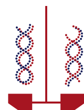
Journal of Bioscience and Bioengineering
VOL. 122 No. 2, 140–145, 2016

www.elsevier.com/locate/jbiosc

Re-characterization of mono-2-ethylhexyl phthalate hydrolase belonging to the serine hydrolase family

Makoto Iwata,[1] Takuya Imaoka,[2] Takashi Nishiyama,[2] and Takao Fujii[2,*]

IMB Co., Ltd., 1070-10 Hitotsugi, Asakura City, Fukuoka 838-0065, Japan[1] *and Department of Applied Life Science, Sojo University, 4-22-1 Ikeda, Nishiku, Kumamoto 860-0082, Japan*[2]

Received 16 November 2015; accepted 13 January 2016
Available online 8 February 2016

A novel bacterium assimilating di-2-ethylhexyl phthalate as a sole carbon source was isolated, and identified as a *Rhodococcus* species and the strain was named EG-5. The strain has a mono-2-ethylhexyl phthalate (MEHP) hydrolase (EG-5 MehpH), which exhibits some different enzymatic features when compared with the previously reported MEHP hydrolase (P8219 MehpH) from *Gordonia* sp. These differences include different pH optimum activity, maximal reaction temperature and heat stability. The K_m and V_{max} values of EG-5 MehpH were significantly higher than those of P8219 MehpH. The primary structure of EG-5 MehpH showed the highest sequence identity to that of P8219 MehpH (39%) among hydrolases. The phylogenetic tree suggested that EG-5 MehpH and P8219 MehpH were categorized in different groups of the novel MEHP hydrolase family. Mutation of a conserved R^{109} residue of EG-5 MehpH to a hydrophobic residue resulted in a dramatic reduction in the V_{max} value towards MEHP without affecting the K_m value. These results indicate that this residue may neutralize the negative charge of a carboxylate anion of MEHP, and thus inhibit the catalytic nucleophile from attacking the ester bond. In other words, the R residue blocks inhibition from the carboxylate anion of MEHP. Recently, registered hypothetical proteins exhibiting 98% or 99% identities for EG-5 MehpH or for P8219 MehpH were found from some pathogens belonging to *Actinomycetes*. The protein may have other activities besides MEHP hydrolysis and function in other physiological reactions in some *Actinomycetes*.
© 2016, The Society for Biotechnology, Japan. All rights reserved.

[**Key words:** Phthalate monoester hydrolase; *Gordonia*; *Rhodococcus*; Diethylhexyl phthalate; Monoethylhexyl phthalate]

Phthalate diesters, such as di-2-ethylhexyl phthalate (DEHP), are used heavily as plasticizers for industrial production of plastics. The annual amount of phthalate diesters circulated in Japan was estimated to be 200 kilotons in 2014. These compounds appear in domestic and industrial wastewater because phthalate diesters readily leak from plastic goods, such as tableware, and may be discharged from plastic-producing industrial plants. Phthalate diesters are recalcitrant compounds and accumulate as environmental contaminants (1), and are suspected to be endocrine-disrupting chemicals (2,3). Reports describing developmental toxicity and teratogenic effects of these compounds on animals are increasing (4,5).

A microorganism named *Gordonia* sp. P8219 that grows with DEHP as a sole carbon source was isolated from a soil sample in previous research (6). An intracellular enzyme catalyzing the hydrolysis of mono-2-ethylhexyl phthalate (MEHP), an intermediate compound in the DEHP degradation system to phthalic acid was purified from the strain and characterized. The enzyme is homodimer composed of a 32,164 Da polypeptide that hydrolyzes effectively phthalate monoesters, such as monoethyl, monobutyl, monohexyl as well as MEHP. The K_m and V_{max} values for MEHP are 26.9 ± 4.3 μM and 18.1 ± 0.9 μmol/min mg protein, respectively.

Furthermore, we showed that the primary structure of the hydrolase exhibits less than 30% homology with C–C hydrolases found in several bacteria, which hydrolyze compounds generated by *meta*-cleavage of phenol rings of biphenyl compounds, carbazole and catechol (7–10). The catalytic triad and pentapeptide motif GX_1SX_2G, conserved in the serine hydrolase family is also present in the MEHP hydrolase sequence. The motif has, however, a distinct basic residue (6). The first X_1 is N or H (N is major, and H is minor). X_2 is usually a hydrophobic residue (F, M or L) in C–C hydrolases and some serine hydrolases, while X_2 of MEHP hydrolase from *Gordonia* sp. P8219 is arginine.

In the previous paper, we discussed that the R residue in the pentapeptide motif may have some interaction with the carboxylic acid of the substrate during the hydrolysis of the phthalate monoesters (6). Currently, the amino acid sequences of several enzymes that use phthalate diesters or monoesters as substrates have been reported (11–14), and all of them belong to the serine esterase family, except for PatE, which is a cysteine esterase from *Rhodococcus jostii* (15). There is no report indicating the presence of a basic residue at X_2 in other enzymes.

In this study, we acclimated and isolated a novel bacterium strain, named *Rhodococcus* sp. EG-5, which assimilates DEHP as a sole carbon source from soil mixed with polishing oil. The strain EG-5 showed MEHP hydrolase activity in a cell-free extract like *Gordonia* sp. P8219. We purified and characterized the EG-5 MEHP hydrolase, and found that the canonical hydrophobic residue at the X_2 position in the pentapeptide motif was replaced with an

* Corresponding author. Tel.: +81 96 326 3948; fax: +81 96 323 1331.
E-mail address: fujii@life.sojo-u.ac.jp (T. Fujii).

1389-1723/$ – see front matter © 2016, The Society for Biotechnology, Japan. All rights reserved.
http://dx.doi.org/10.1016/j.jbiosc.2016.01.008

arginine residue like MEHP hydrolase of *Gordonia* sp. P8219. The importance of this basic residue for MEHP hydrolase activity was investigated. Furthermore, a search of the protein database to identify possible relatives of MEHP hydrolases among registered proteins in the protein database provided phylogenetic insights.

MATERIALS AND METHODS

Chemicals DEHP and MEHP were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Toyopearl Butyl-650M gel was from Tosoh (Tokyo, Japan), and DE 52 and Superdex 200 pg gels were GE Healthcare (Little Chalfont, UK). The Isoplant kit for DNA extraction and restriction endonucleases were purchased from Nippon Gene Co. Ltd. (Tokyo, Japan). KOD-plus-DNA polymerase and pCold I were purchased from Toyobo (Osaka, Japan) and Takara Bio (Shiga, Japan), respectively. Other chemicals were from Wako Pure Chemical Industries Ltd.

Isolation of the DEHP-assimilating microorganism A M9 mineral medium with appropriate amount of DEHP as a sole carbon source, whose composition was reported previously (6), was used to acclimatize the DEHP-assimilating microorganisms. The acclimatized microorganisms were isolated as described previously (6). Colonies were screened to isolate novel strains by focusing on colony appearance that differed from that observed for the P8219 strain.

Determination of cell morphology and the 16S rRNA gene sequence The morphology was determined by differential interference microscopy, following growth on M9 medium with DEHP as the sole carbon source. Extraction of DNA was performed after cultivation of the strain in NB medium containing 2% glucose and the 16S rRNA gene was amplified using the extracted DNA as the template. The amplified gene was sequenced with an ABI Prism 3130x1 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Cultivation for preparation of the cells NB liquid medium containing 2% glucose was used as a pre-culture of a DEHP-assimilating strain. A portion of isolated cells was inoculated into 20 ml of the medium in a 100-ml Erlenmeyer flask and incubated at 25°C on a rotary shaker for 2 d. The culture was then transferred to 200 ml of medium containing 150 μl of DEHP in liquid state in a 500-ml flask and incubated at 25°C on a rotary shaker for 25 h.

Purification of the MEHP hydrolase of *Rhodococcus* sp. EG-5 The culture of strain EG-5 was centrifuged 15,000 ×*g* for 15 min to precipitate the cells, which were disrupted with an Astrason ultrasonic processor XL2020 (Misonix, Farmingdale, NY, USA). After removing cell debris by centrifugation, the supernatant was then super-centrifuged at 160,000 ×*g* for 1 h to prepare cell extracts. Purification of the enzyme was performed by column chromatography using DE 52, Toyopearl Butyl-650M and Superdex 200 pg columns, according to methods reported previously (6).

Determination of the activity of MEHP hydrolase The standard reaction mixture (1.5 ml) consisted of 100 mM Tris–HCl buffer, pH 8.0, an appropriate amount of enzyme solution and 0.015 ml of a 20 mM MEHP–methanol solution. The reaction was performed in a quartz cuvette incubated at 45°C. The decrease in absorbance at 242 nm of the reaction mixture against that without the enzyme (control) was monitored and recorded with a spectrophotometer MPS-2400 (Shimadzu Co. Ltd., Kyoto, Japan). The initial reaction rates were calculated with an extinction coefficient difference between MEHP and phthalic acid at 242 nm of 1.83 mM^{-1} cm^{-1}. To determine the hydrolysis activity to 1-naphthyl esters, the increase in absorbance at 322 nm was observed, and the initial reaction rates were calculated with an extinction coefficient difference between 1-naphthyl esters and 1-naphthol at 322 nm of 2.20 mM^{-1} cm^{-1}.

Effects of pH and temperature on the activity of MEHP hydrolase The EG-5 MehpH was kept in 150 mM potassium phosphate buffer and Tris–HCl buffer at various pH values and 20°C for 30 min to study the pH stability. The enzyme was incubated at various temperatures between 0°C and 60°C for 10 min at pH 8.0, and the activity was then measured at 45°C to study temperature stability. The reaction was performed at various pH values and temperatures for determination of optimum pH and temperature, respectively.

Analysis of protein concentration Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was used with BSA as the standard.

Determination of peptide sequence Peptides in the SDS-PAGE gel were electro-blotted onto a PVDF membrane, which were sequenced using an amino acid sequencer (model PPSQ-21A, Shimadzu Co. Ltd.).

Identification of the MEHP hydrolase gene The sequences of the N-termini of three polypeptides generated by BrCN decomposition of the MEHP hydrolase were determined. Degenerate primers were synthesized on the basis of an appropriate part of the each sequence (Table S1). To amplify regions of the MEHP hydrolase gene, many DNAs fragments were PCR-amplified using chromosomal DNA of the strain EG-5 and by combining arbitrarily pairs of the primers. The resulting PCR products were sequenced. One of the products amplified by using RM-2F and RM-3R was selected and used for designing alternative primer pairs for inverse PCR. An inverse PCR reaction was performed with the newly designed primer pair to amplify a longer part of the gene, as described previously (6). Repeating the inverse PCR and sequencing enabled the isolation of DNA potential for encoding the full-length gene. To clone the gene, PCR amplification, that was not inverse PCR, was performed with chromosomal DNA as the template using a primer pair designed based on terminal sequences of the DNA determined by the last inverse PCR. The DNA fragment (1387 bp) (accession no. LC094142) obtained was cloned with pBluescript II KS+ and *Escherichia coli* DH5α. The gene encoding MEHP hydrolase was called EG-5 *mehpH*.

Construction of site-directed mutants of EG-5 *mehpH* The DNA fragment containing EG-5 *mehpH* was PCR-amplified using chromosomal DNA of the strain EG-5 as a template with the forward primer 5′-CAT**ATG**AACACAGACCTGTCCGTGA-3′ (underline, NdeI restriction site; bold letters, initiation codon changed from TTG to ATG) and the reverse primer 5′-TCTAGAACGACGCGTCGCATAG-3′ (underline, XbaI restriction site). The amplified fragment was incorporated into the EcoRV site of pBluescript II KS+. *E. coli* DH5α cells were transformed with the plasmid, the cloned plasmid was sequenced to confirm the accuracy of amplified fragment, and this construct was used to mutate R^{109} to a hydrophobic residue (i.e., R109F, R109M and R109A). One of the forward primers was combined with the reverse primer shown in Table 1 to amplify the gene by PCR and site-directed mutagenesis. The amplified PCR products carrying the mutated gene were self-ligated and transformed into *E. coli* DH5α cells. Sequencing was performed to confirm the mutations.

Expression of recombinant wild-type and mutant MehpH enzymes The plasmids carrying wild-type and mutant EG-5 *mehpH* gene fragments were extracted from the individual host cells and digested with NdeI and XbaI endonucleases, which were ligated into the same endonuclease cutting sites of the pCold I expression vector. *E. coli* BL21(DE3) was transformed with each plasmid. Each transformant was grown on Luria–Bertani broth with 50 mg/L ampicillin and cold-shocked according to the manufacturer's instructions. The cells were harvested, suspended in 20 mM Tris–HCl (pH 8.0) and 200 mM NaCl, and disrupted by sonication. The cell extracts were centrifuged at 10,000 ×*g* for 30 min and the supernatant was super-centrifuged at 100,000 ×*g* for 1 h. The recombinant wild-type and mutant EG-5 MehpH enzymes were purified by Ni-NTA column chromatography.

RESULTS

Characterization of cells of a novel bacterium The newly isolated colony was dry, light red and rough, which contrasts *Gordonia* sp. P8219. The cells were gram-positive, not acid fast, non-motile, rod shaped or coccoid, and strictly aerobic. The cells occurred in pairs and very often in a typical coryneform V shape, as observed for strain P8219. We amplified the 16S rRNA gene of an isolated bacterium. The amplified gene was cloned and sequenced. The 1441-bp sequence was determined (registration no. LC094141) and homology searching was carried out against the nucleotide database by the Blastn program. The results demonstrated that the gene shared >99% of the 16S rRNA gene sequence of *Rhodococcus erythropolis*, indicating the isolated bacterium is a novel strain that belongs to the genus *Rhodococcus*. Thus, this strain was called *Rhodococcus* sp. EG-5.

Characterization of the EG-5 MehpH The purified MehpH enzyme had a specific activity of 26 μmol/min·mg protein. The purity of the isolated protein was confirmed by SDS-PAGE; a single protein band with a corresponding mass of ∼33 kDa (Fig. S1). Hereafter, MEHP hydrolases from the strain EG-5 and P8219 were called EG-5 MehpH and P8219 MehpH, respectively. The molecular mass of the EG-5 MehpH was estimated to be approximately 66 kDa by gel filtration chromatography. Thus, EG-5 MehpH is a homodimer like P8219 MehpH. The EG-5 MehpH

TABLE 1. Oligonucleotide primer used for site-directed mutagenesis.

Primer[a]	Sequence[b]	Target site in the ORF
EG5-Arg109Phe-F	5′-**TT**CGGTGGCCTGCTGGCGAG-3′	325–344[c]
EG5-Arg109Met-F	5′-**ATG**GGTGGCCTGCTGGCGA-3′	325–343[c]
EG5-Arg109Ala-F	5′-**GC**CGGTGGCCTGCTGGCGA-3′	325–343[c]
EG5-MehpH-R	5′-CGAGTGCCCGACCAGAATCATGT-3′	324–302[c]

[a] F and R, forward and reverse primers, respectively.
[b] Bold letters indicate mutated nucleotide residues.
[c] The number shows the site from the first nucleotide of the start codon of ORF encoded by EG-5 mehpH gene.

دانلود مقاله

http://daneshyari.com/article/19992

- ✓ امکان دانلود نسخه تمام متن مقالات انگلیسی
- ✓ امکان دانلود نسخه ترجمه شده مقالات
- ✓ پذیرش سفارش ترجمه تخصصی
- ✓ امکان جستجو در آرشیو جامعی از صدها موضوع و هزاران مقاله
- ✓ امکان پرداخت اینترنتی با کلیه کارت های عضو شتاب
- ✓ دانلود فوری مقاله پس از پرداخت آنلاین
- ✓ پشتیبانی کامل خرید با بهره مندی از سیستم هوشمند رهگیری سفارشات