Characterization of Aspartate Aminotransferase Purified from *Streptomyces fradiae*

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Aspartate aminotransferase (ASAT) (L-aspartate:2-oxoglutarate, EC 2.6.1.1) from *Streptomyces fradiae* NRRL 2702 has been purified by acetone precipitation, DEAE-cellulose, hydroxyapatite, and preparative electrophoresis (Prep cell), of which the last was the most effective step in the purification of ASAT. The molecular mass was estimated to be 54,000 dalton by SDS-PAGE and 120,000 dalton by gel filtration chromatography. Preparative isoelectric focusing of purified ASAT resulted in one polypeptide band with a pI of 4.2, showing homogeneity and indicating that the enzyme is composed of two identical subunits. The enzyme was specific for L-aspartate as an amino donor; the K_m values were determined to be 2.7 mM for L-aspartate, 0.7 mM for 2-oxoglutarate, 12.8 mM for L-glutamate, and 0.15 mM for oxaloacetate. The enzyme was relatively heat-stable, having maximum activity at 55°C, and it had a broad pH optimum ranging from 5.5 to 8.0. The activity of the purified enzyme was not inhibited by ammonium ions. This paper reports the first purification and characterization of the aspartate aminotransferase from a species of *Streptomyces*.

KEY WORDS □ ***Streptomyces fradiae*, aspartate aminotransferase**

Aspartate aminotransferase (ASAT) is the best-known pyridoxal phosphate-dependent enzyme, playing a central role in nitrogen metabolism (5, 8, 9, 21). Two genetically distinct isoenzymes of ASAT occur in animal cells (cytosolic and mitochondrial ASAT), whereas only a single ASAT has been found in microorganisms (16, 18, 23, 28, 29). As described previously, cell growth is associated with high activities of ASAT and citrate synthase, while the biosynthesis of tylosin, a macrolide antibiotic produced by *Sterptomyces fradiae*, is not accompanied by the activities of the ASAT and citrate synthase but by the high activity of methylmalonyl-CoA carboxytransferase (11, 12, 14). Aspartate auxotroph (SMF 305) defective in ASAT is inferior to the wild strain (Asp$^+$) in both cell growth and biosynthesis of tylosin, whereas the revertant (SMF 306) is superior to the wild strain (15). These results indicated that the formation of aspartate from oxaloacetate by ASAT is essential for both cell growth and tylosin biosynthesis. Threonine dehydratase (TDT) was purified and was found to be a key enzyme involved in the repression of tylosin biosynthesis by ammonium ions (15, 20, 25). To compare ASAT with TDT related to tylosin biosynthesis, we describe the purification and characterization of ASAT from *S. fradiae*.

MATERIALS AND METHODS

Microorganisms and preservation

Streptomyces fradiae NRRL 2702 was grown aerobically in a seed culture medium consisting of (w/v) 2.0% glucose, 1.0% corn steep liquor, 0.5% yeast extract, and 0.3% $CaCO_3$. The pH was adjusted to 7.2 before sterilization and sterilized $CaCO_3$ was added aseptically after sterilization (22). Cells in the exponential growth phase of the batch culture were harvested aseptically, suspended in sterile 10% (v/v) glycerol in nutrient broth and then kept in a deep freezer controlled to −70°C.

Culture conditions

The frozen stock cells were thawed at the ambient temperature and were then used to inoculate the seed cultures which were cultured in baffled flasks at 30°C for 3 days. The seed cultures were inoculated to main cultures (5% of inoculum size, v/v), which were carried out in a fermentor (5 *l* and *in situ* steam sterilizable; Chemap CF). A synthetic medium consisting of (w/v) 2.0% soluble starch, 0.5% glucose, 0.27% sodium lactate, 0.05% $MgSO_4 \cdot 7H_2O$, 0.47% $(NH_4)_2SO_4$, 0.3% $CaCO_3$, and 0.0003% trace elements ($CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, and $MnCl_2 \cdot 4H_2O$) were used for the main

cultures (25). Solutions of $(NH_4)_2SO_4$ were sterilized separately and added before inoculation. The temperature was maintained at 30°C and the pH was adjusted to 7.0 before inoculation, while aeration was fixed to 1 volume per volume per min and agitation was controlled to give a dissolved oxygen tension above 40% (air saturation).

Enzyme and protein assays

Aspartate aminotransferase (ASAT) was assayed in both directions by modifications of coupled dehydrogenase reactions (24, 29). In the forward reaction, ASAT activity was coupled with 4 units of malate dehydrogenase with 50 mM L-aspartate and 5 mM 2-oxoglutarate as substrates for ASAT. In the reverse reaction, ASAT was coupled with 4 units of glutamate dehydrogenase with 50 mM L-glutamate and 5 mM oxaloacetate as substrates for ASAT and 3.1 mM NH_4Cl as a substrate for glutamate dehydrogenase. In both cases, the assay mixture contained 15 μM pyridoxal phosphate, 227 μM NADH, and 100 mM potassium phosphate buffer (pH 7.6). One unit was defined as the amount of ASAT required for the production of 1 μmole of NAD^+ in 1 min at 25°C, and protein concentrations were determined by the Bradford method (4).

Purification of aspartate aminotransferase

All purification steps were done at 4°C, while potassium phosphate buffer (pH 7.2) containing 20 μM pyridoxal phosphate and 0.01% 2-mercaptoethanol was used throughout the purification steps.

Cells were harvested from 5 *l* of culture broth by centrifugation (12,000×g for 10 min). They were washed twice with physiological saline solution, once with distilled water, and suspended in 20 mM of the above buffer. The cells were disrupted by sonication (100 W, 5 min; Model 9100, Lab-Line) at 2~5°C. The intact cells and cell debris were removed by centrifugation (24,000 ×g, 30 min) and the resulting supernatant was used as the cell-free extract.

Acetone (−20°C) was slowly added to the cell-free extract with gentle stirring to give a concentration of 45% (v/v). After gentle stirring for 30 min followed by centrifugation at 24,000×g for 30 min, the precipitate was discarded, and acetone (−20°C) was further added to the supernatant to give a final concentration of 65% (v/v). Again after gentle stirring for 30 min, the precipitate was collected by centrifugation at 24,000×g for 30 min and was suspended in 50 mM buffer. The suspension was used as the crude enzyme and concentrated by ultrafiltration (PM-10, 10,000 dalton cutoff; Amicon).

The concentrated solution was loaded onto a DEAE-cellulose column (2.7×40 cm) equilibrated with 50 mM buffer. The column was washed extensively with 4-bed volumes of the buffer, and elution was carried out with a linear gradient ranging from 0 to 0.7 M NaCl in 50 mM buffer.

The active fractions obtained from the ion-exchanger were concentrated and desalted by Centriprep-10 concentrator (10,000 dalton cutoff; Amicon) in 5 mM buffer. The desalted enzyme solution was then applied to a hydroxyapatite column (2.7×20 cm) equilibrated in 5 mM buffer. The hydroxyapatite column was washed with 4-bed volumes of 5 mM buffer before retained proteins were eluted with a phosphate gradient ranging from 5 mM to 300 mM potassium phosphate.

The active fractions obtained from the hydroxyapatite column were concentrated and desalted using a Centriprep-10 concentrator (10,000 dalton cutoff; Amicon). The desalted enzyme solution (1 m*l*) was made to 10% (v/v) in glycerol and loaded onto a pre-made gel (3.7× 11.4 cm) of Prep Cell apparatus (Bio-Rad). Preparative electrophoresis was carried out at 40 mA constant current (210~270 v) in a 10% (w/v) gel. The flow rate of the elution buffer (25 mM Tris/HCl buffer containing 192 mM glycine, pH 8.3) was 60 m*l* h^{-1} and the volume of each fraction was set at 2.5 m*l* and the running time was 9~10 h.

Characteristics of purified ASAT

SDS-PAGE and native PAGE were performed using the Laemmli method (13). The concentration of the running gel was 12% (w/v), whereas that of the stacking gel was 5% (w/v). After electrophoresis, the gel was silver-stained (19) in addition to staining with Commassie brilliant blue for protein (8) and with Fast blue BB salt for ASAT activity (1). The molecular mass markers obtained from Sigma were rabbit muscle phosphorylase *b* (97,400 dalton), bovine serum albumin (66,200 dalton), hen egg white ovalbumin (45,000 dalton), bovine carbonic anhydrase (31,000 dalton), and soybean trypsin inhibitor (21,500 dalton).

Molecular mass was determined at room temperature by high-performance liquid chromatography (HPLC) with a Waters HPLC system in the Protein-Pak™ 300 SW column at a flow rate of 1 m*l* min^{-1} and in an elution buffer consisting of 100 mM potassium phosphate buffer (pH 7.6). A calibration curve was made with the following proteins obtained from Pharmacia: ovalbumin (43,000 dalton), bovine serum albumin (67,000 dalton), aldolase (158,000 dalton), catalase, (232,000 dalton), and ferritin (440,000 dalton). The molecular mass of the subunit was estimated by 12% polyacrylamide gel electrophoresis containing 0.1% sodium dodecyl sulfate.

Isoelectric focusing was performed on a preparative rotating horizontal (Rotofor) ampholine column (Bio-Rad). The ampholine concentration was 2% and the pH gradient ranged from 4.0 to

6.5. The run lasted for 4~6 h at 12 W of constant power, and 2.5 ml fractions were collected and determined for their activity and pH.

The K_m values for L-aspartate, 2-oxoglutarate, L-glutamate, and oxaloacetate were determined at various concentrations ranging from 0.02 to 10 mM. For each K_m determination, one substrate concentration was varied while the other substrate concentrations were maintained near saturation. One unit of the purified ASAT was added to reaction mixture (3 ml) in the following buffers: 50 mM sodium acetate (pH 3.5~6.0), 50 mM potassium phosphate (pH 6.0~8.0), and 50 mM glycine/NaOH (pH 8.0~10.5). Enzyme activities at different pH values were compared after incubation at 25°C for 20 min and enzyme activities at different temperatures (10~90°C) were determined after incubation at pH 7.6 for 20 min.

Chemicals

NADH and all amino acids were purchased from Sigma. All other chemicals were of reagent grade.

RESULTS

Purification of ASAT from *S. fradiae*

The results of the purification procedure are shown in Table 1. Purification of the enzyme resulted in an approximately 600 fold enhancement of specific activity. All purification steps showed only one peak of enzyme activity, and only one protein band was observed when the final preparation of the enzyme was subjected to SDS-PAGE and the gel was silver-stained (Fig. 1). The purified enzyme had a specific activity of 91 U/mg, and when the purified ASAT was subjected to native polyacrylamide gels, only one band was observed by Commassie blue-staining (Fig. 2a) and activity staining (Fig. 2b).

Molecular mass and subunit structure

The molecular mass of the native enzyme was determined to be 120,000 dalton by gel filtration on the Protein-Pak™ 300 SW column. The subunit structure was examined by SDS-PAGE (0.1% sodium dodecyl sulfate), in which the denatured enzyme migrated as a single band of stained protein, and its molecular mass was determined to be about 54,000 dalton on the basis of its mobility relative to those of the standard proteins (Fig. 1).

Isoelectric point

When the purified fraction of ASAT was subjected to preparative isoelectric focusing (Rotofor), only one band was observed (Fig. 1), and the pI of the purified enzyme was measured to be 4.2 (Fig. 3).

K_m value and reaction mechanism

K_m values for the substrates L-aspartate, 2-oxoglutarate, L-glutamate, and oxaloacetate were individually determined (data not shown). The K_m values of ASAT are 2.7, 0.7, 12.8, and 0.15

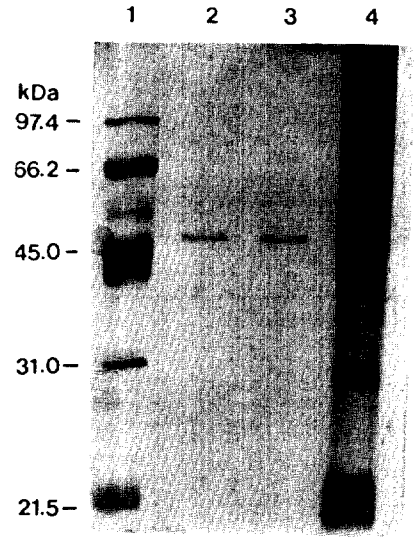

Fig. 1. *Silver-stained SDS-PAGE electrophoregram of fractions from the purification and isoelectric focusing of ASAT (Table 1).*
Lanes 1, molecular mass standards (see Methods); 2, preparative isoelectric focusing (3 ng); 3, Prep Cell (2 ng); 4, cell-free extract (10 ng). The acrylamide concentration was 12 % (w/v).

Table 1. *Purification of ASAT from S. fradiae*

Purification step	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg-protein)	Purification (fold)	Yield (%)
Cell-free extract	2,559	376	0.15	1	100
Acetone fraction	1,680	323	0.2	1.3	86
DEAE-cellulose	65	149	2.3	15.3	40
Hydroxyapatite	17	123	7.2	48	33
Preparative electrophoresis (Prep Cell)	0.22	20	91	607	5

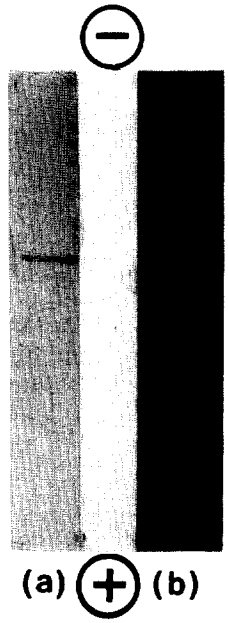

Fig. 2. *Native PAGE of purified ASAT from S. fradiae.* The purified ASAT (2 μg in each lane) was applied to a 12% polyacrylamide gel. The samples were stained with Coomassie brilliant blue (a) for protein and with Fast blue BB salt for ASAT activity (b).

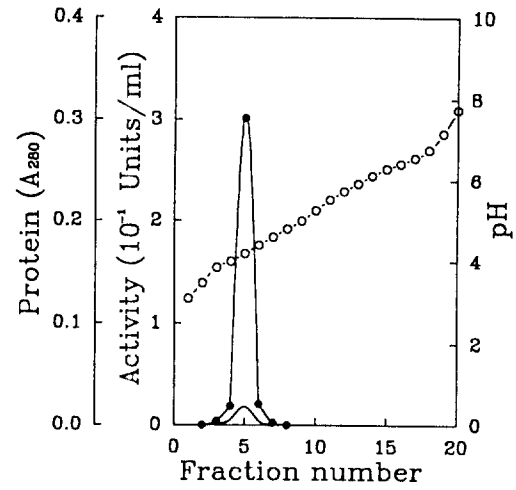

Fig. 3. *Preparative isoelectric focusing of the purified ASAT from S. fradiae.* The ampholine concentration was 2%. The pH gradient ranged from 4.0 to 6.5. 20 Units of purified ASAT in 1 m*l* (total protein, 0.22 mg) were applied on the preparative rotating horizontal (Rotofor) ampholine column (50 m*l*). The run lasted 4~6 h at 12 W constant power. 2.5 m*l* fractions were collected and determined for activity (●), protein concentration (−), and pH (○).

mM for L-aspartate, 2-oxoglutarate, L-glutamate, and oxaloacetate, respectively. The K_m values of ASAT from *S. fradiae* were compared to those of the other eubacteria and a member of the archaebacteria (Table 2). A series of steady-state kinetic analyses were carried out to investigate the reaction mechanism. Double-reciprocal plots of initial velocity against concentrations of L-asparate and 2-oxoglutarate in the presence of various fixed concentrations of 2-oxoglutarate and L-aspartate, respectively, gave two sets of parallel lines (Fig. 4). Product inhibition studies showed that L-glutamate was a competitive inhibitor (Fig. 5a) with respect to L-aspartate and a noncompetitive inhibitor (Fig. 5b) with respect to 2-oxoglutarate.

Reaction rate of ASAT to different substrates

The ability of the enzyme to catalyze transamination between various amino acids and 2-oxoglutarate was examined in the presence of kinetically saturating amounts (20 or 50 mM) of substrates (Table 3). The purified ASAT from *S. fradiae* showed high reaction rate to L-aspartate but showed low reaction rate to L-threonine.

Effects of pH and temperature on stability and activity

Table 2. *Values of K_m for ASAT from different sources*

Source	K_m value (mM)			
	L-aspartate	2-oxoglutarate	L-glutamate	Oxaloacetate
Streptomyces fradiae	2.7	0.7	12.8	0.15
Escherichia coli B[a]	1.3	2.24	15.0	0.01
Pseudomonas striata[b]	0.45	0.29	12.5	0.1
Bacillus sp. strain YM-2[c]	3.0	2.6	ND[e]	ND
Sulfolobus solfataricus[d]	ND	0.3	ND	ND

a, From Yagi *et al.* (29); b, From Yagi *et* (28); c, From Sung *et al.* (23); d, From Marino *et al.* (16); e, Not determined.

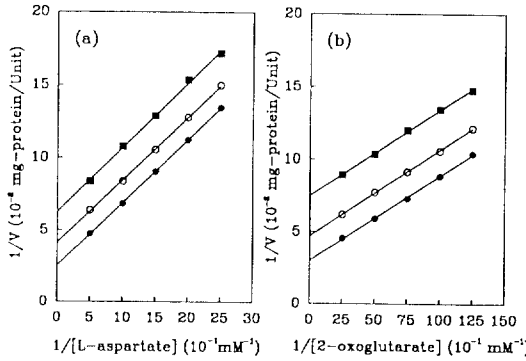

Fig. 4. *Effects of concentrations of L-aspartate and 2-oxoglutarate on the initial velocities of ASAT from S. fradiae.*
The reaction mixture contained variable amounts of the substrates as indicated and the enzyme was assayed as described in the Methods. (a) Double-reciprocal plots of initial velocity versus L-aspartate concentration at 0.16 (■), 0.32 (○) or 0.64 (●) mM 2-oxoglutarate. (b) Double-reciprocal plots of initial velocity versus 2-oxoglutarate at 0.8 (■), 1.6 (○) or 3.2 (●) mM L-aspartate.

Table 3. *Reaction rate of ASAT from S. fradiae to different substrates*

Substrate[a]	Specific activity (Units/mg-protein)	Relative activity (%)
L-Aspartic acid	81.6	100
L-Threonine	2.3	2.8
L-Alanine	1.6	2.0
L-Leucine	0.9	1.1
L-Methione	0.7	0.9
L-Cysteine	0.7	0.9
L-Arginine	0.2	0.2
L-Tryptophan	ND[b]	ND
L-Phenylalanine	ND	ND
L-Tyrosine	ND	ND
L-Valine	ND	ND
L-Isoleucine	ND	ND
L-Lysine	ND	ND

[a]Substrates were used at a concentration of 50 mM except for L-tryptophan (20 mM); [b]Not detected.

The enzyme exhibited maximum activity at pH 7.6 when examined in the presence of 50 mM sodium acetate, potassium phosphate, and glycine/NaOH buffers (Fig. 6a). The enzyme activity retained more than 90% of its maximum activity over the pH range of 5.5 to 8.0. When the enzyme was assayed at various temperatures ranging from 10 to 90°C (Fig. 6b), maximum activity was found

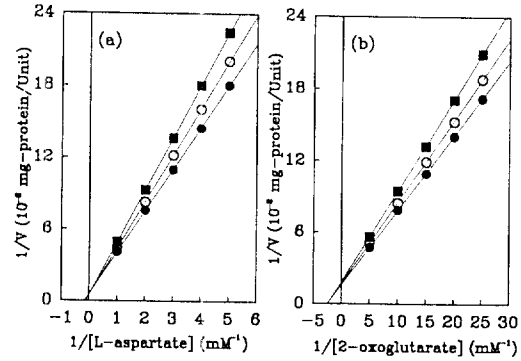

Fig. 5. *Product (L-glutamate) inhibition of ASAT from S. fradiae.*
(a) Double-reciprocal plots of the initial velocity versus L-aspartate concentration varied from 0.2 to 1.0 mM at fixed concentrations of L-glutamate (●, 0 mM; ○, 5 mM; ■, 10 mM) with a constant 2-oxoglutarate concentration of 0.64 mM. (b) Double-reciprocal plots of the initial velocity versus 2-oxoglutarate concentration varied from 0.04 to 0.2 mM at fixed concentrations of L-glutamate (●, 0 mM; ○, 5 mM; ■, 10 mM) with a constant L-aspartate concentration of 2.5 mM.

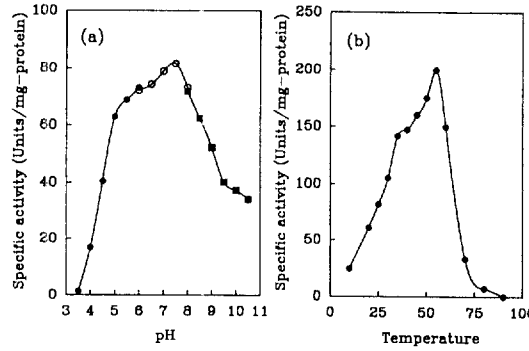

Fig. 6. *Effect of pH and temperature on the specific activity of ASAT from S. fradiae.*
(a) A constant amount of purified ASAT (1 unit) was assayed at the indicated pH: 50 mM sodium acetate buffer (●, pH 3.5~6.0), 50 mM potassium phosphate buffer (○, pH 6.0~8.0), 50 mM glycine/NaOH buffer (■, pH 8.0~10.5). (b) A constant amount of purified ASAT (1 unit) was assayed at the temperature indicated (● 10~90°C).

at 55°C. After incubation at 60°C for 20 min, the enzyme activity was about 75% of the maximum activity but, at 70°C, decreased to about 17%.

DISCUSSION

This paper represents the first purification of ASAT from a *Streptomyces* species. The purified enzyme had a specific activity of 91 Units/mg, which is comparable to that of the ASAT from *Sulfolobus solfataricus* (84 Units/mg) (16). The significant increase in purification fold obtained by preparative electrophoresis (Prep Cell) may have been due to the removal of inhibitory substances and various acidic proteins eluted together with ASAT in the hydroxyapatite column (2). This is the first application of Prep Cell to purify ASAT. Prep Cell is a preparative electrophoresis apparatus which purifies specific proteins from complex mixtures by continuous-elution electrophoresis. A dialysis membrane (6,000 dalton cutoff), directly underneath the elution frit, traps proteins within an elution chamber consisting of a thin frit. The purified enzyme migrated in SDS-PAGE as a single protein band of 54,000 dalton and in native PAGE as one band, indicating that the enzyme from Prep Cell was purified to homogeneity. This band connected with that for enzyme activity and hence the purified enzyme can be assigned as L-aspartate aminotransferase. The molecular mass of the native enzyme was 120,000 dalton. Thus, the purified enzyme appears to be a dimer composed of two identical subunits, the structure of which is common to all ASAT reported so far except for the existence of a monomer in rat heart mitochondria (27) and of a tetramer in chicken heart (3). However, the noticeable discrepancy between the molecular mass determined with the native enzyme and that predicted from the subunit (54,000 dalton×2) is left unexplained as in the *Bacillus* species (23). The molecular mass of the native enzyme or the subunit is higher than any of the ASAT reported until now and similar to that of the subunit (53,000 dalton) from *Sulfolobus solfataricus* (16).

The pI of the purified enzyme was 4.2. This is higher than the minor band (pI 4.1) of the *Bacillus* species (23) and lower than that of the *Escherichia coli* Crookes strain (pI 4.55) (17), carrot (pI 5.2) (24), and *Sulfolobus solfataricus* (pI 6.3 and 6.8) (16). Rotofor (Bio-Rad) is a preparative isoelectric focusing apparatus which is better than analytic isoelectric focusing in terms of protein recovery and rapidity (7). One band upon isoelectric focusing may indicate that the enzyme consists of two subunits, showing homogeneity.

The K_m values for ASAT was 2.7, 0.7, 12.8, and 0.15 mM for L-aspartate, 2-oxoglutarate, L-glutamate, and oxaloacetate, respectively. The K_m values of ASAT from *S. fradiae* were compared to those of the other eubacteria and a member of the archaebacteria. Like the other ASAT, the K_m values for the oxo acids are lower than those for the amino acids. These data indicate that all the aspartate aminotransferases identified to date may perform similar functions. The K_m values for the oxo acids was 4 to 85 times lower than those for the amino acids, and the K_m value for L-glutamate was fivefold higher than that for L-aspartate. This result may be due to competition for oxaloacetate between citrate synthase and aspartate aminotransferase and the low intracellular concentration of oxaloacetate.

Based on the presence of parallel lines on double-reciprocal plots from initial velocity studies for ASAT, the ASAT from *S. fradiae* does not follow the rapid equilibrium random bi bi and ordered bi bi mechanism and does not form ternary complex with substrates. These results have been reported for other aminotransferases (26). When we studied product inhibition, L-glutamate is a competitive inhibitor with respect to L-aspartate and a noncompetitive inhibitor with respect to 2-oxoglutarate. When the concentration of ammonium ion was increased from 5 to 25 mM, enzyme activity was not inhibited (data not shown), in contrast to that of threonine dehydratase, which is repressed and inhibited by ammonium ion and which is concerned with biosynthesis of tylosin, a macrolide antibiotic (15).

The purified enzyme from *Streptomyces fradiae* showed high reaction rate to L-aspartate. The activity of ASAT from *Escherichia coli* Crookes strain showed 22% of the total tyrosine aminotransferase (17), but in ASAT from *S. fradiae*, aromatic amino acids such as L-tryptophan, L-phenylalanine and L-tyrosine were not active as amino donors. The low specific activity of ASAT toward L-threonine indicated that ASAT is partially needed for tylosin biosynthesis because of the formation of 2-oxobutyrate (precursor for tylosin biosynthesis) from L-threonine by ASAT rather than threonine dehydratase.

The enzyme activity over a pH range of 5.5 to 8.0 was more than 90% of the maximum activity. This is similar to the ASAT from *Sulfolobus solfataricus* (16), which is stable in the pH range of 6~9, but different from the ASAT from *Pseudomonas striata* (pH 8.3~8.6) (28). Activity of the purified ASAT was found to be maximal at 55°C. However, enzyme assays were carried out at 25°C because the optimum temperatures of malate dehydrogenase and glutamate dehydrogenase are 25°C, and the values of ε for NADH at 25°C at 340 nm are sufficiently close enough for practical purpose (<1% error at 340 nm), while being independent of the other measurement conditions. After incubation at 60°C for 20 min, the enzyme activity was about 75% of the maximum activity but, at 70°C, decreased to about 17%. This indicates that the thermostability of ASAT from *S. fradiae* is similar to that of the

Bacillus species (maximum activity at 70°C) (23), lower than that of *Sulfolobus solfataricus* (increase of enzyme activity until 90°C) (16), and higher than that of *Escherichi coli* (maximum activity at 30°C) (3). Therefore, aspartate aminotransferase from *S. fradiae* can be considered to be an interesting model for the study of structural requirements necessary to achieve thermostability in enzymes.

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초 록: *Streptomyces fradiae*에서 분리된 Aspartate Aminotransferase의 특성

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Streptomyces fradiae NRRL 2702에서 acetone 분획, DEAE-cellulose, hydroxyapatite와 preparative electrophoresis를 통해 aspartate aminotransferase를 순수 분리하였다. 순수 분리된 효소는 120,000 dalton으로, 54,000 dalton의 크기를 갖는 두 개의 subunit로 이루어지고, pI 값은 4.2로 밝혀졌다. L-aspartate, 2-oxoglutarate, L-glutamate, 그리고 oxaloacetate에 대한 K_m값은 각각 2.7, 0.7, 12.8, 0.15 mM이었다. 본 효소는 55°C에서 최대 활성을 나타내었으며 비교적 열에 안정하였고 pH 5.5에서 8.0 사이가 최적 pH였다. 암모늄 이온에 의하여 저해를 받지 않았다.