.0
F. E+0.1% yeast extract	1.0	0.3	/
G. 1.0% creatine	1.8	0.5	7.2

TABLE V. CHEMICAL AND PHYSICAL EXTRACTIONS OF CREATININE DECOMPOSING ENZYMES FROM THE INDUCED CELLS OF STRAIN C-83

The washed cells of 15 hr's culture in the standard creatinine-enriched medium at 37°C were suspended in 20 mM phosphate buffer (pH 7.2) to give the optical density of 2.6 at 660 nm, and the 10 ml aliquots were: (A), treated with 0.8 saturation of $(\text{NH}_4)_2\text{SO}_4$ for 3 hr at 4°C and then dialyzed against 20 mM borate buffer (pH 9.0) overnight at 4°C; (B), mixed with 5 mg of hen egg-white lysozyme in 10 ml of 20 mM phosphate buffer-20 mM EDTA (pH 7.2) and incubated for one hr at 37°C; (C), mixed with 10 g of glass beads (0.3 mm in diameter) and the mixture was subjected to treatment with a Vibrogen Cell Mill twice for 5 min at 10°C; (D), freeze-dried at -20°C overnight and thawed at room temperature. The clear solutions obtained after centrifugation of the disrupted cells in (A) to (D) at 12,000 rpm for 30 min at 4°C were used as the crude enzyme preparations.

Treated with	pH	Volume (ml)	Absorbance at 280 nm	Creatininase			Creatinase			Sarcosine dehydrogenase		
				Activity (u/ml) $\times 10^2$	Recovery	Specific activity $\times 10^2$	Activity (u/ml) $\times 10^2$	Recovery	Specific activity $\times 10^2$	Activity (u/ml) $\times 10^2$	Recovery	Specific activity $\times 10$
A. Salt	7.2	44.0	0.57	0.12	3	0.21	0.4	10	0.72	0.30	15	0.57
B. Lysozyme	7.4	20.0	3.09	1.80	20	0.59	2.33	24	0.75	6.46	140	3.23
C. Vibrogen cell mill	7.8	20.6	2.80	7.85	88	2.80	5.95	66	2.10	1.86	41	0.66
D. Freezing & thawing	7.4	20.6	0.73	/	/	/	0.73	6	0.10	0.40	1	0.05
E. None	7.4	20.0	/	9.05	100	/	9.35	100	/	4.61	100	/

Recovery: % of the activity before cell disruption.
Specific activity: Units/ml/absorbance at 280 nm.

published). Of particular interest is that betaine*⁵ and dimethylglycine*⁵ markedly stimulated the formation of sarcosine dehydrogenase.

The reason why both the compounds are much more effective than sarcosine on the enzyme formation remains unclear now. Inducible formations of these enzymes were also observed in the washed suspension system of the resting cells. The results are shown in Table IV. Formations of three enzymes took place in the resting cells by the supplement of creatinine or creatine, but the activities of creatinase and sarcosine dehydrogenase were considerably low as compared in the growing cell system.

4. Preparation of cell-free extract and effect of pH on activity and stability of crude enzymes

The creatinine-induced cells were washed and treated chemically or physically to extract the three intracellular enzymes. The results are summarized in Table V. Disruption of the washed cells with a Vibrogen Cell Mill was most effective for the extractions of creatininase and creatinase, and the preparations of high specific activities were obtained. For the extraction of sarcosine dehydrogenase, however, lysozyme-treatment of the bacterial

cells was most effective.

Table VI shows the optimal pH values for the activities and stable pH ranges of three enzymes solubilized. These three enzymes were fairly stable at neutral pH region and could be stored at 4°C for at least 5 days without significant loss in their activities. Creatininase and creatinase were able to be lyophilized without activity loss, but sarcosine dehydrogenase lost the activity by the treatment.

In conclusion, a strain isolated from soil and assigned to *P. putida* was found to produce inducibly the creatinine decomposing enzymes, creatinine amidohydrolase, creatine amidinohydrolase and sarcosine dehydrogenase, by which creatinine was converted to glycine via creatine and sarcosine. During these experiments, a novel assay method of sarcosine dehydrogenase was established, which was more economical and gave higher sensitivity than the previous one.²⁰⁾

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TABLE VI. OPTIMAL pH VALUES FOR ACTIVITIES AND STABLE pH RANGE OF CRUDE PREPARATIONS OF CREATININASE, CREATINASE AND SARCOSINE DEHYDROGENASE OF STRAIN C-83

Buffers used were 50 mM acetate (pH 4~6), phosphate (pH 6~8) ^{a)} and borate (pH 8~10), respectively.		
Enzymes	Optimal pH	Stable pH range ^{b)}
Creatininase	7.5~8.0	5~10
Creatinase	7.5~8.0	6~8
Sarcosine dehydrogenase	8.5~9.0	6~8

^{a)} Tris-HCl buffer was used for sarcosine dehydrogenase.

^{b)} After incubation for 48 hr at 37°C.

*⁵ Dimethylglycine dehydrogenase activity was detected in the cells grown in betaine and dimethylglycine, respectively, but not in those grown in sarcosine medium.

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