〈Brief Note〉

Development of highly efficient variants of *Pyroccocus* hippurate hydrolase for monitoring toluene and xylene exposure

Yoshiaki Nishiya[1,2,*], Satsuki Imai[2], Shota Shinki[1], Kentaro Nagoshi[1] and Toshiaki Baba[3]

Summary Toluene and xylene are used in large quantities as organic solvents in paint industries and are metabolized by the human body to yield hippuric and methyl-hippuric acids (HA and MHA) in urine. Our previous study reported the development of hippurate hydrolase from *Pyrococcus horikoshii* (Hhase1043) and the HA assay method utilizing this enzyme. In the present study, Hhase1043 hydrolysis of HA and three isomers of MHA (*o*-MHA, *m*-MHA, and *p*-MHA) was improved using protein engineering techniques for developing low-cost assays. A tryptophan residue in the putative active center of Hhase1043 was selected using the substrate docking simulation and substituted. As a result, the HA-, *o*-MHA-, *m*-MHA-, and *p*-MHA-hydrolyzing activities of the Hhase1043 mutants increased by approximately 3.4, 2.1, 12, and 26 times those of the wild type, respectively. Moreover, the mutational effects were remarkably enhanced upon combination with the other highly HA-active substitutions obtained previously.

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

1. Introduction

Toluene and xylene, although injurious to health, are used in large quantities in paint industries as organic solvents. They are consequently metabolized in the workers' bodies to produce hippuric acid (HA) and three isomers of methylhippuric acid (MHA; *o*-MHA, *m*-MHA, and *p*-MHA), respectively, and excreted in the urine (Fig. 1). Hence, toluene and xylene exposure in workers is evaluated by measuring urinary HA and MHA levels[1-4]. Due to the low throughput of the currently used HPLC method, a more efficient

[1]Department of Life Science, Setsunan University, 17-8 Ikeda-Nakamachi, Neyagawa, Osaka 572-8508, Japan.
[2]Division of Life Science, Graduate School of Science and Engineering, Setsunan University, 17-8 Ikeda-Nakamachi, Neyagawa, Osaka 572-8508, Japan.
[3]Research and Development Laboratory, Nipro Corporation, 3023 Nojicho, Kusatsu, Shiga 525-0055, Japan.

*Corresponding author: Yoshiaki Nishiya, Department of Life Science, Setsunan University, 17-8 Ikeda-Nakamachi, Neyagawa, Osaka 572-8508, Japan.
Tel: +81-72-800-1151
Fax: +81-72-838-6599
E-mail: nishiya@lif.setsunan.ac.jp

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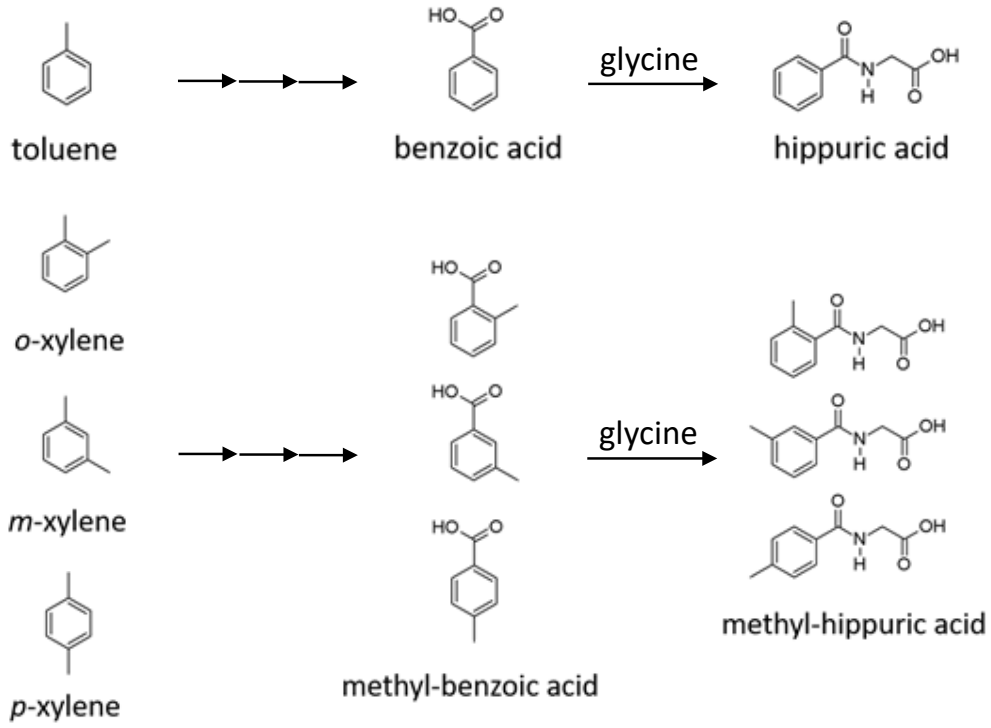

Fig. 1. Reaction steps involved in toluene and xylene metabolism.

method is required[5-7].

Hippurate hydrolase (EC 3.5.1.32, Hhase) catalyzes the reactions involving various N-benzoylamino acids to yield benzoic acid and amino acid (glycine in the case of HA and MHA)[8-11]. Several Hhases from bacteria have been characterized previously. Additionally, our team has identified an Hhase (named Hhase1043) from a hyperthermophilic archaeon *Pyrococcus horikoshii*[12,13]. Moreover, we have developed the Hhase1043-based enzymatic HA assay method for simple and high-throughput estimation of HA and MHA in biological samples[12].

In the present study, we attempted to improve the HA- and MHA-hydrolyzing activities of Hhase1043 using a substrate docking simulation to reduce the assay cost. The relevant hydrolyzing activities of the mutants were elevated by as much as 2–26 times that of the wild type. When the most effective mutation was combined with the other highly HA-active substitutions previously obtained, the activities were further enhanced remarkably.

2. Materials and Methods

Materials, bacterial strain, and plasmid

The compounds and reagents were purchased from Nacalai Tesque (Kyoto, Japan) and FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). Escherichia coli BL21(DE3) was used as a host. The expression plasmid pET24-PH1043HT[12] was used for mutated plasmid construction and recombinant protein preparation.

DNA manipulation

Plasmid isolation, T4 DNA ligase-mediated DNA ligation, and transformation of *E. coli* cells were performed as described previously[12,14]. KOD-Plus Mutagenesis kit (Toyobo Co., Ltd., Osaka, Japan) was used for inverse PCR and site-directed mutagenesis, according to the manufacturer's instruction.

Enzyme purification, assay, and characterization

E. coli BL21(DE3)/pET24-PH1043HT or the mutated plasmid was grown under the same condition as previous study[12]. Purification of the recombinant protein was also performed as previously described[12,14]. The enzymatic properties of the purified product were then characterized. The enzyme assay

was based on the measurement of glycine produced during substrate hydrolysis using the ninhydrin method[12,14]. One unit of activity was defined as the increase in absorbance (570 nm) per minute at 37°C and pH 7.5.

Docking simulation

The Molecular Operating Environment (MOE) software ver. 2019 (Chemical Computing Group Inc., Montreal, Canada) was used to perform molecular visualization and docking simulations[15-20]. The structure model of Hhase1043 used here was built previously through homology modeling using MOE[12]. The molecular structures of the compounds used in the docking simulations were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Molecular docking studies were performed using the induced fit docking protocol in MOE to allow for side chain flexibility. All the compounds used for the docking process were built and saved as MOE files. Next, using the default parameters in MOE, energy minimization was performed after 3D protonation of the proteins. The docking site was defined around the putative active center. Triangle Matcher was used as a placement method. Rescoring was performed using London dG and GBVI/WSA. For each ligand, five conformations were allowed to be formed and the top-ranked reactive conformations were selected based on the docking scores.

3. Results and Discussion

Docking simulations of HA and MHA

To investigate the substrate affinity mechanism, we performed molecular docking simulations of HA and MHA using the Hhase1043 structure model. The reactive substrate-binding state of HA was recognized with the calculated docking score of –4.27 (Fig. 2A). *m*-MHA and *p*-MHA also exhibited stable binding in the reactive states (Fig. 2B, C) with docking scores of –4.59 and –4.03, respectively. Conversely, reactive binding between *o*-MHA and Hhase1043 was not established, suggesting weak activity due to lower interaction between *o*-MHA and the enzyme pocket.

The binding state of *p*-MHA was very similar to that of HA, and the amide bond carbonyl oxygens of both substrates were located in the same direction (Fig. 2A, C). Unexpectedly, the binding conformation of *m*-MHA in Hhase1043 was considerably different from that of *p*-MHA. As a result, the amide bond carbonyl oxygen of *m*-MHA was located in the opposite direction to that of HA and *p*-MHA (Fig. 2). It appears that the position of the methyl group limits the substrate conformations in the active center of Hhase1043 due to its confined enzyme pocket. We assume that the expansion of active site pocket increases hydrolytic activity; therefore, we selected a bulky amino acid, tryptophan, in the active site (W164) as the mutagenesis target (Fig. 2).

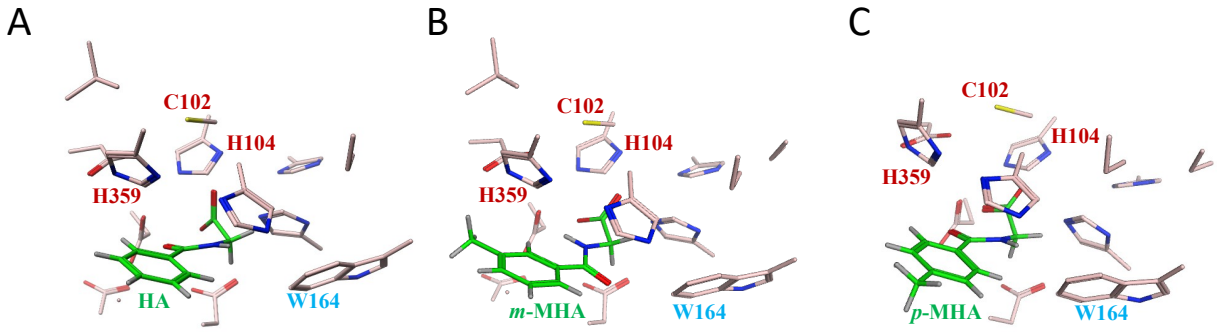

Fig. 2. Docking simulations of HA (A), *m*-MHA (B), and *p*-MHA (C) with Hhase1043. Close-up views of the active sites are shown by stick representations of substrate (green) and amino acid residue side chains (light pink). Sulfur, Oxygen, Nitrogen, and substrate hydrogen are represented by yellow, red, blue, and white, respectively. C102, H104, and H359 are putative metal ion-chelating residues. W164 is the mutagenesis target in the present study.

Effect of W164 mutants on enzyme activities

The wild-type Hhase1043 exhibited weak HA- and even weaker MHA-hydrolyzing activities at 37 °C (Fig. 3); however, highly efficient assays require much higher hydrolyzing activity. Based on the docking simulation, we hypothesized that the substitution of W164 with smaller side chain residues, such as G, A, V, L, and I, may improve the HA- and MHA-hydrolyzing activities of Hhase1043 enzyme.

All the W164 mutant enzymes were purified and their HA, *m*-MHA, and *p*-MHA-hydrolyzing activities were examined. The specific activities of W164G, W164A, W164V, W164L, and W164I for HA, *m*-MHA, and *p*-MHA were found to be 1.9–3.4, 5.9–12, and 4.1–26 times higher than those of the wild type, respectively (Fig. 3). For o-MHA, the specific activity of only W164A increased to two-fold that of the wild type.

Thus, highly active mutants of Hhase1043 were obtained successfully based on the substrate docking simulation and protein engineering. However, the *o*-MHA-hydrolyzing activity was insufficient for the development of an effective and low-cost MHA assay.

Effect of multiple mutations on enzyme activities

To improve the activities further, multiple mutants were constructed. Four mutants (K193S, P200S, T236I, and N307P) were selected; all four were obtained previously by site-directed saturation mutagenesis and shown to enhance the HA-hydrolyzing activity[12]. The structure of Hhase1043 is composed of a larger catalytic domain and a smaller satellite domain. The selected mutation points exist in the loop regions and spaces between secondary structures of the satellite domain, except for N307P. These mutations were combined with W164A and four multiple mutants were constructed.

All enzymes were purified and characterized. As shown in Fig. 4, the multiple mutants demonstrated elevated enzyme activities. In particular, the W164A + K193S + N307P enzyme exhibited increases in specific activities of 12-, 3.9-, 31-, and 29-fold for HA, *o*-MHA, *m*-MHA, and *p*-MHA, respectively compared to those of the wild type (Fig. 4). Thus, the effect of the W164A mutant was

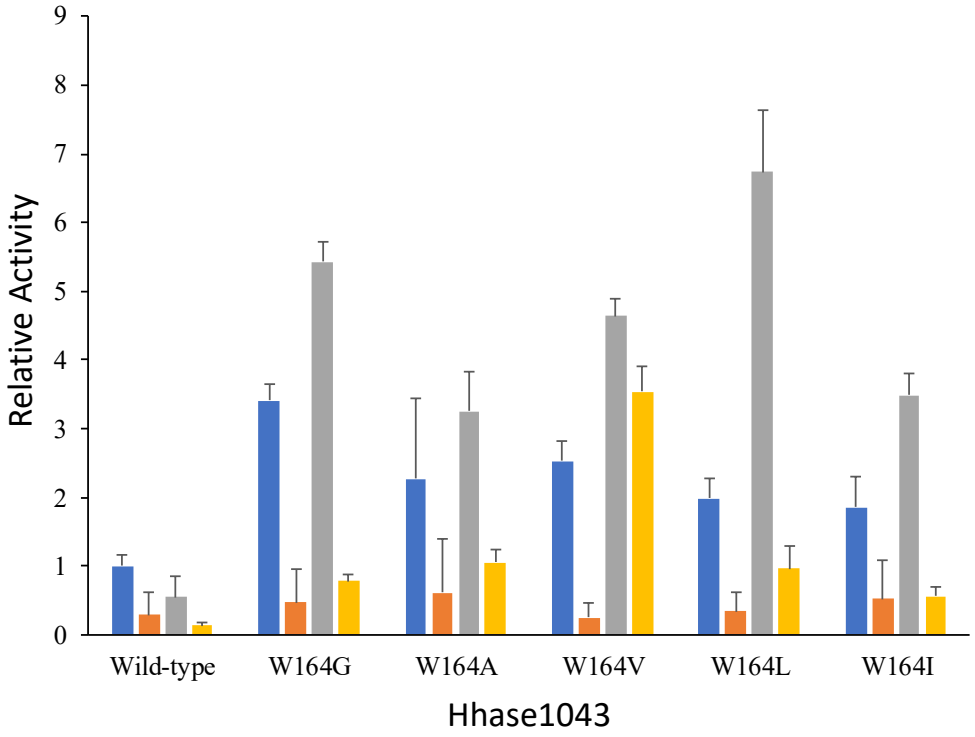

Fig. 3. HA- and MHA-hydrolyzing activities of the wild type and W164 mutants. Relative activities to the HA- and MHA-hydrolyzing activity of the wild-type enzyme were estimated. Activities for HA, *o*-MHA, *m*-MHA, and *p*-MHA are represented by blue, orange, gray, and yellow, respectively. Error bar represents standard deviation, n = 3.

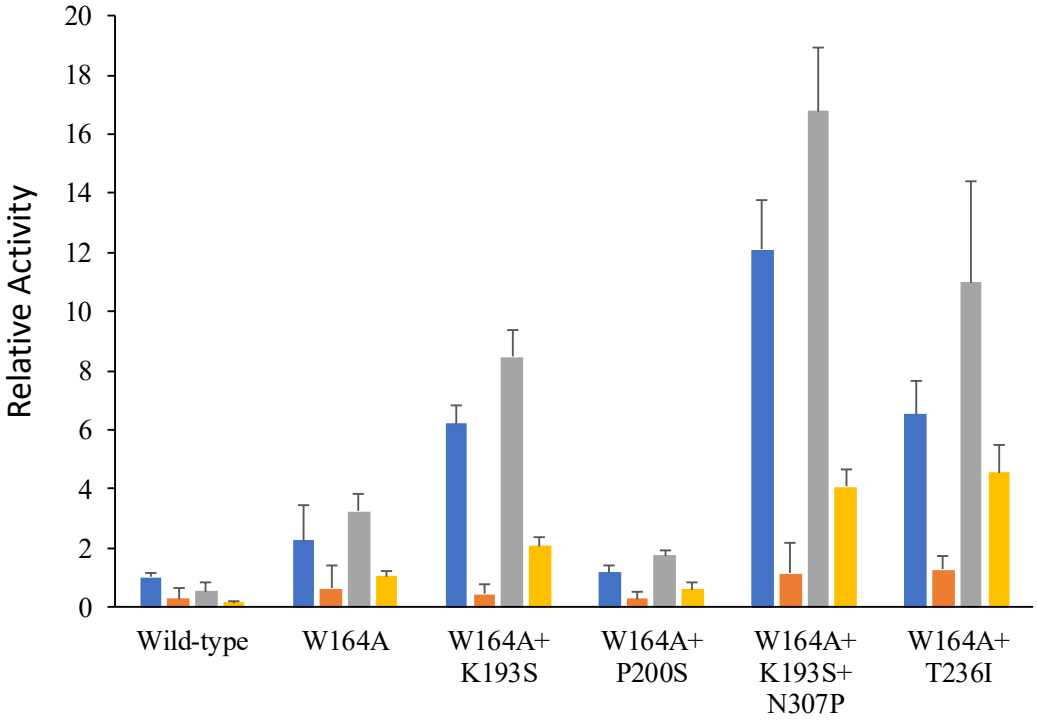

Fig. 4. HA- and MHA-hydrolyzing activities of the wild type, W164A, and multiple mutants. Relative activities to the HA- and MHA-hydrolyzing activity of the wild-type enzyme were estimated. Activities for HA, *o*-MHA, *m*-MHA, and *p*-MHA are represented by blue, orange, gray, and yellow, respectively. Error bar represents standard deviation, n = 3.

enhanced remarkably by combining the other highly HA-active substitutions.

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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References

1. Duydu Y, Süzen S, Erdem N, Uysal H and Vural N: Validation of hippuric acid as a biomarker of toluene exposure. Bull Environ Contam Toxicol, 63:1-8, 1999.
2. Zhao F, Wang Z, Wang H and Ding M: Determination of hippuric acid in human urine by ion chromatography with conductivity detection. J Chromato B, 879:296-298, 2011.
3. Behbahani M, Bagheri S, Omidi F and Amini MM: An amino-functionalized mesoporous silica (KIT-6) as a sorbent for dispersive and ultrasonication-assisted micro solid phase extraction of hippuric acid and methylhippuric acid, two biomarkers for toluene and xylene exposure. Microchimica Acta, 185: Article No.505, 2018.
4. Yuan Y, Han Y, Yang C, Han D and Yan H: Deep eutectic solvent functionalized graphene oxide composite adsorbent for miniaturized pipette-tip solid-phase extraction of toluene and xylene exposure biomarkers in urine prior to their determination with HPLC-UV. Microchimica Acta, 187: Article No.387, 2020.
5. Hao JN and Yan B: Recyclable lanthanide-functionalized MOF hybrids to determine hippuric acid in urine as a biological index of toluene exposure. Chem Commun, 51:14509-14512, 2015.
6. Mohammed NH, Hussien ARME, Hussien MO and Hassan YM: Determination of hippuric acid and

methyl hippuric acid in urine as indices of toluene and xylene exposure by HPLC MS/MS spectrometry. Int J Res Pharm Chem, 5:10-16, 2015.

7. Yadav A, Basu A and Chakarbarti A: Method for estimation of hippuric acid as a biomarker of toluene exposure in urine by high-performance liquid chromatography after extraction with ethyl acetate. Environ Dis, 4:17-22, 2019.
8. Ferrieri P, Wannamaker LW and Nelson J: Localization and characterization of the hippuricase activity of group B Streptococci. Infect Immunity, 7:747-752, 1973.
9. Ottow JCG: Detection of hippurate hydrolase among *Bacillus* species by thin layer chromatography and other methods. J Appl Bacteriol, 37:15-30, 1974.
10. Steele M, Marcone M, Gyles C, Chan VL and Odumeru J: Enzymatic activity of *Campylobacter jejuni* hippurate hydrolase. Protein Eng Des Sel, 19:17-25, 2006.
11. Zolg W and Ottow JCG: Thin layer chromatography methods for detecting hippurate hydrolase activity among various bacteria (*Pseudomonas, Bacillus*, Enterobacteriaceae). Experientia, 29:1573–1574, 1973.
12. Nishiya Y, Nagoshi K, Shinki S, Imai S and Baba T: Development of a hippuric acid-hydrolysing enzyme for monitoring toluene exposure. Int J Anal Bio-Sci, 9:15-21, 2021.
13. Ishikawa K, Ishida H, Matsui I, Kawarabayasi Y and Kikuchi H: Novel bifunctional hyperthermostable carboxypeptidase/aminoacylase from *Pyrococcus horikoshii* OT3. Appl Environ Microbiol, 67:673-679, 2001.
14. Nishiya Y, Torii G, Suginaka M, Imai S and Baba T: Analysis of a hippurate hydrolase homolog from *Acetomicrobium mobile*. Int J Anal Bio-Sci, 9:30-35, 2021.
15. Nishiya Y: Homology modeling and docking study of creatinine deiminase. Int J Anal Bio-Sci, 1: 55-59, 2013.
16. Nishiya Y, Yamamoto M, Takemoto J, Kano S and Nakano S: Monomeric sarcosine oxidase exhibiting high substrate affinity and thermostability. Int J Anal Bio-Sci, 4:55-62, 2016.
17. Nishiya Y and Shimozawa Y: Properties of *Geobacillus stearothermophilus* malate dehydrogenase used as a diagnostic reagent and its characterization by molecular modeling. Int J Anal Bio-Sci, 4:21-27, 2016.
18. Shimozawa Y, Yoshida S, Ikeda K, Kato Y, Toyama F and Nishiya Y: Easy preparation of a stable membrane-bound lactate dehydrogenase for application on lactate biosensor. Int J Anal Bio-Sci, 8:65-70, 2020.
19. Shimozawa Y, Aiba H and Nishiya Y: Structural prediction and analysis of the highly reactive alkaline phosphatase from *Shewanella* sp. T3-3. Int J Anal Bio-Sci, 8:39-43, 2020.
20. Sumida Y, Yamasaki M, Nishiya Y, Kumagai S, Yamada T and Azuma M: Protein Engineering of D-Succinylase from *Cupriavidus* sp. for D-Amino Acid Synthesis and their Structural Implications. Adv Synth Catal, 363: 4770-4778, 2021.