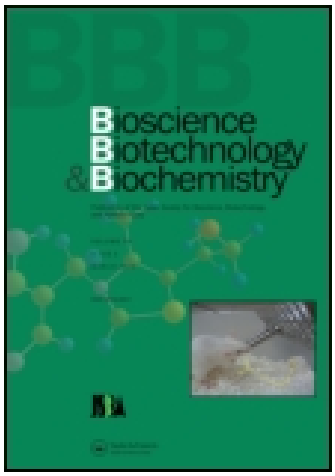

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Purification and Properties of Crystalline 3-Methylaspartase from Two Facultative Anaerobes, *Citrobacter* sp. Strain YG-0504 and *Morganella morganii* Strain YG-0601

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3-Methylaspartase (3-methylaspartate ammonia-lyase, EC 4.3.1.2) from two facultative anaerobes from soil, *Citrobacter* sp. strain YG-0504 and *Morganella morganii* strain YG-0601, were purified and crystallized from their crude extracts. Both of the *Citrobacter* and *Morganella* enzymes appeared to be a dimer of subunits of M_r 40,000 and 44,000, respectively. The enzymes had similar enzymological properties: optimum pH for the deamination reaction of (2*S*,3*S*)-3-methylaspartic acid, substrate specificity, inhibitor, divalent and monovalent cation requirement, and N-terminal amino acid sequence homology. However, some differences were detected in pH and temperature stability, optimum pH for the amination reaction of mesaconic acid, optimum temperature, specific activity, and stability during electrophoresis. Both enzymes had similar enzymological properties to the known 3-methylaspartase from an obligate anaerobic bacterium, *Clostridium tetanomorphum* H1, except kinetic constants and substrate specificities.

A variety of amino acid ammonia-lyases, which are useful in the enzymatic synthesis of several amino acids, have been reported.[1] The enzyme 3-methylapasrtase (3-methylaspartate ammonia-lyase (MAL), EC 4.3.1.2) is associated with an anaerobic degradation of the (*S*)-glutamic acid in obligate anaerobes and catalyzes reversible amination-deamination reactions between mesaconic acid and (2*S*,3*S*)-3-methylaspartic acid.[2,3] MAL began to draw our attention for the enzymatic synthesis of several 3-substituted (*S*)-aspartic acids from their corresponding fumaric acid derivatives. However, the application of MAL for organic synthesis is quite limited, because in the cultivation of MAL-producing obligate anaerobic bacteria, including *Clostridium tetanomorphum* H1, it is very tedious to keep the anaeobic condition. Additionally, we have little information on MAL because it has been prepared to homogeneity[2,3] and characterized[4-7] only from *C. tetanomorphum*.

The occurrence of MAL activity in facultative anaerobes has been investigated in our laboratory.[8] We found that some soil bacteria in the family of *Enterobacteriaceae* produced MAL when they were grown statically in a medium containing (*S*)-glutamic acid, and used them for the synthesis of several 3-substituted (*S*)-aspartic acids.[8] This paper describes the preparation and characterization of crystalline MAL from cell extracts of *Citrobacter* sp. strain YG-0504 and *Morganella morganii* strain YG-0601, and comparison of the properties of the enzymes with MAL from *C. tetanomorphum*.

Materials and Methods

Chemicals and biochemicals. DEAE-Toyopearl 650 M, Butyl-Toyopearl 650 M, and HPLC columns G-3000 SW were purchased from Tosoh (Japan); Superdex 200 was from Pharmacia (Sweden); Coomassie Brilliant Blue R-250 and mesaconic acid were from Tokyo Kasei (Japan); and dimethylsuberimidate dihydrochloride was from Wako Pure Chemicals (Japan). Chlorofumaric acid[9] and ethylfumaric acid[10,11] were synthesized from (2*S*,3*S*)-tartaric acid and ethyl acetoacetate, respectively. The compounds, (2*S*,3*S*)-3-methylaspartic, (2*S*,3*S*)-3-ethylaspartic, and (2*R*,3*S*)-3-chloroaspartic acids were enzymatically synthesized using a cell-free extract of *Clostridium tetanomorphum* H1 NCIMB 11547 as described by Akhtar *et al.*[12] All other chemicals were from commercial sources and used without further purification.

Microorganisms. The microorganisms used were *Citrobacter* sp. strain YG-0504 and *Morganella morganii* strain YG-0601, which were isolated from soil by us.[8] According to "Bergey's Manual of Systematic Bacteriology",[13] strain YG-0601 may be classified as *M. morganii* from its detailed taxonomic studies. However, the strain could be classified as *Proteus* sp. using the minitek identification system.[8]

Growth conditions. Both strains were cultivated in a 2-liter Sakaguchi flask filled with 2 liters of a medium containing 10 g of yeast extract, 80 mmol of potassium phosphate buffer (pH 7.4), 27 g of monosodium (*S*)-glutamate, 0.5 g of $MgSO_4 \cdot 7H_2O$, 22 mg of $FeSO_4 \cdot 7H_2O$, 4.8 mg of $MnSO_4 \cdot 4$–$6H_2O$, 4.8 mg of $Na_2MoO_4 \cdot 2H_2O$, and 30 mg of $CaCl_2 \cdot 2H_2O$. Cultivation was done at 37°C for 40 h without shaking.

Enzyme assay and protein measurement. The enzyme activity in the deamination reaction was assayed at 20°C by measuring the rate of the formation of fumaric acid derivative at 240 nm from their corresponding 3-substituted (*S*)-aspartic acid by the method of Barker *et al.*[2] using appropriate extinction coefficients,[6] ε(M^{-1}, cm^{-1}), for mesaconic, ethylfumaric, chlorofumaric, and fumaric acids were 3850, 3323, 4434, and 2530, respectively. The reaction mixture (1 ml) containing 50 μmol of ethanolamine–HCl buffer (pH 9.7), 10 μmol of KCl, 1 μmol of $MgCl_2$, 5 μmol of monosodium salt of 3-substituted (*S*)-aspartic acid, and enzyme solution. The enzyme activity in the amination reaction was measured at 20°C by the disappearance of fumaric acid derivative at 240 nm in the reaction mixture (1 ml) containing 200 μmol of Tris–HCl buffer (pH 8.5), 10 μmol of KCl, 1 μmol of $MgCl_2$, and 0.5 μmol of disodium salt of fumaric acid derivative, and enzyme solution. One unit of MAL activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of mesaconic acid from (2*S*,3*S*)-3-methylaspartic acid during the deamination reaction per min at 20°C. Protein was assayed by the method of Bradford *et al.*[14] using a dye reagent concentrate (Bio-Rad Laboratories) with bovine serum albumin as the standard, or by measuring the absorbance

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Abbreviation: MAL, 3-methylaspartate ammonia-lyase (3-methylaspartase).

at 280 nm.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) in the absence of SDS was done by the method of Davis[15] using a vertical slab gel apparatus (NPG-520L; 5–20% gradient acrylamide, ATTO Corporation (Japan)). The standard proteins used were (M_r) thyroglobulin (669,000), ferritin (443,000), lactate dehydrogenase (139,850), albumin (66,267), and trypsin inhibitor (20,100) (Daiichi Kagaku Yakuhin (Japan)). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was done as described by Laemmli,[16] with 12.5% acrylamide. The standard proteins used for a calibration were (M_r) β-galactosidase (116,000), fructose-6-phosphate kinase (85,200), glutamate dehydrogenase (55,600), aldolase (39,200), and triosephosphate isomerase (26,600) (Boehringer Mannheim GmbH (Germany)). Protein was stained with Coomassie Brilliant Blue R-250.

Measurement of molecular weight. The molecular weight of the enzyme was measured by HPLC with a G-3000 SW gel filtration column (0.5 × 60 cm) at a flow rate of 0.7 ml/min with an elution buffer consisted of 0.1 M of potassium phosphate buffer (pH 7.0) and 0.2 M NaCl. A calibration curve was made with the following proteins (M_r): glutamate dehydrogenase (290,000), lactate dehydrogenase (142,000), enolase (67,000), adenylate kinase (32,000), and cytochrome *c* (12,400) (Oriental Yeast (Japan)).

Amino terminal amino acid sequencing. To sequence NH_2-terminal amino acids, the enzyme sample was covalently bound to Sequelon-AA and -DITC membranes using the procedures supplied by the manufacturer. Sequelon-AA and -DITC membranes are made of a PVDF (polyvinylidene difluoride) matrix that have been derivatized with aryl amine and 1,4-phenylene diisothiocyanate groups, which react with both the C-terminal and side-chain carboxyl groups, and both N-terminal and side-chain amino groups of the enzyme, respectively. The enzyme samples on the membranes were analyzed with an automatic protein sequencer 6625 Prosequencer (Milligen/Biosearch) using phenylthiohydantoin derivatives.

Purification of the enzymes. All the enzyme purification procedures were done at 4°C. Potassium phosphate buffer (pH 7.0) containing 0.5 mM 2-mercaptoethanol was used throughout the purification.

Purification of MAL from Citrobacter sp. strain YG-0504.

Step 1. Preparation of crude extract. The washed cells (70 g, wet weight) from 40 liters of culture were suspended in 300 ml of the 0.1 M buffer. The cells were disrupted for 20 min by a Kubota-Shoji 9 kHz ultrasonic oscillator. The disrupted cells were removed by centrifugation at 32,000 × *g* for 25 min.

Step 2. Ammonium sulfate fractionation. The supernatant solution (350 ml) was brought to 30% saturation with solid ammonium sulfate. The precipitate was removed by centrifugation, and ammonium sulfate was added to the supernatant solution to 50% saturation. The active precipitate collected by centrifugation was dissolved in 0.01 M buffer and dialyzed against the same buffer (10 liter × 3).

Step 3. DEAE-Toyopearl column chromatography. The dialyzed enzyme solution was put on a DEAE-Toyopearl 650 M column (6 × 19 cm) equilibrated with 0.01 M buffer. After the column was washed with 0.1 M buffer, the enzyme was eluted with 0.1 M buffer containing 0.1 M NaCl. The active fractions were combined and dialyzed against 0.01 M buffer.

Step 4. Butyl-Toyopearl column chromatography. To the active solution were added ammonium sulfate to 30% saturation. The enzyme solution was placed on a column of Butyl-Toyopeal 650 M (4 × 17 cm), equilibrated with 0.01 M buffer containing ammonium sulfate to 30% saturation. After a column was washed with 0.01 M buffer containing ammonium sulfate to 20% saturation, the active fractions were eluted with a linear gradient of ammonium sulfate (20–10% saturation). The active fractions collected were dialyzed and concentrated by ultrafiltration.

Step 5. High-Load Superdex 200 column chromatography. The enzyme solution was filtered on a High-Load Superdex 200 gel column. The enzyme was eluted with the 0.05 M buffer containing 0.1 M NaCl at a flow rate of 1 ml/min. The active fractions were pooled and concentrated by ultrafiltration.

Step 6. Crystallization. The purified enzyme sample was concentrated to be about 50 mg/ml. Solid ammonium sulfate was cautiously added to the enzyme solution until it became slightly turbid, and the mixture was left at 4°C. Crystallization began after 1 d and was virtually completed within 3 d. The enzyme was isolated in needle form.

Purification of MAL from Morganella morganii strain YG-0601.

Step 1. Preparation of crude extract. Washed cells (50 g, wet weight) from 40 liters of culture were suspended in 250 ml of the 0.1 M buffer, disrupted with a Kubota-Shoji 9 kHz ultrasonic oscillator for 20 min and then centrifugated at 32,000 × *g* for 25 min.

Step 2. Ammonium sulfate fractionation. The supernatant solution (290 ml) fractionated with ammonium sulfate (45–70% saturation), followed by centrifugation. The precipitate was dissolved in 0.01 M buffer and dialyzed against the same buffer (10 liter × 3).

Step 3. DEAE-Toyopearl column chromatography. The dialyzed enzyme solution was put on a DEAE-Toyopearl 650 M column (6 × 20 cm) equilibrated with 0.01 M buffer. The column was washed with the same buffer and then the enzyme was eluted with 0.1 M buffer. The active fractions were combined and dialyzed against 0.01 M buffer.

Step 4. Butyl-Toyopeal column chromatography. Ammonium sulfate was added to 30% saturation to the active solution. The enzyme solution was put on a column of Butyl-Toyopeal 650 M (4 × 18 cm), which had been equilibrated with 0.01 M buffer containing ammonium sulfate to 30% saturation. After the column was washed with 0.01 M buffer containing ammonium sulfate to 25% saturation, the active fractions were eluted with a linear gradient of ammonium sulfate (25–20% saturation). The active fractions collected were dialyzed and concentrated by ultrafiltration.

Step 5. Crystallization. The purified enzyme sample was concentrated to be about 30 mg/ml and solid ammonium sulfate was added gradually until a faint turbidity appeared. On standing at 4°C for 2 wk, plate crystals were formed without increase in the specific activity.

Results

Purification and crystallization of the enzymes

Table I (A) summarizes the purification of the enzyme from the cell-free extract of *Citrobacter* sp. strain YG-0504. The *Citrobacter* enzyme was efficiently purified by crystallization after the usual column chromatography. The enzyme was easily crystallized with solid ammonium sulfate in the concentrated enzyme solution, and isolated in fine needle form. Figure 1 (A) is a photomicrograph of the crystalline enzyme. Twenty-eight-fold purification with 22.5% yield was achieved.

As shown in Table I (B), the enzyme from *Morganella morganii* strain YG-0601 were purified to homogeneity in 39-fold with an overall recovery of 62% from the cell-free

Table I. Purification of 3-Methylaspartases from *Citrobacter* sp. Strain YG-0504 (A) and *Morganella morganii* Strain YG-0601 (B)

(A)

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Cell-free extract	7200	42,300	5.84	100
Ammonium sulfate (30–50%)	4100	43,300	10.5	102
DEAE-Toyopearl	630	20,900	33.2	50
Butyl-Toyopearl	81.0	10,400	128	24.6
Superdex 200	64.5	9,610	149	22.7
Crystallization	58.6	9,500	162	22.5

(B)

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Cell-free extract	3200	24,500	7.66	100
Ammonium sulfate (45–70%)	1010	18,800	18.6	102
DEAE-Toyopearl	279	15,600	55.9	63.7
Butyl-Toyopearl	51.0	15,100	296	61.6

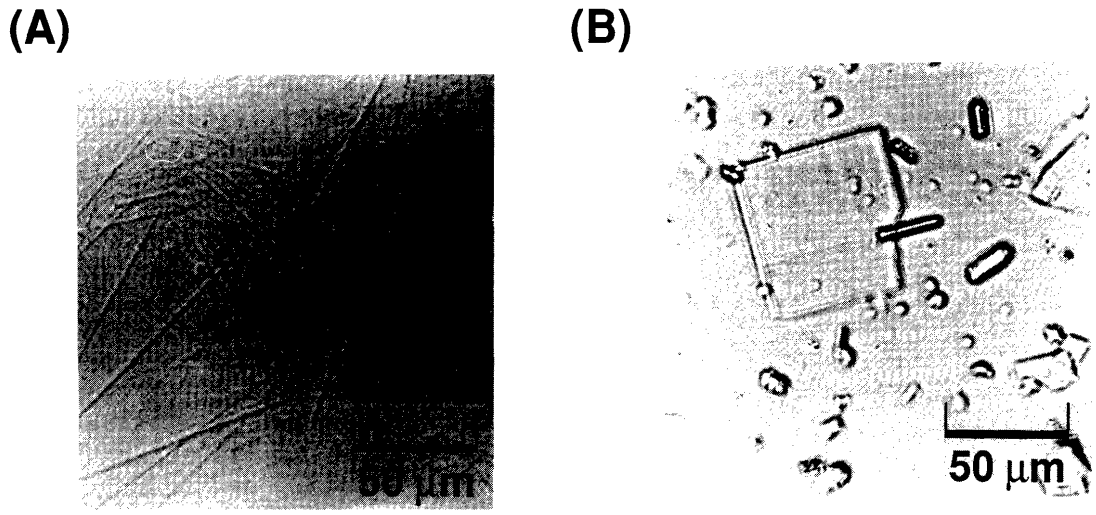

Fig. 1. Photomicrographs of Crystalline MALs.
(A): MAL from *Citrobacter* sp. strain YG-0504. (B): MAL from *Morganella morganii* strain YG-0601.

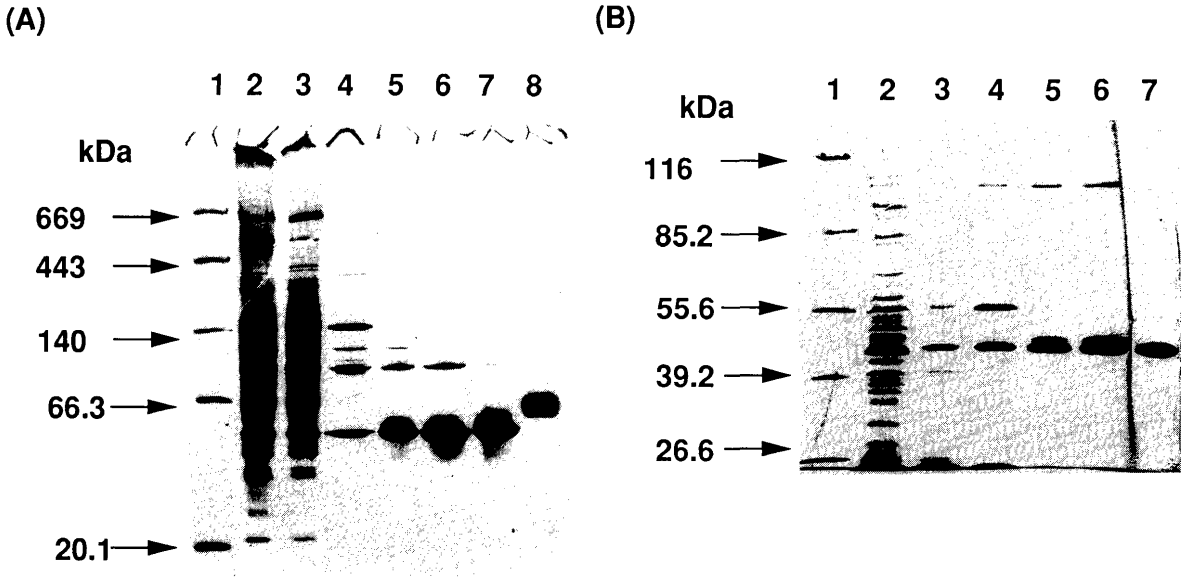

Fig. 2. Polyacrylamide Slab Gel Electrophoresis (PAGE) of the Purified Enzyme from *Citrobacter* sp. Strain YG-0504.
Lane 1, marker proteins (size shown at right in kDa); lane 2, cell-free extract; lane 3, ammonium sulfate fraction; lane 4, DEAE-Toyopearl fractions; lane 5, Butyl-Toyopearl fractions; lane 6, Superdex-200 fractions; lane 7, crystalline enzyme; lane 8, the cross-linked enzyme (10 μg). (A) PAGE in the absence of SDS. (B) PAGE in the presence of 0.1% SDS. To cross-link the enzyme, crystalline enzyme (150 μg) was mixed with 36 μmol of Tris–HCl buffer (pH 8.2) and 1 mg of dimethylsuberimidate hydrochloride in a total volume of 250 μl and incubated at room temperature for 12 h.

extract. The purified enzyme also could be crystallized by the addition of solid ammonium sulfate, and isolated in plate form. A photomicrograph of the crystals is presented in Fig. 1 (B).

Purity of the enzyme

Both enzymes were judged to be homogeneous by the criteria of native- and SDS–PAGE, and HPLC with a TSK G-3000 SW gel filtration column, as all of the results gave a single bands or a single peak. Figures 2 and 3 show the results of PAGE of the *Citrobacter* and *Morganella* enzymes, respectively, in the absence (A) and presence (B) of SDS. The *Citrobacter* enzyme was unstable under native-PAGE conditions and decomposed during electrophoresis (lane 7 of Fig. 2 (A)). The enzyme showed a single band on native-PAGE after a treatment with dimethylsuberimidate, which is a specific cross-linking reagent between protomers of a protein[17] (lane 8 of Fig. 2 (A)). On the other hand, the *Morganella* enzyme was stable during native PAGE and gave a single band.

Molecular weight and subunit structure

The molecular weights of the *Citrobacter* enzyme and *Morganella* one were calculated to be about 79,000 and 70,000 by gel filtration on HPLC, respectively, and their subunit molecular weights were estimated to be about 40,000 and 44,000, respectively, by comparing the mobility on SDS–PAGE to that of standard proteins. Both enzymes might consist of two subunits with an identical molecular weight.

N-Terminal amino acid sequences

The NH_2-terminal 26 amino acid sequences of the *Citrobacter* enzyme and *Morganella* one were MKIKQALF-

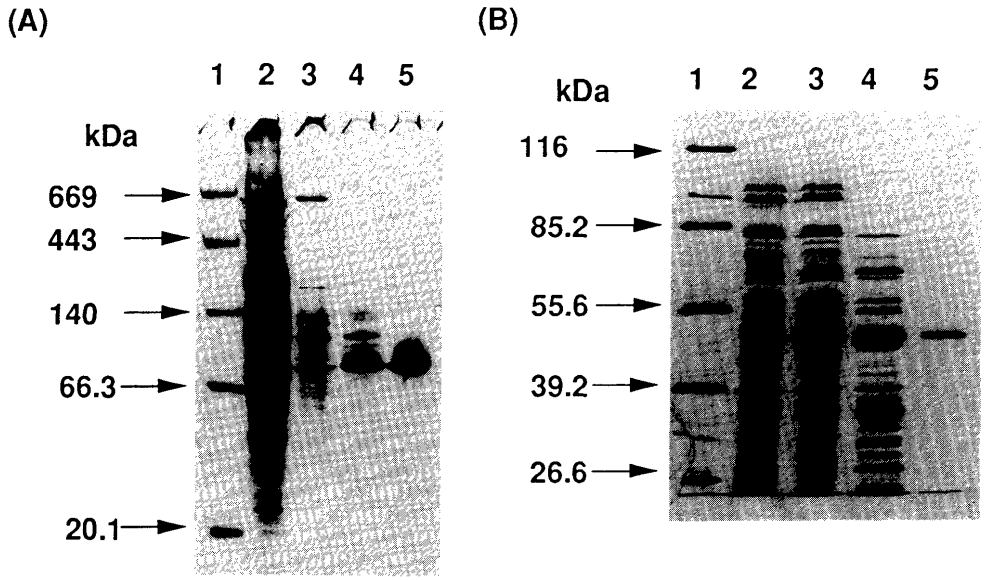

Fig. 3. Polyacrylamide Slab Gel Electrophoresis of the Purified Enzyme from *Morganella morganii* Strain YG-0601.

Lane 1, marker proteins (size shown at right in kDa); lane 2, cell-free extract; lane 3, ammonium sulfate fraction; lane 4, DEAE-Toyopearl fractions; lane 5, Butyl-Toyopearl fractions. (A) PAGE in the absence of SDS. (B) PAGE in the presence of 1% SDS.

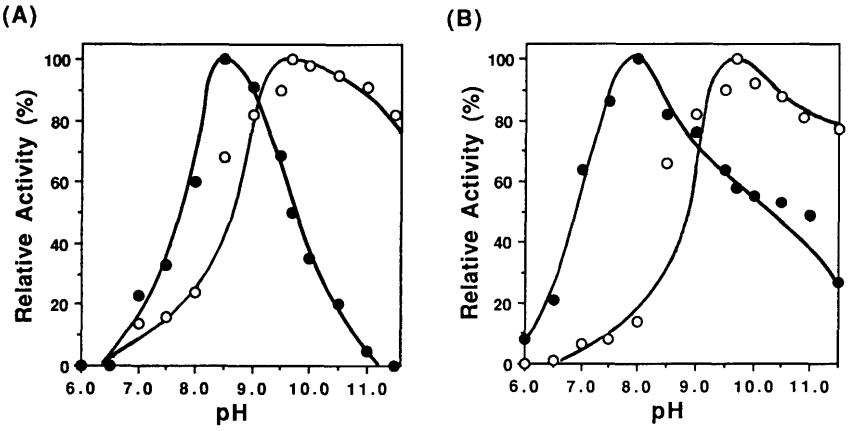

Fig. 4. Effects of pH on Enzyme Activity.

The enzyme activities in the deamination reaction of (2*S*,3*S*)-3-methylaspartic acid (○) and in the amination reaction of mesaconic acid (●) were measured at different pHs with 50 mM sodium acetate buffer (pH 3.5–6.0), potassium phosphate buffer (pH 6.5–8.5), MOPS–NaOH buffer (pH 7.0–7.5), HEPES–NaOH buffer (7.5–8.0), Tris–HCl buffer (pH 7.5–9.0), ethanolamine–HCl buffer (pH 8.5–11.0), glycine–NaCl–NaOH buffer (pH 8.5–11.5). (A): *Citrobacter* enzyme. (B): The *Morganella* one.

TAGYSSFYFDDQQAIKDG and MKIKQALFTAGNSSFYFDDQQAIKDG, respectively.

Effects of pH and temperature on enzyme activity

The enzyme activities were measured at various pHs from 3.5 to 11.5 in several buffer. As shown in Fig. 4, maximal activity in the deamination reaction of (2*S*,3*S*)-3-methylaspartic acid was seen for both enzymes at pH 9.7 in ethanolamine–HCl buffer, although those in the amination reaction were slightly different. The *Citrobacter* enzyme and *Morganella* one had their maximum activity at pH 8.5 and pH 8.0 in Tris–HCl buffer in the amination reaction of mesaconic acid, respectively. The enzyme activities were measured at various temperatures from 5 to 70°C in the deamination reaction of (2*S*,3*S*)-3-methylaspartic acid. As shown in Fig. 5, the *Citrobacter* enzyme and *Morganella* one had their maximum activity at approximately 50°C and 45°C, respectively.

Effects of pH and temperature on the stability of the enzyme

The remaining activities of the enzymes were measured after an incubation for 10 min at 30°C in several buffer at various pHs (final concentration of 50 mM). The *Citrobacter* enzyme and *Morganella* one were stable from pH 5.5 to 10.0 and from 6.0 to 8.5, respectively. Both enzymes were inactivated rapidly below pH 5.0. More than 95% of the activities remained when both enzymes were incubated at 45°C in 0.1 M potassium phosphate buffer (pH 7.0) for 10 min; about 50% of the activities remained at 50°C and most of the enzyme activities were lost at 55°C.

Effects of various compounds on the enzyme activity

As shown in Table II, the enzyme activities in the deamination reaction of (2*S*,3*S*)-3-methylaspartic acid were measured after the enzymes were incubated at 30°C for 30 min with various compounds. Both enzymes were strongly inhibited by heavy metal ions such as Hg^+, Hg^{2+}, and Ag^+, and sulfhydryl reagents such as *p*-chloromer-

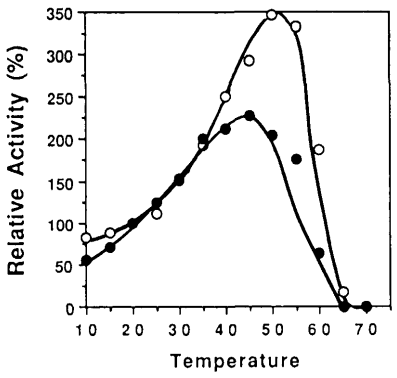

Fig. 5. Effects of Temperature on the Deamination Reaction of Both Enzymes.

The enzyme activities were measured at various temperatures in the deamination reaction of (2*S*,3*S*)-3-methylaspartic acid. Activities of *Citrobacter* MAL (○) and *Morganella* one (●) are expressed relative to those at 20°C.

curibenzoic acid (*p*CMB) and *N*-ethylmaleimide (NEM). Some metal ions such as Ca^{2+}, Fe^{2+}, Fe^{3+}, and Cu^{2+}, and chelating reagents such as 8-hydroxyquinoline also inhibitited both enzymes. Iodoacetic acid and some metal ions such as Co^{2+}, Cd^{2+}, Al^{3+}, Sn^{2+}, and Pb^{2+} inhibited the *Morganella* enzyme but did not inhibit the *Citrobacter* one. 5,5′-Dithiobis(2-nitrobenzoic acid) (DNBA) inhibited the *Citrobacter* enzyme strongly but *Morganella* weakly. No inhibition occurred for both enzymes in the pesence of 10 mM of Mg^{2+}, Ba^{2+}, Cr^{3+}, serine reagents, or carbonyl reagents.

Cation requirements

The enzymes required both divalent and monovalent cations such as Mg^{2+} and K^+, respectively, for their activity. Both enzymes completely lost their activities and were converted to the apoenzymes by dialysis against 1 mM Tris–HCl buffer (pH 7.5) containing 5 mM EDTA for 3 h. The apoenzymes were reactivated by adding divalent and monovalent cations. The enzyme activities were measured under the standard assay conditions in the deamination reaction of (2*S*,3*S*)-3-methylaspartic acid, in which Mg^{2+} was replaced by several divalent cations. Both enzymes required Mg^{2+} as the most desirable divalent cation. The relative activities of the *Citrobacter* enzyme in the presence of 1 mM Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, and Ni^{2+} were estimated to be 100, 26, 22, 18, and 16%, respectively, while those of the *Morganella* one were 100, 45, 25, 27, and 3%, respectively. The apparent K_ms of the *Citrobacter* enzyme and *Morganella* one for Mg^{2+} were calculated to be 3.0×10^{-5} M and 4.2×10^{-5} M, respectively. The enzyme activities were measured under the standard assay conditions in the deamination reaction of (2*S*,3*S*)-3-methylaspartic acid, in which K^+ was replaced with several monovalent cations. Among several monovalent cations, K^+ was the most effective, but several alkaline metal cations were also active in order of decreasing effectiveness. The relative activities of the *Citrobacter* enzyme in the presence of 10 mM Li^+, Na^+, K^+, Rb^+, and Cs^+ were estimated to be 32, 11, 100, 37, and 6%, respectively, while those of *Morganella* one were 33, 6, 100, 25, and 4%, respectively. The K_ms of the *Citrobacter* enzyme and *Morganella* one for K^+ were 2.9×10^{-3} M and 4.4×10^{-3} M, respectively.

Table II. Effects of Various Compounds

Compounds[a]	Concn. (mM)	Relative Activity (%)	
		Citrobacter MAL	*Morganella* MAL
None		100	100
$MgSO_4$	10	100	124
$AlCl_3$	5	100	5
$CaCl_2$	5	17	26
$CrCl_3$	10	111	92
$MnSO_4$	5	83	70
$FeSO_4$	5	44	11
$FeCl_3$	1	25	31
$CoCl_2$	1	100	40
$NiSO_4$	5	94	100
$CuSO_4$	1	33	27
$ZnSO_4$	5	83	81
AgCl	1	0	0
$CdCl_2$	1	100	0
$SnCl_2$	1	100	49
$BaCl_2$	10	100	100
$PbCl_2$	5	100	12
HgCl	1	0	0
$HgCl_2$	1	0	0
o-Phenanthroline	5	100	115
α,α′-Bipyridyl	5	106	118
8-Hydroxyquinoline	5	56	12
EDTA	5	117	130
DNBA	1	0	46
*p*CMB	1	0	0
Iodoacetic acid	1	100	14
NEM	1	0	0
PMSF	10	106	122
KCN	10	100	98
NH_2OH	10	98	118
D-Penicillamine	10	106	153
NaN_3	10	94	110
D-Cycloserine	10	10	138

The enzyme activity in the deamination reaction of (2*S*,3*S*)-3-methylaspartic acid was measured after the enzyme was incubated at 30°C for 10 min with various compounds in 0.1 M potassium phosphate buffer (pH 7.0).

Abbreviations: DNBA, dithiobis (2-nitrobenzoic acid); *p*CMB, *p*-chloromercuribenzoic acid; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride.

Substrate specificity and kinetic properties

The enzyme activities were measured in the deamination and in the amination reactions with various amino acids and conjugated carboxylic acids, respectively. Both enzymes deaminated 3-substituted (*S*)-aspartic acids such as (2*S*,3*S*)-3-methylaspartic, (2*S*,3*S*)-3-ethylaspartic, and (2*R*,3*S*)-3-chloroaspartic acids but not (*S*)-aspartic acid. Both enzymes did not act on other amino acids such as (*S*)-phenylalanine and (*S*)-histidine which are substrates of phenylalanine and histidine ammonia-lyase,[18] respectively. (*S*)-Glutamic acid, which induced the enzyme activity as the strains,[8] and (*R*)-amino acids such as (*R*)-aspartic acid, (*R*)-glutamic acid, (*R*)-phenylalanine, (*R*)-alanine, and (*R*)-valine were also inert for the substrates in the deamination reaction. Both enzymes catalyzed the amination reaction of several *E*-conjugated dicarboxylic acids such as fumaric, mesaconic, ethylfumaric, and chlorofumaric acids. *Z*-Conjugated ones such as maleic and citraconic acids, and other conjugated mono-carboxylic acids such as

Table III. Kinetic Constants of MALs for the Deamination and Amination Reactions

Substrate	MAL from *Citrobacter*			MAL from *Morganella*		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/K_m)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/K_m)
(2*S*, 3*S*)-3-Methylaspartic acid	1.00	233	233	2.95	426	144
(2*S*, 3*S*)-3-Ethylaspartic acid	6.16	313	50.8	12.7	252	19.8
(2*R*, 3*S*)-3-Chloroaspartic acid	23.7	40.0	1.69	35.7	34.6	0.969
Mesaconic acid	0.611	42.3	69.2	0.600	86.7	145
Ethylfumaric acid	2.07	89.6	43.3	1.96	64.1	32.7
Chlorofumaric acid	3.93	68.4	17.4	3.80	44.1	11.6
Fumaric acid	7.33	94.2	12.9	2.50	22.8	9.12
NH_4Cl	60.7	35.6	0.586	50.0	31.9	0.638

cinnamic and 4-methoxycinnamic acids did not act as substrates in the amination reaction for both enzymes.

Kinetic studies were done to calculate the Michaelis constants (K_m), the catalytic center activities (k_{cat}), and k_{cat}/K_m values for several 3-substituted (*S*)-aspartic acids and their corresponding fumaric acid derivatives from double-reciprocal plots. Table III shows the kinetic constants of the enzymes for several substrates. The K_ms of both enzymes for (2*S*,3*S*)-3-methylaspartic acid were lower than those for all the other compounds. The k_{cat} of the *Morganella* enzyme for (2*S*,3*S*)-3-methylaspartic acid was higher than those for the others, although, that of *Citrobacter* one for (2*S*,3*S*)-3-methylaspartic acid was lower than that for (2*S*,3*S*)-3-ethylaspartic acid. The K_ms of both enzymes for mesaconic acid were lower than those for the others. The k_{cat} of the *Morganella* enzyme for mesaconic acid was higher than those for the others, although that of *Citrobacter* one for mesaconic acid was lower than that for the others. The K_ms of both enzymes in the amination reaction seems to be lower than those in the deamination reaction.

Discussion

In this report, MALs from the two facultative anaerobes, *Citrobacter* sp. strain YG-0504 and *Morganella morganii* strain YG-0601, have been purified in their crystalline form from cell-free extracts and characterized. The properties of both enzymes and known MAL of *Clostridium tetanomorphum* H1 are summarized in Table IV. The molecular weights of the *Citrobacter* enzyme and *Morganella* one were 79,000 and 70,000, and those of the subunits were about 40,000 and 44,000, respectively. Both enzymes consist of two identical subunits. These values are similar to that of *Clostridium* MAL, which is a dimeric enzyme with a molecular weight of the subunit of 45,539.[7] The specific activity of the purified *Morganella* enzyme was 296 (units/mg protein) and is similar to that of *Clostridium* MAL, which is 280 (units/mg),[4] while that of the *Citrobacter* enzyme is about one-half those of the *Morganella* enzyme and *Clostridium* MAL. Both enzymes and *Clostridium* MAL[2] had the same optimum pH in the deamination reaction of (2*S*,3*S*)-3-methylaspartic acid, but their optimum pHs in the amination reaction of mesaconic acid and optimum temperatures were different. The

Table IV. Comparison of MALs Purified from *Citrobacter* sp. Strain YG-0504 and *Morganella morganii* Strain YG-0601, and *Clostridium tetanomorphum* H1

Property	Value with enzyme from *Citrobacter* YG-0504	*Morganella* YG-0601	*Clostridium*[b] *tetanomorphum*
Specific activity of final preparation (units/mg)	162	296	280
Molecular weight			
Native	79,000	70,000	91,078
Subunit	40,000	44,000	45,539
Number of subunits	2	2	2
pH optimum			
Deamination	9.7	9.7	9.7
Amination	8.0	8.5	9.0
Temperature optimum (°C)	50	45	55
K_m (mM) value for[a]			
MeAsp	1.00	2.95	2.37
ClAsp	23.7	35.7	No react
Asp	No react	No react	10.5
Mesaconic acid	0.611	0.600	1.24
Mg^{2+}	0.030	0.042	0.12
K^+	2.89	4.39	3.00
NH_4Cl	60.7	50.0	75.0

[a] *Abbreviations*: MeAsp, (2*S*, 3*S*)-3-methylaspartic acid; ClAsp, (2*R*, 3*S*)-3-chloroaspartic acid; Asp, (*S*)-aspartic acid.
[b] Values reported previously.[4,6,7]

Citrobacter enzyme was more stable than the *Morganella* one with respect to pH. Both enzymes might contain a metal ion and an active cysteine residue near the active site of them, because both enzymes were strongly inhibited by sulfhydryl reagents and heavy metal ions, and were partially inhibited by some chelating reagents and divalent cations. The susceptibility of the enzymes against several compounds were different with each other. Several cations such as Fe^{2+}, Co^{2+}, Sn^{2+}, Pb^{2+}, and Al^{3+}, and iodoacetic acid more intensively inhibited the *Morganella* enzyme than the *Citrobacter* one, while dithiobis(2-nitrobenzoic acid) (DNBA) more weakly inhibited the former enzyme than the latter one. *Clostridium* MAL is also inhibited by heavy metal ions and SH-reagents.[2,4] Both divalent and monovalent cations were required for the activities of both

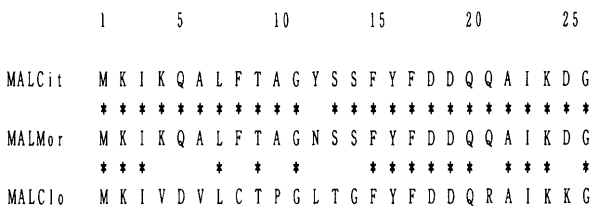

Fig. 6. Comparison of the N-Terminal Amino Acid Sequences of the *Citrobacter* Enzyme (MALCit), *Morganella* One (MALMor), and *Clostridium* MAL (MALClo).

Symbols: (*) identical residue.

enzymes. Their requirements were similar to those of *Clostridium* MAL.[4] Table III suggests that both enzymes most effectively catalyze the reversible amination-deamination reactions between mesaconic acid and (2*S*,3*S*)-3-methylaspartic acid. Both enzymes prefer the amination reaction than the deamination one because the K_ms of both enzymes for mesaconic acid was lower than those for (2*S*,3*S*)-3-methylaspartic acid. *Clostridium* MAL showed the same tendency because the K_ms of the enzyme for mesaconic acid and (2*S*,3*S*)-3-methylaspartic acid were calculated to be 1.24 mM and 2.37 mM, respectively.[6] This phenomenon is also seen in aspartase, which bind to fumaric acid more tightly than (*S*)-aspartic acid.[19,20] Both enzymes and *Clostridium* MAL are different in substrate specificities. The *Citrobacter* and *Morganella* enzymes catalyze the reversible amination-deamination reaction between (2*R*,3*S*)-3-chloroaspartic acid and chlorofumaric acid, but *Clostridium* MAL catalyzes only the amination reaction of chlorofumaric acid (K_m 3.52 mM).[6] On the other hand, *Clostridium* MAL catalyzes the reversible reaction between (*S*)-aspartic acid (K_m 10.5 mM) and fumaric acid (K_m 23 mM),[6] but the *Citrobacter* and *Morganella* enzymes catalyze only the amination reaction of fumaric acid.

Figure 6 shows the comparison of the N-terminal sequences between the *Citrobacter* enzyme, the *Morganella* one, and *Clostridium* MAL.[7] The N-terminal 26 amino acid sequences of the *Citrobacter* and *Morganella* enzymes are identical except for the twelfth residue, those of the former and the latter are tyrosine and asparagine, respectively. The N-terminal sequences of both enzymes are very similar with those of *Clostridium* MAL and have 63% identity with them. It is reported that some lyases such as fumarase and aspartase which catalyzes analogous reaction of MAL involving hydration and amination reaction of fumaric acid,[18] respectively, have close homologies in their primary structures.[21] However, both MALs showed no homology at the N-terminal sequence with the lyases such as fumarases from *Escherichia coli* and *Bacillus subtilis*, and aspartases from *E. coli* and *Pseudomonas fluorescens*.[21]

In this study, it was found that the *Citrobacter* and *Morganella* enzymes are similar in their molecular weight, subunit structure, N-terminal amino acid sequences, and some properties. However, they are different in their specific activities, stabilities, and inhibition by some compounds. We also showed here that the enzymes from Gram-negative facultative anaerobes from the family of *Enterobacteriaceae*, were similar to MAL of a Gram-positive obligate anaerobe, *C. tetanomorphum*, in their enzymological properties and N-terminal amino acid sequences, but not substrate specificities.

References

1) M. J. van der Werf, W. J. J. van den Tweel, J. Kamphuis, S. Hartmans, and J. A. M. de Bont, *Trends in Biotechnol.*, **12**, 95–103 (1994).
2) H. A. Barker, R. D. Smith, R. M. Wilson, and H. Weissbach, *J. Biol. Chem.*, **234**, 320–328 (1959).
3) H. J. Bright and L. L. Ingraham, *Biochim. Biophys. Acta*, **44**, 586–588 (1960).
4) H. A. Barker, R. D. Smyth, H. J. Bright, and L. L. Ingraham, *Methods in Enzymol.*, **5**, 827–832 (1961).
5) M. W. Hsiang and H. J. Bright, *Methods in Enzymol.*, **13**, 347–353 (1969).
6) N. P. Botting, M. A. Cohen, M. Akhtar, and D. Gani, *Biochemistry*, **27**, 2956–2959 (1988).
7) S. K. Goda, N. P. Minton, N. P. Botting, and D. Gani, *Biochemistry*, **31**, 10747–10756 (1992).
8) Y. Asano and Y. Kato, *Biosci. Biotech. Biochem.*, **58**, 223–224 (1994).
9) N. H. Perkin, *J. Chem. Soc.*, **53**, 695–713 (1888).
10) P. Walden, *Chem. Ber.*, **24**, 2035 (1891).
11) E. Demercay, *Ann. Chem.*, **20**, 433–502 (1880).
12) M. Akhtar, N. P. Botting, M. A. Cohen, and D. Gani, *Tetrahedron*, **43**, 5899–5908 (1987).
13) N. R. Krieg and J. G. Holt, "Bergey's Manual of Systemtic Bacteriology," Vol. 1, Williams & Wilkins, Baltimore, London, 1986.
14) M. M. Bradford, *Anal. Biochem.*, **72**, 248–254 (1976).
15) B. J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, Art2, 404 (1964).
16) U. K. Laemmli, *Nature*, **227**, 680–685 (1970).
17) G. E. Davis and G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.*, **66**, 651 (1970).
18) K. R. Hanson and E. A. Havir, in "Enzymes," 3rd Ed., Vol. 7, Academic Press, New Yor, 1972, pp. 75–166.
19) I. I. Nuiry, J. D. Hermes, P. M. Weiss, C.-Y. Chen, and P. F. Look, *Biochemistry*, **23**, 5168–5175 (1984).
20) F. B. Rudolph and H. J. Fromm, *Arch. Biochem. Biophys.*, **147**, 92–98 (1971).
21) J. S. Takagi, M. Tokushige, Y. Shimura, and M. Kanehisa, *Biochem. Biophys. Res. Commun.*, **138**, 568–572 (1986).