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Purification and Characterization of Creatinine Amidohydrolase of *Alcaligenes* Origin

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Creatinine amidohydrolase (creatininase) from *Alcaligenes* sp. nov. was purified to electrophoretic homogeneity by adsorption on diethylaminoethyl-cellulose, affinity chromatography on creatinyl-AH-Sepharose, gel filtration on Sephadex G-200 and hydroxyapatite chromatography. The molecular weight of the enzyme was estimated to be approximately 160000 by gel filtration on Sephadex G-200 and 80000 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the enzyme was assumed to consist of two identical subunits. The enzyme showed maximum activity at pH 7—8 and was stable in the pH range of 8—11.5. The enzyme catalyzed interconversion between creatinine and creatine, and the K_m values for creatinine and creatine were 60.9 mM and 162 mM, respectively. Though the enzyme was markedly inactivated by ethylenediaminetetraacetate (EDTA), *N*-bromosuccinimide, Zn^{2+}, Cu^{2+}, Ni^{2+} or Co^{2+}, activation of the enzyme was only observed in the presence of Mn^{2+}. Furthermore, the reactivation of EDTA-treated inactive enzyme was observed on the addition of Mn^{2+} to the reaction mixture.

Keywords——creatininase; creatinine; creatinyl-AH-Sepharose; *Alcaligenes*; clinical diagnosis; metalloenzyme

Recently, we found that a novel species of genus *Alcaligenes* (deposited at the Fermentation Research Institute of the Agency of Industrial Science and Technology, Japan, with the accession number 5071) produces extracellular catabolic enzymes for creatinine,[1] and the creatinine amidohydrolase (creatininase, EC 3.5.2.10) activity was found to be 20 times greater than the creatine amidinohydrolase (creatinase, EC 3.5.3.3) activity. Since the Folin method,[2] which is most commonly used for clinical diagnostic analysis of creatinine in serum and urine, has the disadvantage of poor specificity for creatinine, an enzymatic measurement of creatinine can be expected to give much higher specificity and reliability. For this purpose, we attempted to purify creatininase produced by *Alcaligenes* sp. using creatinyl-AH-Sepharose as an affinity adsorbent. In this paper, the details of the purification and characterization of the enzyme are described.

Materials and Methods

Materials——1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was obtained from Sigma Chemical Co. Sarcosine oxidase (EC 1.5.3.1) of *Bacillus subtilis* origin was obtained from Toyo Jozo Co. and horseradish peroxidase was a product of Wako Chemical Ind., Ltd. AH-Sepharose and Sephadex G-200 were purchased from Pharmacia Fine Chemicals and hydroxyapatite was obtained from Seikagaku Kogyo Co. All other materials were commercial products of analytical grade.

Production of Creatininase——A seed culture was established in a 500 ml Erlenmeyer flask containing 110 ml of a medium composed of 3.0% soluble starch, 1.0% glucose, 0.75% meat extract, 0.75% polypeptone, 0.3% NaCl, 0.1%

$MgSO_4 \cdot 7H_2O$, 0.0008% $MnCl_2 \cdot 4H_2O$, 0.0002% $ZnSO_4 \cdot 7H_2O$ and 0.0002% $CaCl_2 \cdot 2H_2O$ (pH 7.4) at 28 °C for 48 h on a rotary shaker at 200 rpm. The seed culture (330 ml) was used to inoculate a 10 l jar fermenter (LABOTEC Co., Ltd., LABO Fermentor LF-100) containing 7.5 l of the same medium. The culture was conducted at 28 °C for 48 h with aeration at 8.0 l/min and agitation at 400 rpm. The cultured broth was sonicated at 2 A for 7 min (Kubota, 200 M Insonator) and centrifuged at 4000 rpm for 20 min to give 7.3 l of supernatant (3.8 units/ml, specific activity = 0.56 unit/mg protein) which was treated batchwise with diethylaminoethyl (DEAE)-cellulose (wet volume = 3 l) previously equilibrated with 10 mM glycine–NaOH buffer (pH 7.5) for 30 min with gentle agitation. The resulting DEAE-cellulose cake was washed with 3 l of 10 mM glycine–NaOH buffer (pH 7.5) and eluted with 4.5 l of the same buffer containing 0.6 M NaCl. The eluate was concentrated by ultrafiltration and dialyzed against 10 mM phosphate buffer (pH 7.5) for 15 h. After centrifugation at 4000 rpm for 20 min, 900 ml of crude sample (21.5 units/ml) was obtained.

Assay for Protein Determination——The protein concentration was measured by the method of Lowry *et al.*[3] using bovine serum albumin as a standard.

Assay of Creatininase Activity——Assay Method I: A mixture of an aqueous enzyme solution (0.1 ml) and 0.1 M Tris–HCl buffer (pH 8.0, 0.9 ml) containing 0.1 M creatinine and 1 mM $MnCl_2$ was incubated at 37 °C for 10 min. Creatine formed from creatinine by the enzyme reaction was measured by the α-naphthol-diacetyl method.[4] One unit was defined as the amount of enzyme that catalyzed the transformation of 1 μmol of substrate per min under the conditions described above.

Assay Method II: A mixture of aqueous enzyme solution (0.1 ml) and 0.1 M Tris–HCl buffer (pH 8.0, 0.9 ml) containing 0.1 M creatine and 1 mM $MnCl_2$ was incubated at 37 °C for 10 min. The creatinine formed was measured by the Jaffe method.[5]

Assay of Creatinase Activity——A mixture of 1.0 M Tris–HCl buffer (pH 8.0, 0.1 ml) and 50 mM creatine (0.8 ml) was warmed to 37 °C and the reaction was initiated by the addition of aqueous enzyme solution (0.1 ml). After 20 min of incubation at 37 °C, the reaction was terminated by the addition of 1 mM *p*-chloromercuribenzoate (PCMB, 1 ml). The sarcosine formed was determined by further incubation at 37 °C for 20 min with a reaction mixture (0.5 ml) consisting of 30 mM Tris–HCl buffer (pH 8.0), 2.7 mM 4-aminoantipyrine, 0.3 mg/ml phenol, 30 units/ml horseradish peroxidase, 6 units/ml sarcosine oxidase and 0.2 mM PCMB, and the absorbance at 500 nm was read against the blank (in which heat-denatured enzyme was employed).

Electrophoresis——Electrophoresis was performed in 10% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS) according to the method of Weber and Osborn.[6] Bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), α-chymotrypsinogen (M_r 25700) and lysozyme (M_r 14300) were used to determine the molecular weight of the enzyme.

Preparation of Creatinyl-AH-Sepharose——A mixture of H_2O-swollen AH-Sepharose (12 ml) which had been successively washed with 0.5 M NaCl (600 ml) and distilled H_2O (150 ml, pH adjusted to 4.5 with diluted HCl), 25 mM aqueous creatine solution (pH 4.5, 12 ml) and 1.2 M aqueous 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride solution (1 ml) was kept at room temperature for 15 h with gentle shaking. The resulting gel was washed with 1 M NaCl, 1 M $NaHCO_3$, distilled H_2O, 1 N acetic acid and distilled H_2O in this order and stored at 4 °C until use.

Results

Purification of Creatininase

All procedures were carried out at 4 °C unless otherwise specified. The crude enzyme obtained by DEAE-cellulose treatment was subjected to chromatography on creatinyl-AH-Sepharose (Fig. 1) and then gel filtration on Sephadex G-200 (Fig. 2). Most of the creatinase activity was separated from creatininase activity, as shown in Fig. 2. After chromatography on hydroxyapatite (Fig. 3), the purified enzyme (which was free from creatinase activity) was dialyzed against distilled H_2O for 15 h and stored in a frozen state for further characterization. The purification of the enzyme is summarized in Table I. The hydrolysis of α-pyrrolidone, 1-methylhydantoin or hydantoin could not be detected by qualitative analysis on a silica gel thin layer plate[7] in the range of enzyme concentrations employed in the following experiments.

Estimation of Molecular Weight

The purified enzyme showed a single band on SDS-polyacrylamide gel electrophoresis and the molecular weight was estimated to be 80000. On the other hand, the molecular weight obtained by gel filtration on Sephadex G-200 was approximately 160000.

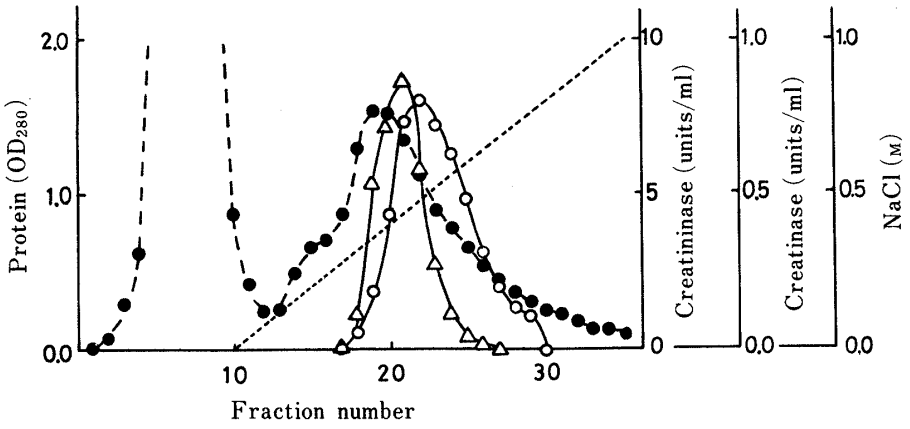

Fig. 1. Chromatography of Creatininase on Creatinyl-AH-Sepharose

The crude enzyme obtained by DEAE-cellulose treatment (141 mg as protein) was charged on a creatinyl-AH-Sepharose column (4.5 × 1.3 cm, i.d.) equilibrated with 50 mM Tris–HCl buffer (pH 8.0). The column was washed with a sufficient amount of the same buffer, then the enzyme was eluted with a linear gradient of NaCl (0—1 M) in the starting buffer. The eluate was collected in 4.0 ml fractions and the creatininase activity was measured by assay method I.

○, creatininase activity; △, creatinase activity; ●, protein; ------, NaCl.

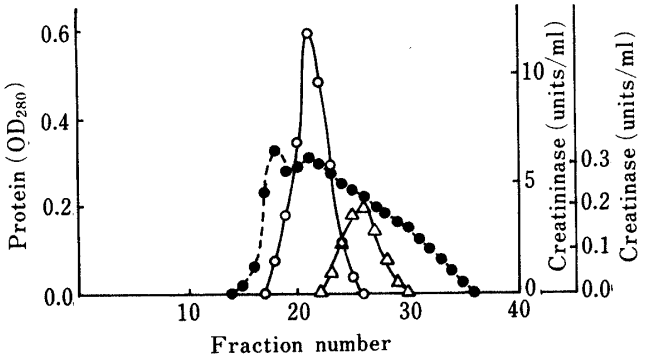

Fig. 2. Chromatography of Creatininase on Sephadex G-200

The partially purified powder from creatinyl-AH-Sepharose chromatography (16.5 mg as protein) was dissolved in 5 mM phosphate buffer (pH 8.0) and charged on a Sephadex G-200 column (86.0 × 1.5 cm, i.d.). Five mM phosphate buffer (pH 8.0) was used for elution of the enzyme and the eluate was collected in 4.0 ml fractions.

○, creatininase activity; △, creatinase activity; ●, protein.

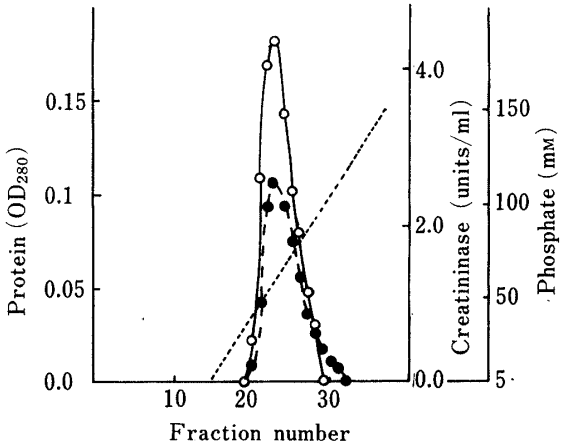

Fig. 3. Chromatography of Creatininase on Hydroxyapatite

The peak fractions of creatininase activity from Sephadex G-200 gel filtration were pooled and charged on a hydroxyapatite column (9.0 × 1.6 cm, i.d.). The column was washed with 5 mM phosphate buffer (pH 8.0) and then the enzyme was eluted with a linear gradient of phosphate buffer (5—150 mM, pH 8.0). The eluate was collected in 3.0 ml fractions.

○, creatininase activity; ●, protein; ------, phosphate.

Effects of pH on Creatininase Activity and Stability

The effects of pH on the enzyme activity and stability were tested by assay method I. The optimum activity was observed near pH 7—8 (Fig. 4). Although the enzyme was found to be stable at pH 8—11.5, it seemed labile at pH below 6 (Fig. 5).

Effects of Various Chemicals and Metal Ions

The creatininase activity was measured by assay method I without the addition of $MnCl_2$ to the reaction mixture. As shown in Table II, the enzyme was markedly inactivated by ethylenediaminetetraacetate (EDTA) and *N*-bromosuccinimide. Among metal ions tested,

TABLE I. Purification of Creatininase

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
DEAE-cellulose	141	538	3.8	100
Creatinyl-AH-Sepharose	16.5	335	20.3	62
Sephadex G-200	2.48	176	71.0	33
Hydroxyapatite	0.87	83.5	96.0	16

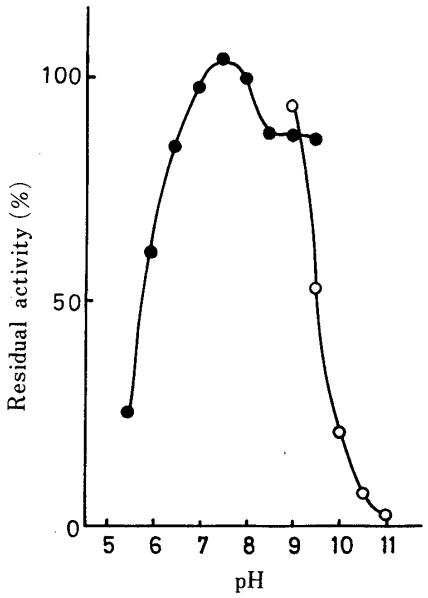

Fig. 4. Effect of pH on Creatininase Activity

The creatininase activity was measured by assay method I, in which aqueous enzyme solution (2.4 units/0.1 ml) was incubated with either 0.1 M Tris–HCl buffer (●, pH 5.5—9.5) or 0.1 M carbonate buffer (○, pH 9.0—11.0). The enzyme activity under the standard conditions was defined as 100%.

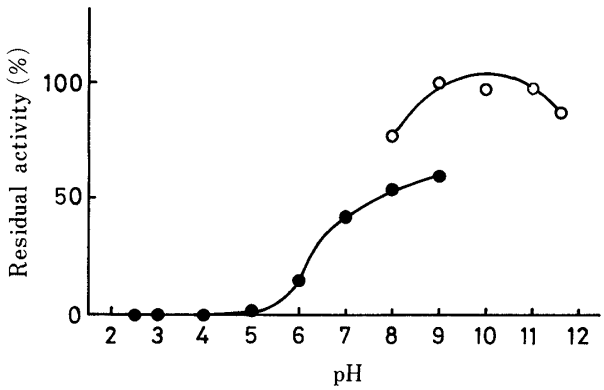

Fig. 5. Effect of pH on the Stability of Creatininase

A mixture of aqueous enzyme solution (11.9 units/0.1 ml) and 0.1 M buffer solution (0.1 ml) was allowed to stand at room temperature for 1 h. The pH was adjusted to 8.0 with 1.0 M Tris or 1.0 N HCl and the volume to 1.0 ml with distilled H_2O, then the residual activity was measured by assay method I.

●, citrate buffer (pH 2.5—9.0); ○, glycine–NaOH buffer (pH 8.0—11.5).

only Mn^{2+} enhanced the enzyme activity (Table III). In contrast, the enzyme was adversely affected by Zn^{2+}, Co^{2+}, Cu^{2+} or Ni^{2+}, and negligible effects were observed with other metal ions. The enzyme was treated with EDTA and subjected to gel filtration on a Sephadex G-15 column (50.0 × 1.5 cm, i.d.). Each fraction from the column was assayed for creatininase activity by assay method I in the absence of any metal ions, or in the presence of Mn^{2+} or Zn^{2+}. It is clearly shown in Fig. 6 that the enzyme was reactivated by Mn^{2+}, but not Zn^{2+}.

Kinetics

Since the enzyme catalyzed interconversion between creatinine and creatine, the kinetic parameters were obtained from Lineweaver–Burk plots[8] using creatinine and creatine as substrates. The K_m and V_{max} values for creatinine were calculated to be 60.9 mM and 59 μmol/min/mg protein, respectively, by assay method I using a reaction mixture composed of an aqueous enzyme solution (0.87 unit/0.1 ml) and 0.1 M Tris–HCl buffer (pH 8.0, 0.9 ml) containing 20—90 mM creatinine. The corresponding values for creatine were calculated to be 162 mM and 95 μmol/min/mg protein, respectively, by assay method II using a reaction

Table II. Effects of Various Chemicals on Creatininase Activity

Chemical (1 mM)	Residual activity (%)
None	100
Ethylenediaminetetraacetate	26
8-Hydroxyquinoline	89
L-Cysteine	78
o-Phenanthroline hydrochloride	63
α, α′-Dipyridyl	103
Monoiodoacetate	78
p-Chloromercuribenzoate	63
Glutathione (reduced)	89
Phenylmethanesulfonyl fluoride	82
Hydroxylamine hydrochloride	81
N-Bromosuccinimide	30

A mixture of the purified enzyme solution (20.3 units/0.8 ml) in 0.1 M Tris–HCl buffer (pH 8.0) and 5 mM chemical solution (0.2 ml) in the same buffer was incubated at 37 °C for 30 min and the residual activity was measured by assay method I using Mn^{2+}-free reaction mixture.

Table III. Effects of Various Metal Ions on Creatininase Activity

Metal salt (1 mM)	Residual activity (%)
None	100
$MnCl_2$	154
$CoCl_2$	30
$MgCl_2$	98
$ZnCl_2$	18
$CaCl_2$	86
$FeSO_4$	70
$FeCl_3$	96
$CuSO_4$	14
$NiCl_2$	16

A mixture of the purified enzyme solution (20.3 units/0.8 ml) in 0.1 M Tris–HCl buffer (pH 8.0) and 5 mM aqueous metal salt solution (0.2 ml) was incubated at 37 °C for 30 min and the residual activity was measured by assay method I using Mn^{2+}-free reaction mixture.

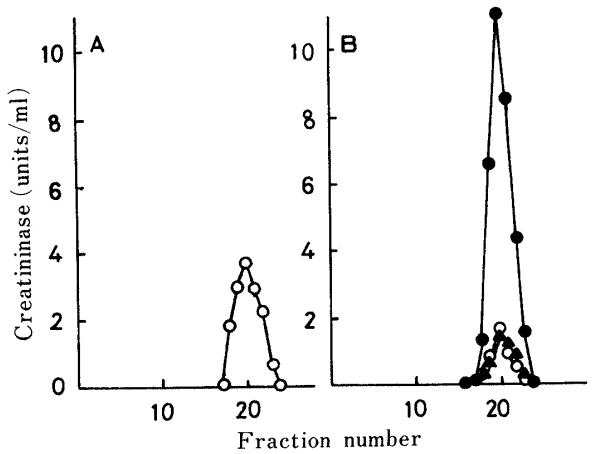

Fig. 6. Chromatography of Native and EDTA-Treated Creatininase on Sephadex G-15

(A) Native enzyme: The purified enzyme solution from hydroxyapatite chromatography (27.7 units/1.0 ml) was charged on a Sephadex G-15 column (50.0 × 1.5 cm, i.d.). The column was eluted with 0.1 M Tris–HCl buffer (pH 8.0) and the eluate was collected in 2.0 ml fractions. The creatininase activity was measured by assay method I using Mn^{2+}-free reaction mixture. (B) The purified enzyme solution (27.7 units/0.9 ml) was mixed with 10 mM EDTA (0.1 ml) and incubated at 37 °C for 20 min prior to gel filtration. The eluate with 0.1 M Tris–HCl buffer (pH 8.0) was collected in 2.0 ml fractions and the creatininase activity was measured by assay method I using Mn^{2+}-free reaction mixture in the absence of any metal ions (○), or in the presence of 1 mM $MnCl_2$ (●) or 1 mM $ZnCl_2$ (▲).

mixture composed of aqueous enzyme solution (0.81 unit/0.1 ml) and 0.1 M Tris–HCl buffer (pH 8.0, 0.9 ml) containing 20—90 mM creatine.

Discussion

Since the intracellular creatininase inducibly produced by *Pseudomonas putida* var. *naraensis* strain C-83 had been purified and extensively studied by Rikitake *et al.*,[9] the characteristics of the creatininase from *Alcaligenes* sp. were compared with those of *Pseudomonas* creatininase. Though metal ions were intrinsic to both enzymes, native *Pseudomonas* creatininase was not inactivated by EDTA. Besides Mn^{2+}, metal-free inactive creatininase of *Pseudomonas* origin was reactivated by other metal ions such as Co^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+}, in remarkable contrast to *Alcaligenes* creatininase, of which Mn^{2+} was the only activating factor. With regard to molecular weight, *Alcaligenes* creatininase was composed of two subunits with a molecular weight of 80000, while a molecular weight of 175000 was obtained for *Pseudomonas* creatininase, which consisited of eight subunits of

equal molecular weight. In addition to these two enzymes, creatininases produced by *Anthrobacter ureafaciens*[10] and *Flavobacterium*[11] had been reported. Inactivation was observed by heavy metal ions for the former, and by heavy metal ions and PCMB for the latter.

Creatinyl-AH-Sepharose was employed as an affinity adsorbent for the creatininase. As can be seen in Fig. 1, *Alcaligenes* creatininase was eluted from the column by increasing the NaCl concentration to 0.5 M, while the majority of protein retained on a column was collected in fractions of around 0.4 M NaCl. Delayed elution of the enzyme was, however, not observed in column chromatographies on DEAE-Sephadex and QAE-Sephadex. Due to the positive charge carried by the guanido group, the adsorbent worked as a strong anion-exchanger, and this limited the maximum capacity of creatinyl-AH-Sepharose to about 50 units of *Alcaligenes* creatininase per ml of the packed gel.

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