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Sarcosine Oxidase from *Arthrobacter ureafaciens*: Purification and Some Properties

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Sarcosine oxidase (EC 1.5.3.1) of *Arthrobacter ureafaciens* was purified to homogeneity by means of sequential chromatography on DEAE-Toyopearl 650M, Toyopearl HW-55, and hydroxyapatite. The purified enzyme was most active at pH 8.3 and was stable at pH 6.5—9.5 for 20 h at 20 °C. The molecular weight of the enzyme was estimated to be 185000 by gel filtration. Sodium dodecylsulfate-polyacrylamide gel electrophoresis indicated that the enzyme was composed of four dissimilar subunits with molecular weights of 96000, 45000, 23000, and 14000 daltons. The isoelectric point was 4.5 as checked by isoelectrofocusing. The K_m and k_{cat} values for sarcosine were 6.4 mM and 5.8 s^{-1}. The enzyme was strongly inactivated by heavy metal ions such as Ag^+, Zn^{2+}, Cu^{2+}, Hg^{2+}, Co^{2+}, and Cd^{2+}, and an SH-blocking reagent, *p*-chloromercuribenzoate. Spectrophotometric and fluorogenic analyses suggested that a flavin cofactor associated with the 45000-dalton subunit participates in the enzyme reaction.

Keywords——*Arthrobacter ureafaciens*; sarcosine; sarcosine oxidase; flavoprotein; dissimilar subunit

Some microorganisms have been found to inducibly produce intracellular sarcosine oxidase[1-6] which is involved in the metabolism of creatinine[3] and choline.[1] The enzyme is very useful for clinical analysis of creatinine and related metabolites, since the reaction product, hydrogen peroxide, is easily detectable by quinone dye formation in the 4-amino antipyrine–peroxidase system. The enzymes from *Cylindrocarpon didymum* M-1,[1] *Corynebacterium* sp. U-96,[2,7-11] *Alcaligenes denitrificans* subsp. *denitrificans*,[4] *Corynebacterium* P-1[5] and *Bacillus* sp. B-0618[6] were highly purified and characterized as flavin-containing proteins. However, the detailed properties of sarcosine oxidase from *Arthrobacter* have remained unclear.

The present report deals with the purification and characterization of sarcosine oxidase from *Arthrobacter ureafaciens* and compares its enzymatic and physicochemical properties with those of the enzymes from other microorganisms.

Materials and Methods

Materials——A commercial preparation (Toyobo, 0.46 units/mg) was used as the starting material for the purification of *Arthrobacter* sarcosine oxidase. 4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole (AHMT) was purchased from Wako Pure Chemicals Co., Ltd., Osaka. Hydroxyapatite was prepared by the method of Bernardi.[12] DEAE-Toyopearl 650 M and Toyopearl HW-55 were products of Toyo Soda Co., Tokyo. Other chemicals were commercial products of analytical grade.

Disc Gel Electrophoresis——This was carried out using 7.5% gel of pH 9.4 according to the method of Davis.[13] The enzyme preparations (50 μg as protein) were loaded on a gel and a current of 3 mA per gel was supplied for 1.5 h in the cold. Protein was stained with 1% Amide Black 10B and the activity band was visualized by incubating the gel in

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10 ml of 50 mM phosphate buffer, pH 8.0, containing 50 mM sarcosine and 50 μg each of phenazine methosulfate (PMS) and nitro blue tetrazolium (NBT) at 30 °C for 1 h in the dark. This treatment led to the formation of diformazan, whose blue color showed the position of sarcosine oxidase.

Estimation of Molecular Weight of Enzyme and Subunit Components——The molecular weight of the enzyme was estimated by the gel filtration method of Andrews[14] and the sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis method of Weber and Osborn.[15] Gel filtration was carried out using a column (3.0 × 100 cm) of Sepharose 6B equilibrated with 50 mM phosphate buffer, pH 7.2 containing 0.2 M KCl. Thyroglobulin, apoferritin, alcohol dehydrogenase, bovine serum albumin, and carbonic anhydrase were used as marker proteins (Sigma). In SDS-polyacrylamide gel electrophoresis, the subunit molecular weight was estimated by comparison with parallel runs of the marker proteins, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin (Pharmacia Fine Chemicals).

Isoelectric Focusing——This was done by the method of Vesterberg and Svensson[16] using an LKB apparatus. The enzyme solution (5 ml) was dialyzed against 0.2% carrier ampholyte (pH 4—6) for 24 h, applied to a column (110 ml in volume) with a sucrose density gradient and then subjected to isoelectric focusing at 500 V for 48 h.

Assay of Enzyme Activity——The reaction mixture was composed of 0.125 M sarcosine and the enzyme in 1 ml of 30 mM phosphate buffer, pH 8.3, and was incubated at 37°C for 10 min. The reaction was terminated by the addition of 1 ml of 5 N KOH, and the formaldehyde produced was determined by the AHMT-KIO_4 method[17]; 1 ml of 0.5% AHMT was added to the reaction mixture and the mixture was left to stand at room temperature for 15 min, then mixed with 1 ml of 0.75% KIO_4. The resulting 6-mercapto-*S*-triazolo[4,3-6]-*S*-tetrazine (MTT) was measured by monitoring the absorbance at 550 nm. One unit of the activity was defined as the amount of enzyme that produces 1 μmol of formaldehyde per min under these conditions. The production of hydrogen peroxide was checked by using the 4-aminoantipyrine peroxidase system as described by Kim *et al.*[3] Protein concentration was estimated spectrophotometrically assuming than $A^{1\%}_{1\,cm}$ at 280 nm is 13.5. The specific activity was defined as the enzyme units per milligram of protein.

Results and Discussion

Purification of Sarcosine Oxidase from *Arthrobacter ureafaciens*

All the procedures were carried out at 4 °C. A partially purified enzyme preparation, 2 g, was dissolved in 100 ml of 10 mM phosphate buffer, pH 8.0, and fractionated by 0.3 to 0.7 saturation of ammonium sulfate. The precipitate was dissolved in 10 ml of the same buffer and desalted by gel filtration on Sephadex G-25. The active fractions were combined and applied to a column (2.4 × 35 cm) of DEAE-Toyopeal 650M equilibrated with the above buffer. The column was washed with the buffer and the enzyme adsorbed was eluted with an increasing linear gradient of NaCl concentration. The enzyme appeared in the 0.25 M NaCl eluate. The active fractions were combined, concentrated by ultrafiltration with an Amicon PM-10, and desalted by gelfiltration. After this procedure was repeated, the active fractions were subjected to gel filtration on a column (3.0 × 100 cm) of Toyopearl HW-55 equilibrated with 10 mM phosphate buffer, pH 8.0, and, after concentration, the enzyme fraction was applied to a hydroxyapatite column (2.0 × 15 cm) equilibrated with the above buffer. The enzyme was eluted at 0.1 M phosphate buffer concentration as a symmetric protein peak with a constant specific activity (7.5 units/mg). The purification process is summarized in Table I. The activity recovery was 23%. On disc gel electrophoresis, the purified preparation gave a single protein

TABLE I. Summary of Purification Procedure of *Arthrobacter* Sarcosine Oxidase

Steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Salted out with $(NH_4)_2SO_4$	1250	650	0.52	100
Desalted with Sephadex G-25	1150	600	0.53	92
1st DEAE-Toyopearl 650M	430	500	1.17	77
2nd DEAE-Toyopearl 650M	280	430	1.54	66
Toyopearl HW-55	180	390	2.17	60
Hydroxyapatite	20	146	7.3	23

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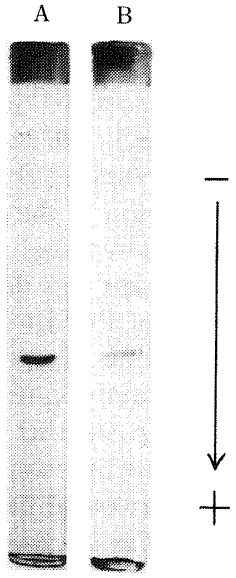

Fig. 1. Disc Gel Electrophorogram of *Arthrobacter* Sarcosine Oxidase

The enzyme (50 μg as protein) was subjected to electrophoresis on 7.5% gels. The gels were stained after a run at 3 mA per gel at pH 8.3 for 1.5 h. A) Stained with 1% Amido Black 10B. B) Detected by utilizing color development based on enzyme reaction. The experimental details are given in the text.

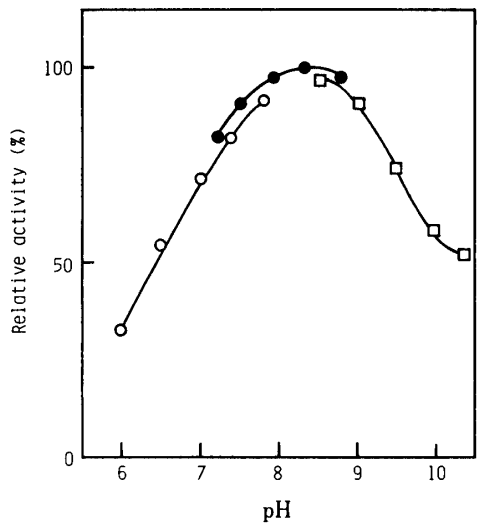

Fig. 2. Effect of pH on *Arthrobacter* Sarcosine Oxidase

The reaction mixture contained 125 μmol of sarcosine and 0.02 unit of sarcosine oxidase in 1 ml of 30 mM buffer at various pH values. The assay was carried out at 37 °C for 10 min. Buffers used were: ○, potassium phosphate (pH 6—7.8); ●, Tris–HCl (pH 7.2—8.8); □, Na_2CO_3–$NaHCO_3$ (pH 8.5—10.5).

TABLE II. Effects of Various Chemicals and Metal Ions on *Arthrobacter* Sarcosine Oxidase

Chemical (1 mM)	Relative activity (%)
None	100
PCMB (0.1 mM)	0
Iodoacetate	60
Iodoacetamide	19
N-Ethylmaleimide	33
Dithiothreitol	76
2-Mercaptoethanol	86
o-Phenanthroline	67
EDTA	88
AgCl	2
$ZnCl_2$	1
$CuCl_2$	0
$HgCl_2$	5
$CoCl_2$	4
$NiCl_2$	92
$CaCl_2$	94
$Cd(CH_3COO)_2$	0
$Ba(CH_3COO)_2$	92

The reaction mixture contained 0.02 unit of the enzyme in phosphate buffer, pH 7.0, and the indicated amounts of chemicals and metals. After preincubation for 10 min at 37 °C, the enzyme activity was assayed by the determination of formaldehyde as described in the text.

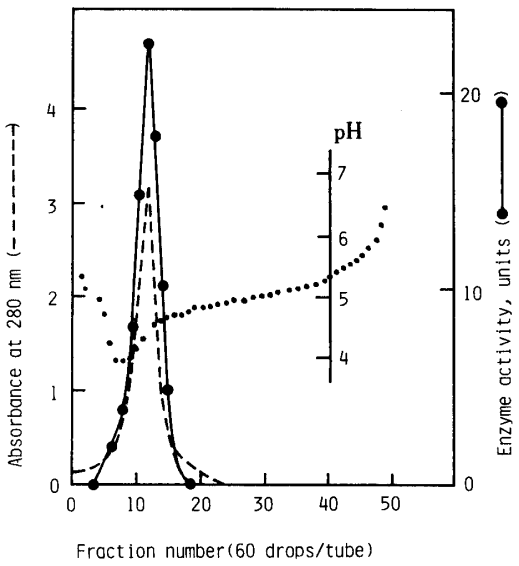

Fig. 3. Isoelectric Focusing Pattern of *Arthrobacter* Sarcosine Oxidase

About 5 mg of the enzyme was applied. See the text for experimental details.

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band with the activity of sarcosine oxidase (Fig. 1).

Some Enzymatic Properties

The enzyme was most active at pH 8.3 at 37 °C (Fig. 2) and the optimal temperature for the activity was 37 °C for a 10 min reaction in phosphate buffer, pH 8.3. The enzyme (0.2 unit) was stable between pH 6.5—9.5 at 20 °C for 24 h. Incubation of the enzyme (0.2 unit) at 40°C in 20 mM phosphate buffer, pH 8.0 for 24 h, resulted in 50% reduction of the original activity. The enzyme was markedly inactivated by incubation with 1 mM Ag^{+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Co^{2+}, and Cd^{2+}. *p*-Chloromercuribenzoate (PCMB) at 0.1 mM was also strongly inhibitory for the enzyme activity (Table II). The inhibitory effect of PCMB suggests that a sulfhydryl group(s) is located at or near the active site of the enzyme. However, it remains unclear whether the sulfhydryl group is directly involved in the catalytic reaction, since it is possible that the modification of the enzyme by a bulky group such as mercuribenzoate results in steric hindrance to make the enzyme–substrate interaction impossible. These heavy metal and PCMB inhibitions are common properties of sarcosine oxidases derived from microorganisms, except for those of the enzyme from a *Bacillus* sp. B-0618.[6]

Some Physicochemical Properties and Subunit Structure

The isoelectric point was 4.5 as estimated by the isoelectric focusing method (Fig. 3). The molecular weight of *Arthrobacter* sarcosine oxidase was estimated to be 185000 by the gel filtration method. SDS-polyacrylamide electrophoretic analysis indicated that the enzyme consists of four dissimilar subunits with molecular weights of 96000, 45000, 23000 and 14000 daltons as shown in Fig. 4. Among these four subunits, the component with a molecular weight of 45000 daltons showed fluorescence under ultraviolet (UV) illumination. Figure 5 shows the absorption spectra of the enzyme in the UV and near UV regions. Besides the

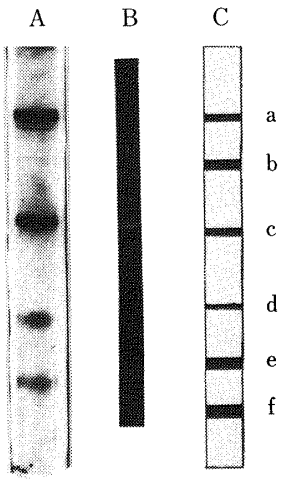

Fig. 4. SDS-Polyacrylamide Gel Electrophoregram of *Arthrobacter* Sarcosine Oxidase

The purified enzyme (100 μg as protein) in 1 ml of 10 mM phosphate buffer, pH 7.0, containing 25% glycerol, 1% SDS, and 5% 2-mercaptoethanol, was treated at 70 °C for 1 h, then 0.1 ml aliquots were applied to 7.5% gels and run at a constant current of 8 mA per gel for 4 h at room temperature. (A) Stained with 1% Amido Black 10B. (B) Photographed under ultraviolet illumination. (C) Marker proteins, stained with 1% Amido Black 10B, such as (a) phosphorylase b, 94000; (b) bovine serum albumin, 67000; (c) ovalbumin 43000; (d) carbonic anhydrase, 30000; (e) soybean trypsin inhibitor, 20000; (f) α-lactalbumin, 14400.

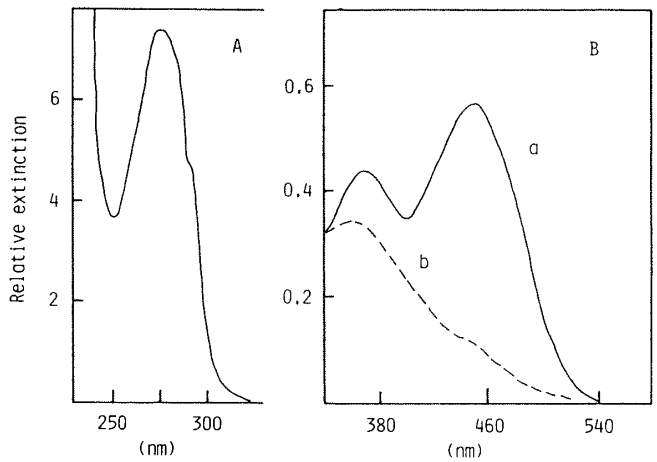

Fig. 5. Absorption Spectra of *Arthrobacter* Sarcosine Oxidase

(A) Spectrum of the native enzyme in 10 mM phosphate buffer, pH 8.0, in the UV region. (B) Spectra of the native enzyme in near ultraviolet and visible regions before (a) and after (b) incubation with 40 mM sarcosine.

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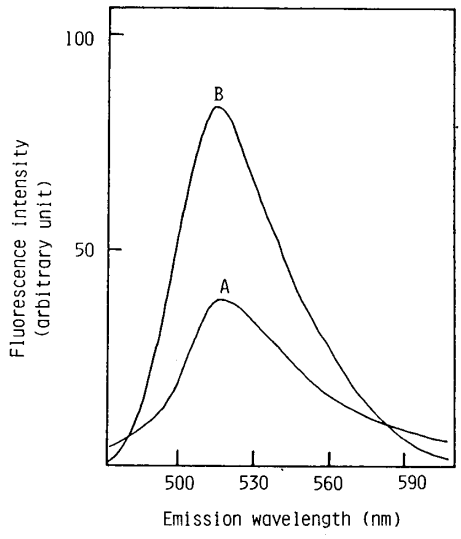

Fig. 6. Fluorescence Spectra of *Arthrobacter* Sarcosine Oxidase and Riboflavin

(A) The native enzyme (1 mg/ml) in 20 mM acetate buffer, pH 4.5. (B) Riboflavin (5 μg/ml) in the same buffer. The excitation wavelength was 450 nm.

TABLE III. Kinetic Parameters of *Arthrobacter* Sarcosine Oxidase

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m
Sarcosine	6.4	6.8	1.06
N-Methyl-DL-alanine	16.5	4.1	0.25

The enzyme was inert toward the following substrate analogues: *N*-methyl-DL-valine, *N*,*N*-dimethylglycine, betaine, choline, L-alanine, L-valine, glycine, 1,3-dimethylurea, 1-methylguanidine, 1-methylhydantoin.

absorption maximum at 278 nm, two minor absorption peaks at 375 and 455 nm were observed. On addition of substrate, the latter peaks disappeared. The fluorescence spectrum of the enzyme is shown in Fig. 6. The enzyme showed an emission spectrum with a maximum at 520 nm under excitation at 450 nm. Treatment of the enzyme in 10% trichloroacetic acid for 15 min in a boiling water bath gave a yellow-colored material in the soluble fraction, suggesting the existence of noncovalently bound flavin. Morevoer, trypsin digestion of the resulting precipitate also gave some peptides which show absorption peaks at 350 and 450 nm, suggesting the existence of covalently bound flavin. These characters of *Arthrobacter* sarcosine oxidase resemble those of prosthetic groups in sarcosine oxidase from *Corynebacterium* sp. U-96.[2,7,8] These results together with that of SDS-polyacrylamide gel electrophoresis analysis suggest that *Arthrobacter* sarcosine oxidase has two types of flavin components different in their binding mode to protein (presumably covalently bound and noncovalently bound flavins), and these flavins are involved in the oxidation reaction of sarcosine.

Substrate Specificity

Substrate specificity of the enzyme is summarized in Table III. *Arthrobacter* sarcosine oxidase was active toward sarcosine and *N*-methyl-DL-alanine, but completely inert toward the other amino acids and related compounds tested. Sarcosine was the best substrate of the enzyme, and the K_m and k_{cat} values for sarcosine were 6.4 mM and 6.8 s^{-1}, respectively. The bacterial sarcosine oxidases from *Arthrobacter*, *Corynebacterium*,[2] *Alcaligenes*,[4] and *Bacillus*[6] resemble each other in substrate specificity, whereas fungal sarcosine oxidase from *Cylindrocarpon*[1] was highly specific to sarcosine. Very low activity was found toward *N*-methyl-DL-alanine. In Table IV, some properties of microbial sarcosine oxidases hitherto reported are compared. The enzymatic properties and multi-subunit structure of *Arthrobacter* sarcosine oxidase are quite similar to those of the *Corynebacterium* enzyme. The sarcosine oxidases from fungus, *Cylindrocarpon didymum* M-1,[1] and from bacterium, *Bacillus* sp. B-618[6] have molecular weights of 45000, and 42000 daltons, respectively, whereas the enzyme from bacteria, *Corynebacterium* sp. U-96[2] and *Alcaligenes denitrificans*,[3,4] were reported to have molecular weights of 174000 and 190000 daltons, respectively. Therefore, bacterial

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TABLE IV. Sarcosine Oxidases from Various Origins

Origin	Bacteria				Fungus
Strain	*Arthrobacter ureafaciens*	*Corynebacterium* sp. U-96[2]	*Alcaligenes denitrificans*[4]	*Bacillus* sp. B-0618[6]	*Cylindrocarpon didymum* M-1[1]
M.W.	185000	174000	190000	42000	45000
Subunit	Tetramer (hetero)	Tetramer (hetero)	Dimer (hetero)	Monomer	Monomer
Cofactor	Covalent flavin Noncovalent flavin	Covalent FAD Noncovalent FAD	Flavin	Covalent flavin	Covalent FAD
Opt. pH	8.3	7.5—8.5	8.0	8.5—9.0	7.5—9.0
K_m					
Sarcosine	6.4 mM	3.4 mM	4.2 mM	12.2 mM	1.8 mM
N-Methyl-DL-alanine	16.5 mM	8.7 mM[a]	(oxidized)[a]	6.8 mM	(very low activity)
Inhibitors	PCMB Ag^+, Cu^{2+}, Hg^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}	PCMB, iodoacetamide Ag^+, Cu^{2+}, Hg^{2+}, Zn^{2+}, Cd^{2+}	— —	NBS Hg^{2+}, Zn^{2+}, Fe^{3+}	PCMB, iodoacetate Ag^+, Cu^{2+}, Hg^{2+}

FAD, flavin adenine dinucleotide; NBS, *N*-bromosuccinimide; PCMB, *p*-chloromercuribenzoate. *a*) Used as *N*-methyl-L-alanine form.

sarcosine oxidases have a variety of molecular weights. The value of molecular weight, 18500 daltons, of *Arthrobacter* sarcosine oxidase is very close to those of the enzymes from *Corynebacterium*[2] and *Alcaligenes*.[4] Among these enzymes, *Arthrobacter* and *Corynebacterium* sarcosine oxidases are composed of four dissimilar subunits, but only two protein bands were detected on SDS-polyacrylamide gel electrophoresis of *Alcaligenes* sarcosine oxidase.

The structure of the flavin component in *Arthrobacter* sarcosine oxidase and its binding mode to protein are under investigation, and will be reported elsewhere.

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