〈Research Article〉

Development of a hippuric acid-hydrolysing enzyme for monitoring toluene exposure

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Summary Toluene is used in large quantities as an organic solvent in paint industries. Following toluene exposure, it is metabolized in the body of workers to yield hippuric acid in urine. An enzymatic assay for measuring hippuric acid is desired for health checks of workers. In this study, the carboxypeptidase/aminoacylase from the thermophilic archaeon was demonstrated as a new hippuric acid-hydrolysing enzyme. A hippuric acid assay method using this enzyme and the cycling reaction was developed, and standard solutions were effectively measured. However, cost-saving assays require considerably higher hydrolysing activities. To improve the practicality of this enzyme, various mutants constructed by structure prediction and site-directed saturation mutagenesis were screened, and the highly active enzymes were successfully obtained.

Key words: Toluene, Hippuric acid, Hydrolase, Enzymatic assay, Mutagenesis

1. Introduction

In paint industries, toluene is handled in large quantities as an organic solvent and is consequently metabolized in the body of workers to yield hippuric acid (HA) in urine (Fig. 1). Therefore, it is important to evaluate toluene exposure in workers and prevent health damage by measuring urinary HA[1-3]. However, the HPLC method currently used to measure HA[4] is complicated, time consuming, and has low-throughput. Hence, there is a need to develop a highly efficient method for measuring the levels of HA in urine.

HA-hydrolysing enzyme [hippuricase or hippurate hydrolase (EC 3.5.1.32), Hhase] catalyzes the reaction with various N-benzoylamino acids to yield benzoic acid and amino acid (glycine in the case of HA)[5,6]. The enzyme can be exploited for the construction of an enzymatic assay that is capable of simple and high-throughput analysis of HA levels in biological samples. Several Hhases from bacteria,

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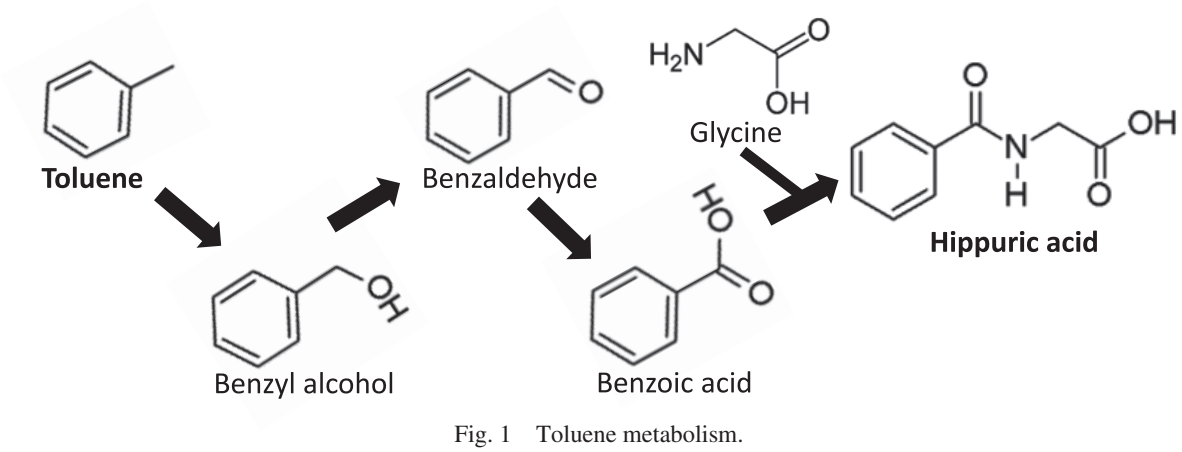

Fig. 1 Toluene metabolism.

such as *Campylobacter jejuni* and *Pseudomonas putida*, have been characterized[7,8], however, an Hhase with industrial stability has been not found yet.

In this paper, the hyperthermostable carboxypeptidase/aminoacylase bifunctional enzyme from the thermophilic archaeon *Pyrococcus horikoshii* (gene name: PH1043)[9] was found to be a new Hhase. A new method of HA assay was developed using this enzyme. Moreover, the mutants with higher hydrolysing activities were successfully obtained based on structural prediction and protein engineering.

2. Materials and Methods

Materials

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

Bacterial strains, plasmid, and culture conditions

Escherichia coli strains DH5α and BL21(DE3) and the plasmid pET24a(+) (K_m^r) 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% K_2HPO_4, and 0.22% KH_2PO_4) broth, or on LB agar (LB broth plus 1.5% agar) at 30°C. The antibiotic used was kanamycin (30 μg/mL).

General DNA manipulation

Plasmid isolation, cleavage of DNA with restriction enzymes, and ligation of DNA with T4 DNA ligase were carried out as described previously[10]. Transformation of *E. coli* was carried out according to the competent cell method[11]. The PH1043 gene was artificially synthesized by the manufacturer (Eurofins Genomics, Tokyo, Japan). The codon usage was adapted to the codon bias of *E. coli* genes. In addition, regions of very high (>65%) or very low (<35%) GC content were avoided where possible. The 1,164-bp synthesized DNA was ligated between the *Nde*I and *Eco*RI sites of pET24a(+). An additional His-tag sequence was then introduced into the C-terminus of the gene product by inverse PCR using the KOD-Plus Mutagenesis kit (Toyobo Co., Ltd., Osaka, Japan) and the following primers: 5'-GAATTCGAGCTCCGTCGACAAGCTTG CGGC-3' (sense primer, corresponding and following the sequence that encodes the 6×His-tag plus spacer peptide NSSSVDKLAAALEHHHHHH and the stop codon) and 5'-AGGGAGAGG TAGTGATAAGTCAGAAGAGAA-3' (antisense primer, complementary to the sequence that encodes the C-terminal peptide SLLTYHYLSL). This construct was designated pET24-PH1043HT. The DNA sequences of the constructs were verified by sequencing. The pET24-PH1043HT carrier was induced to synthesize the gene product by the addition of isopropyl-L-D-thiogalactopyranoside (IPTG) to the culture medium.

Enzyme purification

E. coli BL21(DE3)/pET24-PH1043HT was grown in TB medium (100 mL) containing 30 μg/mL of kanamycin and 0.1 mmol/L IPTG at 30°C for 48 h with rotation at 180 rpm. The cells were harvested by centrifugation and re-suspended in 20 mmol/L potassium phosphate buffer (pH 7.5). A crude extract was prepared by sonication of the cells following centrifugation. The supernatant was then loaded onto a His GraviTrap immobilized metal affinity chromatography column (Cytiva, Uppsala, Sweden). The gene product was eluted using 20 mmol/L potassium phosphate buffer (pH 7.5) containing 100-500 mmol/L imidazole and 500 mmol/L NaCl. Pooled fractions were finally purified by dialysation. The enzymatic properties of the purified product were then characterized.

Enzyme assay and characterization

The enzyme assay was based on the measurement of glycine produced during substrate hydrolysation. A ninhydrin method was used for glycine quantification. Five microliters of the enzyme solution in 20 mmol/L potassium phosphate buffer (pH 7.5) was mixed with 100 μL of 1% HA solution and was then incubated at 37°C for 10 min by shaking. Subsequently, 50 μL of the ninhydrin solution [1.75 g of ninhydrin in 50 mL of acetone and butanol (1:1, v/v)] was added and reacted at 37°C for 10 min by shaking. The ninhydrin reaction with glycine formed by the enzyme reaction was measured spectrophotometrically at 570 nm (secondary wavelength: 700 nm) against a sample blank. One unit of activity was defined as the increased absorbance per minute at 37°C and pH 7.5. Various concentrations of substrate solution were used to determine the K_m value. Molecular weight was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Enzymatic HA assay

A spectrophotometrical HA assay method using Hhase was newly developed. The final assay mixtures were composed of reagent 1 and 2 (R1 and R2). R1 contained 3 mg/mL Hhase, 7.5 U/mL horseradish peroxidase, 4.5 mmol/L N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS), and 50 mmol/L Tris-HCl buffer (pH 8.5). R2 contained 7.2 U/mL glycine N-methyltransferase, 40 U/mL sarcosine oxidase, 1.2 mmol/L 4-aminoantipyrine, 10 mmol/L S-adenosyl-L-methionine, and 50 mmol/L Tris-HCl buffer (pH 8.5). Standard HA solution (2 μL) was incubated with R1 (180 μL) at 37°C for 5 min. Subsequently, R2 (60 μL) was added and re-incubated at 37°C for 5 min. The amount of quinoneimine dye formed by the coupling of 4-aminoantipyrine, TOOS, and horseradish peroxidase was continuously measured at 548 nm against a sample blank.

Homology modeling

Homology modeling was used to build the structure model of the PH1043 gene product. MOE (Molecular Operating Environment) software (Chemical Computing Group Inc., Montreal, Canada) was used to generate a three-dimensional protein model based on the structure of the similar protein[12-14]. Pymol software was used for structure visualization[15].

Site-directed saturation mutagenesis

A KOD-Plus Mutagenesis kit (Toyobo Co., Ltd.) was used for the site-directed saturation mutagenesis of the PH1043 gene. Inverse PCR primers for mutagenesis are shown in Fig. 2.

Screening of highly active Hhase

Hhase mutants created were grown using 96-deep well plates in 1/9 concentration of LB medium (200 μL) containing 30 μg/mL kanamycin and 5.5% glycerol at 37°C overnight with rotation at 150 rpm. The cells were lysed by two cycles of freezing and thawing at -80°C and room temperature (25°C), and crude extracts were prepared. Highly active Hhase mutants were screened by the enzyme assay with a microplate reader (Tecan Japan Co., Ltd., Kawasaki, Japan).

Mutation residue	Primer sequence	
E11	Forward:	NNNTTGCAGGGGTATATAGTTGAGAAGAGG
	Reverse:	TTTTGCTCTTTTAATTATAAACTCATCCAT
K193	Forward:	NNNGGAGGTCACGGAGCAGCACCGCAC
	Reverse:	CCCCTTTATCAAAACCCTAAATGCATCTGC
P200	Forward:	NNNCACCTCTCGATAGACCCAATAGCTTTA
	Reverse:	TGCTGCTCCGTGACCTCCCTTCCCCTT
P206	Forward:	NNNATAGCTTTAGCTGTAGATTTAGTGAATGCCT
	Reverse:	GTCTATCGAGAGGTGCGGTGCTGCTCC
I221	Forward:	NNNTCGAGGGAAGTAGATCCACTACAACCA
	Reverse:	TATCTTCTGATAGGCATTCACTAAATCTAC
P227	Forward:	NNNCTACAACCAGCGGTTCTTAGCGTTACC
	Reverse:	ATCTACTTCCCTCGATATTATCTTCTGATA
T236	Forward:	NNNTCGATAAAAGCTGGAACGACATTTA
	Reverse:	AACGCTAAGAACCGCTGGTTGTAGTGGATC
P248	Forward:	NNNGAATCCGCGGAGATACTTGGAACCATA
	Reverse:	AATTACATTAAATGTCGTTCCAGCTTTTAT
E249	Forward:	NNNTCCGCGGAGATACTTGGAACCATAAGG
	Reverse:	TGGAATTACATTAAATGTCGTTCCAGCTTT
T276	Forward:	NNNGAAAACTTTGCCAATGGGATGAGGT
	Reverse:	AATCTCCTTCATTCTCCTTACTATATAATC
N281	Forward:	NNNGGGATGAGGTGCGAGGGGAAGTTTG
	Reverse:	GGCAAAGTTTTCAGTAATCTCCTTCATTCT
I293	Forward:	NNNGAGCATATACCCCCAACTATAAATAAT
	Reverse:	CGTTAGTTCAAACTTCCCCTCGCACCTCAT
N307	Forward:	NNNTTTGCGAGGGATGTGCTAAAGGTTCTG
	Reverse:	CGCCAGCTTTTCATTATTTATAGTTGGGGG
E318	Forward:	NNNATTAGGGAGCCTAAACCAACGATGGGA
	Reverse:	GCCCAGAACCTTTAGCACATCCCTCGC
P324	Forward:	NNNACGATGGGAGCTGAAGATTTTGCCTTT
	Reverse:	TTTAGGCTCCCTAATTTCGCCCAGAAC

Fig. 2 Mutagenesis primers. Mutation points are underlined.

3. Results and Discussion

Development of new Hhase and characterization

The development of a practical enzymatic HA assay requires sufficiently stable Hhase. We focused on the hyperthermostable carboxypeptidase/aminoacylase encoded in the PH1043 gene of *P. horikoshii* OT3. As previously reported, this enzyme reacts well with N-benzyloxycarbonylglycine[9], and therefore, we expected it to react with HA. The PH1043 gene product was produced from the recombinant *E. coli* strain carrying the expression plasmid pET24-PH1043HT and was purified, as described in the Materials and Methods section. A HA-hydrolysing activity of the purified enzyme was accurately detected to be 0.020 U/mg. Hence, the PH1043 gene product was found to be a new Hhase, named Hhase1043 in this paper.

The purified Hhase1043 was characterized by the HA-hydrolysing activity. It is a homodimer possessing one zinc ion per subunit, as described previously[9]. Subunit molecular weight was estimated to be 43 kDa from SDS-PAGE, corresponding to the value calculated from the amino acid sequence (45 kDa). Enzymatic properties of Hhase1043 are shown in Fig. 3. The K_m value for HA was estimated to be 4.1 mM (Fig. 3A). The optimum temperature and pH were above 90°C and 6.2, respectively (Fig. 3B, C).

Development of enzymatic HA assay method

An enzymatic assay method using Hhase1043 was developed for the colorimetric measurement of HA concentrations. The cyclic reaction and 4-aminoantipyrine-peroxidase system following the Hhase1043 reaction quantified the hydrolysed product glycine (Fig. 4A). The cyclic reaction was composed of glycine methylation and sarcosine (N-methylglycine) oxidation by glycine N-methyltransferase and sarcosine oxidase, respectively. The assay method worked well in analysis of standard HA solutions (Fig. 4B). It is expected to be applicable on high-throughput monitoring of HA for health checks of those working with toluene.

Construction of Hhase1043 model structure

A three-dimensional structural model of Hhase1043 was constructed by homology modeling (Fig. 5), using a template structure with the highest sequence identity [X-ray structure of indole-3-acetic acid-amino acid hydrolase from the plant *Arabidopsis thaliana* (PDB ID: 1XMBa), amino acid identity: 45.1%][16]. The overall structures of Hhase1043 and 1XMBa superimpose well, with a root mean square deviation for atomic Cα positions of 1.07 Å. The structure provided a basis for identifying sites for mutagenesis designed to alter the activity of the enzyme. It will also enhance understanding of the structure-function relationship in Hhase1043.

Screening of highly active Hhase1043

The wild-type Hhase1043 exhibited a weak HA-hydrolysing activity at 37°C. Highly efficient assays required much higher hydrolysing activities.

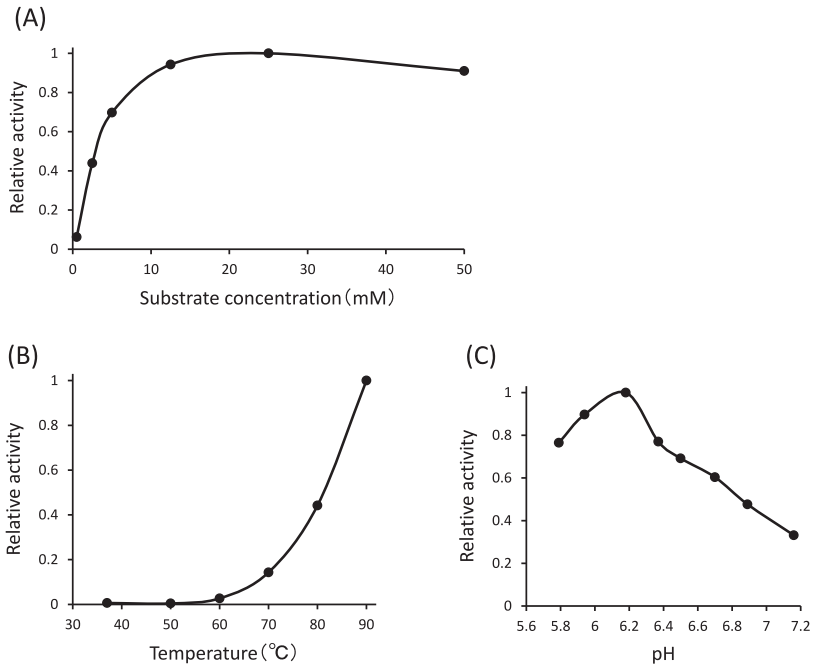

Fig. 3 Properties of Hhase1043. (A) Substrate (HA)-activity profile at increased concentrations. (B) Effect of temperature on HA-hydrolysing activity. (C) Effect of pH on HA-hydrolysing activity. Potassium phosphate buffers with various pH were used.

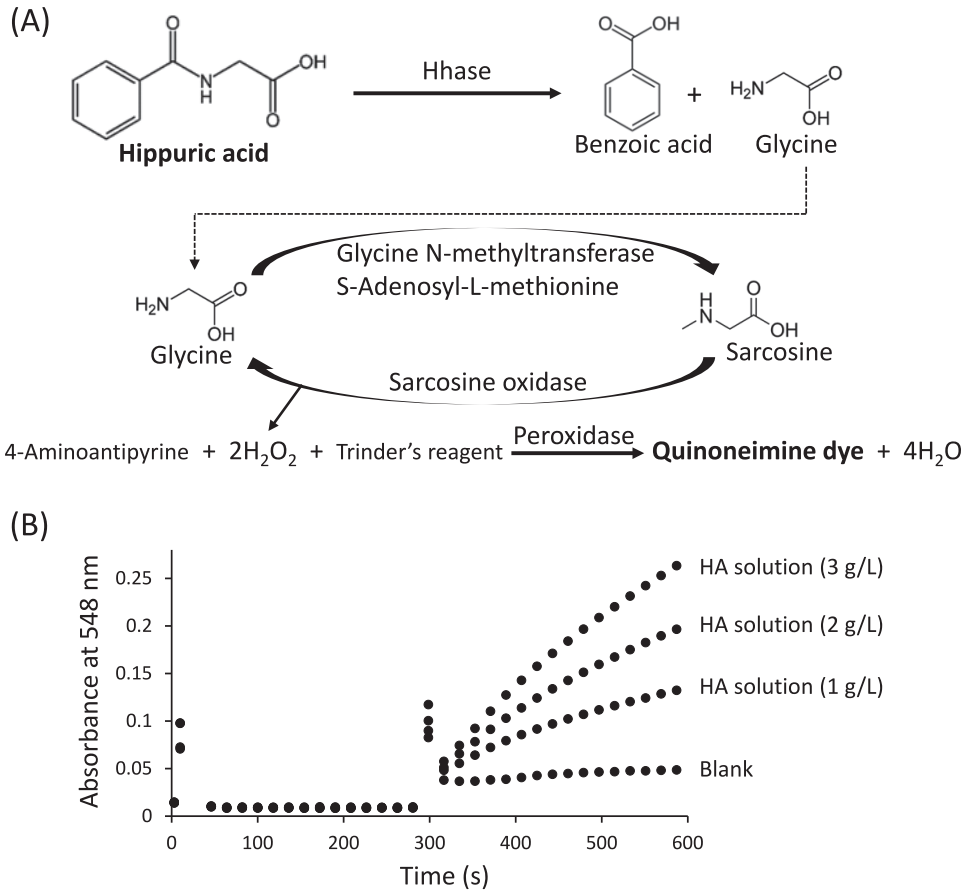

Fig. 4 Enzymatic HA assay. (A) Assay principle. (B) Measurement of standard HA solutions.

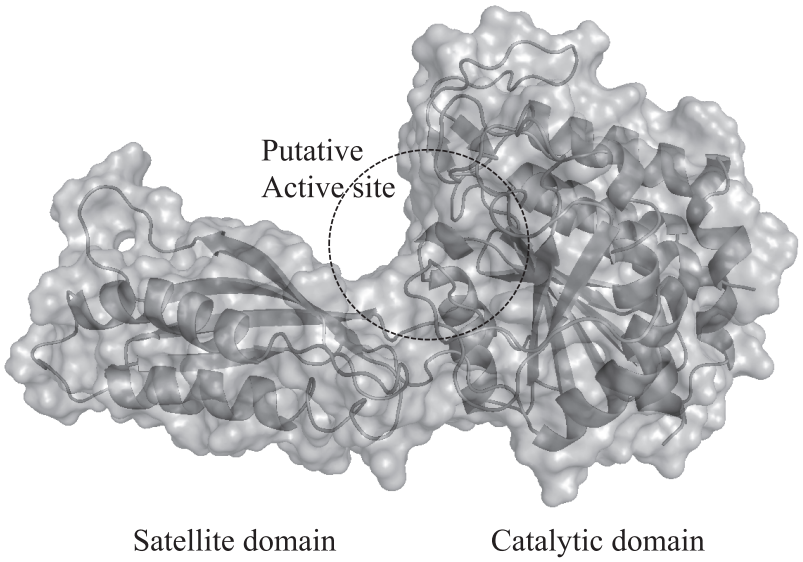

Fig. 5 A model structure of Hhase1043. The overall structure was shown in ribbon and transparent surface representations.

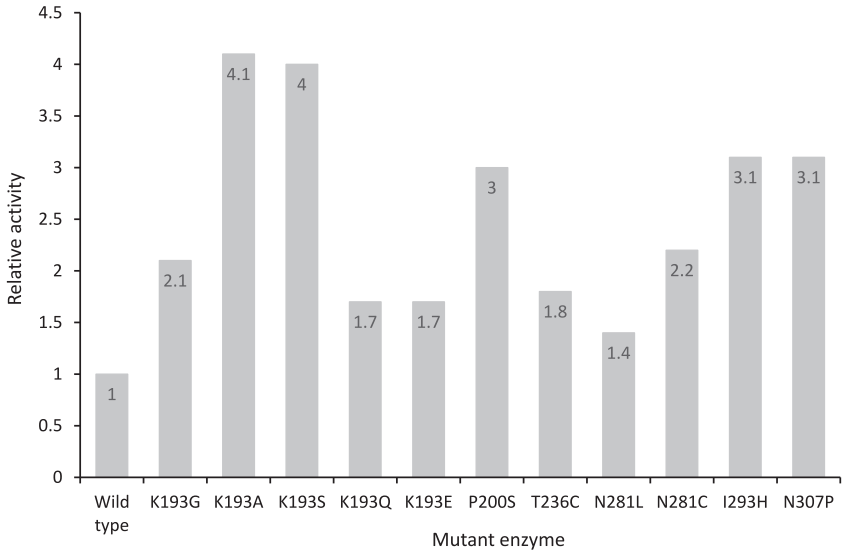

Fig. 6 Hhase1043 mutants with higher HA-hydrolysing activities and their relative activities to wild-type enzyme.

To improve the practicality of Hhase1043, screening of highly active mutant enzymes was planned using protein engineering techniques.

As shown in Fig. 5, the structure of Hhase1043 was composed of a larger catalytic domain with aminopeptidase topology and a smaller satellite domain, like 1XMBa[19]. Fifteen amino acid residues (E11, K193, P200, P206, I221, P227, T236, P248, E249, T276, N281, I293, N307, E318, and P324), that were mainly in loop regions and spaces between secondary structures of the satellite domain, were selected as mutational targets (Fig. 2). Each residue was randomly mutated by site-directed saturation mutagenesis as described in the Materials and Methods section. A total of approximately 3,000 mutants constructed were screened, and effective mutants on the HA-hydrolysing rate were confirmed by secondary and tertiary screenings. Finally, 11 mutants with higher HA-hydrolysing activities than that of the wild-type Hhase1043 were obtained.

These were changed in 6 amino acid residues (K193, P200, T236, N281, I293, and N307). All mutant enzymes were purified, and their specific activities were 1.4-4.1 times higher than that of the wild-type (Fig. 6).

Thus, highly active mutants of Hhase1043 were successfully obtained based on structural prediction and protein engineering. These enzymes will be applied to the development of an effective and cost-saving HA assay.

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. 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.
4. 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.
5. Ferrieri P, Wannamaker LW, and Nelson J: Localization and characterization of the hippuricase activity of group B Streptococci. Infect Immunity, 7: 747-752, 1973.
6. Ottow JCG: Detection of hippurate hydrolase among *Bacillus* species by thin layer chromatography and other methods. J Appl Bacteriol, 37: 15-30, 1974.
7. 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.
8. 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.
9. 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.
10. Aiba H, Nishiya Y, and Azuma M: Over-expression, characterization and modification of highly active alkaline phosphatase from a *Shewanella* genus bacterium. Biosci Biotechnol Biochem, 81: 1994-2001, 2017.
11. 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.
12. 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.
13. 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.
14. 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.
15. Nishiya Y: Homology modeling and docking study of creatinine deiminase. Int J Anal Bio-Sci, 1: 55-59, 2013.
16. Bitto E, Bingman CA, Bittova L, Houston NL, Boston RS, Fox BG, and Phillips Jr. GN: X-ray structure of ILL2, an auxin-conjugate amidohydrolase from *Arabidopsis thaliana*. Proteins, 74: 61-71, 2009.