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Purification and Characterization of Creatine Amidinohydrolase of *Alcaligenes* Origin

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Extracellular creatine amidinohydrolase (creatinase, EC 3.5.3.3) produced by *Alcaligenes* sp. nov. was purified to electrophoretic homogeneity by ion exchange chromatography on diethylaminoethyl-cellulose, gel filtration on Sephadex G-75 and hydrophobic chromatography on phenyl-Sepharose CL-4B. The molecular weight of the enzyme was estimated to be 51000 by gel filtration on Sephadex G-200 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme showed the maximum activity at pH 8 and was stable at pH 5—9. The pI value was 4.7 as determined by isoelectric focusing. The enzyme catalyzed hydrolysis of creatine to sarcosine and urea, and the K_m and V_{max} values for creatine were 17.2 mM and 105 μmol/min/mg protein, respectively. The enzyme was markedly inactivated by *p*-chloromercuribenzoate (PCMB). Besides PCMB, the enzyme was inactivated by *N*-bromosuccinimide, Zn^{2+}, Cu^{2+} or Hg^{2+}.

Keywords——creatine amidinohydrolase (EC 3.5.3.3); creatine; *Alcaligenes*; clinical diagnosis; hydrophobic chromatography

Since the Folin method,[1] which is most commonly used in the clinical diagnostic analysis of creatinine in serum and urine, has the disadvantages of poor specificity and requirement for deproteinization, enzymatic measurement systems[2-5] using creatinine amidohydrolase (creatininase, EC 3.5.2.10) and creatine amidinohydrolase (creatinase, EC 3.5.3.3) in combination with other enzymes (for example, sarcosine oxidase and peroxidase) are replacing the Folin method. However, the creatininases and creatinases involved have not yet been characterized completely except for those from *Pseudomonas putida*.

In our screening for creatinine catabolic enzymes, a novel species of *Alcaligenes* was found to be an adequate source for creatiniase and creatinase.[6] The former catalyzes interconversion between creatinine and creatine, and the latter hydrolyzes creatine to sarcosine and urea. The purification and characterization of creatininase produced by *Alcaligenes* have been reported previously.[7] The present report describes the purification and characterization of creatinase of the same origin.

Materials and Methods

Materials——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. Phenyl-Sepharose CL-4B, Sephadex G-75, Sephadex G-200 and Pharmalyte were purchased from Pharmacia Fine Chemicals. All the other materials were commercial products of analytical grade.

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

$MgSO_4 \cdot 7H_2O$, 0.001% $MnCl_2 \cdot 4H_2O$, 0.01% $ZnSO_4 \cdot 7H_2O$ and 0.001% $CuSO_4 \cdot 2H_2O$ (pH 7.4) at 28 °C for 72 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 LF-100 fermenter) containing 5 l of a production medium consisting of 2.0% glucose, 2.0% soybean meal, 0.5% creatine, 0.2% polypepton, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl and 0.0001% $FeSO_4 \cdot 7H_2O$. The culture was conducted at 28 °C for 48 h with aeration at 0.8 l/min and agitation at 400 rpm.

Assay for Protein Determination——The protein concentration was measured by the method of Lowry *et al.*[8] using bovine serum albumin as a standard.

Assay of Creatinase Activity——Assay Method I: A mixture of an aqueous enzyme solution (0.1 ml) and 50 mM Tris–HCl buffer (pH 8.0, 0.9 ml) containing 50 mM creatine was incubated at 37 °C. After 20 min, 2 ml of dimethylsulfoxide containing 1.7% (w/v) *p*-dimethylaminobenzaldehyde and 13% (v/v) conc. HCl was added to produce a yellow dye from urea which was formed by the enzyme reaction. The mixture was allowed to stand at room temperature for 20 min, and the absorbance at 453 nm was measured against the blank, in which the heat-denatured enzyme was employed.

Assay Method II: A mixture of 0.1 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 started by adding an aqueous enzyme solution (0.1 ml). After incubation for 20 min at 37 °C, the reaction was stopped by adding 1 mM *p*-chloromercuribenzoate (PCMB, 1 ml). The sarcosine formed was determined by further incubation at 37 °C for 20 min with a mixture (0.5 ml) of 30 mM Tris–HCl (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 both methods, one unit was defined as the amount of enzyme which catalyzed the hydrolysis of 1 μmol of substrate per min under the conditions described above.

Electrophoresis——Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed in 10% polyacrylamide according to the method of Weber and Osborn.[9] Bovine serum albumin (M_r 68000), α-chymotrypsinogen (M_r 25700) and lysozyme (M_r 14300) were used as references to determine the molecular weight of the enzyme. Polyacrylamide disc gel electrophoresis was performed according to the method of Davis[10] and specific staining of creatinase activity in the gel was carried out by the formation of diformazan.[11c] Isoelectric focusing was conducted according to the method of Låås and Fast-Johansson[12] using Pharmalyte in a gel rod.

Results

Purification of Creatinase

All the procedures were carried out at 4 °C unless otherwise specified. The culture broth was centrifuged at 3500 rpm for 20 min to remove the mycelial cake. The broth filtrate was brought to 75% saturation with $(NH_4)_2SO_4$ at pH 7.8 and allowed to stand for 15 h. The precipitate collected by centrifugation at 3500 rpm for 20 min was dialyzed against 0.02 M Tris–HCl buffer (pH 7.8) for 15 h. Then 2% aqueous protamine sulfate was added to the retentate (0.7 ml/100 mg protein) with stirring and the whole was allowed to stand for 15 h. The supernatant obtained by centrifugation at 3500 rpm for 20 min was dialyzed against 0.02 M Tris–HCl buffer (pH 7.8) for 15 h. The crude sample was concentrated by salting out with 75% saturation of $(NH_4)_2SO_4$ and redissolving in 0.02 M Tris–HCl buffer (pH 7.8), and charged on a column (21 cm × 3.0 cm) of diethylaminoethyl (DEAE)-cellulose (Whatman, DE 23) which had been equilibrated with 0.02 M Tris–HCl buffer (pH 7.8). The column was thoroughly washed with 1 l of the same buffer containing 0.15 M NaCl, then the enzyme was eluted with a linear gradient of NaCl (0.15—0.7 M) in the same buffer. The partially purified enzyme from DEAE-cellulose chromatography was further subjected to Sephadex G-75 column chromatography (Fig. 1) and then to hydrophobic chromatography on phenyl-Sepharose CL-4B (Fig. 2). The purified enzyme was dialyzed against 0.02 M Tris–HCl buffer (pH 7.8) for 15 h and stored in a frozen state for further characterization. The purification of the enzyme is summarized in Table I.

The purified enzyme showed a single band on polyacrylamide gel electrophoresis in the absence or presence of SDS (Fig. 3). On polyacrylamide gel electrophoresis, the relative mobility of the protein band was identical with that of the band stained with diformazan.

Estimation of Molecular Weight

By both SDS-polyacrylamide gel electrophoresis and gel filtration on Sephadex G-200,

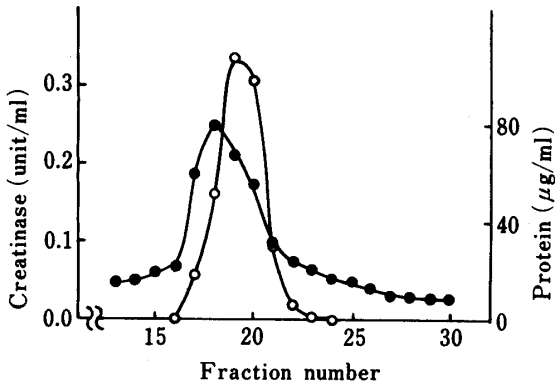

Fig. 1. Gel Filtration of Creatinase on Sephadex G-75

The crude enzyme obtained by DEAE-cellulose chromatography (2.3 mg as protein) was charged on a column of Sephadex G-75 (84 cm × 1.5 cm), and 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl was used for elution of the enzyme. The eluate was collected in 3.8 ml fractions. Creatinase activity was measured by assay method II.

○, creatinase activity; ●, protein.

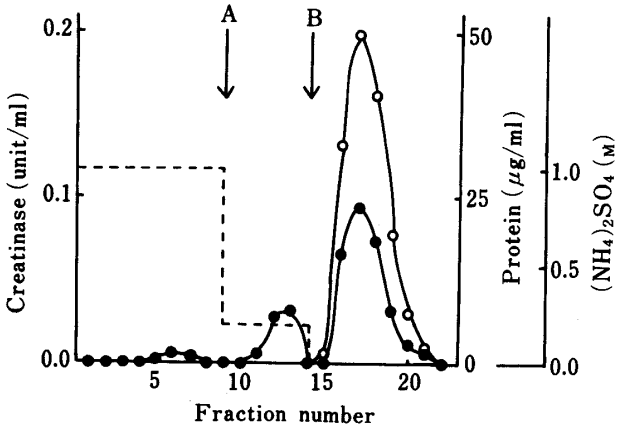

Fig. 2. Hydrophobic Chromatography of Creatinase on Phenyl-Sepharose CL-4B

The peak fractions of creatinase activity from Sephadex G-75 gel filtration were pooled and, after addition of solid $(NH_4)_2SO_4$ to a final concentration of 1 M, charged on a column of phenyl-Sepharose CL-4B (5.5 cm × 1.2 cm) equilibrated with 50 mM phosphate buffer (pH 7.0) containing 1 M $(NH_4)_2SO_4$. The column was washed with the same buffer and then 50 mM phosphate buffer (pH 7.0) containing 0.2 M $(NH_4)_2SO_4$ (buffer was changed at arrow A). Arrow B indicates a further change to 50 mM phosphate buffer (pH 7.0). The eluate was collected in 2.5 ml fractions and the creatinase activity was measured by assay method II.

○, creatinase activity; ●, protein; ----, $(NH_4)_2SO_4$.

TABLE I. Purification of Creatinase

Fraction	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Recovery (%)
Culture broth	49820	3890	0.08	100
Ammonium sulfate ppt.	44850	3690	0.08	95
Protamine sulfate sup.	8580	3350	0.39	86
Ammonium sulfate ppt.	7750	3180	0.41	82
DEAE-cellulose	730	2550	3.49	66
Sephadex G-75	220	1270	5.77	33
Phenyl-Sepharose CL-4B	110	935	8.50	24

Fig. 3. Electrophorograms of Purified Creatinase

a) Polyacrylamide gel electrophorogram. b) SDS–polyacrylamide gel electrophorogram.

the molecular weight of the enzyme was estimated to be 51000.

Isoelectric Point (pI) of Creatinase

The pI value was determined to be 4.7 by isoelectric focusing.

Effects of pH on Creatinase Activity and Stability

The effects of pH on the enzyme activity and stability were tested by assay methods I and

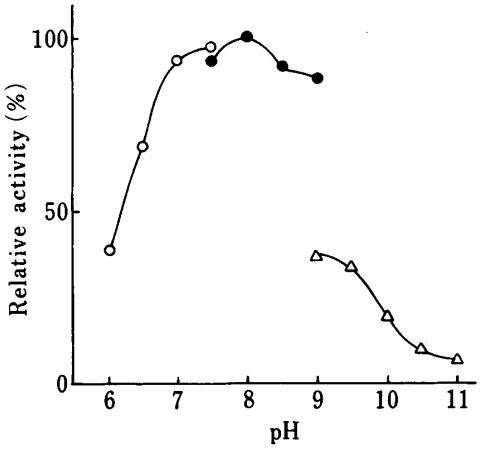

Fig. 4. Effect of pH on Creatinase Activity

The creatinase activity was measured by assay method I. An aqueous enzyme solution (0.39 unit/0.1 ml) was incubated with either 0.1 M phosphate buffer (○, pH 6.0—7.5), 0.1 M Tris–HCl buffer (●, pH 7.5—9.0) or 0.1 M carbonate buffer (△, pH 9.0—11.0). The enzyme activity under the standard conditions was defined as 100%.

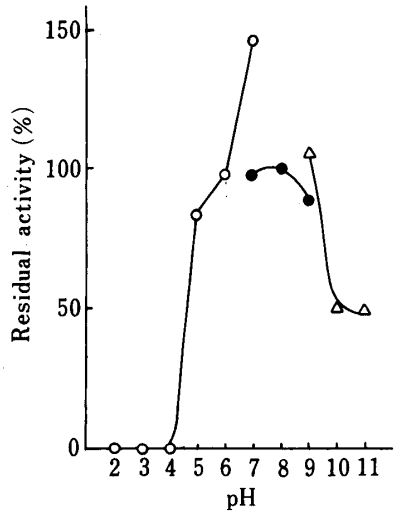

Fig. 5. Effect of pH on the Stability of Creatinase

A mixture of an aqueous enzyme solution (0.063 unit/0.15 ml) and 0.1 M buffer solution (0.15 ml) was incubated at 37 °C for 1 h. The pH was adjusted to near 8 with 0.5 M Tris–HCl buffer (pH 8.0, 0.7 ml), then the residual activity was measured by assay method II. The activity of untreated enzyme was defined as 100%.

○, citrate buffer (pH 2.0—7.0); ●, Tris–HCl buffer (pH 7.0—9.0); △, carbonate buffer (pH 9.0—11.0).

Table II. Effects of Various Chemicals on Creatinase Activity

Chemical (1 mM)	Residual activity (%)
None	100
Ethylenediaminetetraacetate	108
8-Hydroxyquinoline	91
o-Phenanthroline hydrochloride	106
Monoiodoacetate	99
p-Chloromercuribenzoate	0
Glutathione (oxidized)	104
p-Toluenesulfonyl chloride	102
Phenylmethanesulfonyl fluoride	99
N-Bromosuccinimide	0

A mixture of the purified enzyme solution (0.276 unit/0.2 ml) in 0.1 M Tris–HCl buffer (pH 8.0) and 5 mM inhibitor solution (0.05 ml) in the same buffer was incubated at 37 °C for 30 min and the residual activity was measured by assay method I.

Table III. Effects of Various Metal Ions on Creatinase Activity

Metal salt (1 mM)	Residual activity (%)
None	100
$MnCl_2$	96
$CoCl_2$	77
$ZnCl_2$	14
$CaCl_2$	98
$FeSO_4$	91
$MgSO_4$	100
$CuSO_4$	51
$HgCl_2$	0

A mixture of the purified enzyme solution (0.260 unit/0.2 ml) in 0.1 M Tris–HCl buffer (pH 8.0) and 5 mM aqueous metal salt solution (0.05 ml) was incubated at 37 °C for 30 min and the residual activity was measured by assay method I.

II, respectively. The optimum activity was observed near pH 8 (Fig. 4). Although the enzyme was stable at pH 5—9, it seemed especially labile at pH below 4 (Fig. 5).

Effects of Various Chemicals and Metal Ions

The creatinase activity was measured by assay method I. As shown in Table II, the enzyme was completely inactivated by PCMB and *N*-bromosuccinimide. The inhibition by PCMB, however, was reversed by 50% by the further addition of 10 mM dithiothreitol.

Among metal ions tested, the enzyme was adversely affected by Zn^{2+}, Cu^{2+} or Hg^{2+} and negligible effects were observed with the other metal ions (Table III).

Kinetics

Since the enzyme catalyzed the hydrolysis of creatine to sarcosine and urea, the kinetic parameters were obtained from Lineweaver–Burk plots,[13] using creatine as a substrate. The K_m and V_{max} values were calculated to be 17.2 mM and 105 μmol/min/mg protein, respectively, by assay method II, using a reaction mixture composed of an aqueous enzyme solution (0.043 unit/0.1 ml), 0.1 M Tris–HCl buffer (pH 8.0, 0.1 ml) and 2—50 mM aqueous creatine solution (0.8 ml).

Discussion

The creatinase and creatininase were produced by the parental strain of *Alcaligenes* sp. nov. at the ratio of 1 : 20 in terms of enzymatic activities (0.1 and 2.0 units/ml, respectively). By repeated selections for substrains producing a high titer of creatinase, the productivity of creatinase (0.4 unit/ml) was increased approximately 4 times over that of the parental strain, while the creatininase activity (0.3 unit/ml) was decreased to approximately one-seventh of that of the original strain.

Although creatinyl-AH-Sepharose was used as an affinity adsorbent for the creatininase from *Alcaligenes*, the creatinase could not be separated from impurities which were retained on the column by the strong ionic interaction with guanide groups of the adsorbent. However, hydrophobic chromatography on phenyl-Sepharose CL-4B was found to be effective for the isolation of the creatinase. Furthermore, the creatininase coadsorbed with the creatinase on phenyl-Sepharose CL-4B was not eluted from the column with 50 mM phosphate buffer (pH 7); the enzyme was finally eluted with H_2O.

To date, creatinases from *Arthrobacter ureafaciens*[14] and *Pseudomonas putida*[11] have been reported. The former has not yet been purified completely, though the molecular weight was estimated to be 100000. The latter was purified extensively by Yoshimoto *et al.*[11b,c] and found to be composed of two subunits with a molecular weight of 47000. Like the creatinase from *Alcaligenes*, the enzyme from *Pseudomonas* was inactivated by PCMB. The pI values of both enzymes are 4.7. However, the creatinase from *Alcaligenes* is a monomer with a molecular weight of 51000. The K_m values for creatine of the enzymes from *Pseudomonas* and *Alcaligenes* are 1.3 and 17.2 mM, respectively. In addition, on polyacrylamide gel electrophoresis, the enzymes showed different mobilities. It is worth emphasizing that the specific activity of the creatinase from *Alcaligenes* is 65 times higher than that of the creatinase from *Pseudomonas*.

The purified enzymes of *Alcaligenes* origin should be useful in the enzymatic determination of creatinine and/or creatine in clinical samples.

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