〈Brief Note〉

Analysis of a hippurate hydrolase homolog from *Acetomicrobium mobile*

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Summary Toluene and xylene exposure among workers can be evaluated by measuring the levels of urinary hippuric acid and methylhippuric acid, to prevent health problems. We previously identified a new hippurate hydrolase from the hyperthermophilic archaeon (Hhase1043), and developed a hippuric acid assay using this enzyme. In this study, we discovered and analyzed an Hhase1043 homolog (HhaseAm) from the moderately thermophilic bacterium *Acetomicrobium mobile* that exhibited hippuric acid- and methylhippuric acid-hydrolyzing activity. The maximum level of HhaseAm was observed at 70°C and pH 7.0. Based on a tertiary structure predicted using homology modeling, mutants of HhaseAm with altered activity and substrate specificity were obtained by mutagenesis of an active center residue.

Key words: Toluene, Xylene, Hippuric acid, Methylhippuric acid, Mutagenesis

1. Introduction

In the paint industry, toluene and xylene are handled in large quantities as organic solvents. They are consequently metabolized in the bodies of workers yielding hippuric acid (HA) and three isomers of methylhippuric acid (MHA), *o*-MHA, *m*-MHA, and *p*-MHA, in urine. Hence, toluene and xylene exposure in workers is evaluated to prevent health problems by measuring urinary HA and MHA levels[1,2]. A highly efficient method for measuring these levels is desired, because the HPLC method currently used[3] has low throughput.

Hippurate hydrolase (EC 3.5.1.32, Hhase) catalyzes reactions with various N-benzoylamino acids to yield benzoic acids and amino acids (glycine, in the cases of HA and MHA)[4,5]. Several Hhases from bacteria, such as *Campylobacter jejuni* and *Pseudomonas putida*, have been characterized[6,7]. We also have found an Hhase from the hyperthermophilic archaeon *Pyrococcus horikoshii* (Hhase1043)[8,9].

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Moreover, we have developed an enzymatic HA assay using Hhase1043 for simple high-throughput analysis of biological samples[8].

There have been few reports of the MHA isomer-hydrolyzing activity of Hhase. In this study, an Hhase1043 homolog from the moderately thermophilic bacterium *Acetomicrobium mobile* was found and analyzed. This protein showed HA-, *m*-MHA-, and *p*-MHA-hydrolyzing activities. Mutants obtained based on structural prediction and protein engineering were also investigated.

2. Materials and Methods

Materials, bacterial strains, plasmids, and culture conditions

Compounds and reagents were purchased from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries (Osaka, Japan).

Escherichia coli strains DH5α and BL21(DE3) and the previously constructed expression plasmid pET24-PH1043HT[8] were used for recombinant strain preparation and plasmid construction, respectively. Bacteria were grown in LB [1% Tryptone, 0.5% yeast extract, and 1% NaCl (pH 7.2)] or TB (1.2% Tryptone, 2.4% yeast extract, 1% glycerol, 0.94% K2HPO4, and 0.22% KH2PO4) broth, or on LB agar (LB broth plus 1.5% agar) at 30°C[8]. The antibiotic used was kanamycin (30 μg/mL).

General DNA manipulation

Plasmid isolation, DNA cleavage with restriction enzymes, DNA ligation with T4 DNA ligase, and *E. coli* transformation were performed as described previously[8].

An Hhase homolog gene from *A. mobile* was artificially synthesized by Eurofins Genomics (Tokyo, Japan). The codon usage was adapted to match the codon bias of *E. coli*. The 1,181 bp synthesized DNA was ligated between the *Nde*I and *Eco*RI sites of the plasmid pET24-PH1043HT to replace PH1043 gene[8]. The DNA sequence of the construct, which was designated pET24-Am993HT, was confirmed by sequencing.

The KOD-Plus Mutagenesis kit (Toyobo Co., Ltd., Osaka, Japan) was used for site-directed mutagenesis.

Protein purification, enzyme assay, and characterization

E. coli BL21(DE3)/pET24-Am993HT was grown under the same conditions used in a previous study[8]. Purification of the homolog protein was performed as previously described[8]. The enzymatic properties of the purified product were then characterized.

The enzyme assay was performed by measuring the amount of glycine produced during substrate hydrolyzation, using the ninhydrin method[8] at pH 7.0 instead of 7.5. One unit of activity was defined as the increase in absorbance (at 570 nm, secondary wavelength: 700 nm) per minute at 37°C and pH 7.0. Various concentrations of substrate solutions were used to investigate the enzyme kinetics. All data were averaged over three independent experiments. Molecular weight of the homolog protein was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Homology modeling

The Molecular Operating Environment (MOE) software (Chemical Computing Group Inc., Montreal, Canada) was used to generate a three-dimensional protein model based on the structure of similar proteins[10-13].

3. Results and Discussion

Hhase1043 homology search

We previously identified the thermostable HA-hydrolyzing enzyme Hhase1043 from the hyperthermophilic archaeon *P. horikoshii*[8,9]. To identify new Hhases, putative orthologs of Hhase1043 were detected using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. An amino acid sequence homology search indicated that several homologs of two archaeal genera, *Pyrococcus* and *Thermococcus*, had high sequence identity to Hhase1043 (Table 1). *Thermococcus* is hyperthermophilic and closely related to the genus *Pyrococcus*.

Table 1 Homologous proteins of Hhase 1043

Homolog	Origin (belonging to Bacteria/Archaea)	Amino acids	Identity (%)	Overlap
OCC_03928	*Thermococcus litoralis* (Arc)	389	83.6	385
CHITON_1060	*Thermococcus chitonophagus* (Arc)	385	75.1	381
Anamo_0993	*Acetomicrobium mobile* (Bac)	395	57.1	387
A0127_02395	*Thermococcus peptonophilus* (Arc)	384	61.4	383
PAB0873	*Pyrococcus abyssi* (Arc)	383	60.5	380
TK0494	*Thermococcus kodakarensis* (Arc)	384	60.1	383
TERMP_02178	*Thermococcus barophilus* (Arc)	385	58.6	384
A3L14_03905	*Thermococcus thioreducens* (Arc)	381	58.9	384
A3L08_04570	*Thermococcus pacificus* (Arc)	383	59.2	382
A3L11_06320	*Thermococcus siculi* (Arc)	383	59.2	382

Unexpectedly, we found a non-archaeal homolog with high identity (57.1%). Especially, the deduced amino acid residues chelating to metal ion cofactors and their surrounding residues were highly conserved (data not shown). This homolog, Anamo_0993, belonged to *A. mobile*, a moderately thermophilic bacterium (Table 1).

Various enzymes from moderately thermophilic microorganisms are used in practical assays because they retain both high stability and activity at working temperatures. Therefore, this homolog gene was cloned and expressed in a recombinant *E. coli* strain carrying the expression plasmid pET24-Am993HT. The gene product was then purified to homogeneity.

Characterization of the homolog protein

The HA-hydrolyzing activity of the purified homolog protein from *A. mobile* was 0.0093 U/mg at 37°C, whereas that of Hhase1043 was 0.020 U/mg[8]. Hence, this protein was determined to be a new Hhase, designated HhaseAm. Based on the results of SDS-PAGE, the subunit molecular weight of HhaseAm was estimated to be 43 kDa, which corresponded to the value calculated from the amino acid sequence (45 kDa). The optimum temperature and pH for HA-hydrolyzing activity were 70°C and 7.0, respectively. The specific activity at 60 and 70°C was 4.8 and 7.8 times greater than that at 37°C, respectively. The residual activity was 41% after 10 min of incubation at 80°C, although the activity was almost entirely retained after 20 min of incubation at 70°C. The effects of temperature on the activity and stability of HhaseAm corresponded to the general enzymatic properties of moderate thermophiles.

HhaseAm exhibited hydrolyzing activity not only toward HA but also toward *m*-MHA and *p*-MHA. No activity toward *o*-MHA was detected. The K_m values for HA and two of the three MHA isomers could not be estimated because HhaseAm exhibited non-Michaelis-Menten kinetics (Fig. 1A). The substrate-activity curves for *m*-MHA and *p*-MHA revealed remarkable substrate inhibition at lower concentrations.

Construction of an HhaseAm structural model

A three-dimensional structural model of HhaseAm was constructed by homology modeling, using the template structure with the highest sequence identity (PDB ID: 4EWTa, amino acid sequence identity: 45.1%)[14]. 4EWTa is the structure of an amidohydrolase from methicillin-resistant *Staphylococcus aureus* belonging to the M20D family of peptidases. The overall structures of HhaseAm and 4EWTa superimposed well, with a root-mean-square deviation for atomic Cα positions of 1.07 Å. As shown in Fig. 2, the overall configuration of the major amino acid residues was conserved when the both active centers were compared. Especially, the deduced HhaseAm chelate residues C109, H111, H169, and H366 had similar conformations, while E144 did not. HhaseAm was predicted by amino acid sequence alignment to have two metal

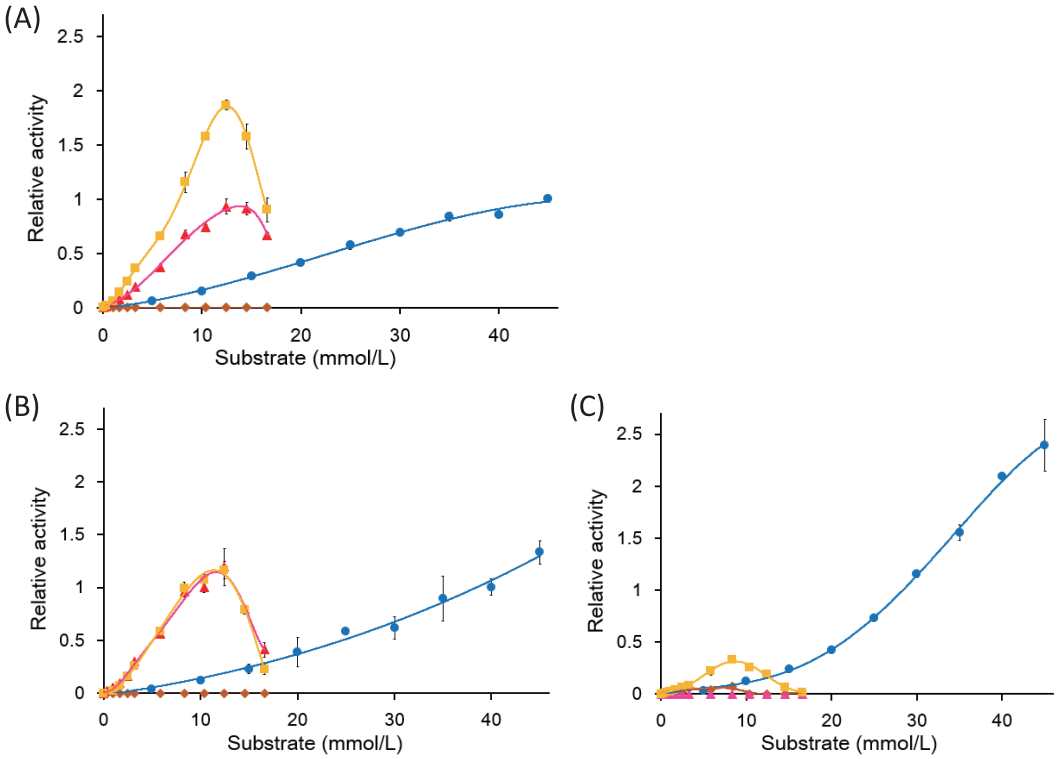

Fig. 1 Substrate-activity curves of HhaseAm and its W171 mutants. The relative activity of each mutant to the specific activity of the wild-type toward HA was estimated. Curves for HA-, *o*-MHA-, *m*-MHA-, and *p*-MHA-hydrolyzing activity are represented by blue, brown, magenta, and yellow, respectively. (A) Wild-type, (B) W171A, (C) W171G. Error bar; standard deviation, n = 3.

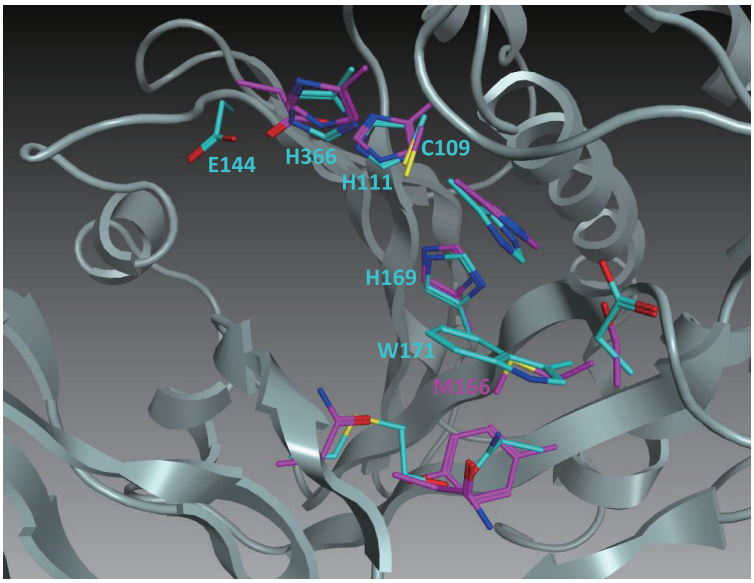

Fig. 2 Active site regions of an HhaseAm model and 4EWTa structures. The both structures are superimposed, and the ribbon diagram represents the backbone structure of HhaseAm. Amino acid residues of HhaseAm and 4EWTa are represented by cyan and magenta, respectively.

binding triads: C109, H111, and H169; and C109, E144, and H366. In terms of the effects on enzymatic properties, it should be noted that E144 was located at a slight distance from the other chelate residues of the triad.

Conversely, W171 of HhaseAm was markedly bulky compared with M166, the corresponding residue of 4EWTa (Fig. 2). Therefore, the substitution of W171 by smaller amino acids was predicted to influence the enzyme activity and substrate specificity of HhaseAm.

Construction of mutants and comparison of their kinetics with the wild-type

Two HhaseAm mutants, W171A and W171G, were constructed by site-directed mutagenesis as described in the Materials and Methods section. The substrate-activity curves of the purified mutant enzymes are presented in Fig. 1B and C. The specific activity of W171G toward HA was approximately two times higher than those of the wild-type and W171A. In contrast, the acitivities of W171 mutants toward *m*-MHA and *p*-MHA were lower than that of the wild-type. The *p*-MHA-hydrolyzing activity was decreased as the side-chain at position 171 became smaller. Moreover, *m*-MHA-hydrolyzing activity was not detected in W171G. These drastic mutational effects were difficult to explain structurally.

In summary, a novel Hhase from a moderate thermophile was found, and mutants possessing altered activity and substrate specificity were obtained. To increase the number of practical applications of this Hhase, the screening of highly active mutant enzymes using protein engineering techniques is now in progress.

Conflicts of interest

The authors have no conflicts of interest. Toshiaki Baba is an employee of Nipro Corporation and has a legitimate salary from Nipro Corporation.

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