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Crystalline 3-methylaspartase from a facultative anaerobe, *Escherichia coli* strain YG1002

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Abstract: Crystalline 3-methylaspartase (EC 4.3.1.2) from *Escherichia coli* strain YG1002 that had been isolated from soil was characterized. The enzyme activity was induced when the organism was grown statically on medium containing (*S*)-glutamic acid. Its molecular mass is about 84 kDa, and it may be composed of two identical subunits of 42 kDa. The enzyme requires both divalent and monovalent cations such as Mg^{2+} and K^{+}, respectively. The enzyme catalyzes reversible amination–deamination between mesaconic acid and (2*S*,3*S*)-methylaspartic acid, which is the best substrate.

Key words: 3-Methylaspartase; *Escherichia coli*

Introduction

The enzyme 3-methylaspartase (3-methylaspartate ammonia-lyase (MAL), EC 4.3.1.2) is an enzyme which catalyzes reversible amination–deamination between several 3-substituted (*S*)-aspartic acid and corresponding fumaric acid derivatives. The enzyme activity was first detected in a cell-free extract of the obligate anaerobic bacterium *Clostridium tetanomorphum* H1 by Barker et al. [1]. Mechanistic studies of the enzyme [2–8] and the nucleotide sequence of the MAL gene [9] have been reported. The enzyme is believed to be distributed very narrowly in obligate anaerobic microorganisms [10–12].

To study enzyme-catalyzed organic synthesis, we required an ammonia lyase that acted on a wide range of aspartic acid derivatives. Recently, we discovered that MAL activities are also distributed among facultative anaerobic bacteria [13]. This communication describes the first preparation, crystallization and some properties of the enzyme from *Escherichia coli* strain YG 1002, an isolate from soil that has the enzyme activity.

Materials and Methods

Materials

All chemicals were purchased from commercial sources and used without further purification. DEAE-Toyopearl 650 M, Butyl-Toyopearl 650 M, and HPLC columns G-3000 SW were purchased from Tosoh Corp. (Japan).

Synthesis of substrates and authentic samples

Chlorofumaric acid and ethylfumaric acid were synthesized according to the method of Perkin

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[14] and Walden [15], respectively. The compounds (2*S*,3*S*)-3-methylaspartic acid, (2*S*,3*S*)-3-ethylaspartic acid, and (2*R*,3*S*)-3-chloroaspartic acid were enzymatically synthesized using a cell-free extract of *Clostridium tetanomorphum* H1 NCIMB 11547 described by Akhtar et al. [16].

Microorganisms, media and cultivation

We isolated *Escherichia coli* strain YG 1002 from soil [13] and cultivated it in a 2-l Sakaguchi flask containing 2 l of a medium consisting of 10 g yeast extract, 80 mmol potassium phosphate buffer (pH 7.4), 27 g monosodium (*S*)-glutamate, 0.5 g $MgSO_4$, 22 mg $FeSO_4$, 4.8 mg $MnSO_4$, 4.8 mg Na_2MoO_4 and 30 mg $CaCl_2$ at 37°C for 40 h without shaking.

Enzyme assays

MAL activity for deamination was assayed at 20°C by measuring the rate of the formation of fumaric acid derivatives at 240 nm from the corresponding 3-substituted aspartic acid derivatives using the appropriate extinction coefficients according to the method of Botting et al. [6]. The reaction mixture contained 50 μmol of ethanolamine · HCl buffer (pH 9.7), 10 μmol of KCl, 1 μmol of $MgCl_2$, 5 μmol of monosodium salt of aspartic acid derivatives and enzyme solution in a total volume of 1.0 ml. The enzyme activity for amination was measured at 20°C by the disappearance of fumaric acid derivatives at 240 nm in a reaction mixture consisting of 200 μmol of Tris · HCl buffer (pH 8.5), 1 μmol of $MgCl_2$, 0.5 μmol of sodium salt of fumaric acid derivatives and enzyme solution in a total volume of 1.0 ml. 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 deamination. Protein was assayed by the method of Bradford et al. [17] using a dye reagent concentrate (Bio-Rad Laboratories) with bovine serum albumin as the standard or by measuring the absorbance at 280 nm.

Other methods

Polyacrylamide slab gel electrophoresis (PAGE) in the absence and presence of SDS were carried out according to the method by Davis [18] and Laemmli [19], respectively. The molecular mass was determined by gel-filtration with HPLC using a TSK G-3000 SW column.

Results and Discussion

Purification of MAL

Washed cells (64 g, wet weight) from 40 l of culture were suspended in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.5 mM 2-mercaptoethanol (250 ml), and disrupted for 20 min by an ultrasonic oscillator (19 kHz, 10 min, 4°C). All the subsequent purification procedures were carried out at 4°C. After the cell debris was removed by centrifugation (14000 × *g*, 25 min), the supernatant was fractionated with ammonium sulfate (30–50%), followed by dialysis against 0.01 M potassium phosphate buffer (pH 7.0). The dialyzate was applied to a DEAE-Toyopearl (Tosoh Corp., Japan) 650 M column (6 × 18 cm) equilibrated with 0.01 M buffer. After washing with 0.01 M buffer, the enzyme was eluted with 0.1 M buffer. Active fractions were combined and dialyzed against 0.01 M buffer. The active solution was loaded onto a Butyl-Toyopearl (Tosoh Corp., Japan) 650M column that was equilibrated with 0.01 M buffer 25% saturated with ammonium sulfate, and eluted with a linear gradient of ammonium sulfate (25–15% saturation). The active fractions were dialyzed and concentrated by ultrafiltration. Solid ammonium sulfate was added to the enzyme solution to 30% saturation, then more ammonium sulfate was added until the solution became faintly turbid. After standing for a few weeks, crystals in a plate form appeared (Fig. 1). Table 1 summarizes a typical purification procedure. The enzyme migrated as a single band on native- and SDS-PAGE (Fig. 2), and on HPLC with a TSK G-3000 SW column. The specific activity of the enzyme was 179 (U (mg protein)$^{-1}$). This value was increased to 282 (U mg^{-1}) by incubating the enzyme with 5 mM EDTA in 5 mM potassium phosphate buffer (pH 7.0) for 3 h. This increase in specific activity was attributed to contamination of the enzyme by divalent metal ions during the purification.

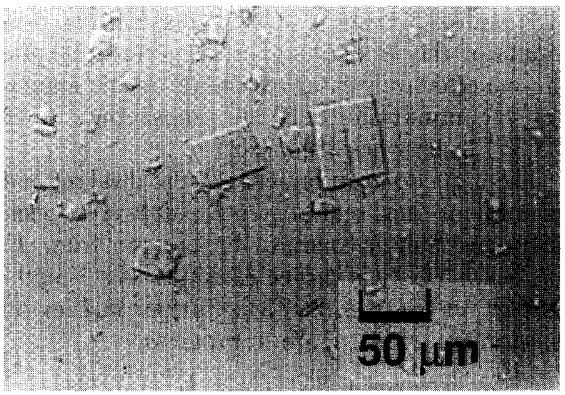

Fig. 1. Photomicrograph of crystalline MAL from *E. coli* strain YG 1002.

Table 1
Summary of purification of MAL from *E. coli* strain YG 1002

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)
Cell-free extract	5900	43600	7.40	100
Ammonium sulfate (30–50%)	2500	34000	13.6	78
DEAE-Toyopearl	260	27000	104 [a]	62
Butyl Toyopearl	61	11000	179 [a]	25

[a] This value can be increased to 282 (U mg^{-1}) by 3-h treatment with 5 mM EDTA in 5 mM potassium phosphate buffer, pH 7.0.

Properties of the enzyme

The molecular mass of the native enzyme was estimated to be 84 kDa by G-3000 SW gel filtration on HPLC under the conditions previously described [20]. When the enzyme was treated with SDS and 2-mercaptoethanol, the molecular mass of subunit was about 42 kDa according to SDS-PAGE. The native enzyme probably consists of two identical subunits. The enzyme showed an absorption peak at 281 nm.

A divalent cation is required for the activity of the enzyme. The holoenzyme was completely converted to the apoenzyme by dialysis in 1 mM buffer containing 5 mM EDTA for 3 h. The apoenzyme was reactivated by adding divalent

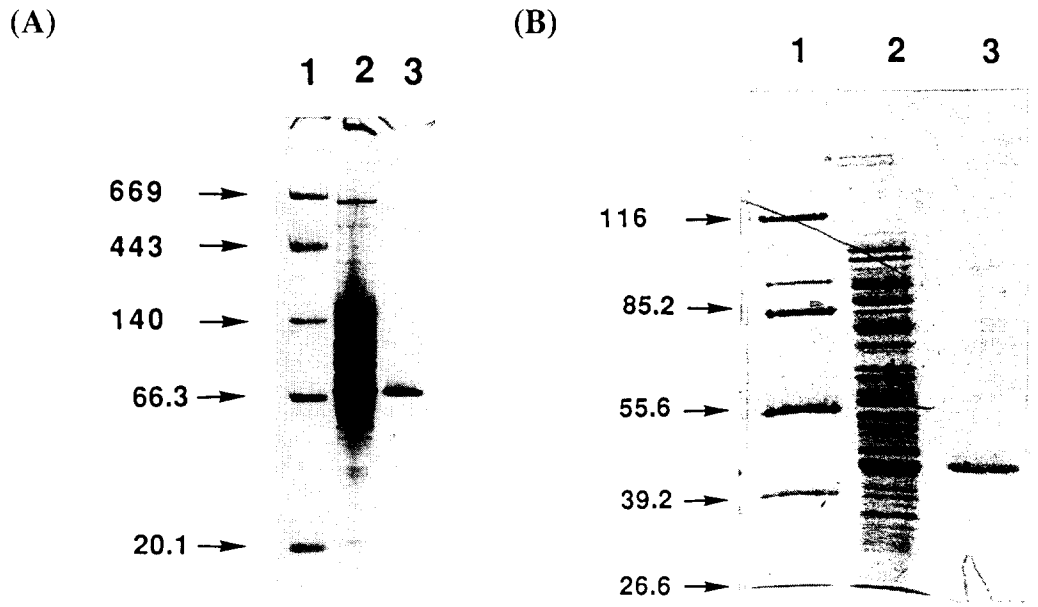

Fig. 2. Polyacrylamide slab gel electrophoresis of the purified enzyme from *E. coli* strain YG 1002. Lane 1 molecular size markers (size shown at the right in kDa); lane 2, crude enzyme after DEAE-Toyopearl column chromatography; lane 3, purified enzyme. Protein bands were stained with Coomassie brilliant blue R-250. **(A)** The enzymes were electrophoresed in the absence of SDS. **(B)** The enzymes were incubated in the presence of 1% SDS and 3% 2-mercaptoethanol at 105°C for 5 min, then electrophoresed in the presence of 0.1% SDS.

cations. The divalent cation requirement was as follows: $Mg^{2+} \gg Mn^{2+} > Co^{2+} > Zn^{2+} > Ni^{2+}$. A monovalent cation is also required for the enzyme. The monovalent cation requirement was estimated as follows; $K^+ > Rb^+ > Li^+ > Na^+ > Cs^+$. These metal ion requirements of the enzyme are similar to those of MAL from *C. tetanomorphum* [21,22]. The apparent K_m values for Mg^{2+} and K^+ of the enzyme were 8.1×10^{-5} M and 3.6×10^{-3} M, respectively, while those of *C. tetanomorphum* MAL are reported to be 1.2×10^{-4} M and 3.0×10^{-3} M, respectively [21,22].

The enzyme catalyzed reversible amination–deamination between several 3-substituted (*S*)-aspartic acids and corresponding fumaric acid derivatives. The enzyme catalyzed the deamination of (2*S*,3*S*)-3-methylaspartic acid, (2*S*,3*S*)-3-ethylaspartic acid, and (2*R*,3*S*)-3-chloroaspartic acid. In the reverse reaction, the enzyme catalyzed the amination of mesaconic acid, ethylfumaric acid, chlorofumaric acid, and fumaric acid. The enzyme could not catalyze the deamination of (*S*)-aspartic acid which was deaminated by a clostridial enzyme [6], but catalyzed the amination of fumaric acid. The product of the amination reaction was isolated and identified as (*S*)-aspartic acid. The enzyme did not catalyze the deamination of other amino acids such as (*S*)-phenylalanine, and (*S*)-histidine, which are phenylalanine and histidine ammonia lyase substrates, respectively [23]. (*S*)-Glutamic acid, which induced the enzyme activity for the strain [13], and (*R*)-amino acids were also inert as substrates. Z-Unsaturated dicarboxylic acids, such as maleic and citraconic acids, and other conjugated carboxylic acids, such as cinnamic and 4-methoxycinnamic acids, did not act as a substrate for the enzyme. The apparent K_m values for the deamination of (2*S*,3*S*)-3-methylaspartic, (2*S*,3*S*)-3-ethylaspartic and (2*R*,3*S*)-3-chloroaspartic acids were calculated to be 0.76, 5.3 and 7.9 mM, respectively, and the V_{max}/K_m values for those were 443, 51.9, and 14.9 U mg^{-1} mM^{-1}, respectively. In the amination reactions, the K_m values for mesaconic, ethylfumaric, chlorofumaric and fumaric acids were calculated to be 0.13, 0.69, 0.36 and 0.52 mM, respectively, and the V_{max}/K_m values for them were calculated to be 223.8, 108.1, 81.7, and 65.3 U mg^{-1} mM^{-1}, respectively. The results of the kinetic studies suggested that the enzyme effectively catalyzes the reversible amination–deamination between mesaconic and (2*S*,3*S*)-3-methylaspartic acid.

The induction conditions [13], the divalent and monovalent ion requirements, substrate specificity, and kinetic studies, the enzyme from *E. coli* strain YG 1002 can be classified as a 3-methylaspartase (EC 4.3.1.2) [23]. Further studies on the primary structure are in progress.

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