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Purification and Characterization of an Alkaliphilic Choline Oxidase of *Fusarium oxysporum*

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A novel choline oxidase found in a fungus, *Fusarium oxysporum* strain V2, was purified to homogeneity as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme has a molecular mass of 128 kDa and consists of two identical subunits. The purified enzyme showed adsorption peaks at 340 nm and 450 nm. It showed alkaliphilic pH characteristics: its optimum pH was 9.0–10.0, and it was stable at pH 8.0–10.2. The Michaelis constant (K_m) values for choline and betaine aldehyde were 0.28 mM and 0.39 mM respectively. Trimethylamino-alcohols, dimethylamino-alcohols, and diethylaminoethanol were substrates for the enzyme, but the K_m values for them increased with decreasing numbers of methyl groups on the ammonium headgroup. A marked decrease in the maximum velocity (V_{max}) and V_{max}/K_m values was observed when *N*-replaced choline analogs were used as substrate instead of choline. The enzyme had a remarkably higher affinity for choline and betaine aldehyde than do previously reported enzymes. The enzyme oxidized these two substrates more quickly than a choline oxidase from *Arthrobacter globiformis*, and oxidation by the V2 enzyme was accompanied by an increase in the stoichometric amount of hydrogen peroxide.

Key words: alkaliphilic; choline oxidase; flavoprotein; *Fusarium oxysporum*; phospholipid

Microbial oxidation of choline has been investigated using various microorganisms, and the processes of choline oxidation have been reported. It has been suggested that two different enzymes are involved in the oxidation of choline to glycine betaine. *Pseudomonas aerginosa*, for example, utilizes choline dehydrogenase, a phenazine methosulfate (PMS)-dependent enzyme, in the oxidation of choline to betaine aldehyde.[1,2] Moreover, the intermediate, betaine aldehyde, is oxidized to glycine betaine by betaine aldehyde dehydrogenase, a nicotinamide adenine dinucleotide (NAD)-dependent enzyme.[3] It is also known that some bacteria such as *Alcaligenes* species[4] and *Arthrobacter globiformis*,[5,6] and a fungus, *Cylindrocarpon didymum* M-1,[7] oxidize choline to glycine betaine *via* betaine aldehyde using a single enzyme called choline oxidase that consumes oxygen and produces H_2O_2. It has been found that choline oxidases are flavoproteins.[4,6,7] Choline oxidases from *A. globiformis* and *Alcaligenes* sp. are also commonly used for the enzymatic determination of phospholipids coupled with phospholipase D and peroxidase.[8,9] It has been reported that the accumulation of betaine in the cytoplasm of cells protects plants and bacteria from salt stress.[10–12] Because choline oxidases have the ability to convert choline to betaine, the *codA* gene encoding the enzyme from *A. globiformis* is utilized to develop transgenic plant species with higher stress tolerance.[13–16] Many studies of microbial choline oxidase have reported that this enzyme shows optimal activity at neutral pH, but no studies have reported choline oxidases produced by alkaliphilic microorganisms. In this study, strain V2, identified as *Fusarium oxysporum*, which is able to grow on an alkaline medium containing choline chloride as sole source of carbon and nitrogen, was isolated. The purification and characterization of a novel choline oxidase from this fungus is described below.

Materials and Methods

Materials. Choline chloride and betaine hydrochloride were purchased from Nacalai Tesque (Kyoto, Japan). Betaine aldehyde chloride was from Sigma (St. Louis, MO). β-Methylcholine chloride, *N*,*N*-dimethylglycine methylester, 3-dimethylamino-1-propanol, and 4-dimethylamino-1-butanol were from Tokyo Kasei (Tokyo). Trimethylamino-1-propanol and trimethylamino-1-butanol were donated by Prof. N. Mori of the Faculty of Agriculture, Tottori University. DEAE-Toyopearl was purchased from Tosoh (Tokyo). Sephacryl S-200 HR and Hydroxyapatite Bio-gel HTP gel were from Amersham Pharmacia Biotech AB (Uppsala, Sweden) and Bio-Rad Laboratories (Hercules, CA) respectively. Peroxidase was from Wako Pure Chemical Industries (Osaka, Japan). Multi Gel II mini 7.5 and Multi Gel II 10/20 were from Cosmo Bio (Tokyo). All other chemicals used were of analytical grade and were commercially available.

Screening. Soil samples collected from different areas were suspended in distilled water. The suspensions were cultured on agar plates (basal medium) containing 1% choline chloride as sole source of carbon and nitrogen, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% yeast extract, 1% Na_2CO_3, and 2% agar. The initial pH of the medium was about 10 with the addition of 1% Na_2CO_3 for the isolation of alkaliphilic microorganisms. Cultivation was done at 30 °C for 4 to 7 d. The colonies that appeared were picked out and isolated in storage medium containing 1% choline chloride, 0.1% K_2HPO_4, 0.2% polypeptone, 0.2% yeast extract, 0.02% $MgSO_4 \cdot 7H_2O$, 1% Na_2CO_3, and 2% agar, pH 10. The isolates were cultivated in choline liquid medium (identical to the basal medium except that no agar was used) for 5 to 7 d at 30 °C in a reciprocal shaker. Cell growth was estimated visually. Cells were harvested by centrifugation at 10,000 *g* for 15 min and washed with 0.9% NaCl. The washed cells were suspended in 50 mM potassium phosphate buffer (pH 8.0) and disrupted with glass beads (diameter 0.1–0.5 mm) using Mini-Beadbeater Model 3110BX

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(Wakenyaku, Japan) at 5 °C for 3 min for cell disruption. Cell debris was removed by centrifugation, and the supernatant was assayed for choline oxidase activity. The degree of cell growth in the choline liquid medium, the productivity, and the pH requirements of the enzyme were examined.

Identification. Identification of the isolated strain was done by the National Collections of Industrial, Food and Marine Bacteria (NCIMB Japan, Shizuoka). The morphological characteristics and the 28S rDNA-D1/D2 sequence of strain V2 were investigated. A homology search for the 28S rDNA-D1/D2 sequence was performed by BLAST.[17)]

Organism and cultivation. Strain V2 isolated from soil was used. The culture medium contained 1% choline chloride, 0.3% K_2HPO_4, 0.3% KH_2PO_4, 0.05% yeast extract, and 0.02% $MgSO_4 \cdot 7H_2O$ at pH 8.0. Cultivation was carried out at 30 °C for 5 d in a 500 mL shaking flask containing 100 mL of the medium, with reciprocal shaking.

Enzyme assay. Choline oxidase activity was assayed by the quantity of betaine aldehyde or H_2O_2 formed. For estimation of the formation of betaine aldehyde, the reaction mixture contained 90 µmol of glycine-NaOH buffer (pH 9.5), 10 µmol of choline chloride, and the enzyme in a total volume of 1 mL. The reaction was carried out with shaking at 30 °C for 10 min. The quantity of betaine aldehyde in the reaction mixture was determined by a modification of a procedure described by Jellinek *et al.*,[18)] as follows: One mL of the reaction mixture was mixed with 0.1% 2,4-dinitrophenylhydrazine in 1 M HCl and heated in a boiling water bath for 7 min. The mixture was cooled to room temperature, and then 6.4 mL of water was added. NaOH (2 M, 1.6 mL) was added, and then the absorbance at 440 nm was measured. To measure the formation of H_2O_2, a method involving peroxidase, phenol, and 4-aminoantipyrine was used.[19)] The reaction mixture contained 60 µmol of glycine-NaOH buffer (pH 9.5), 3 µmol of phenol, 2.25 µmol of 4-aminoantipyrine, 5 U of peroxidase, 15 µmol of choline chloride, and a suitable amount of the enzyme in a total volume of 1.5 mL. The reaction was carried out at 30 °C, and the increase in absorbance at 505 nm was measured using a Hitachi U-2000A spectrophotometer (Hitachi, Tokyo). Enzyme activity was calculated using an extinction coefficient of 9.2 mM^{-1} cm^{-1} for quinine-imine dye at 505 nm, pH 9.5. One unit of enzyme activity was defined as the quantity of enzyme necessary to catalyze the formation of 1 µmol of betaine aldehyde or H_2O_2 per min. Choline oxidase activity was estimated by measurement of the H_2O_2 formed in the reaction mixture, unless otherwise stated.

Protein determination. Protein levels were determined by Bio-Rad Protein Assay (Hercules, CA) with bovine serum albumin as standard, or by the absorbance at 280 nm.

Purification of choline oxidase. Purification of choline oxidase from strain V2 was carried out at 5 °C. Tris–HCl buffer (50 mM, pH 8.0) containing 0.1 mM dithiothreitol (DTT) was used, and centrifugation was performed at 40,000 *g* for 20 min throughout the enzyme purification procedure, unless otherwise stated.

Preparation of cell-free extract. The washed cells, harvested from 4 L of culture medium, were suspended in 1 L of Tris–HCl buffer and disrupted for 90 min using an ultrasonic oscillator (19 kHz) on ice. The disrupted cells were centrifuged, and the supernatant was used as cell-free extract.

Ammonium sulfate fractionation. During this procedure, the pH of the solution was kept at about 8.0 with 14% ammonium hydroxide solution. Ammonium sulfate was added to the cell-free extract to 50% saturation and the precipitate was discarded. A precipitate was formed by further addition of ammonium sulfate to 80% saturation, and was collected by centrifugation. The precipitate recovered was dissolved in Tris–HCl buffer and dialyzed against the buffer (2 L) for 15 h.

DEAE-Toyopearl column chromatography. The dialyzed enzyme solution was applied to a DEAE-Toyopearl column (2.4 × 26.5 cm) equilibrated using the buffer. The column was washed with the equilibrated buffer, and the enzyme was eluted with a linear gradient of NaCl (0–0.5 M) in the buffer. The active fractions were pooled and concentrated by the addition of ammonium sulfate to 80% saturation. The precipitate formed was collected by centrifugation and dissolved in a minimal volume of the buffer.

Sephacryl S-200 column chromatography (1st). The enzyme solution was applied to a Sephacryl S-200 column (1.4 × 76 cm) equilibrated with the buffer, and was eluted with the buffer. Active fractions were combined and concentrated by the addition of ammonium sulfate to 80% saturation. The precipitate formed was collected by centrifugation and dissolved in 10 mM potassium phosphate buffer (pH 8.0) containing 0.1 mM DTT. The enzyme solution was dialyzed against the buffer.

Hydroxyapatite column chromatography. The dialyzed solution was applied to a hydroxyapatite column (1.4 × 3.2 cm) equilibrated with 10 mM potassium phosphate buffer (pH 8.0) containing 0.1 mM DTT. The column was washed with the equilibrated buffer, and the enzyme was eluted using a linear gradient of phosphate buffer (0.01–0.2 M). Active fractions were combined and concentrated by the addition of ammonium sulfate to 80% saturation. The precipitate obtained was dissolved in a minimal volume of the buffer.

Sephacryl S-200 column chromatography (2nd). The enzyme solution was applied to the column as in step 4, and was eluted with the buffer as in step 4. Fractions with enzyme activity were stored at −80 °C.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed on commercially available gel plates (Multi Gel II mini 7.5 for native-PAGE and Multi Gel II mini 10/20 for SDS–PAGE). The gels were stained with Coomassie Brilliant Blue R-250.

Determination of molecular weight. The molecular weight of the choline oxidase was estimated by gel filtration on a Sepharose 6B column equilibrated with 0.05 M Tris–HCl buffer, pH 8.0, containing 0.1 mM DTT. The standard proteins used were thyroglobulin (669 kDa), ferritin (450 kDa), catalase (240 kDa), bovine albumin (68 kDa), chymotrypsinogen A (25 kDa), and cytochrome c (12.5 kDa).

Results and Discussion

Screening and identification of the microorganism

A total of 244 microorganisms that were grew on choline as sole carbon and nitrogen source at pH 10 were isolated from 50 Japanese soil samples. Among these isolates, three strains of filamentous fungi showed significantly faster and more profuse growth on choline liquid medium at pH 10. A fungus designated strain V2 exhibited the highest specific choline oxidase activity and highest productivity in the cell-free extract. Hence it was selected for the present study. It was identified by NCIMB Japan as *F. oxysporum* in that its 28S rDNA-D1/D2 sequence showed more than 99% similarity to that species.

Choline oxidase production by strain V2

The production of choline oxidase by strain V2 was examined under the conditions described above. Strain V2 showed better growth at pH 8.0 than at pH 10.2 when a large culture was grown in a 2 L shaking flask containing 300 mL of medium, with reciprocal shaking (data not shown). Choline oxidase activity increased with cell growth, reaching a maximum at 4–5 d.

Table 1. Summary of the Purification of Choline Oxidase from *Fusarium oxysporum* Strain V2

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free extract	436	554	0.79	1	100
$(NH_4)_2SO_4$ fractionation	314	160	1.96	2.5	72
DEAE-Toyopearl	198	8.1	24.4	31	45
Sephacryl S-200 (1st)	143	5.3	27	34	33
Hydroxyapatite	98	3.2	30.6	39	22
Sephacryl S-200 (2nd)	61	1.9	32.1	41	14

Purification of choline oxidase

The purification steps and results are summarized in Table 1. The enzyme was purified 41-fold, yielding a preparation with a specific activity of 32.1 units/mg. The purified enzyme gave a single protein band on native- and SDS-PADE (Fig. 1). Analysis of the N-terminal amino acid sequence was unsuccessful, probably due to N-terminal blocking.

Protein characterization

The molecular mass of the native enzyme was estimated by gel filtration to be about 128 kDa. SDS–PAGE suggested that the molecular mass of the enzyme subunit was 64 kDa, and thus the native enzyme appeared to be a homodimer. The molecular masses of choline oxidases purified from the bacteria *A. globiformis*[6] and *Alcaligenes* sp.[4] have been reported to be 83 kDa and 72 kDa under non-denaturing conditions, and 71 kDa and 66 kDa in the presence of SDS, respectively. On the other hand, it has been found that the choline oxidase from *C. didymum* M-1, the only one known of fungal origin, exhibited a molecular mass of 120 kDa by gel filtration and 64 kDa by SDS–PAGE.[7] The molecular mass and the number of subunits of the enzyme from strain V2 (*F. oxysporum*) were remarkably similar to those of *C. didymum* M-1. It has been reported that a recombinant choline oxidase of *A. globiformis* expressed in *Escherichia coli* exists as a homodimer of 120 kDa, as found by size-exclusion chromatography and amino acid composition analysis.[20] Thus it may be that bacterial and fungal choline oxidases have similarities in the molecular masses of their subunits and their oligomerized states, although the molecular mass of the subunit and the oligomerized state of the reported recombinant enzyme did not agree with previous results for the native choline oxidase from *A. globiformis*.

Adsorption spectrum of choline oxidase

The absorption spectrum of the purified choline oxidase is shown in Fig. 2. Peaks at 340 nm and 450 nm were detected, and the latter had a shoulder at a longer wavelength (Fig. 2a). This spectrum is typical of flavoproteins. The addition of choline to the enzyme resulted in a decline of the peak at 450 nm (Fig. 2b). The spectrum was similar to the spectra of choline oxidases of other origins reported to date.[4,6,7,20] The prosthetic group of the enzyme remains to be identified.

Effects of temperature and pH

The optimum temperature of the enzyme was about 35–40 °C (Fig. 3a). Temperature stability was measured after incubation of the enzyme for 10 min at various temperatures. It was stable at below 35 °C (Fig. 3b). Its optimum pH was about 9.0–10 (Fig. 3c). Its pH stability was tested by incubation at various pHs for 10 min at 30 °C. It was stable in an alkaline pH range of 8.0 to 10.2 (Fig. 3d). The optimum pH values and stabilities of the choline oxidases to pH changes were as follows: *A. globiformis*, 7.5–8.0 and about 8.0 at 37 °C for 30 min, and *C. didymum* M-1, 9.0 and 7.0–9.0 at 40 °C for 10 min.[7,21] Thus the choline oxidase from strain V2 exhibited significantly alkaliphilic pH characteristics.

Effects of metal ions and various reagents

The effects of various metal ions and reagents on enzyme activity were examined. The enzyme was pre-incubated at 30 °C for 10 min with various compounds

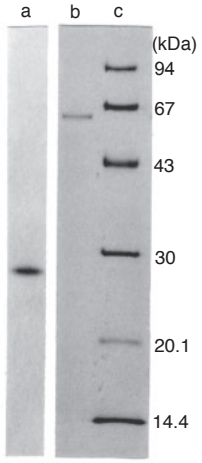


Fig. 1. Polyacrylamide Gel Electrophoresis of the Purified Choline Oxidase from *Fusarium oxysporum* Strain V2.

Purified enzyme and marker proteins were subjected to electrophoresis. a, native-PAGE; b, SDS–PAGE; c, marker proteins: phosphorylase b (94 kDa); albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20.1 kDa); α-lactalbumin (14.4 kDa). An LMW electrophoresis calibration kit (Pharmacia) was used as molecular weight standard for SDS–PAGE.

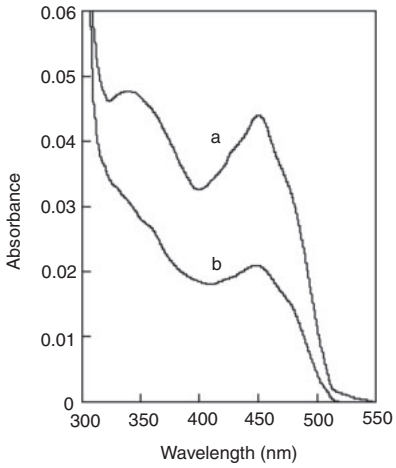


Fig. 2. Absorption Spectrum of the Purified Choline Oxidase.

a, the native enzyme solution at a concentration of 0.25 mg/mL in 50 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM DTT; b, the reduced enzyme after the addition of choline at a final concentration of 40 mM at 25 °C for 20 min.

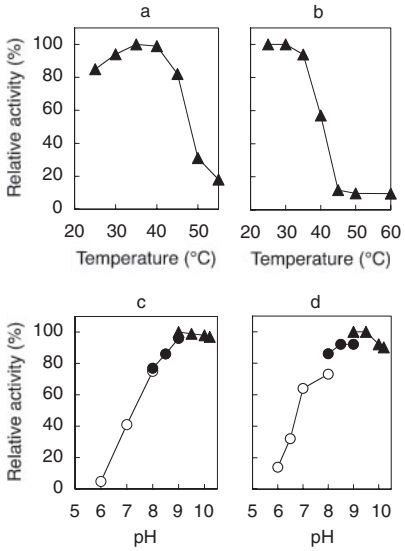


Fig. 3. Effects of Temperature and pH on the Activity (a, c) and Stability (b, d) of the Purified Enzyme.

Enzyme activity was assayed by measuring the formation of betaine aldehyde. Symbols: ○ potassium phosphate buffer; ● Tris–HCl buffer; ▲ Glycine-NaOH buffer.

(1 mM) before the addition of substrate. Residual activity was then assayed. Hg^{2+} caused substantial inhibition of 94%. Cu^{2+}, Zn^{2+}, and Mn^{2+} caused 29%, 10%, and 7% inhibition respectively. Ba^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, and Fe^{3+} did not significantly affect enzyme activity. No metal ions enhanced enzyme activity. Sulfhydryl reagents such as *p*-chloromercuribenzoate and iodoacetamide inhibited the reaction, by 75% and 10% respectively, suggesting that the sulfhydryl groups of the enzyme are involved in the enzyme activity. The enzyme was also inhibited 20% by quinine, a flavoprotein inhibitor. It was not sensitive to metal chelators such as *o*-phenanthroline, EDTA, α,α'-diphenyl, and Tiron.

Substrate specificity

Various choline analogs were tested to investigate the substrate specificity of the enzyme. The results are summarized in Table 2. The enzyme showed greatest activity on choline. Betaine aldehyde was oxidized by 49%. Dimethylaminoethanol and 2-methylaminoethanol were oxidized, but the relative activities markedly decreased with decreasing numbers of methyl groups on the substrate's amine moiety. Trimethylamino-1-propanol, trimethylamino-1-butanol, dimethylamino-propanol, and dimethylamino-butanol were slightly reactive. The enzyme did not react with betaine, monoethanolamine, *N*,*N*-dimethylglycine methylester, β-methylcholine, L-carnitine, or primary alcohols such as propanol, ethanol, and methanol. K_m values for the substrates were calculated from Lineweaver–Burk plots at pH 9.5. The kinetic parameters are shown in Table 2. The K_m values for choline and betaine aldehyde were 0.28 mM and 0.39 mM respectively. Both are significantly lower than previously reported by other sources, including *A. globiformis* (1.2 mM, 8.7 mM),[6] *Alcaligenes* sp. (0.87 mM, 6.2 mM),[4] and *C. didymum* M-1 (1.3 mM, 5.8 mM).[7] For previously described enzymes, the affinities to betaine aldehyde tend to be 4 to 7 times lower than the affinities to choline. In contrast, the enzyme from strain V2 (*F. oxysporum*) exhibited a significantly high affinity to betaine aldehyde as well as to choline. This is a remarkable characteristic.

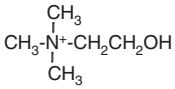
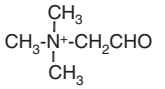
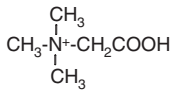
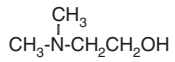
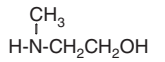
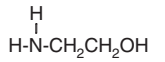
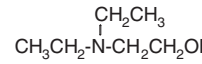
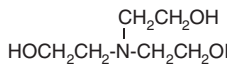
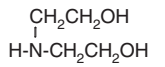
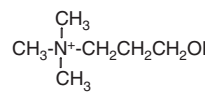
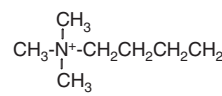
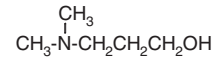
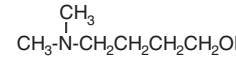
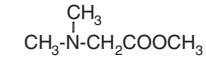
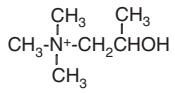
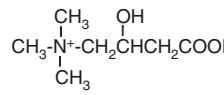
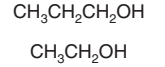
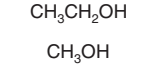
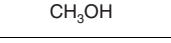
Gadda *et al.* have reported that using a recombinant enzyme from *A. globiformis*, expressed in *Escherichia coli*, the specificity of choline oxidase for its substrate was defined by the presence of the trimethylammonium headgroup on choline.[22] Similar results were obtained for an enzyme from *F. oxysporum* strain V2. When choline was replaced by dimethylaminoethanol or 2-methylaminoethanol, the K_m values for these substrates increased about 3 times. Marked decreases in the V_{max} and V_{max}/K_m values for these substrates were observed with decreasing numbers of methyl groups on the ammonium headgroup of the substrate. These results indicate that the methyl group on the amine portion of the substrate strongly affects the catalytic activity of the enzyme.

The effect of the acetyl side chain attached to the trimethylammonium moiety of choline on enzyme activity was examined using a number of choline analogs as substrates. Compounds containing methyl-ester, a methyl group, or a carboxylate residue on the acetyl site of choline were not oxidized. Relative activities against trimethylamino-1-propanol and trimethylamino-1-butanol, which have longer carbon chains than choline, were very low. The K_m values for trimethylamino-1-propanol and trimethylamino-1-butanol increased when the carbon chain become much longer than that of choline. Moreover, the V_{max} and V_{max}/K_m of these compounds significantly decreased with increasing numbers of carbon atoms in the main chains. Thus, the nature of the side chain attached to the trimethylammonium moiety of choline was also important for binding at the active site of the enzyme.

Estimation of oxidation products of choline by choline oxidase from strain V2

During the oxidation of choline (0.1 μmol) to betaine by the purified enzyme from strain V2, the intermediate betaine aldehyde accumulated, reaching a maximum of about 0.05 μmol after 2 min in the reaction mixture, then rapidly decreasing. It may be that the fast decrease in the intermediate was caused by the low K_m value of betaine aldehyde. After 20 min, betaine aldehyde could no longer be detected, and about 0.2 μmol of H_2O_2 was produced from 0.1 μmol of choline by the enzyme reaction (Fig. 4a). During the oxidation of betaine aldehyde (0.1 μmol), the rate of disappearance of the substrate corresponded to the rate of production of H_2O_2, and finally a stoichiometric amount of H_2O_2 (0.1 μmol) was generated (Fig. 4b). These results indicate that the enzyme from stain V2 also catalyzed the sequential oxidation of choline to betaine aldehyde and of betaine aldehyde to betaine in reactions coupled with H_2O_2 generation. This suggests that the enzyme showed the same oxidation process of choline to betaine as reported for other microorganisms such as *A. globiformis*[6,23] and *C. didymum* M-1.[7] The same experiment to estimate the oxidation products of choline by choline oxidase has been reported using an enzyme from *A. globiformis*.[6] According to that report, when 0.1 μmol of choline was oxidized completely to betaine *via* betaine aldehyde, using 0.25 U of a choline oxidase

Table 2. Substrate Specificity of Choline Oxidase from *Fusarium oxysporum* Strain V2

Substrate (10 mM)	Structure	Relative activity (%)	K_m (mM)	V_{max} (μmol/min/mg)	V_{max}/K_m
Choline		100	0.28	2.04	7.27
Betaine aldehyde		49	0.39	1.07	2.74
Betaine		0	—	—	
Dimethylaminoethanol		12	0.92	0.29	0.32
2-Methylaminoethanol		4.4	0.94	0.09	0.10
Monoethanolamine		0	—	—	
Diethylaminoethanol		7	0.48	0.18	0.37
Triethanolamine		1	—	—	
Diethanolamine		1	—	—	
Trimethylamino-1-propanol		14	0.96	0.27	0.28
Trimethylamino-1-butanol		5	1.80	0.11	0.06
Dimethylamino-1-propanol		9	0.76	0.20	0.27
Dimethylamino-1-butanol		2	—	—	
N,*N*-Dimethylglycine methylester		0	—	—	
β-Methylcholine		0	—	—	
L-Carnitine		0	—	—	
Propanol		0	—	—	
Ethanol		0	—	—	
Methanol		0	—	—	

Enzyme activity was estimated by measuring the amount of H_2O_2, as described in the text. —, not determined.

from *A. globiformis*, a reaction time of about 30 min was required. For the complete oxidation of 0.1 μmol of betaine aldehyde, a reaction time of about 30 min was needed. In contrast, as shown in Fig. 4, when 0.25 units of the enzyme from strain V2 was used, more than 90% of the initial concentration (0.1 μmol) of choline or of betaine aldehyde was oxidized within 15 min, generating a stoichiometric amount of H_2O_2. The oxidation reactions of the substrates were completed in 20 min. It appears that the strain V2 enzyme oxidizes these substrates to betaine much faster than any previously tested enzymes. This is confirmed by the high affinity of the enzyme for choline and betaine aldehyde, as described above.

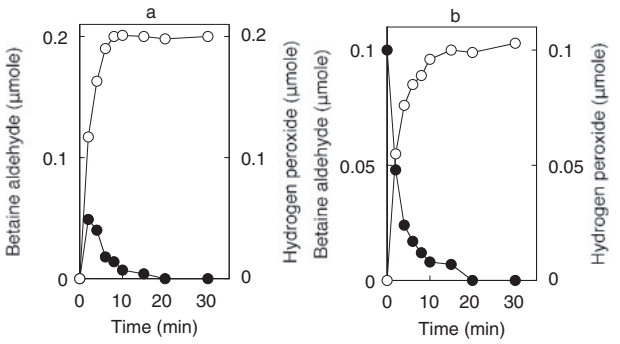


Fig. 4. Time Course of the Oxidation of Choline (a) and Betaine Aldehyde (b) by the Purified Enzyme.

The oxidation reactions of the substrates were carried out at 30 °C with 0.25 U of the purified enzyme, and were stopped for the indicated periods by the addition of HCl. The estimation of H_2O_2 generated and betaine aldehyde utilized was carried out in the same reaction mixture. Symbols: ○ H_2O_2; ● betaine aldehyde.

In conclusion, this study succeeded in isolating *F. oxysporum* strain V2, producing alkaliphilic choline oxidase. This is the first report of the purification and characterization of the enzyme from *F. oxysporum*. Although many studies of microbial choline oxidase have been reported, I have found that the enzyme from *F. oxysporum* exhibits remarkably higher affinity for choline and betaine aldehyde than do previously reported enzymes. In addition, it has alkaliphilic pH characteristics in the oxidation of substrates and pH stability. These properties should carry an advantage in the enzymatic measurement of choline.

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