

A Thermostable Aspartate Aminotransferase from *Aeropyrum pernix* K1

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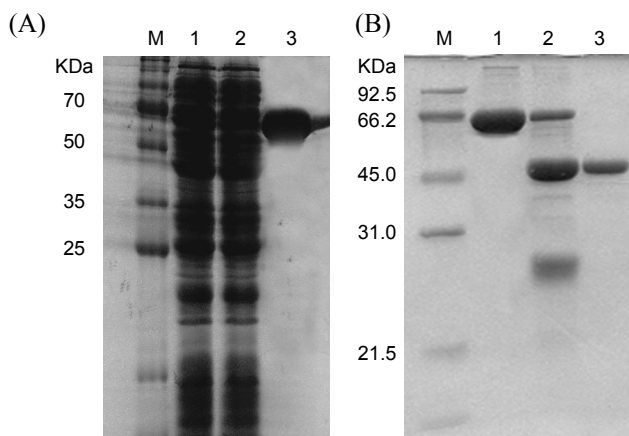
Aspartate aminotransferase (AAT, EC 2.6.1.1), the best known pyridoxal 5'-phosphate dependent enzyme, is an essential component of nitrogen and carboxylic cellular metabolism,<sup>1,2</sup> where it catalyzes the reversible transamination between dicarboxylic amino and  $\alpha$ -keto acids. Aminotransferases from many species are classified into four subgroups. Family I aminotransferases transfer the amino acids of aspartate, alanine, histidine, and aromatic compounds to other proteins, and have been further subdivided into seven subfamilies ( $I_{\alpha}$ ,  $I_{\lambda}$ ,  $I_{\beta}$ ,  $I_{\Omega}$ ,  $I_{\delta}$ ,  $I_{\gamma}$ , and  $I_{\phi}$ ).<sup>3,4</sup> The AATs of subfamily  $I_{\alpha}$  include enzymes from *Escherichia coli*, yeasts, plants, and animals,<sup>5</sup> and have been more extensively investigated than other subfamilies to elucidate the structure and functions of the enzymes. Subfamily  $I_{\gamma}$  includes AATs from *Bacillus* sp. YM-2, *Rhizobium meliloti*, *Sulfolobus solfataricus*, and *Thermus thermophilus* HB8, with sequences that are < 16% identical between the subfamilies.<sup>3,6,7</sup> According to the three-dimensional structures of the AATs as determined by X-ray crystallography, both the overall and active

site structures are essentially conserved between the AATs of subfamilies  $I_{\alpha}$  and  $I_{\gamma}$ .<sup>8</sup> Despite the available research, the biochemical properties and functions of the prokaryotic AATs remain to be elucidated.

*Aeropyrum pernix* K1 is an aerobic hyperthermophilic crenarchaeon isolated from a coastal solfataric thermal vent in Kodakara-Jima island of Kagoshima, Japan that grows optimally at 90 ~ 95 °C.<sup>9</sup> Genome sequencing of *Aeropyrum pernix* K1 at the National Institute of Technology and Evaluation in Japan revealed that the open reading frame APE2423, composed of 1149 base pairs, encodes a protein homologous to AATs from *Hyperthermus butylicus*, *Caldicellulosiruptor saccharolyticus*, and *Methanocaldococcus jannaschii* (approximately 37 ~ 59% identity, Fig. 1), as judged by homolog searches in the NCBI database. The amino acid sequences of the APE2423 gene showed  $\geq$  25% amino acid sequence identity to these thermophilic AATs (results not shown). These results suggest that the product of the APE2423 gene may belong to the  $I_{\gamma}$

ApAAT.	1	MA --- RLLMIPGPTPVDPPEVLLAMARPVISHTSPPEFDIHSSTVSMLOKLFRRSGKVF
HbAAT.	1	MVH -- GKVLLMTPGPTPVDPPEVLLAMAKPTINHWPEFDEIHSSEVLMKMLSEVFAKGRVV
CsAAT.	1	MR --- KPRLLMTPGPTPVDPPEVLLAMSQQLIHRTRKESAEIHSFQVNNENLKKVFOKKNMVL
MjAAT.	1	MKIDAVKRLMTPGPTPVDPPEVLLAMALPFGHREKDSNLEBETIEKLEKVFIPENDTF
Chhydrogenase.	1	MQ --- KRVMIPGPTPVDPPEVLLAMSRPMLGRSGDPAKLEHRRVVAKLEKQVFKQNDIE
ApAAT.	56	LIPGSGSLGMEFAVRSVAGRCGRALVLLKACVFCGYERHLRSLCASVTVESPVCRGFTG
HbAAT.	59	VIPGSGTGAEMELDRSVVVKPGSKVVLKACFFGDYLRGVEALCGNAVIEAPIGRGFTA
CsAAT.	58	TFAASGTGAMASAVNFFSEGDVLLVSVGVFQDRFINCKTFGLNVEKKVYHGSAANI
MjAAT.	61	LITGSGTAAAMDANSNLIKRGDKVLLVVTGNFGERFANLVKAYGGAIRLDVWEGDMAEP
Chhydrogenase.	57	LLTNGTGGEMAVANVNVNPGDEVLAISTGNFGERFAKIAKREYGARVDMVDFGQNAVDL
ApAAT.	116	ADVEEALDR - AGVDVYVVFQHVETSTGVANPVEELAGVVKKNGRLVVDGLIASVGGMDLR
HbAAT.	119	SDLEAVLRK - HPDAEYVAFQHVDTSTGVANPLRELAEAARHGKVLVVDVGVGGGQEVRR
CsAAT.	118	DELIDILENDKSNIKGVFITHNETSTGVNTPLEKLRVYKDKDKLILVYDVSLLGADLKL
MjAAT.	121	EAWKSLDK - YDDKAVTVVHNETSTGARNPIMEHGEVVVDYDLYVYDVSLLGQVYVN
Chhydrogenase.	117	AVVEKLRQ - NPNYKAVLATHNETSTGVKNDIAGIAALTKNHRVLLVYDVSLLGGMDFD
ApAAT.	175	LDWGVVDSLTGSQKALSTPPGLAVVAFSSEYSVELEKKE - PSGIYDFLSLSR - EMEST
HbAAT.	178	LDDWGVVDCVTGSQKALGVPGLAVVAFSREYSSELEGRGEPWSLVENMPKLLK - EMEST
CsAAT.	178	TDEWGVVYVVTGSQKALMSPPGLAVVSVSDKAWFYYKTSKLR - KFYVDFKXQYDNLLES
MjAAT.	180	VKRFHDECVTGSQKCHAAAPPGLAAITVSKAWVEVKKNDKVGFFDGLLAYKR - YVBEK
Chhydrogenase.	176	TDEWGVVYVVTGSQKALMLPPGLAFISFSQKALAAQDNKNP - RFFYSLFAAKK - ALA - E
ApAAT.	233	RNYHTPAVNLVPAFNASLKRILSEGLNRFERHRLARAFSTAMEALGLRVLVABEPPFA
HbAAT.	237	RNYVTPAVNLVVALRESRIILAEGLSEBRFKRHRIMAEAVRAALEAMGLKLVABEGPFA
CsAAT.	237	QDTPPTPAVNLIRAVDVGKLLLDYGLENNFKRHTLAKLTLQAVDLNLEBELLPEKVESS
MjAAT.	239	KQTPPTPQVNLVAVLNVAIDLVLREGLENVFRHRERLAKATRAGLEAMGCELFPAKREAS
Chhydrogenase.	233	WNTAYTPAVSIFFGLEAAIDLILREGLDKVIKRKLLAKACREGVRLGLKELFPABENAS
ApAAT.	293	WVVTAVYLFQGIWRSRFSYVMK - RGVETAGGLGGFKGKIFRUGHMGVNDANDLITATAA
HbAAT.	297	DRVTAAYTPQGVWPFVLSGMRRA - RGVETAGGLGGFKGKIFRIGHMGQTYVDIATAAA
CsAAT.	297	AVITLAKAPGTDIEVVRKINNKYDINVAAGGQANLKKGIFRIGHMGVYDDELKKTQC
MjAAT.	299	VTVTSAKYVEGIDSKFRGILSNKYNVAVAGGQKHLKGGKIFRIGHMGICGEVLAIDTAC
Chhydrogenase.	293	DRVTVVAGDDRYDPEQLKVLRTKYGVTFAGGQKDLKGGKIFRIGHMGVYDKLIDIVAECA
ApAAT.	352	IERSLVELGVREANLGGLEAAQKELSSLGL Identity
HbAAT.	356	LERTLKSLGVD - TELSGGLRALQELKHEHGV 59%
CsAAT.	357	FELSLLEGGYKNEFVEPEPTKILQELAKEVV 37%
MjAAT.	359	VELLAKKELGFE - VK - ESGVEVAKVVDL - KE 39%
Chhydrogenase.	353	LEMAKKEFGYF - VELGAGVKKRAMEVITGGEA 42%

**Figure 1.** Comparison of amino acid sequences of ApAAT and other AATs. Sequence alignment was performed by T-Coffee and Boxshade software (<http://www.ch.embnet.org>). Dashes indicate gaps, and consensus identical (black) or similar (gray) amino acids are shaded. The following sequences were aligned: ApAAT, AAT from *Aeropyrum pernix* K1 (GenBank accession number, BAE96897); HbAAT, AAT from *Hyperthermus butylicus* (YP\_001013233); CsAAT, AAT from *Caldicellulosiruptor saccharolyticus* (YP\_001180096); MjAAT, AAT from *Methanocaldococcus jannaschii* DSM 2661 (AAB98961); ChHydrogenase, Hydrogenase from *Carboxydotherrus hydrogeniformans* (ABB15912).



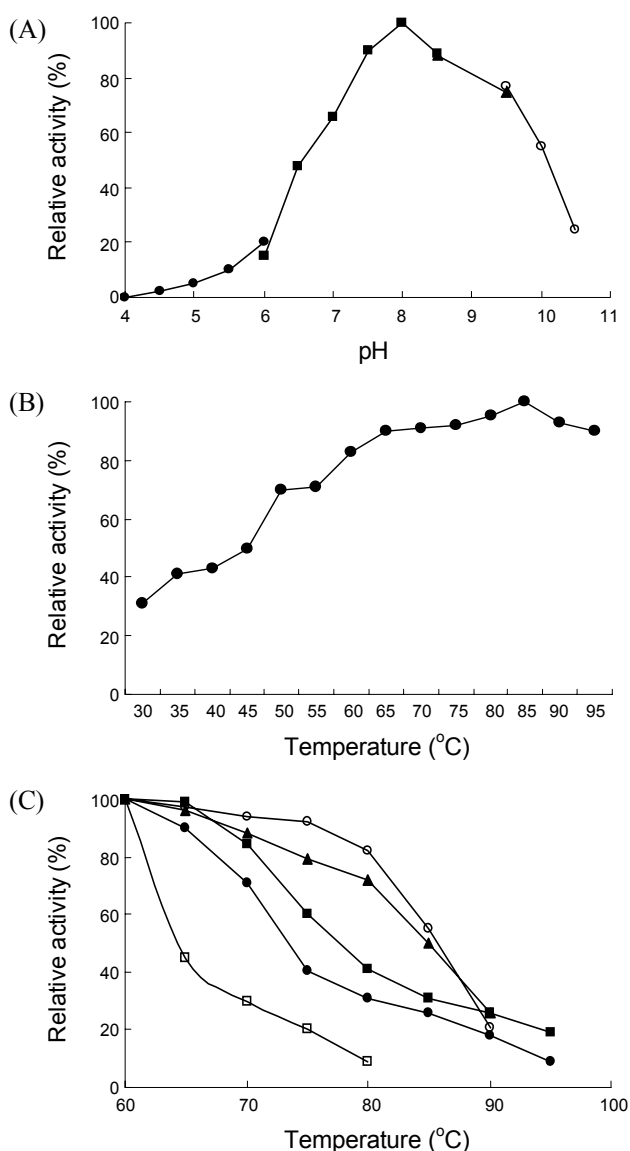
**Figure 2.** SDS-PAGE analysis of GST-ApAAT (A) and ApAAT (B). Denaturing SDS-PAGE was carried out using the method of Laemmli (1970) in 12.5% gels. The molecular-mass makers were SDS molecular weight standard markers and Coomassie Blue R-250 was used for staining. (A) Lane M, Protein size marker; lane 1, cell-free crude extract; lane 2, flow-through fraction; lane 3, purified GST-ApAAT; (B) Lane M, Protein size marker; lane 1, purified GST-ApAAT; lane 2, thrombin treatment; lane 3, purified ApAAT.

**Table 1.** Substrate specificities of ApAAT towards amino acids and  $\alpha$ -keto acids

Substrates	Concentration (mM)	Relative activity (%)
<i>Amino donors</i>		
L-Aspartate	50	100
L-Alanine	50	0.60
L-Phenylalanine	50	0.03
L-Tyrosine	50	0.04
<i>Amino acceptors</i>		
$\alpha$ -Ketoglutarate	5	100
$\alpha$ -Ketobutyrate	5	0.3
Pyruvate	5	1.2

subfamily. The amino acid sequences encoded by the APE2423 gene also demonstrated approximately 42% identity to hydrogenase from *Carboxydotherrnus hydrogeniformans* (Figure 1). When hydrogenase activity was assayed spectrophotometrically using reduced methyl viologen dichloride as a substrate,<sup>10</sup> however, the recombinant protein of APE2423 had no hydrogenase activity. Based on these results, we selected the APE2423 gene as a candidate for an AAT. Until now, there have been no reports concerning the product of this gene, and so we cloned and expressed the APE2423 gene in *Escherichia coli*.

In general, it is difficult to express cloned genes from hyperthermophilic bacteria in *E. coli*, because the expressed proteins are insoluble.<sup>11</sup> To effectively overexpress and purify the APE-2423 gene product from *Aeropyrum pernix* K1 (ApAAT), a recombinant plasmid was designed using the expression vector pGEX-KG containing glutathione *S*-transferase (GST) as the fusion partner. The recombinant ApAAT was efficiently purified 10-fold with a yield of 52% by GSH-agarose affinity chromatography and thrombin treatment. Approximately 6 mg of the recombinant ApAAT protein was purified from 1 L of the transformant culture medium. The purified ApAAT yielded a single band on 12.5% sodium dodecyl sulfate-polyacrylamide



**Figure 3.** (A) Effect of pH on ApAAT. ApAAT buffer conditions were as follows: pH 4.0 - 6.0: 200 mM citrate-sodium phosphate buffer (-●-), pH 6.0 - 8.5: 200 mM potassium phosphate buffer (-■-), pH 8.5 - 9.5: 200 mM tris-HCl buffer (-▲-), pH 9.5 - 10.5: 200 mM glycine-NaOH buffer (-○-). (B) Effect of temperature on ApAAT. The optimal temperature of the enzyme was determined in the 100 mM Tris-HCl buffer (pH 8.0) at temperatures ranging from 30 to 95 °C, at a concentration of 0.1 mg/mL. (C) Thermostability of ApAAT. After incubation at various temperatures and with various additives in 100 mM Tris-HCl (pH 8.0) for 20 min (-●-, no additive; -■-, 10 mM  $\alpha$ -ketoglutarate; -▲-, 30 mM pyridoxal phosphate; -○-, 10 mM  $\alpha$ -ketoglutarate and 30 mM pyridoxal phosphate; -□-: 100 mM L-aspartate), the remaining enzyme activity was measured after chilling the enzyme on ice.

gel electrophoresis (SDS-PAGE) that indicated an approximate molecular mass of 42 kDa (Figure 2).

Substrate specificities of the recombinant ApAAT were studied with the transamination reaction between amino donors and acceptors. ApAAT was specific for dianionic amino acid, with L-aspartate as an amino donor, but showed very low activity towards L-alanine (Table 1). Aromatic amino acids such as L-phenylalanine and L-tyrosine were negligibly active as amino donors. Substrate specificity for amino acceptors was also

performed with L-aspartate as an amino donor and a variety of  $\alpha$ -keto acids (Table 1). ApAAT was specific for  $\alpha$ -ketoglutarate as an amino acceptor. Pyruvate and  $\alpha$ -ketobutyrate served only slightly as substrates.

To further determine the function of ApAAT, we investigated the kinetic parameters of the enzyme toward L-aspartate and  $\alpha$ -ketoglutarate. The  $K_m$  values of ApAAT were  $3.5 \pm 0.2$  mM for L-aspartate and  $3.0 \pm 0.3$  mM for  $\alpha$ -ketoglutarate, respectively, which were similar to those of AAT from thermophilic *Bacillus* sp. Strain YM-2 (3.0 mM for L-aspartate and 2.6 mM for  $\alpha$ -ketoglutarate).<sup>2</sup> ApAAT also showed an affinity for L-aspartate that was higher than AAT from *Rhizobium meliloti*, with a  $K_m$  of 5.3 mM.<sup>12</sup>

We then determined the optimum pH and temperature for ApAAT with the transamination reaction toward L-aspartate and  $\alpha$ -ketoglutarate. The optimum pH for ApAAT was 8.0 (Fig. 3A), with more than 60% of maximum activity in the pH range of 6.5 to 10. These findings were similar to AAT of *Rhizobium meliloti* (pH 8.0 - 8.5).<sup>12</sup> At the same time, acidic or neutral ranges of optimum pH have been observed for AATs from *Sulfolobus solfataricus* (pH 5.8) and *Bacillus* YM-2 (pH 7.2).<sup>13,14</sup> Activity of ApAAT increased steadily in the temperature range from 30 to 85 °C (Fig. 3B), with an optimum temperature of 85 °C. The thermostability of ApAAT was investigated by incubation of the enzyme for 20 min from 60 to 95 °C. The midpoint of the temperature-stability curve was approximately 74 °C (Fig. 3C). The enzyme was fairly stable at temperature up to 65 °C. Moreover, ApAAT was protected against heat activation by the addition of pyridoxal phosphate or/and  $\alpha$ -ketoglutarate, which significantly increased its heat stability by approximately 5 ~ 10 °C. In contrast, the addition of L-aspartate decreased the heat stability of ApAAT. This thermostability is comparable with those of AATs purified from other thermophilic bacteria (2,4,13).

In conclusion, we expressed the hypothetical protein of the APE2423 gene from *Aeropyrum pernix* K1 and characterized the purified recombinant protein. The hypothetical protein first reported herein is a novel AAT displaying high substrate specificities towards L-aspartate as an amino donor and  $\alpha$ -ketoglutarate as an amino acceptor. The recombinant ApAAT was highly stable at high temperatures, making it comparable to other available AATs. Further studies are underway to elucidate the detailed physiological functions of ApAAT and to utilize this recombinant enzyme in medicine and bio-industrial processes.

### Experimental Section

Cloning of the APE2423 gene and construction of expression plasmid. The cDNA of *Aeropyrum pernix* K1 was prepared as described by Sako *et al.*<sup>15</sup> The gene APE2423 of *A. pernix* was amplified by the polymerase chain reaction (PCR) using the cDNA as template. The sequences of the PCR primers were: 5'-ggaattccaATGGCTCGTCTGCTGATGAT-3' (upper primer, containing an *Eco*R I digestion sequence as underlined); 5'-cccaagcttTCAGAGGCCTAGGCTAGAGA-3' (lower primer, containing a *Hind* III digestion sequence as underlined). The PCR product was cloned into an expression vector pGEX-KG

using *Eco*R I and *Hind* III sites. The resulted vector was designated as pGEX-ApAAT and used to transform the *E. coli* strain BL21 (DE3). The colony containing the appropriate insert was identified by DNA sequencing.

Expression and purification of the recombinant enzyme. The *E. coli* strain BL21 (DE3) harboring each of the constructed plasmids was grown in Luria-Bertani (LB) broth containing ampicillin (25.0  $\mu$ g/mL) at 37 °C and induced at OD<sub>600</sub> = 0.9 - 1.0 with 0.4 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) for 8 h.<sup>16</sup> The induced cells were harvested by centrifugation at 10,000 g for 10 min at 4 °C and resuspended in 50 mM phosphate-buffered saline (PBS) buffer (pH 7.3) containing 20 mM pyridoxal-5'-phosphate and 20% glycerol. The resuspended cells were subjected to sonication using an ultrasonic processor (Sonics & Materials Inc., Newtown, CT, USA) until lysed. The cell-free extract was obtained by centrifugation at 40,000 g for 60 min, the supernatant was collected and stored at 4 °C.

The supernatant containing the enzyme bearing the glutathione *S*-transferase (GST)-tag was subjected to affinity column chromatography with glutathione (GSH)-agarose. The column was washed with 50 mM PBS buffer (pH 7.3) containing 50 mM KCl. The fusion protein, GST-ApAAT was then eluted with 50 mM Tris-HCl buffer (pH 9.6) containing 10 mM GSH. Free GSH was removed by dialysis against 50 mM Tris-HCl (pH 8.0) containing 20 mM pyridoxal-5'-phosphate and 5% glycerol. Approximately 2 mg of the fusion protein was incubated with 10 units of thrombin (Amersham pharmacia Biotech, Uppsala, Sweden) at room temperature for 16 h and loaded to GSH-agarose affinity column chromatography. The eluted ApAAT was subjected to dialysis for 24 h at 4 °C with three successive changes against 50 mM Tris-HCl (pH 8.0) containing 20 mM pyridoxal-5'-phosphate and 5% glycerol.

Enzyme assay and kinetic studies. The transamination reaction activities between amino donors of L-aspartate, L-alanine, L-tyrosine, L-phenylalanine and amino acceptors of  $\alpha$ -ketoglutarate,  $\alpha$ -ketobutylate, pyruvate were measured spectrophotometrically at 340 nm with a HITACHI U-2000 double-beam spectrophotometer (Hitachi CO., Tokyo, Japan) using cuvettes of 10<sup>-2</sup> m path-length (Sigma-Aldrich). The reaction mixture contained the following, in a final volume of 1.0 mL: 10 mM Tris-HCl buffer (pH 8.0); 50 mM L-aspartate; 5 mM  $\alpha$ -ketoglutarate; 0.1 mM NADH and 2 units of malate dehydrogenase; an appropriate amount of enzyme. The reaction was then initiated by addition of the enzyme preparation. One unit of activity was defined as the amount of enzyme producing 1  $\mu$ M of oxaloacetate per 1 min. To measure the kinetic parameters, reaction rates were measured at a series of L-aspartate concentrations (0.2 ~ 50 mM) and the rates at various  $\alpha$ -ketoglutarate concentrations (0.4 ~ 32 mM) finally fit by Lineweaver-Burk plots. The parameters (with standard deviation) were determined by five separate experiments. The protein concentration was determined by the method of Bradford using  $\gamma$ -globulin as a standard.

**Thermostability of the enzyme.** The thermostability of the enzyme was measured at 60 ~ 95 °C. The enzyme was incubated in 100 mM Tris-HCl buffer (pH 8.0) for 20 min. After chilling the sample on ice, residual activity was determined under standard conditions.

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