

Gene Identification and Characterization of the Pyridoxine Degradative Enzyme α -(N-Acetylaminomethylene)succinic Acid Amidohydrolase from *Mesorhizobium loti* MAFF303099

Baiqiang YUAN¹, Nana YOKOCHI¹, Yu YOSHIKANE¹, Kouhei OHNISHI², Fei GE¹,
and Toshiharu YAGI^{1,*}

¹Department of Bioresources Science, Faculty of Agriculture, and ²Research Institute of Molecular Genetics,
Kochi University, Nankoku, Kochi 783–8502, Japan

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Summary We have found for the first time that a chromosomal gene, mlr6787, in *Mesorhizobium loti* encodes the pyridoxine degradative enzyme α -(N-acetylaminomethylene)succinic acid (AAMS) amidohydrolase. The recombinant enzyme expressed in *Escherichia coli* cells was homogeneously purified and characterized. The enzyme consisted of two subunits each with a molecular mass of $34,000 \pm 1,000$ Da, and exhibited K_m and k_{cat} values of $53.7 \pm 6 \mu M$ and $307.3 \pm 12 \text{ min}^{-1}$, respectively. The enzyme required no cofactor or metal ion. The primary structure of AAMS amidohydrolase was elucidated for the first time here. The primary structure of the enzyme protein showed no significant identity to those of known hydrolase proteins and low homology to those of fluoroacetate dehalogenase (PDB code, 1Y37), haloalkane dehalogenase (1K5P), and aryl esterase (1VA4).

Key Words vitamin B₆, pyridoxine, degradation pathway, α -(N-acetylaminomethylene)succinic acid, amidohydrolase

α -(N-Acetylaminomethylene)succinic acid (AAMS) amidohydrolase catalyzes degradation of AAMS, which is produced from 2-methyl-3-hydroxypyridine-5-carboxylic acid (MHPC) dioxygenase as shown in Fig. 1A, to acetic acid, ammonia, carbon dioxide, and succinic semialdehyde as shown in Fig. 1B. The enzyme reaction is the final step in degradation pathway I for vitamin B₆ (pyridoxine, pyridoxamine, and pyridoxal) (1). The enzyme was purified from the crude extract of *Pseudomonas* MA-1 cells grown on pyridoxine as a sole carbon source, and some properties were characterized (2, 3). However, the primary structure of the enzyme or the gene encoding the enzyme has not been reported.

Recently we found that a nitrogen-fixing symbiotic bacterium, *Mesorhizobium loti* MAFF303099 possessed several enzymes involved in degradation pathway I, and characterized their properties. The first enzyme for degradation of pyridoxine is pyridoxine 4-oxidase (4, 5) and that for pyridoxamine is pyridoxamine-pyruvate aminotransferase (6). The second, third, seventh enzymes are pyridoxal 4-dehydrogenase (7), 4-pyridoxolactonase (8), and MHPC dioxygenase (9), respectively. These enzymes are encoded by genes (from mlr6785 to mlr6807) clustered on the chromosome of the bacterium. The numbering of the genes is described in the RhizoBase (10). Among the enzymes in the cluster, gene mlr6787 is the only one which can be deduced to be a hydrolase based on the predicted amino acid sequence.

Here, we identified the protein encoded by the mlr6787 gene as the AAMS amidohydrolase, and elucidated the primary structure of the enzyme for the first time. The recombinant AAMS amidohydrolase was over-expressed and characterized.

MATERIALS AND METHODS

Bacterial strains, plasmids and chemicals. *Escherichia coli* strains BL21(DE3) and JM109 were purchased from Novagen (San Diego, USA). *M. loti* MAFF303099 was obtained from MAFF GenBank (Tsukuba, Japan). The plasmids pTrc99A (Pharmacia Biotech, Uppsala, Sweden), pTA2 (TOYOBO, Osaka, Japan), pNEB205A (New England BioLabs, Beverly, USA), pET-21a (Novagen), and pKY206 (11) were used for cloning and expression. MHPC was prepared from the culture media of *M. loti* as described previously (9). AAMS was prepared from MHPC by bioconversion with recombinant *E. coli* cells as described below.

Preparation of the recombinant *E. coli* cells for bioconversion of MHPC to AAMS, and preparation of AAMS. As shown in Fig. 1A, AAMS was produced from MHPC by a dioxygenation reaction catalyzed by MHPC dioxygenase. The reaction uses NADH as a hydrogen donor. Thus, the regeneration system of NADH was required to make the conversion reaction complete. An ethanol-active medium-chain dehydrogenase/reductase alcohol dehydrogenase (12) gene (*adh*) was amplified by PCR with the chromosomal DNA from *E. coli* K12 as a template, and primers 5'-GGAGACAdUATGAAGGCTGCAG TTGTTACGAAGG-3' (primer adh-F1, the boldfaced letters show *Nde*I restriction site) and 5'-GGGAAAGdUTT

*To whom correspondence should be addressed.

E-mail: yagito@kochi-u.ac.jp

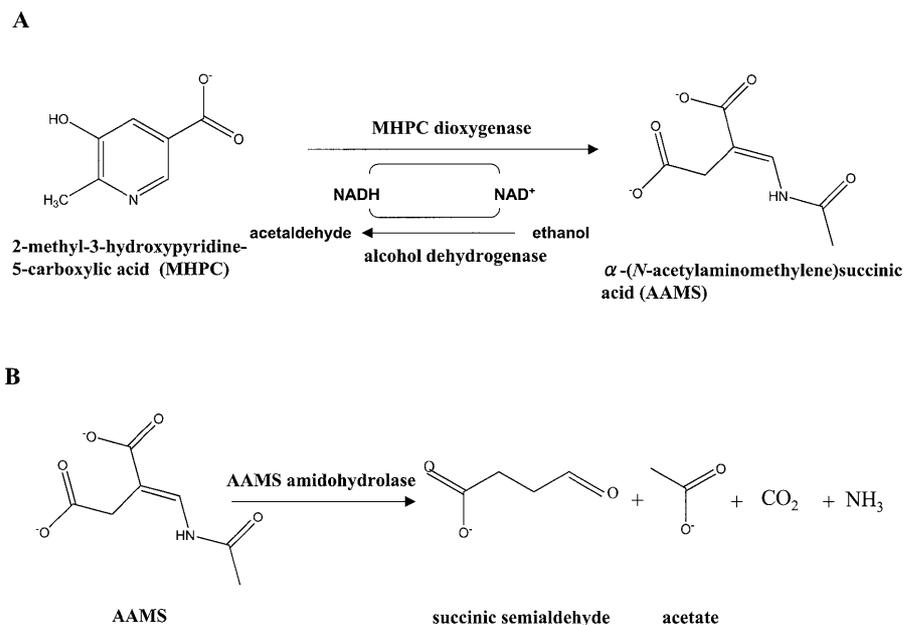


Fig. 1. A: the principle of bio-synthesis of AAMS with recombinant *E. coli* cells. B: the reaction catalyzed by AAMS amidohydrolase.

AGTGACGGAAATCAATCACCATGC-3' (primer adh-R1). The reaction mixture (50 μ L) for PCR consisted of LA PCR Buffer II, 20 nmol dNTP, 2.5 U LA Taq polymerase, 125 nmol MgCl₂, 3.8 ng of the template DNA, and 20 pmol of each primer. The PCR condition was heating to 94°C for 5 min, 30 cycles of 94°C for 1 min, 54.5°C for 1 min, and 72°C for 1 min. The PCR product was ligated with a USER™ FRIENDLY Cloning Kit into the pNEB205A, to construct pNEB205A-adh. After the sequence of the introduced fragment had been verified by DNA sequencing of cloned *adh* gene with an ABI PRISM 3100 genetic sequencer, pNEB205A-adh was digested with *Nde*I and *Eco*RI, and *adh* gene was re-cloned into the *Nde*I and *Eco*RI sites of pET21a. The constructed plasmid was designated as pET21a-adh. The plasmid pET21a-adh was digested with *Xba*I and *Hind*III, and the DNA fragment containing the *adh* gene was isolated from an agarose gel. The isolated fragment was ligated into pTrc99A to make pTrc99A-adh. pET6788 plasmid harboring MHPC dioxygenase gene (9) was digested with *Eco*RI, and then subjected to alkaline phosphatase treatment. The pTrc99A-adh was digested with *Eco*RI, and then the *adh* gene isolated from an agarose gel was inserted into the *Eco*RI site of pET6788 to construct pET6788-adh. *E. coli* BL21(DE3) cells were co-transformed with pET21a-adh-pld and pKY206 carrying the *groES* and *groEL* genes encoding chaperonins (11). The transformed cells were used for the bioconversion.

The cotransformed cells (100 mg) were incubated at 30°C for 24 h in 1 mL of 50 mM potassium phosphate buffer (pH 7.5) containing 0.5 M ethanol and 5 mM MHPC. Then the reaction mixture was applied to an ion-exchange HPLC column IEC QA825 (8.0 \times 75 mm) (Showa Denko, Tokyo, Japan). The AAMS was eluted at 0.2 M formic acid by linear gradient (0–0.5 M formic acid) elution. The formic acid in the elution was

removed thoroughly by evaporation at 60°C and then AAMS was crystallized from hot water. The AAMS preparation showed the same absorption spectrum as described previously (2). Its structure was verified by the NMR spectrum. Its concentration was determined with an extinction coefficient of 17.5 mM⁻¹cm⁻¹ (pH 7.0) at 261 nm.

Cloning and expression of the mlr6787 gene. The *mlr6787* gene was amplified by PCR from the chromosomal DNA of prepared *M. loti* MAFF303099. The primers used were 5'-CATATGGACATGGCGGCAGA CATA-3' (*mlr6787*-F) with a *Nde*I site (underlined) and a start codon (bold type), and 5'-GGATCCT**C**AGGCGTC TATGAAGTT-3' (*mlr6787*-R) with a *Bam*HI site and a stop codon (bold type). PCR was performed in a 50- μ L reaction mixture containing KOD-plus buffer, 0.2 mM dNTPs, 1.0 mM MgSO₄, 0.3 μ M of each primer, 2 ng *M. loti* chromosome and 1.25 U KOD-plus DNA polymerase (TOYOBO). The reaction mixture was heated at 94°C for 2 min, then subjected to 30 cycles of heating (94°C, 15 s; 55°C, 30 s; and 68°C, 90 s). Finally, the reaction mixture was heated at 68°C for 10 min. The amplified DNA fragment (846 bp) was cloned into the pTA2 vector, to construct pTA6787. After the sequence of the introduced fragment had been verified by DNA sequencing with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, USA), pTA6787 was digested with *Nde*I and *Bam*HI, and then the digested DNA fragment was inserted into the *Nde*I/*Bam*HI sites of pET-21a to construct the expression plasmid pET6787.

E. coli BL21(DE3) cells were transformed with pET6787. The transformed cells were grown aerobically at 37°C in 5 mL of LB medium containing ampicillin (50 μ g mL⁻¹) until the absorbance at 600 nm reached 1.0. The broth was then added to 200 mL of the same medium, and the inoculated cells were grown

at 37°C for 16 h. The bacterial cells were harvested by centrifugation at 8,000 × *g* for 10 min at 4°C, washed with 0.9% (w/v) sodium chloride, and then stored at -20°C until use.

Purification of recombinant AAMS amidohydrolase. All steps were done at 4–10°C. The transformant *E. coli* BL21(DE3)/pET6787 cells (5.5 g) were suspended in 25 mL of 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% (v/v) 2-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride) (Buffer A containing phenylmethylsulfonyl fluoride). The cell suspension was sonicated on ice for 6 min with a Heatsystems-Ultrasonics sonicator W-220. After centrifugation at 10,000 × *g* for 20 min at 4°C, the supernatant (44 mL) was used as the crude extract. The crude extract was fractionated with ammonium sulfate, and the precipitate obtained on centrifugation of 40–55% saturated solution was dissolved in 15 mL of Buffer A, and the solution was dialyzed thoroughly against Buffer A. To the dialyzed solution (20 mL) was added 1 M ammonium sulfate, and then the enzyme solution was applied to a Butyl-Toyopearl 650 M column (3.0 × 17.0 cm, Tosoh Corporation, Tokyo, Japan) equilibrated with Buffer A containing 1 M ammonium sulfate. The enzyme was eluted at around 0.4 M ammonium sulfate with a linear gradient (1.0–0 M ammonium sulfate). The active fractions were pooled and dialyzed thoroughly against Buffer B (50 mM Tris-HCl, pH 7.0, containing 0.1% 2-mercaptoethanol). The dialyzed solution was applied to a hydroxylapatite column (1.5 × 16.0 cm) equilibrated with Buffer B. The enzyme was eluted at around 10 mM potassium phosphate (pH 7.0) with a linear gradient (0–50 mM potassium phosphate). The active fraction was dialyzed thoroughly against 20 mM potassium phosphate buffer containing 0.1% 2-mercaptoethanol and 10% glycerol, and used as the homogeneous enzyme preparation.

Enzyme and protein assays. AAMS amidohydrolase activity was determined by measuring the initial decrease in A_{261} of AAMS at 25°C in a reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0), 0.025 mM AAMS and the enzyme. One unit of enzyme was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of the substrate per min. The substrate concentration was near the K_m value to make the original absorbance at 261 nm precisely measurable. The optimum pH of the enzyme was determined with a 100 mM GTA buffer (pH 5.49–8.50), which is a universal one consisting of 33.3 mM 3,3-dimethylglutaric acid, 33.3 mM Tris, and 33.3 mM 2-amino-2-methyl-1,3-propanediol.

Protein concentration was measured by the dye-binding method with bovine serum albumin as a standard (13). The concentration of the purified enzyme was also determined from the molecular absorption coefficient ($\epsilon=23,040 \text{ M}^{-1} \text{ cm}^{-1}$) at 280 nm determined on the basis of the amino acid composition (14). The reaction conditions for measuring the effect of pH and temperature on the enzyme activity are shown in the legends of the figures.

When the effect of metal ions on enzyme activity was determined, the enzyme was incubated with 10 mM EDTA on ice for 3 h and then the incubated solution was applied to a Sephadex G25 column (PD-10, GE Healthcare). The eluted enzyme solution was incubated with 1 mM of Zn^{2+} , Fe^{2+} or Ni^{2+} at 30°C for 1 h, and then the activity was assayed.

Other analytical methods. The molecular masses of the native enzyme and the subunit were determined by gel filtration and SDS-PAGE, respectively, as described previously (7). The N-terminal amino acid sequence of the recombinant enzyme was determined with an Applied Biosystems 492 protein sequencer. NMR spectrum was measured with a Varian MERCURY 300 spectrophotometer.

RESULTS AND DISCUSSION

Preparation of AAMS with the recombinant *E. coli* cells

The time course of bioconversion of MHPC to AAMS was spectrophotometrically followed. MHPC with absorption maximum at 241 nm ($3.7 \text{ mM}^{-1} \text{ cm}^{-1}$) in 0.1 M HCl was completely changed to AAMS with that at 265 nm ($21.3 \text{ mM}^{-1} \text{ cm}^{-1}$) after 24 h under the reaction conditions described above. Then, AAMS in the reaction mixture was purified and crystallized. The over-all yield of AAMS was about 15%.

Molecular cloning and expression of the *mlr6787* gene

The *E. coli* BL21(DE3) cells transformed with the expression plasmid, pET6787, grown at 37°C showed high AAMS amidohydrolase activity: the activity ($0.38 \pm 0.13 \text{ U/mg}$) was about 42-fold higher than the value for *M. loti* cells grown in the synthetic medium

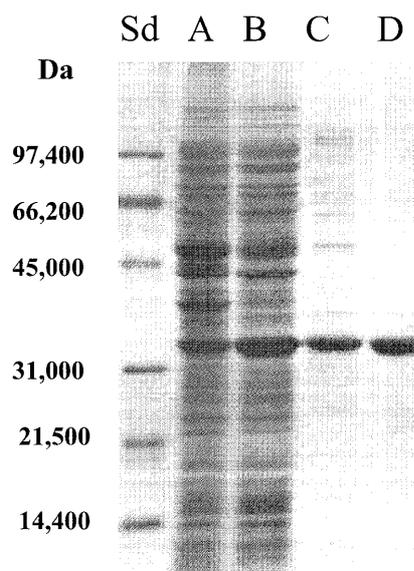


Fig. 2. SDS-PAGE patterns of the crude extract and the purified enzyme fractions from *E. coli* BL21(DE3)/pET6787 cells. Lane A, cell extract (20 μg); lane B, 40–55% saturated ammonium sulfate fraction (20 μg); lane C, the fraction (5.0 μg) from the Butyl-Toyopearl column chromatography; lane D, the fraction (2.8 μg) from the hydroxylapatite column chromatography. The standard proteins were applied to lane Sd.

Table 1. Purification of AAMS amidohydrolase from the transformant *E. coli* cells.

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	654	306	0.468	100
Ammonium sulfate	370	263	0.711	83.6
Butyl-Toyopearl	57.4	65.0	1.14	21.0
Hydroxylapatite	20.9	38.7	1.89	12.4

	1	99
AAMSAH	<u>MDMAADIASDHF</u> <u>SRRVD</u> GR <u>TLNVREK</u> <u>SGSGLMLFFHG</u> <u>TSNSAVFEPLM</u> <u>IRLSDR</u> - <u>FTT</u> <u>IAVDQRGHGLSDKPE</u> ----- <u>TGYEANDYADD</u> <u>AGL</u> <u>IRTLARGH</u>	
AryIE	----- <u>STFVAKDGT</u> - <u>QIYFKDWGSGKPVLF</u> <u>SHGWLLDADMWYQMEYL</u> <u>SSRGYRT</u> <u>AFDRRGFGRSDQPW</u> ----- <u>TGNDYDTFADD</u> <u>AQL</u> <u>IEHLDLKE</u>	
γ Lac	----- <u>GY</u> <u>TVGNENSTP</u> <u>ELYEDQGGSGQPVL</u> <u>HGYPLDGHSWERQ</u> <u>TRELLAQGYRV</u> <u>TYDRRGFGGSSKVN</u> ----- <u>TGYDYDTFAADLHTVLETLDLRD</u>	
FADH	----- <u>MFEGFERRLV</u> <u>DVGDVT</u> <u>INCVVGGSGPALLLLHGFPQNLHMWAR</u> - <u>VAPLLANEYTVV</u> <u>CADLRGYGGSSKPGV</u> <u>GAPDHANYSFRAMASDQRELMRTLGFER</u>	
HADH	---- <u>SLGAKPFGEKFK</u> <u>EIKGRRMAY</u> <u>DEGTGDP</u> <u>LFQHGNTSSYLWRN</u> - <u>IMPHCAGL</u> <u>GRL</u> <u>ACDL</u> <u>GMGSDSKLDPSG</u> - <u>PERYAYAEHRDYL</u> <u>DALWEALDLGD</u>	
	100	192
AAMSAH	- <u>A</u> <u>ILVGHSLGARN</u> <u>SVTAAAKY</u> ----- <u>PDLVRSVVA</u> <u>IDFTPY</u> <u>ETEALDALEARNAGS</u> <u>QLFED</u> <u>KAVEAYLAGRYPN</u> <u>PADA</u> <u>IR</u> <u>RAESGYQP</u> ----- <u>VDGGLR</u>	
AryIE	- <u>VTLVGF</u> <u>SMGGGDVARY</u> <u>ARHGSARVAGL</u> <u>VLLGAVT</u> <u>PLFGQKPDYPQGVPLD</u> <u>VFAFARKTELLKDRAQF</u> <u>ISDFNAPFYG</u> <u>INK</u> - <u>GQVVSQGVQ</u> <u>TQ</u> - <u>TLQIAL</u>	
γ Lac	- <u>VVLVGF</u> <u>SMGTGELARV</u> <u>VARYGHERVAKLAFLASLEPFL</u> <u>VQRDDNPEGVPQVFDG</u> <u>EAAAKGDRFAW</u> <u>FTDFYKNFYNL</u> <u>DENLGSR</u> <u>SEQAVTG</u> - <u>SWNVA</u>	
FADH	- <u>FHLVGHDRGGRTGHRMALDHPDSVLSLAVLD</u> <u>IPTYVMFEEDVRFVARAYWHWYFLQQPAPYPEKV</u> <u>GADPDTFYEGLFGWGATGADGDFPEQLEERYKQWRD</u>	
HADH	<u>RVVLVVHDWGSALGFDWARRHRERVQGI</u> <u>AYMEA</u> <u>AMP</u> <u>EWADFP</u> <u>EQDRDLFQA</u> <u>FRSQAGEELVLQDNVFEQVLPGL</u> <u>ILRPLSEAEMAAYREPF</u> - <u>LAAGEAR</u>	
	193	278
AAMSAH	<u>PLASSAAMAQTARGLRSDLV</u> <u>PAYRDVTKP</u> - <u>VL</u> <u>VRGESSKLVSAALAKTS</u> - <u>RLRPDL</u> <u>PVVVPGA</u> - <u>DHYVNEVSPE</u> <u>TLKA</u> <u>TNF</u> <u>IDA</u> -----	
AryIE	<u>LASLKATVDCVTAFAETDFRPM</u> <u>AKIDVP</u> - <u>TLV</u> <u>IHGDDQI</u> <u>VPFETT</u> <u>GKVAEL</u> <u>I</u> <u>KGAELK</u> <u>VYKDA</u> - <u>PHGFAVTHAQQLNEDLLAFLKRGSHHHHHH</u> -	
γ Lac	<u>GSAPVAAYAVVPAW</u> <u>IE</u> - <u>DFRSDVEAVRAAGKPTL</u> <u>ILHGTKDN</u> <u>ILP</u> <u>DATARRFHQAVPEADYVEVEGA</u> - <u>PHGLLWTHADEVNAALKTFLAK</u> -	
FADH	<u>PAAIHGSCCDYRAGGT</u> <u>IDFELDHGDLGRVQCPALVFSGSAGLMHSLFEMQVWVAPRLANMRFASLPG</u> - <u>GHFFVDRFPDDTAR</u> <u>ILREFLSDARSG</u> <u>HQTERRES</u>	
HADH	<u>RPTLSWPRQIP</u> <u>IAGTPADVVA</u> <u>IARDYAGWLS</u> <u>SESP</u> <u>IPKLF</u> <u>INAEPGAL</u> <u>TGRRMDFC</u> <u>RTWPNQTE</u> <u>ITVAGAHF</u> <u>IQEDSPDE</u> <u>GAA</u> <u>IAAFVRRLRPA</u> -	

Fig. 3. The primary and predicted secondary structures of AAMS amidohydrolase, and alignment of amino acid sequences of the enzyme and some other similar enzymes. In the sequence of AAMS amidohydrolase (AAMSAH), the amino-terminal amino acid residues determined by the sequencer, and β -sheet and α -helix structures predicted by JPRED are shown by underlined, double-underlined, and wave-underlined letters, respectively. The secondary structures determined in aryl esterase (AryIE) from *P. fluorescens* are marked in the same way as those for AAMSAH. The amino acids involved in the catalytic triad in aryl esterase are shown in red letters. γ -Lactamase (γ Lac) from *Aureobacterium* sp., fluoroacetate dehalogenase (FADH) from *Burkholderia* sp., and haloalkane dehalogenase (HADH) from *Sphingomonas paucimobilis* are aligned together. Amino acid residues common in 5 sequences are shown in blue letters.

containing pyridoxine. The *E. coli* cells transformed with pET21a showed no measurable AAMS amidohydrolase activity. Thus, mlr6787 gene encodes AAMS amidohydrolase.

Purification and properties of the recombinant AAMS amidohydrolase

The recombinant AAMS amidohydrolase was purified to homogeneity from the transformed *E. coli* by the ammonium sulfate fractionation and two steps of column chromatography (Fig. 2 and Table 1). The purified enzyme preparation gave a single protein band corresponding to a molecular weight of $34,000 \pm 1,000$ (an average of three experiments \pm SD) on SDS-PAGE. The value was slightly higher than that ($M_r = 29,896$) of the predicted protein encoded by mlr6787 gene. The molecular weight of the native enzyme was found to be $64,000 \pm 2,000$ on gel filtration. The amino terminal

10-amino acid sequence was MDMAADIASD, coinciding with that deduced from the nucleotide sequence of the mlr6787 gene. Thus, the enzyme was a dimeric protein.

The primary structure and predicted secondary structure of AAMS amidohydrolase is shown in Fig. 3. The recombinant enzyme consisted of 273 amino acid residues. The predicted secondary structure contained 8 β -strands and 9 α -helices.

The optimum pH of the enzyme was 7.0 in the 100 mM GTA buffer (Fig. 4A). When the enzyme activity was determined in various kinds of buffers, different optimum pH values were obtained: pH 8.0 in Tris-HCl; pH 7.0 in sodium pyrophosphate buffer; and pH 7.5 in potassium or sodium phosphate buffer. Thus, the *M. loti* enzyme also showed an ionic environment-dependency like the *Pseudomonas* one (2).

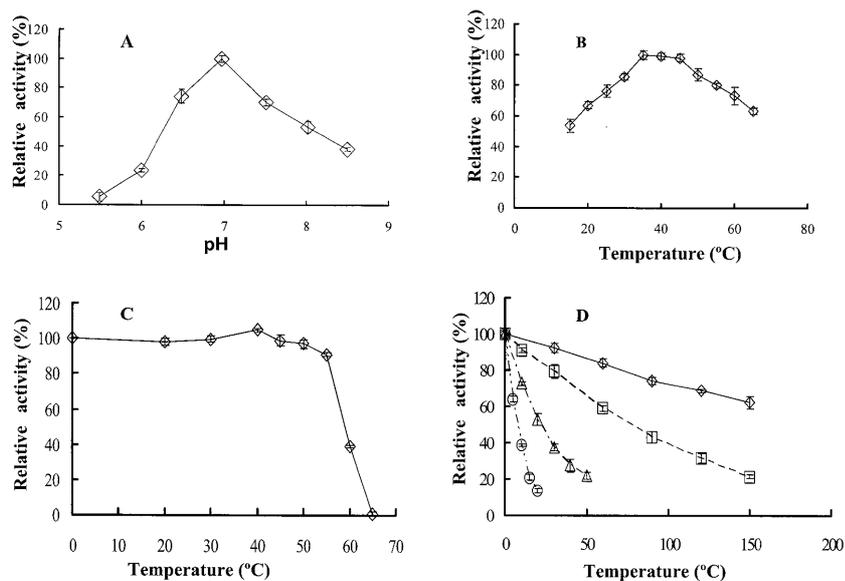


Fig. 4. Optimal pH and temperature, and temperature stability of AAMS amidohydrolase. A: the effect of pH on activity. The reaction was carried out in the GTA buffer (pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5). B: the optimum temperature. The reaction was carried out in 0.1 M potassium phosphate buffer (pH 7.0) using 0.025 mM AAMS as a substrate. The activity was determined under the standard condition at 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, and 65°C. C: temperature stability. The enzyme solution was incubated at various temperatures for 10 min. The activity of the enzyme incubated at 0°C was 100%. D: the half lives at various temperatures. The enzyme was incubated at 50°C (diamonds), 55°C (squares), 58°C (triangles), and 60°C (circles).

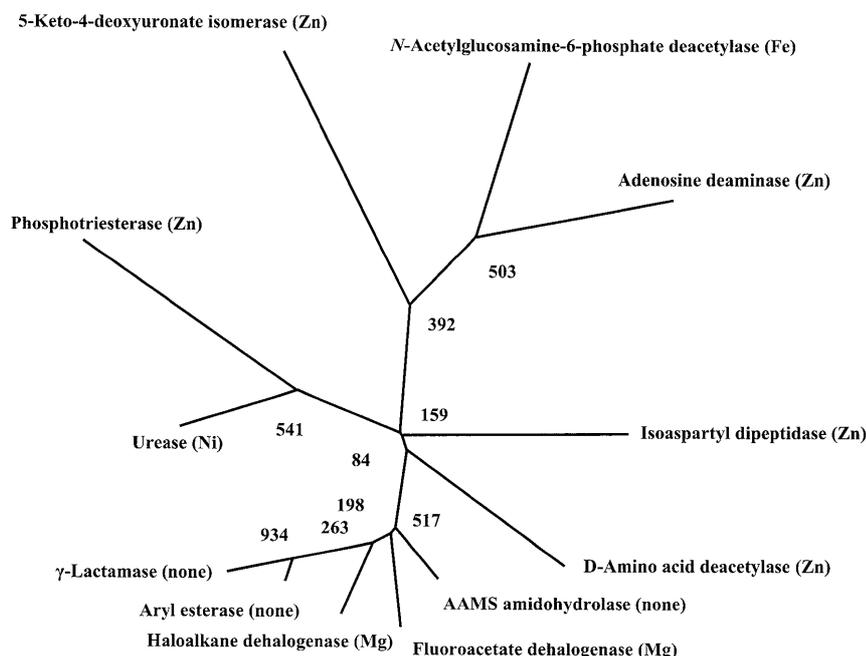


Fig. 5. Unrooted phylogenetic tree of hydrolases. Amino acid sequences of eleven hydrolases, whose substrate specificity is characterized, were aligned by CLUSTALW, and an unrooted tree was determined. The numbers show bootstrap values. Metals required by them are also shown. PDB ID of the enzymes are *N*-acetylglucosamine-6-phosphate deacetylase, 1O12; adenosine deaminase, 1A4M; *D*-amino acid deacetylase, 1M7J; aryl esterase, 1VA4; fluoroacetate deacetylase, 1Y37; haloalkane dehalogenase, 1K5P; isoaspartyl dipeptidase, 1ONW; 5-keto-4-deoxyuronate isomerase, 1XRU; γ -lactamase, 1HKH; phosphotriesterase, 1DPM; and urease, 1IE7.

The enzyme showed the maximum activity at 35°C in 0.1 M potassium phosphate buffer (pH 7.0) (Fig. 4B). An Arrhenius plot of the optimum temperature gave $5,250 \pm 360 \text{ cal mol}^{-1}$ of the activation energy for the hydrolysis of AAMS. The enzyme was stable at 50°C or lower for 10 min (Fig. 4C). The half life times of the

enzyme were 193.6, 77.3, 21.6, and 13.6 min at 50, 55, 58, and 60°C, respectively (Fig. 4D).

Typical Michaelis-Menten type kinetics were observed when the effect of the AAMS concentration on the reaction rate was examined. The K_m and k_{cat} for AAMS were $53.7 \pm 6 \mu\text{M}$ and $307.3 \pm 12 \text{ min}^{-1}$, respec-

tively. Thus, the recombinant *M. loti* and *Pseudomonas* AAMS amidohydrolases shared the K_m value, the ionic environment-dependency of optimum pH, the metal ion-independency, and molecular weight of the subunit (3).

Under the assay conditions no activation or inhibition was observed upon addition of any one of the following metal ions: Zn^{2+} , Fe^{2+} , or Ni^{2+} (each 1 mM). The enzyme activity was not affected when it was incubated at 30°C for 30 min in Buffer A containing 100 mM of EDTA. The results suggested that the enzyme contains no metal.

Amino acid sequence comparisons

The amino acid sequence of AAMS amidohydrolase was compared with other proteins in the PROSITE database. The sequence has no signature sequence of defined protein families, including amidohydrolase family proteins. Then, the sequence was analyzed by BLAST: the enzyme showed no identity to amidohydrolases so far reported. However, it showed low but significant (about 27%) homology to fluoroacetate dehalogenase (PDB code, 1Y37) (15), haloalkane dehalogenase (1K5P) (16), and aryl esterase (1VA4) (17). Amino acid sequences of eleven hydrolases, whose substrate specificity is characterized, were aligned by CLUSTALW, and an unrooted tree was determined (Fig. 5). AAMS amidohydrolase made a cluster with fluoroacetate dehydrogenase, haloalkane dehalogenase, aryl esterase, and γ -lactamase, showing that these enzymes have low but significant amino acid sequence identity.

The alignment of the amino acid sequences of the enzymes in the cluster is shown in Fig. 3. Although overall identity in amino acid sequence is low, several amino acid residues are common in these enzymes, showing that they play essential roles in the catalysis of hydrolytic reaction. Interestingly, the distribution of secondary structures in AAMS amidohydrolase and aryl esterase is quite similar, suggesting that they have a similar tertiary structure. The aryl esterase, which is a metal-independent hydrolase, has the catalytic triad (Ser94, Asp222, and His251) (17) which is shown in red colors in Fig. 3. AAMS amidohydrolase has two corresponding amino acid residues (His258 and Ser106) but lacks one. Because AAMS amidohydrolase is also metal-independent as shown here, and should have the catalytic triad, aspartic acid residue corresponding to Asp222 in aryl esterase should exist. We are continuing our investigation into the tertiary structure and reaction mechanism of AAMS amidohydrolase.

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