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# Molecular Characterization of a Novel Bacterial Aryl Acylamidase Belonging to the Amidase Signature Enzyme Family

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**In seeking aryl acylamidase (EC 3.5.1.13) acting on an amide bond in *p*-acetaminophenol (Tylenol™), we identified a novel gene encoding 496 residues of a protein. The gene revealed a conserved amidase signature region with a canonical catalytic triad. The gene was expressed in *E. coli* and characterized for its biochemical properties. The optimum pH and temperature for the activity on *p*-acetaminophenol were 10 and 37°C, respectively. The half-life of enzyme activity at 37°C was 192 h and 90% of its activity remained after 3 h incubation at 40°C. Divalent metals was found to inhibit the activity of enzyme. The $K_m$ values for various aryl acylamides such as 4-nitroacetanilide, *p*-acetaminophenol, phenacetin, 4-chloroacetanilide and acetanilide were 0.10, 0.32, 0.83, 1.9 and 19 mM, respectively. The reverse reaction activity (amide synthesis) was also examined using various chain lengths ($C_1$~$C_4$ and $C_{10}$) of carboxylic donors and aniline as substrates. These kinetic parameters and substrate specificity in forward and reverse reaction indicated that the aryl acylamidase in this study has a preference for aryl substrate having polar functional groups and hydrophobic carboxylic donors.**

## INTRODUCTION

Amidase signature (AS) enzymes belong to one of the amidase superfamilies catalyzing the hydrolysis of amide bonds (-CO-NH-). The sequences and functions of these proteins are found in almost all living organisms across three domains of life (Chebrou et al., 1996). The AS sequence, as the name indicates, is an approximate 160-residue conserved region found in all AS enzymes (Chebrou et al., 1996). AS enzymes are divided into several subfamilies by their molecular function, including peptide amidases (Neumann and Kula, 2002; Valina et al., 2004), fatty acid amide hydrolases (McKinney and Cravatt, 2005), malonamidases (Shin et al., 2002), and subunit A of Glu-tRNA amidotransferases (Kwak et al., 2002). These enzymes show a broad spectrum of substrate specificity and sequence diversity, but the structural architecture of the catalytic triad (Lys-Ser-Ser) is highly conserved, indicating that these enzymes are non-classical serine hydrolase and possess a remote evolutionary relationship among subfamilies (Chebrou et al., 1996; Shin et al., 2002).

Aryl acylamidase (EC 3.5.1.13; AAA) is an enzyme that acts on the amide bond between aryl and acyl compounds. The most typical reaction is the hydrolysis of an anilide, producing a carboxylate and aniline, which is reversible (Fig. 1). Since the AAA activity has been identified in many living organisms ranging from bacteria (Engelhardt et al., 1973; Heumann, 2009; Hoagland and Zablotowicz, 1995; Vaughan et al., 1990; Yoshioka et al., 1991) to plants (Gaynor and Still, 1983; Fukuda et al., 1997) to animals (Fujimoto, 1974; George et al., 1985; Masson et al., 2007), the biochemical characteristics of many AAAs have been reported but detailed molecular information (e.g. sequences or structures of proteins by the name of AAA) is lacking. Only a few gene sequences can be found in public databases such as Genbank or Uniprot under the name of AAA or the same EC number (3.5.1.13). The activities of bacterial AAAs have been identified from several microorganisms including *Arthrobacter* (Villarreal et al., 1994), *Bacillus* (Engelhardt et al., 1973), *Corynebacterium* (Villarreal et al., 1994), *Pseudomonas* (Hammond et al., 1983), *Rhodococcus* (Vaughan et al., 1990), and *Nocardia* (Sonja Heumann, 2009), but only a few AAAs are elucidated at the gene level (Kizaki et al., 2006; Sonja Heumann, 2009).

In contrast to the activities of eukaryotic AAAs, which have mainly been studied in relation to neural development in animals (Boopathy and Layer, 2004; Fujimoto, 1974; Masson et al., 2008; Montenegro et al., 2008) or herbicide resistance in plants (Fukuda et al., 1997; Gaynor and Still, 1983; George et al., 1985), bacterial AAAs have been highlighted for several biotechnological applications. One attempt is the biodegradation of toxic chemicals such as xenobiotic herbicides in the environment (Hoagland and Zablotowicz, 1995; Villarreal et al., 1994; Zablotowicz et al., 2001). Since acyl anilide herbicides (e.g. the rice herbicide, propanil) can contaminate the soil environment, bacterial AAAs, by attacking the amide bonds in those chemicals, reduce their concentrations in soil and can be used for bioremediation (Hoagland and Zablotowicz, 1995; Yasuhira et al., 2007; Zablotowicz et al., 2001). Another application is for the detection of *p*-acetaminophenol (Tylenol™) in clinical tests. *p*-Acetaminophenol has an amide bond conforming from acetic

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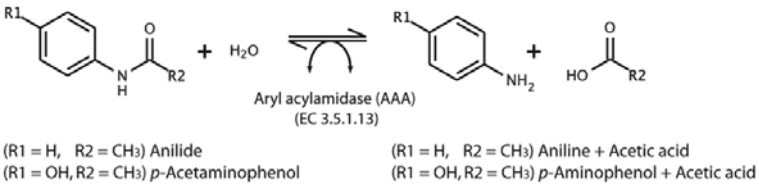



**Fig. 1.** A typical reaction mediated by aryl acylamidase (EC 3.5.1.13; AAA).

acid and *p*-aminophenol. Because AAA acts only on non-peptide amide bonds, the bioassay of *p*-acetamino-phenol in biological fluids using AAA has been proposed and provided as a template for a biosensor system (Hammond et al., 1981; Morris et al., 1990; Vaughan et al., 1990). The other application is the synthesis of an aryl acylamide using the reverse reaction of AAA, which can provide a biocatalytic method to prepare a novel aryl acylamide substance. One known example is the synthesis of aniline analgesics (*p*-acetaminophenol) by AAA (Hwang, 1995).

Here, we present a novel bacterial AAA gene identified from a genomic library of a soil bacterium, which was isolated using a selection media having *p*-acetaminophenol as a sole carbon source, and deposited in Genbank (accession number: FJ 755834). The sequence analysis of the gene revealed that it belongs to the AS enzyme family. We examined the evolutionary relationship with other known AS enzymes and categorized them into six subfamilies by their core AS regions. In order to inspect the biochemical properties, we overexpressed the gene in *E. coli* and examined the enzymatic characteristics and kinetic parameters of the gene on various substrates, suggesting potential biotechnological applications using the AAA gene.

## MATERIALS AND METHODS

### Bacterial strains and plasmids

We used *E. coli* NM522 and *E. coli* DH5$\alpha$ for construction of a genomic library and as general cloning hosts. *E. coli* BL21(DE3) was used as a host for protein expression. For construction of the genomic library, we employed pBluescript II KS (+) (Stratagene, USA). A modified pET21a vector for ligation independent cloning (LIC) was used for recombinant protein production, which was kindly provided by Berkeley Structural Genomics Center (BSGC). *E. coli* NM522 and *E. coli* DH5$\alpha$ were routinely cultured at 37°C in Luria-Bertani (LB) (Difco, USA) medium with ampicillin (100 $\mu$g /ml), and *E. coli* BL21(DE3) harboring the recombinant plasmid was cultured at 25°C in auto-inducing medium ZYM-5052 with ampicillin (100 $\mu$g/ml), according to the Studier's protocol provided by BSGC (Kim et al., 2008; Studier, 2005).

### Cloning of an aryl acylamidase gene from the genomic library of a soil bacterium

A soil bacterium was isolated as described in a previous study (Hwang, 1995). Briefly, the procedures were as follows. Soil samples were collected from various areas in southern Korea. The samples were suspended in saline solution and filtered with Whatman No.1 filter paper. The filtrates were inoculated into selection media containing 0.1% *p*-acetaminophenol as a sole carbon source. A single isolate was separated from the selection medium plate after a series of transfers of active cultures. To construct a genomic library for screening a gene showing aryl acyl amidase (AAA) activity, we extracted the chromosomal DNA using a commercial kit (Comso Genetech, Korea). The genomic DNA was digested with *Bam*HI (NEB, USA) and ligated with pBluescript II KS (+) treated with the same restriction enzyme by T4 ligase (NEB, USA), according to the manufacturers protocol. The library was screened in LB media containing 0.1% (wt/vol) *p*-acetaminophenol. A colony having AAA activity was chosen by the release of a purple-red color that originated from the color of the reaction product, *p*-aminophenol. In order to acquire the entire nucleotide sequence information from the colony, an insert DNA fragment of the clone was sequenced (Macrogen, Korea) with serial sequencing primer design. The nucleotide sequence of the potential AAA gene was deposited into Genbank (accession number: FJ755834).

### Sequence analysis of the aryl acylamidase gene

The nucleotide and protein sequence of the AAA was searched against the NCBI Genbank non-redundant database using TBLASTX and BLASTP (Altschul et al., 1990). To compare with the amidase signature (AS) enzyme family, the multiple sequence alignment of representative members of the AAA was collected from the Pfam database (Finn et al., 2008) (seed sequences of PF01425). The Phylip package (Retief, 2000) was used for phylogenetic tree construction as follows: 1) bootstrapped the aligned sequences 100 times with 'Seqboot', 2) calculated the distance matrix by 'Protdist', and 3) built the tree by the neighbor-joining method (Neighbor) with default parameters. The consensus tree was determined by 'Consense' program.

### Expression and purification of recombinant aryl acylamidase

In order to study the functional characteristics of the AAA protein, we cloned the gene in a modified pET21a having a 6-histidine tag at the carboxy terminus for affinity chromatography purification. *E. coli* BL21(DE3), having the plasmid harboring the recombinant AAA gene, was grown in 1 L of autoinducing media with ampicillin (100 $\mu$g/ml) for 16 h at 25°C and 200 rpm. The cells were harvested by centrifugation at 5,000 $\times$ g for 30 min at 4°C, resuspended in 0.1 M Tris/HCl buffer (pH 7.4), and disrupted by sonication for 15 min at 4°C. The crude cell extracts were centrifuged at 15,000 g (50 min and 4°C) to remove the cell debris. The resulting supernatant solution was placed on a histidine affinity column (HiTrap HP, GE Healthcare, USA) equilibrated with 20 mM Tris/HCl buffer (pH 7.4) in an LP system (Bio-Rad, USA). The rate of sample loading and column elution was maintained at 3.0 ml/min by the LP system. The enzyme was eluted by a linear gradient of imidazole (0-0.5 M) included in the same buffer, and the active fractions were collected. The combined fractions were concentrated with a 30 kDa MWCO Centricon (3,000 g for 1-2 h) and stored at 4°C for the enzymatic assay.

### Determination of aryl acylamidase activity

The enzymatic activity was determined by the protocol described by Hammond et al. (Hammond et al., 1981). The sample containing the recombinant AAA (rAAA) in 0.1 ml was added to 0.9 ml of reaction mixture (0.1 M Tris buffer pH 9.0 and 100 mM *p*-acetaminophenol). The reaction was carried out for 10 min at 37°C and stopped with the addition of 2 ml of 1%

(vol/vol) *o*-cresol and 0.2 ml of 0.2% (wt/vol) $CuSO_4$ in 1.6% (vol/vol) $NH_4OH$, in which *p*-aminophenol reacted with *o*-cresol releasing a blue color. After 10 min of incubation at room temperature, the amount of *p*-aminophenol was determined by a spectrophotometer (615 nm). A unit of enzyme was defined as the amount of enzyme hydrolyzing 1 μmole of *p*-acetaminophenol for 1 min at 37°C. The enzymatic thermostability was examined under the various temperature conditions by thermal inactivation. The proteins were incubated for 3 h at different temperatures between 4 and 80°C and determined the activity remained. For the stability test, the proteins were incubated at 37°C for 10 days measuring the residual activity at time interval.

**Activity assay on various substrates and kinetic analysis**

In order to check the substrate specificity, we additionally tested for acetanilide, 4-chloroacetanilide, 4-nitroacetanilide and phenacetin using same reaction condition above (10-100 ng of enzymes in 0.1 M Tris buffer pH 9.0 with 100 mM of each substrate). All substrates were purchased from Sigma Co. The hydrolysis products were measured by a modified diazocoupling method (aniline and 4-chloroaniline) described in (Hiramatsu, 1982). The reaction was stopped by adding 750 μl of acid mixture (acetic acid : HCl : water = 4 : 1 : 1), 50 μl of 2% (wt/vol) $NaNO_2$ and 1 ml of cold water. After standing for 5 min at 0°C for diazotization, 100 μl of 10% (wt/vol) sulfamic acid, 50 μl of 1% (wt/vol) 1-naphthylethylene diamine and 300 μl of water were added and incubated for 20 min at room temperature. The purple color developed in the mixture was determined spectrophotometerically at 540 nm. Phenetidine (the hydrolysis product of phenacetine) was determined by the molar extinction coefficients (phenetidine; $\varepsilon_{300}$ = 1.696 $mM^{-1}cm^{-1}$ in assay buffer) (Alt et al., 1975). One unit of activity was defined as the amount of enzyme which catalyses the hydrolysis of 1 μmole substrate for 1 min at 37°C. The kinetic analysis was carried out in a 96-well microplate with the same reaction mixture varying the concentrations of enzyme and substrate. Kinetic parameters ($K_m$ and $V_{max}$) were calculated with a conventional Lineweaver-Burk plot (Lineweaver and Burk, 1934).

**Activity assay for the reverse reaction (amide synthesis)**

To test the reverse reaction activity (amide synthesis), we used 6.2 μg of the protein and 80 mM of substrates (aniline and various mono-carboxylic acids) in 250 μl of reaction buffer (0.1 M citrate buffer, pH 5.0). The reaction was carried out for 3 h at 37°C. The amount of decreased aniline was determined by a modified diazocoupling method as described above.

## RESULTS

**Identification and cloning of a novel aryl acylamidase**

Amidases are ubiquitous enzymes found in living organisms, and are divided into various subfamilies depending on molecular function and substrate specificity. Among those subfamilies, we focused on the activity of aryl acylamidase (AAA) acting on the hydrolysis of amide bonds in aryl acylamides for potential biotechnological applications. The typical reaction of AAA is shown in Fig. 1. In order to clone an AAA gene, we considered a soil bacterium having AAA activity using an enrichment media with *p*-acetaminophenol as a sole carbon source (Hwang, 1995). We extracted genomic DNA from the soil bacterium and constructed a genomic library. The isolated microorganism was initially identified as *Pseudomonas* sp. but accidentally lost during the experiment before performing 16S rRNA analysis and thus the taxonomic origin remained unidentified except it was a Gram-negative bacterium. Because AAA hydrolyzes the amide bond in aryl acylamides such as *p*-acetaminophenol, we developed a quick and easy screening method based on the color of reaction product. *p*-Acetaminophenol is basically a colorless compound, however, its reaction product by AAA, *p*-aminophenol, gives a purple-red color. As a result, a clone presenting AAA activity from the genomic library gave a color change around the colony when it was grown on solid media enriched with *p*-acetaminophenol (Supplementary Fig. S1). We chose a single colony changing the color by visual inspection, extracted the plasmids, and sequenced the entire insert DNA fragment. The size of the insert was 2.3 kb and we identified 496 residues (53.2 kDa) in an open reading frame (ORF) of a potential AAA gene within the insert fragment. The Pfam domain analysis (Finn et al., 2008) of the ORF sequence indicated that the ORF belonged to the amidase signature (AS) enzyme family (Pfam id: PF01425; amidase). The ORF was also searched against the NCBI Genbank using BLAST (Altschul et al., 1990). The top hit of the results was a glutamyl-tRNA(Gln) amidotransferase subunit A (Glu-ADT subunit A) of a Gram-negative extreme thermophile, *Thermomicrobium roseum* DSM 5159 (*E*-value = $4 \times 10^{-132}$; 50% of sequence identity. Although the Glu-ADT subunit A is known as a subfamily of the AS enzymes (Chebrou et al., 1996), the annotation of the hit sequence seemed to be an error, common in a high throughput annotation pipeline, because the majority of blast hits were indicating other amidases belonging to different subfamilies of AS enzymes. In addition, the sequence identity in high scoring pairs among these hits was less than 50%, revealing that the ORF could be sequentially diverged from other known AS enzymes. Interestingly, amidase domains (PF01425) are found in 927 species spanning three domains of life and the number of members is 4,978, revealing sequence and substrate diversity (average sequence identity among domains is 26%) as one of the largest protein family (Finn et al., 2008). The whole nucleotide sequence of the potential AAA gene was deposited into a public database (Genbank accession number: FJ755834).

**Phylogenetic analysis of the novel aryl acylamidase gene**

Even though the AAA gene had many Blast hits and identified as a member of the AS enzyme family, the molecular function designated by the high scoring hits was ambiguous to describe the activity of the cloned gene. In order to locate the identified AAA gene among the AS enzyme subfamilies, and thus make a more precise annotation of the molecular function, we built a phylogenetic tree using the seed sequences employed in the Pfam model (PF01425) of the AS enzyme family. The seed sequences in the tree showed low sequence identity with the AAA gene (ranging 17-32%) but the AS region was conserved in those sequences. The AS enzymes were initially divided into five subfamilies depending on sequence similarity: 1) AMD (bacterial amidases), 2) IAAH (indole-acetamide hydrolases), 3) EAI (eukaryote amidases 1), 4) EA2 (eukaryote amidases 2) and 5) AH (6-arninohexanoate-cyclic-dimer hydrolases) (Chebrou et al., 1996). The phylogenetic tree based on the amino acid sequence built in this study clearly indicated those five subfamilies represented as indole-acetamide hydrloases (IAAH), 6-aminohexanoate-cyclic-dimer hydrolases (AH), eukaryotic amidase group I (EA1), and eukaryotic amidase group II [EA2; eukaryotic fatty acid amide hydrolases (FAAH)], respectively (Fig. 2). The 'subunit A of Glu-tRNA(Gln) amidotransferases' (GATA) subfamily was not assigned as a subfamily in previous report (Chebrou et al., 1996) but appeared as a distinct subfamily in this study. The cloned AAA gene was closely located to the AH subfamily (Fig. 2).

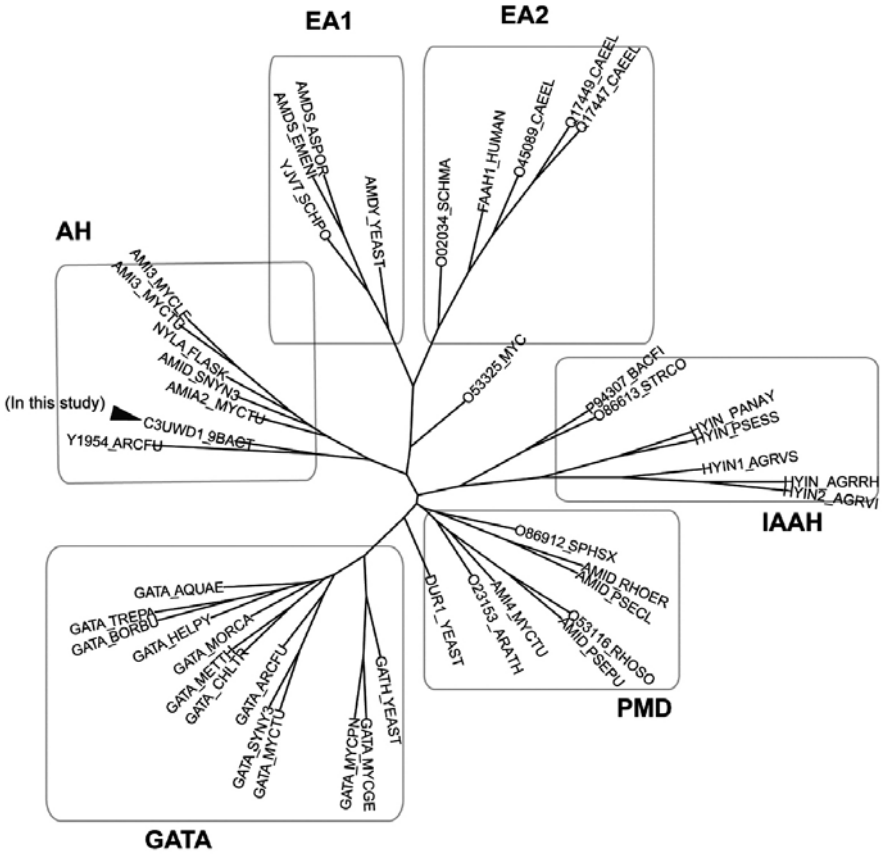



**Fig. 2.** Phylogenetic analysis of the aryl acylamidase (AAA) gene with representative members in the AS enzyme family. The sequences were collected from seed sequences used to build a hidden Markov model in the Pfam database (PF012540) (Finn et al., 2008). The multiple alignment was extracted from the full alignment of PF012540 and used for tree construction by 100 boot strapped neighbor-joining methods in the Phylip package (Retief, 2000). Each box in the tree indicates the subfamilies of the AS enzymes, which were based on the previous classification (Chebrou et al., 1996) and manually determined. The AAA gene in this study is indicated by an arrow in the tree. The UNIPROT database identifier of each member used in the tree is as follows. Subunit A of the Glu-tRNA (Gln) amidotransferase (GATA) family: GATA_MYCGE, GATA_MYCPN, GATH_YEAST, GATA_MYCTU, GATA_ARCFU, GATA_SYNY3, GATA_MORCA, GATA_AQUAE, GATA_HELPY, GATA_BORBU, GATA_TREPA, GATA_METTH and GATA_CHLTR; Peptide amidase (PMD) family: AMID_RHOER, AMID_PSECL, AMID_PSEPU, O23153_ARATH, Q53116_RHOSO, O86912_SPHSX, AMI4_MYCTU and DUR1_YEAST; Indole-acetamide hydrloase (IAAH) family: HYIN2_AGRVI, HYIN_AGRRH, HYIN1_AGRVS, HYIN_PANNY, HYIN_PSESS, P94307_BACFI and O86613_STRCO; 6-aminohexanoate-cyclic-dimer hydrolase (AH) family: C3UWD1_9BACT (AAA in this study), Y1954_ARCFU, AMID_SYNY3, AMIA2_MYCTU, NYLA_FLASK, AMI3_MYCLE, AMI3_MYCTU and C3UWD1_9BACT; Eukaryotic amidase 1 (EA1): AMDS_ASPOR, AMDS_EMENI, AMDY_YEAST and YJV7_SCHPO; Eukaryotic amidase 2 (EA2, fatty acid amide hydrolase (FAAH) family): Q17449_CAEEL, Q17447_CAEEL, O45089_CAEEL, FAAH1_HUMAN and O02034_SCHMA.

### Biochemical characteristics of recombinant aryl acylamidase

To characterize the biochemical properties of the AAA gene, we attempted to overexpress the protein in an *E. coli* system. Initially, the recombinant AAA (rAAA) was mainly expressed as inclusion bodies in *E. coli* BL21 (DE3) with 0.1 mM isopropyl thiogalactoside (IPTG) induction either at 37°C or 16°C in LB media. However, after we changed the LB media to autoinduction media [originally developed by Studier (2005) and modified at Berkeley Structural Genomics Center (BSGC) (Kim et al., 2008)], we could obtain soluble forms and successfully purified them with His-tag affinity chromatography for further biochemical study. Using the purified protein, we determined the AAA activity at various pHs and temperatures using *p*-acetaminophenol as a substrate. The optimum pH of the recombinant protein was around 10 (Fig. 3A). The maximum activity of the protein was reached at 37°C and abruptly decreased over 42°C (Fig. 3B).

### Stability of recombinant aryl acylamidase

Although the optimum temperature for the activity was around 37°C, the protein was maintained its initial activity at 4°C for 6 months without any protective agent when we stored the protein in 0.1 M Tris buffer pH 9.0. In order to check the stability of rAAA, we determined the half-life ($T_{1/2}$) of enzymatic activity at 37°C. The activity was maintained almost 10 days and $T_{1/2}$ was

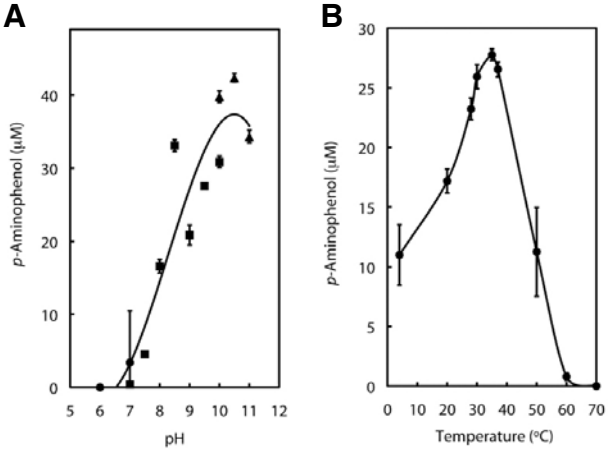



**Fig. 3.** The effect of pH (A) and temperature (B) on aryl acylamidase (AAA) activity. The activity is expressed as the amount of *p*-aminophenol produced when *p*-acetaminophenol was used as the substrate at the given conditions. Different compositions of buffers were used depending on the pH condition: pH 4-7, 100 mM $KH_2PO_4$ (solid circle); pH 7-10, 100 mM Tris Cl (solid rectangle); pH 10-11, 100 mM $H_3BO_3$ (solid triangle). All measures were in triplicate samples and the error bar indicates the standard deviation of those measures.

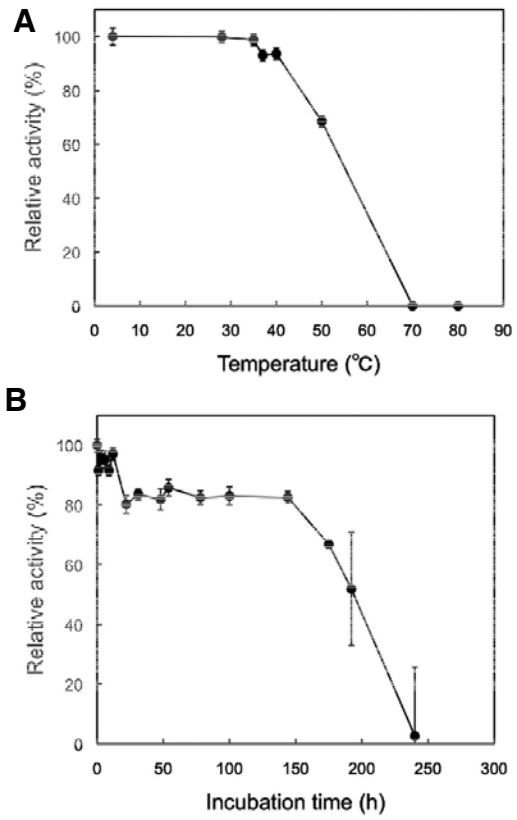



**Fig. 4.** Stability of aryl acylamidase. (A) thermal inactiviation after incubating for 3 h at various temperatures and (B) stability of the protein at 37°C. The activity was expressed as the residual activity comparing to that of the starting point.

around 8 days (192 h) (Fig. 4B). We also tested its thermostability by incubating proteins for 3 h at various temperatures. The activity was remained more than 90% of initial activity at 40°C but became partially inactivated at 50°C and completely inactivated above 70°C after 3-h incubation (Fig. 4A).

### Inhibition by divalent metal cations

Because heavy metals were known to inhibit the activity of AS enzymes (Hammond et al., 1983; Toogood et al., 2004), we examined the effect of various divalent metal cations to check whether they can be potential inhibitors for AAA. As seen in Table 1, most divalent cations decreased the relative activity of the enzyme in the range of 0.1-10 mM but could not completely inhibit the activity. Among divalent cations tested, $Zn^{2+}$ gave the strongest inhibition at 10 mM.

### Kinetic analysis of recombinant aryl acylamidase and substrate specificity for amide synthesis

To examine the substrate specificity, we measured the $K_m$ value against several aryl acylamide compounds structurally similar to *p*-acetaminophenol. As shown in Table 2, 4-nitroacetanilide and *p*-acetaminophenol showed $K_m$ values of 0.10 mM and 0.32 mM, respectively, which were comparable to 0.11 mM (*p*-acetaminophenol) for *Rhodococcus* AAA (Vaughan et al., 1990), but a little higher than 0.069 mM (nitroacetanilide) for *Pseudomonas* AAA (Hammond et al., 1983).

Since most amidases were known to have reverse reaction activity, one potential application of AAA could be synthesis of various aryl acylamide compounds (amide synthesis). In order to find optimum pH for the reverse reaction activity, *p*-acetaminophenol was synthesized by AAA using *p*-aminophenol and acetate as substrate at various pH and found most active at weak acidic pH range between 5.0 and 7.0 (Fig. 5). We also tested the reverse reaction activity on various lengths of carboxylic acids [formate ($C_1$) to decanoate ($C_{10}$)] as acyl donors to aryl compound, aniline. The relative activity slightly increased along with the number of carbons in the carboxylic acids used, implying that hydrophobic substrates are more preferred for amide synthesis; however, we could not test beyond decanoate ($C_{10}$) due to solubility problems (Table 3).

Since known eukaryotic AAAs are intrinsically esterases (Balasubramanian and Bhanumathy, 1993; Boopathy and Layer, 2004; Montenegro et al., 2008), we tested the esterase activity of the cloned AAA on an aryl acylester (phenylacetate) as a substrate in which no esterase activity was detected (data not shown).

**Table 1.** Effect of various divalent metal cations on the activity of aryl acylamidase (AAA) using *p*-acetaminophenol as substrate. The enzymatic activity of AAA was expressed as the relative activity to the reaction condition having no metal cations.

| Divalent Cations | Concentration (mM) | | |
|---|---|---|---|
| | 0.1 | 1 | 10 |
| $Ca^{2+}$ | 88.6 | 78.9 | 78.5 |
| $Ni^{2+}$ | 78.0 | 74.0 | 56.8 |
| $Co^{2+}$ | 90.9 | 85.9 | 82.6 |
| $Zn^{2+}$ | 67.7 | 60.0 | 24.2 |
| $Cu^{2+}$ | 90.6 | 86.6 | 54.3 |

## DISCUSSION

In this report, we developed a simple but robust screening strategy for the AAA gene. Because colorless *p*-acetaminophenol gives a purple-red colored *p*-aminophenol by AAA activity and the color change can be easily detected visually or spectrophotometrically, we may employ the cloned AAA gene to develop a simple reporter system using an inexpensive chromogenic substrate (*p*-acetaminophenol; Tylenol™). For instance, we designed a heavy metal detection system in *E. coli* using AAA as a reporter gene and presented in iGEM2009 (international Genetically Engineered Machine) which is a premier undergraduate 'synthetic biology competition' held yearly by MIT (http://2009.igem.org). The AAA reporter system showed visual color changes on *p*-acetaminophenol by the AAA expression under the heavy metal sensing promoters (http://2009.igem.org/Team:KU_Seoul).

The molecular function of AS enzymes is to hydrolyze amide bonds in various substrates in the natural environment. Although many proteins have been annotated to the AS enzyme family (at present 4,978 proteins by the Pfam database, release 24.0), only a small number of members have been functionally examined, and the molecular functions of others are not yet determined. Since AS enzymes are regarded as non-classical serine hydrolase having invariant catalytic triad (Lys-Ser-Ser) and the sequences are highly diverged except the AS region (Shin et al., 2002), the substrate specificity and biological functions of these enzymes may not be inferred by a simple sequence comparison. Initial annotation of the AAA gene based on the BLAST search revealed that the gene might be a member of GATA subfamily but the annotation of hit seemed to be wrong because other hits were mainly annotated as putative

**Table 2.** The kinetic parameters of aryl acylamidase on various substrates (acetanilide, 4-nitroacetanilide, 4-chloroacetanilide, penacetin and *p*-acetaminophenol). The parameters were obtained from the Lineweaver-Burk plot for the hydrolysis of aryl amide compounds by aryl acylamidase as described in "Materials and Methods".

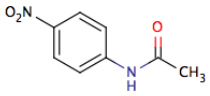
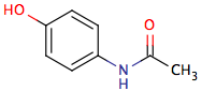
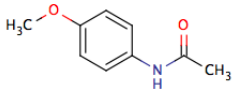
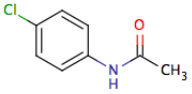
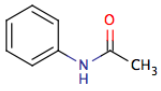
| Substrate | | $K_m$ (mM) | $V_{max}$ (μmole μg$^{-1}$ min$^{-1}$) | $V_{max}/K_m$ |
|---|---|---|---|---|
|  | 4-Nitroacetanilide | 0.10 ± 0.03 | 1.71 ± 0.03 | 17.75 ± 5.17 |
|  | *p*-Acetaminophenol | 0.32 ± 0.03 | 0.31 ± 0.23 | 1.02 ± 0.77 |
|  | Phenacetin | 0.83 ± 0.02 | 0.135 ± 0.001 | 0.16 ± 0.12 |
|  | 4-Chloroacetanilide | 1.9 ± 0.6 | 16.29 ± 1.34 | 9.10 ± 2.51 |
|  | Acetanilide | 19.00 ± 13.64 | 0.066 ± 0.018 | 0.005 ± 0.002 |

**Table 3.** Substrate specificity of carboxylic donors on amide synthesis by the reverse reaction of aryl acylamidase. Compounds with increasing numbers of carbons [formate ($C_1$) to decanoate ($C_{10}$)] were used as acyl donors in amide formation.

| Substrate | Amide synthesis rate (μmole mg$^{-1}$ min$^{-1}$) | Relative activity (%) |
|---|---|---|
| $C_1$ (HCOOH) | N.D.[a] | N.D.[a] |
| $C_2$ ($CH_3COOH$) | 2.2 ± 0.1 | 100 |
| $C_3$ ($CH_3CH_2COOH$) | 2.4 ± 0.0 | 106 |
| $C_4$ ($CH_3(CH_2)_2COOH$) | 2.4 ± 0.2 | 108 |
| $C_{10}$ ($CH_3(CH_2)_8COOH$) | 2.5 ± 0.3 | 110 |

[a]Not detectable

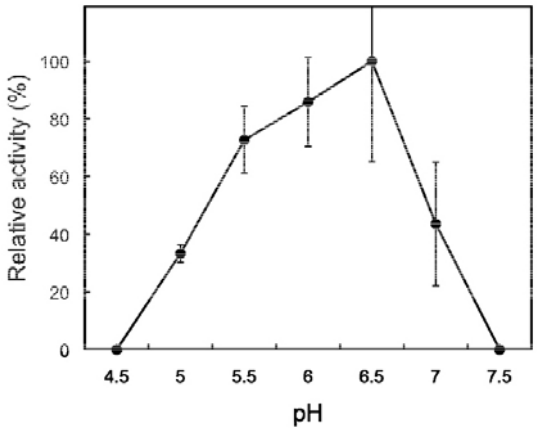



**Fig. 5.** The effect of pH on the reverse activity of aryl acylamidase (AAA). We used 6.2 μg of protein and 80 mM of substrates (aniline and acetic acid) in 250 μl of reaction buffer having various pHs. The reaction was carried out for 3 h at 37°C and the amount of decreased aniline was also determined by a modified diazocoupling method. The relative activity was expressed as the rate of substrate (aniline) removal.

amidases belonging to AS enzymes. As a result, we inspected closely related proteins because the molecular function of a gene can be inferred by correct orthology relationship. Based on phylogenetic analysis, while updating previous classification of AS enzymes, we clustered the representative AS enzymes into six subfamilies. The result was the extension of the previous observation of five subgroups in the AS enzyme family (Chebrou et al., 1996). The isolated AAA gene was categorized into AH, which has not known for any AAA activity until a recent study showed a novel aryl acylamidase in *Nocardia farcinica* belonging to the AH subfamily (Sonja Heumann, 2009) (see Fig. 2; Supplementary Fig. S2 for the multiple sequence alignment). However, the sequence identity with that of *N. farcinica* was pretty low (only 29%) showing high sequential divergence. Although these AS enzymes have a common motif (about 160 residues of AS) and similar molecular function such as amide hydrolysis, the overall sequence similarity among members is low (average of 26%) and thus the cellular functions of subfami-

lies might be highly diverse depending on the exact biochemical reaction they perform. According to these two AAA instances, we may deduce one potential molecular function of the AH subfamily as the AAA activity. On the other hand, although we did not examine AH activity, one may assume that the AAA gene can have a similar activity as other members of the AH subfamily, suggesting a potential application of the AAA gene for nylon degradation (Heumann, 2009) and xenobiotic degradation in environmental bioremediation process (Yasuhira et al., 2007).

The hydrolysis of amide bond by AAA can be reversible depending on the reaction conditions. Considering that the substrate (an anilide compound) degrades into aniline and a carboxylic acid, it is predictable that the optimum pH for a forward reaction would favor alkaliphilic condition to make more carboxylic acids (products). As predicted, the optimum pH of the protein was around 10. In the same way, we can predict that amide synthesis would be preferably achieved at acidic pH and found the optimum pH for the reverse reaction was between 6 and 7. The kinetic parameters of rAAA indicated a substrate preference for an aryl group having polar functional groups [$K_m$ values: 4-nitroacetanilide (0.10 mM) < *p*-acetaminophenol (0.32 mM) < phenacetin (0.83 mM) < 4-chloroacetanilide (1.9 mM) < acetanilide (19 mM)] (Table 1). Also, values of $V_{max}$ $K_m^{-1}$ for each substrate indicated that catalytic activity was affected by size and polarity of side chain in meta-position of aryl moiety such that substrates having polar side chain had higher catalytic activities in some degree (H- < $CH_3O$- < OH- < Cl- < $NO_2$-). If these catalytic activities can be considered with the residues consisting active sites in the enzyme, it will provide valuable information to modulate the substrate specificity by protein engineering. To examine application of the enzyme to the synthesis of the amide bond, we tested the reverse reaction activity. The cloned AAA had a preference for long chain carboxylic acids indicating the active site pocket might be wide enough to be stabilized by hydrophobic interactions with substrates. By these results, we suggest that the AAA in this study can be used as a biocatalyst for the specific synthesis of an amide bond in aryl compounds having a polar functional group in an aryl donor, and long chain carboxylic acids for a carboxylic donor (e.g. fatty acids). For clinical application of AAA as a bioassay of *p*-acetaminophenol, the substrate specificity should be considered. In particular, the range of concentration of *p*-acetaminophenol in a clinical sample is known to be 0-3 mM (Vaughan et al., 1990). The $K_m$ value of the cloned AAA on *p*-acetaminophenol was 0.32 mM, which is appropriate for use in the determination of the compound in a clinical sample.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

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