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ARTICLE

Cloning of an organophosphorus hydrolase (opdD) gene of *Lactobacillus sakei* WCP904 isolated from chlorpyrifos-impregnated *kimchi* and hydrolysis activities of its gene product for organophosphorus pesticides

Md. Azizul Haque[1,3] · Soo Young Hong[2] · Chung Eun Hwang[3] · Su Cheol Kim[3] · Kye Man Cho[3]

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Abstract Chlorpyrifos (CP) residues are absorbed from soil and often found in Korean cabbages that are being used to make *kimchi*. *Lactobacillus sakei* WCP904, harboring the organophosphorus (OP) hydrolase gene *opd*D, was isolated from CP-impregnated *mulkimchi*. The cloned gene *opd*D from strain CP904 comprises 825 base-pair nucleotides that encode 274 amino acids. The recombinant *Escherichia coli* harboring the *opd*D gene depleted 73% of CP after 6 days in M9 medium. In fact, the OpdD protein is a novel member of the GHSQG family of esterolytic enzymes or lactic acid bacterial Opd groups. The molecular weight of the OpdD protein was estimated to be 31 kDa using SDS-PAGE. Broad-spectrum activities of the OpdD protein were obtained against OP insecticides containing both P–O and P–S bonds. The OpdD protein exhibits maximum activity at 30 °C with pH 6. No enzyme activities of the mutated OpdD (Ser116 → Ala116) protein toward *ρ*-nitrophenyl butyrate and CP substrates were observed. These results suggested that the strain WCP904 scavenges insecticide residues from *mulkimchi* vegetables, thus abolishing health hazards by secreting OP hydrolase during fermentation.

Keywords Biodegradation · *Kimchi* · *Lactobacillus sakei* WCP904 · *opd*D gene · Organophosphorus insecticides

Introduction

Chlorpyrifos (CP) is an organophosphorus (OP) insecticide being extensively applied on agricultural crops and vegetables worldwide [1]. Unfortunately, CP residues are gradually impregnated into the environment and persist for an unpredictable period [2], which poses a great threat to human health [3]. The fate of CP in the environment has been widely studied, and its half-life in soil varies from 10 to 120 days [3, 4], resulting in 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product. Hwang and Moon [5] recently published data, reporting that the residual CP ratios in Korean cabbage were 0.93–6.01 and 0.57–2.61%, respectively. The CP-degrading fungal strain *Verticillium* sp. was used in the detoxification of these insecticides on vegetables [6]. Moreover, Cho et al. [7] reported that lactic acid bacteria (LAB) such as *Leuconostoc mesenteroides* WCP907, *Lactobacillus brevis* WCP902, *Lactobacillus plantarum* WCP931, and *Lactobacillus sakei* WCP904 utilize OP insecticides as the sole sources of carbon and phosphorus and scavenged the insecticides from *yeolmu-mulkimchi* vegetables during fermentation.

The taste and character of *kimchi* are usually achieved through the response of LAB during fermentation. Based on changes in acidity, the *kimchi* fermentation process was divided into five phases—initial, immature, optimum ripening, over-ripening, and rancid [7]. In general, *kimchi*

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✉ Kye Man Cho
kmcho@gntech.ac.kr

[1] Department of Biochemistry and Molecular Biology, Hajee Mohammad Danesh Science and Technology University, Dinajpur 5200, Bangladesh

[2] Food Science Research Institute, Kolmar BNH Inc., Sejong 30003, Republic of Korea

[3] Department of Food Science, Gyeongnam National University of Science and Technology, Jinju 52725, Republic of Korea

fermentation is dominated by *Lactobacillus* sp. and *Leuconostoc* sp. [8, 9]. The population density of *L. mesenteroides* had reached its maximum in the ripening phase and then declined as the pH of *kimchi* dropped, but the population of acid-tolerant *lactobacilli* such as *L. plantarum* and *L. sakei* increased until the last phase of fermentation [9]. As stated above, *L. mesenteroides, L. brevis*, *L. plantarum,* and *L. sakei* play a role in the degradation of organophosphorus insecticides during *kimchi* fermentation [7]. In a related study, the role of *L. plantarum* phosphatase on OP insecticide degradation in skimmed milk was reported [10]. Meanwhile, we reported the OP hydrolase (*opd*B) genes from the *L. brevis* WCP902 responsible for the degradation of OP insecticides. However, the relevant gene of *L. sakei* responsible for the degradation of OP insecticides has not been revealed.

In this study, we cloned an OP hydrolase gene named opd from *L. sakei* WCP904 and expressed it in *Escherichia coli*. This is the first report of the cloning of an *opd* gene of *L. sakei* WCP904 isolated from CP-impregnated *mulkimchi.*

Materials and Methods

Strains, plasmids, media, and chemicals

The *E. coli* DH5α, media, and expression vectors used in this study were the same as previously described [11]. The pure grade OP insecticides including coumaphos (CM), CP, cadusafos (CS), diazinon (DZ), dyfonate (DF), ethoprophos (EP), fenamiphos (FA), methylparathion (MPT), parathion (PT), and the decomposition products such as TCP and diethylthiophosphoric acid (DETP) were purchased from Chem Service (West Chester, PA, USA) and Sigma-Aldrich, Inc. (Merck KGaA, Darmstadt, Germany), respectively. All other chemicals and reagents were of the same purity grade as previously described [11].

Isolation and identification of strain WCP904

The strain WCP904 was isolated from *yeolmu-mulkimchi* contaminated with a high concentration of CP [7] as previously described [10]. Specifically, the strain WCP904 was identified according to its 16S rRNA gene sequence and constructed phylogenetic tree [12].

Microbial cell growth and degradation of insecticides in liquid medium

The growth of strain WCP904 was continued in lactobacilli MRS (MRS) broth and was used as the inoculum for the degradation of CP in liquid medium. 250 µL of bacterial suspension (10^6 cfu/mL) was inoculated into 50 mL of 1/25 MRS medium containing 100 mg/L of CP. Recombinant *E. coli* DH5α (clone name: pGCY400) growth was also confirmed in the 1/25 MRS medium containing 100 mg/L of CP. The growth of microbial cells, such as strain WCP904 and *E. coli* DH5α, was identified by spectrophotometer at 600 nm (Spectronic 20, Thermo Fisher Scientific Co., Waltham, MC, USA). Moreover, the cross-feeding of the insecticides CM, CS, DZ, DY, ET, FE, MPT, and PT were also performed using the identical conditions. An individual flask of culture was harvested thereafter, and its decomposition residues and OP insecticides were determined at periodic intervals [11]. The decomposition process of insecticides with the strain WCP904 and recombinant *E. coli* cells was performed in triplicate.

Cloning and sequencing of the *opd*D gene

According to the carboxylesterase database, the forward primer sequence 5′-AAA GGA TCC ATG GTG ATA AGC TAT GTT GCG-3′ and reverse primer sequence 5′-AAA AAA CGT TCT ACG GGG CCC GTA A-3′ (Supplementary Table 1S) were designed to clone the complete open reading frame (ORF) of the *opd*D gene from the genomic DNA of the WCP904 strain. The amplified ORF was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). After that, the recombinant plasmid and pBluescript II SK + vector were digested with *Bam*HI and *Hind*III restriction enzymes separately, followed by cloning. The nucleotide sequence of the cloned opd gene in the plasmid was determined using the PRISM Ready Reaction Dye Terminator/primer cycle sequencing kit (Perkin-Elmer, Norwalk, CT, USA). The BLAST program was used to find the protein coding regions.

Expression and purification of the OpdD enzyme

To facilitate high expression levels of OpdD protein, the PCR product generated with forward primers 5′-AAA AGG ATC CAT GCA AAT TAT GAA TCA A-3′ and reverse primer 5′-AAA AAA CGT TAT GAT GAG CAA TAT CGA T-3′ (Supplementary Table 1S) was cloned into the expression vector pET-32a(+) (Merck KGaA, Darmstadt, Germany), resulting in the addition of a C-terminal $(His)_6$ tag. The expression and purification of the OpdD protein was performed as previously described [11]. The overexpressed protein concentration was determined by the method of Bradford [13].

Characteristics of the OpdD enzyme

The esterase activity of the OpdD enzyme was determined by a spectrophotometric method as previously described [11]. The influences of pH and temperature on the esterase activity of purified recombinant OpdD were examined. At 30 °C, the esterolytic activity of the OpdD enzyme was determined from pH 3.0 to 11.0. Moreover, the effect of temperature on the OpdD enzyme was determined at temperatures ranging from 10 to 70 °C for 1 h. A total of 50 µL of enzyme OpdD sample was added to an individual assay mixture containing 250 µL insecticides such as CS, CP, CM, DZ, DY, ET, FE, MPT, and PT with 700 µL of 200 mM phosphate buffer saline (PBS, pH 6.5). One unit of esterase activity was determined as the amount of enzyme required to release 1 µmol of ρ-nitrophenol (ρNP) per minute under the assay conditions. All the assays were performed in triplicate.

Site-directed mutagenesis

Site-directed mutagenesis was conducted to confirm the location of the catalytic sites of OpdD. The amino acid substitutions were introduced at position 116 (serine to alanine) of OpdD using oligonucleotide primers: 5′- GTA CTC GTT GGT TTC TCA GCT GGT GGG CAT CTA-3′ (sense) and 5′-TAG ATG CCC ACC AGC TGA GAA ACC AAC GAG TAC-3′ (antisense) (Supplementary Table 1S). The positions of the mutated codons are underlined in both primer sequences. The *Dpn*I-treated plasmids were then transformed into *E. coli* DH5α according to the manufacturer's specifications (Stratagene, La Jolla, CA, USA). Briefly, 50 µL of reaction reagents included 1 µL of pET-32a(+)/OpdD (80 ng/µL), 4 µL of 10 *p*mol of each primer, 5 µL of 2 mM dNTP mixture, 5 µL of 10 × *Pfu* DNA polymerase buffer with 20 mM $MgSO_4$, and 2.5 U of *Pfu* DNA polymerase. The PCR products were stored on ice for 5 min, and 1 µL of *Dpn*I restriction enzyme (10 U/µL) was added for 1-h incubation at 37 °C [11].

Analysis of residual nine OPs and TCP

The residual concentration of OP insecticides and TCP was determined using TLC and HPLC methods as previously described [7]. The culture supernatants were centrifuged (MICRO 17R Micro High Speed Centrifuge, Hanil Scientific Industrial Co., Ltd., Seoul, Korea) at 12,000 rpm for 5 min at 4 °C, and the debris-free culture extracts were dried over anhydrous Na_2SO_4 and concentrated under vacuum. To detect CP and TCP, the TLC plate was developed using chloroform, hexane (4:1, v/v) solvents and ethyl acetate, isopropanol, ammonium hydroxide (5:3:2, v/v) solvents, respectively. For HPLC analysis (Perkin-Elmer 200 series, CT, USA), 1 mL of culture supernatant was mixed with 1 mL of methanol and passed through a 0.45-µm PVDF filter (GmbH, Dassel, Germany). Next, 10 µL of the filtrate was injected into a C18 column (250 × 4.6 mm, 5 µm, Phenomenex, CA, USA). The elution was conducted using 0.5% acetic acid and methanol (1:4 (v/v)) at a flow rate of 1 mL/min at 30 °C. The concentration of insecticides and TCP was measured at 214 nm by a UV detector (Perkin-Elmer UV 200 series, Perkin-Elmer Corp., Norwalk, CT, USA). The typical HPLC chromatogram of OPs and TCP peaks is presented in supplementary Fig. S1. The nine OPs and TCP have been confirmed by comparing the previous literatures and standard materials [7], and their retention times are as follows: peak 1 of Fig. S1A (TCP, R.T. = 5.5 min) and peak 2 of Fig. S1A (CP, R.T. = 14.0 min) and peak 1 of Fig. S1B (CS, R.T. = 9.2 min), peak 2 of Fig. S1B (CM, R.T. = 7.0 min), peak 3 of Fig. S1B (DZ, R.T. = 5.4 min), peak 4 of Fig. S1B (DF, R.T. = 8.6 min), peak 5 of Fig. S1B (EP, R.T. = 6.1 min), peak 6 of Fig. S1B (FA, R.T. = 5.9 min), peak 7 of Fig. S1B (MPT, R.T. = 4.6 min), and peak 8 of Fig. S1B (PT, R.T. = 6.4 min).

Results

WCP904 strain identity and degradation of CP

A partial 16S rRNA gene sequence of the WCP904 strain was analyzed. The 16S rRNA gene similarity between the strain WCP904 and the LAB (including *Lactobacillus*, *Leuconostoc,* and *Weissella*) species ranged from 86.4 to 99.6%. The highest level of 16S rRNA gene similarity (99.6%) was observed between the WCP904 strain and *L. sakei* DSM 20017^T (Supplementary Fig. S2). The cell growth of the WCP904 strain and degradation pattern of CP with the strain WCP904 are shown in Supplementary Fig. S3. The strain's growth markedly increased after 1 day of incubation (OD 0.86), but dropped slightly at day 2. Thereafter, cell growth negligibly increased until day 6 (OD 0.91) during incubation (Supplementary Fig. S3A). However, the strain WCP904 exhibited an initial rapid degradation of CP estimated at approximately 67 mg/L during the first 3 days of incubation. After that, CP was degraded slowly, and maximum degradation was found to be 88 mg/L after day 9 of incubation (Supplementary Fig. S3B). The strain WCP904 was able to degrade CP to DETP and TCP and utilized DETP as the sole sources of carbon and phosphorus.

Cloning and sequencing of the *opd*D gene

PCR amplification of the genomic DNA of *L. sakei* WCP904 with specific primers produced product of approximately 1.5 kb size. A total 1500 bp nucleotide sequence was obtained in the open reading frame of *opd*D of *L. sakei* WCP904 (Fig. 1A). The ORF revealed that the gene sequence started with an ATG start codon and ended with a TAA ocher stop codon (Fig. 1B). The gene was predicted to encode 274 amino acids with molecular mass of 31 kDa (http://web.expasy.org/compute_pi/). Analysis of the amino acid sequence of the OpdD enzyme revealed no potential signal sequence. The calculated isoelectric pH (*p*I) of the OpdD enzyme is 5.18.

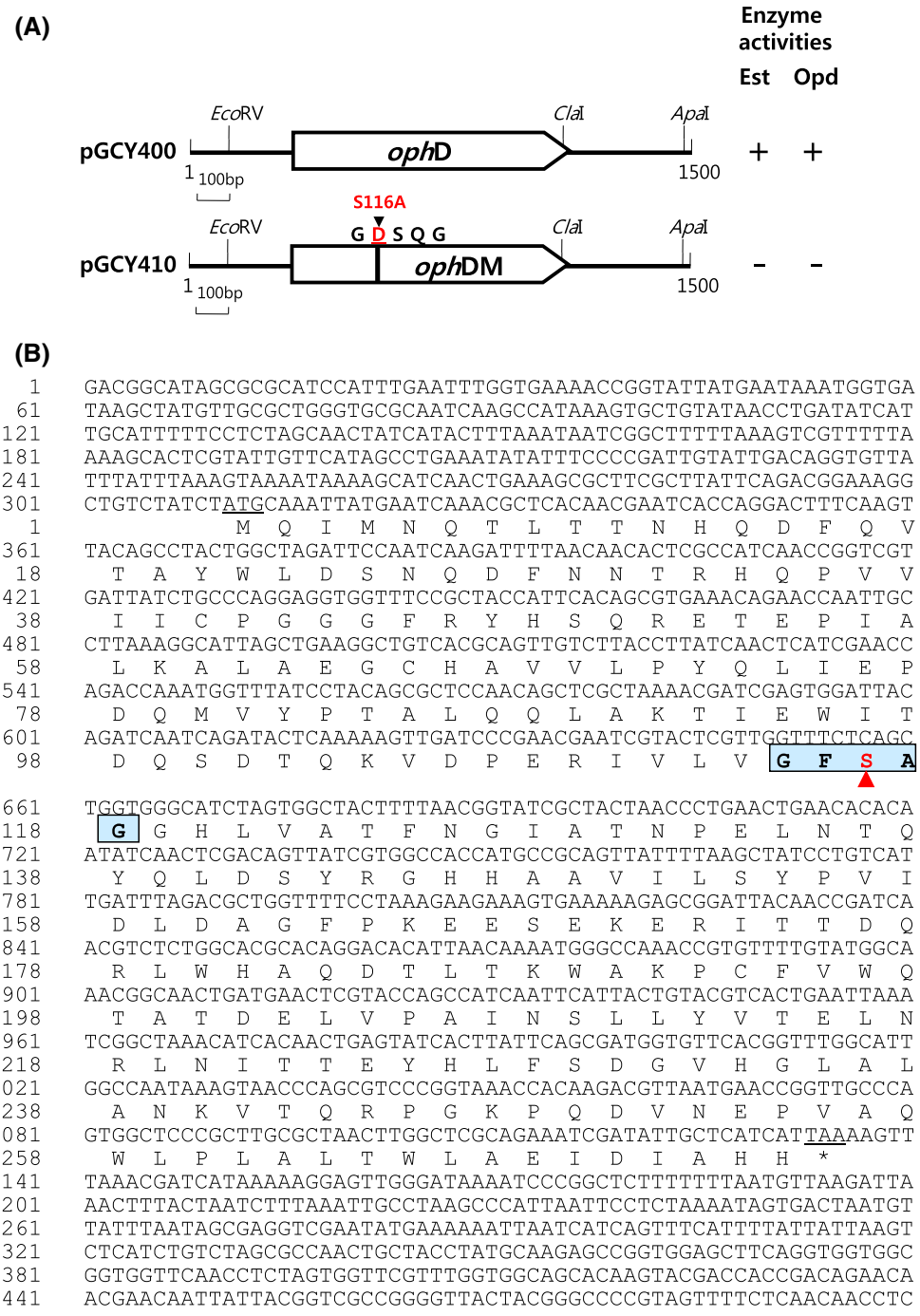

Fig. 1 Restriction mapping (**A**) and nucleotide sequences (**B**) of *the opd*D gene of *L. sakei* WCP904. The start codon is indicated by a bar, and the stop codon is indicated by an asterisk. The box shows the motif of esterolytic and lipolytic enzymes

Degradation of CP by the clone in liquid culture

The CP decomposition pattern of the clone pGCY400 (*opd*D) is shown in Fig. 2. As shown in Fig. 2A, CP and TCP with R_f values of 0.57 and 0.66, respectively, were detected in samples drawn at 0-, 1-, 3-, 6-, and 9-day intervals (Fig. 2A). The clone pGCY400 (*opd*D) gradually decomposed the CP in liquid culture. Particularly noteworthy, the clone decomposed CP markedly within 2 days, and the estimated concentration of CP was found to be 78 mg/L. Thereafter, the CP concentration markedly decreased to 24 mg/L at day 6. In contrast, the TCP concentrations were gradually increased to 32 mg/L at day 3 and then reached 68 mg/L at day 6 (Fig. 2B).

Comparison of *opd*D gene and OpdD protein with organophosphorus hydrolase gene and esterolytic proteins

The multiple sequence alignment had shown 40.74% homology of the *opd*D gene with OP hydrolase gene (JF837335.1) of *Enterobacter* sp. CCF01Et-UAEM, *oph* gene (HM191722.1) of *Pseudomonas* sp. BF1-3, *opd*B gene (FJ550130.1) of *L. brevis* strain WCP902, and *opd*B gene (AY646835.2) of *Burkholderia* sp. FDS-1 (Supplementary Fig. S4), while the amino acid sequence GHSQGS, starting at residue 116 of the OpdD enzyme, fits

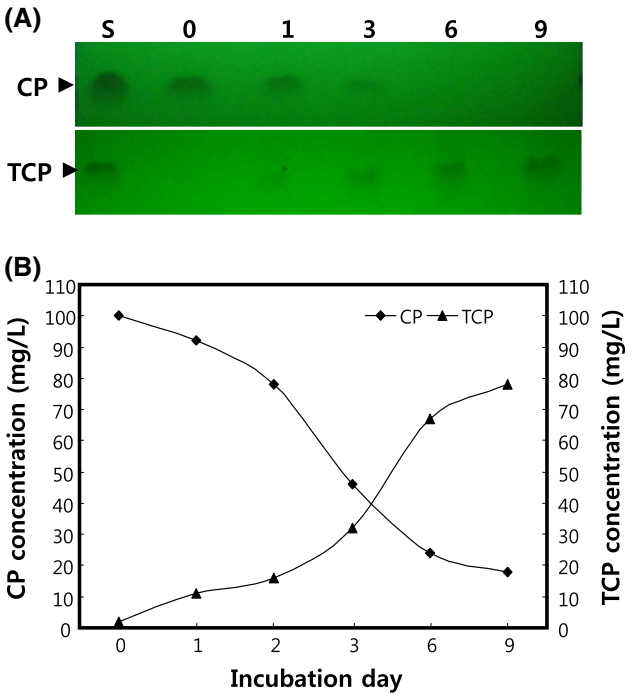

Fig. 2 Profiles of TLC (**A**) and changes of chlorpyrifos (CP) and 3,5,6-trichloro-2-pyridinol (TCP) concentration (**B**) of *opd*D clone (pGCY400) growing in the M9 medium containing 100 mg/L of CP for day 9. The standard errors were within 5% of the mean

with the Gly-X-Ser-X-Gly motif found in most bacterial and eukaryotic serine hydrolases (Figs. 1 and 3). However, a catalytic triad of Ser-Asp/Glu-His which is highly conserved in most esterase and lipase groups is absent in the OpdD enzyme. The homology tree made of esterolytic and lipolytic proteins showed that the OpdD protein does not belong to group I, II, III, or IV (Fig. 3). This deviation of OpdD from the known esterolytic and lipolytic proteins suggested that it may represent a new type of esterase.

Purification and characterization of the OpdD protein

The OpdD protein overproduced in *E. coli* BL21 (DE3) was purified using column filtration techniques. As seen by SDS-PAGE, a protein of 31 kDa was exhibited after the final purification step (Fig. 4). The optimum pH of the OpdD enzyme was determined to be 6 at 30 °C according to its ability to hydrolyze ρ-nitrophenyl butyrate (ρ-NPB) (Fig. 5A). Moreover, the optimum temperature for OpdD hydrolysis of ρ-NPB was determined to be 30 °C at pH 6 (Fig. 5B). Most lipases and carboxyl esterases have the consensus sequence motif Gly-X-Ser-X-Gly that includes the active site. Analysis of the deduced amino acid sequences of OpdD showed a potential serine hydrolase motif such as G-H-S116-Q-G. Site-directed mutagenesis of the serine hydrolase motif S116 of OpdD was performed to confirm whether it is involved in catalytic esterase action. The mutated purified protein did not exhibit enzymatic activity toward ρ-NPB and CP (Supplementary Table S2).

Substrate range test by the strain WCP904 and OpdD protein

All OP insecticides tested in the cross-feeding experiment were degraded by the WCP904 strain. The tested OP insecticides such as CM, CP, DZ, MPT, and PT have DEPT as side chains, but CS, DF, EP, and FA had no DEPT side chains. Except DF, eight other OP insecticides including CM, CP, CS, DZ, EP, FM, MPT, and PT were hydrolyzed at a phosphoester bond by the WCP904 strain. At day 9, the strain WCP904 provided 72–88% degradation of CM, CP, DZ, MPT, and PT, respectively (Table 1). In fact, nine OP insecticides were decomposed by the recombinant OpdD enzyme. The higher enzyme activity was especially observed toward CP, CM, DZ, MPT, and PT by OpdD (Table 1).

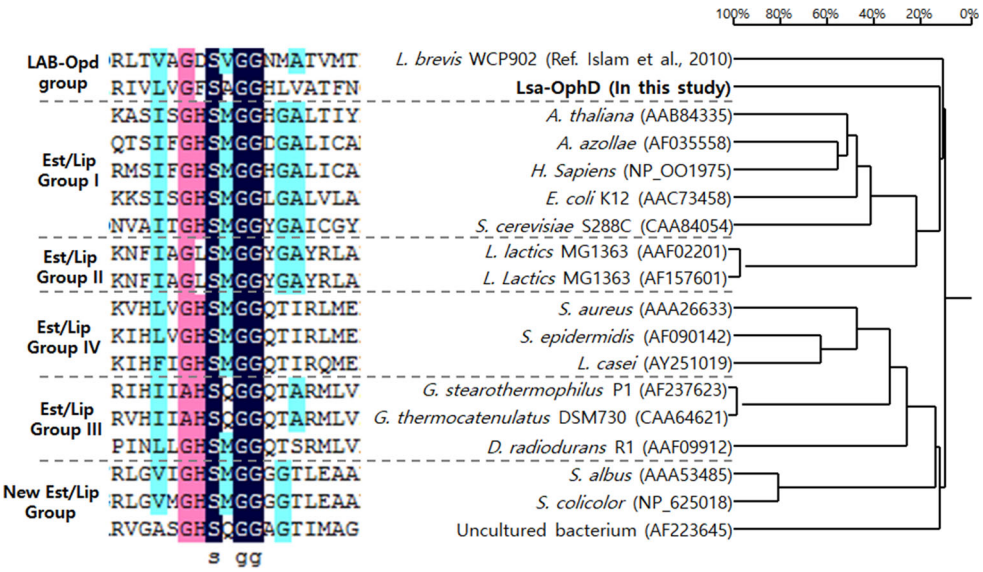

Fig. 3 Homology tree showing the evolutionary relatedness and homology degree between the esterolytic and lipolytic enzyme amino acid sequences, and the alignment of the conserved regions found in the primary esterolytic and lipolytic enzymes

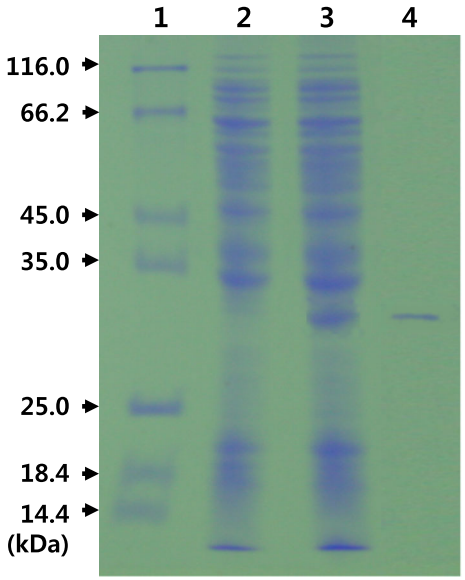

Fig. 4 Electrophoretic analysis of the purified OpdD protein. The separation was performed on a 12.5% (w/v) SDS polyacrylamide gel. The gel was stained with 0.025% Coomassie Blue R-250

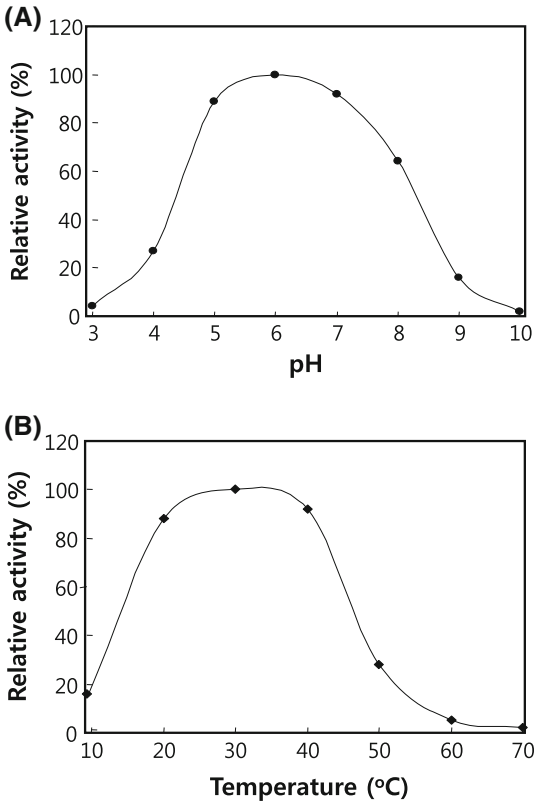

Fig. 5 Effect of pH and temperature on the relative activity of OpdD enzymes. (**A**) The esterase activity of OpdD was assayed using *ρ*-nitrophenyl butyrate as substrate at different pH values at 35 °C for 1 h. (B) The esterase activity of OpdD was assayed using *ρ*-nitrophenyl butyrate as substrate at different temperature values at pH 6.0 or 7.0 for 1 h

Discussion

The isolated *L. sakei* WCP904 was screened for its ability to degrade OP insecticides. The strain WCP904 has been shown to hydrolyze CP and to utilize part of the DEPT compound as its sole source of carbon, and that pathway was confirmed by TLC and HPLC analysis. When the *ye-olmu-mulkimchi* was fermented contaminated with CP, Cho et al. [7] observed a higher population of *L. sakei* WCP904. The strain WCP904 has a potential role for the decontamination of OP insecticides, which has been suggested based on its versatile exhibition in utilizing dimethyl compounds such as MPT and diethyl compounds such as CM, CP, DZ, and PT as its carbon source. In fact, the strain WCP904 degraded 67% of 100 mg/L CP within 3 days and

Table 1 Residual concentration of nine organophosphorus (OPs) insecticides degradation by the strain of *L. sakei* WCP904 and OpdD protein

Organophosphorus (OPs)	Residual OPs concentration (mg/L)[a]	
	WCP904 strain[b]	OpdD protein[c]
Coumaphos (CM)	22 ± 1.11	29 ± 1.30
Chlorpyrifos (CP)	16 ± 0.88	12 ± 0.57
Cadusafos (CS)	83 ± 2.49	88 ± 2.55
Diazinon (DZ)	22 ± 1.03	27 ± 0.98
Dyfonate (DF)	89 ± 3.56	94 ± 3.77
Ethoprophos (EP)	86 ± 2.84	87 ± 2.66
Fenamiphos (FA)	59 ± 2.36	63 ± 2.11
Methylparathion (MPT)	21 ± 0.76	26 ± 1.19
Parathion (PT)	14 ± 0.99	17 ± 1.03

[a]Values indicate the means of three replications. The standard errors were within 5% of the mean

[b]The strain WCP904 incubated in 1/25 MRS containing the 100 mg/L of the 9 OPs (CM, CP, CS, DZ, DF, EP, FA, MPT, and PT) for 9 days

[c]The OpdD activity assayed with CM, CP, CS, DZ, DF, EP, FA, MPT, and PT as substrate (100 mg/L) at pH 7.0 and 30 °C for 12 h, respectively

utilized its degraded products TCP and DEPT as the sole sources of carbon, while the *Enterobacter* strain B-14 degraded 40% of 25 mg/L CP within 48 h [14] and *Synechocystis* sp. strain PUPCCC 64 degraded 93.8% of 5 mg/L CP within 5 days [15]. However, the *Synechocystis* sp. strain PUPCCC 64 and *Enterobacter* strain B-14 only hydrolyzed CP to TCP. In contrast, *Alcaligenes faecalis* strain DSP3 degraded 100% and 93.5% of 100 mg/L CP and TCP within 12 days [16]. The degradative enzymes present in bacteria are responsible for degradation and detoxification of insecticides. The OP insecticide-degrading gene (*opd*D) was isolated from *L. sakei* WCP904, because it showed broad specificity against a range of OP compounds along with enormous potential to provide a versatile gene or enzyme system for the remediation of highly toxic OP insecticides. The *opd*D gene harbored by the recombinant *E. coli* was capable of degrading and utilizing CP as the sole carbon source when grown in M9 medium supplemented with CP instead of glucose. It can be assumed that the CP-degrading ability of the *opd*D clone is stronger than that of the *opd*B clone, because the *opd*D gene had been observed to deplete the CP concentration to 73% in M9 medium after day 6 in the previous study where *E. coli* harboring the *opd*B gene depleted the CP concentration to 67% in M9 medium after day 6 [11].

Four OP hydrolase genes, *opd*, *mpd*, *oph*, and *ophc*, have been reported to date. Among them, the *opd* gene is widely distributed in nature and isolated from several species [11, 17–19]. The reported *opd* genes were found to belong to chromosomes [18, 20] or plasmids [21] of the isolated strains. However, there has as yet been no study reporting the *opd* gene isolated from *L. sakei* of *kimchi* origin. Therefore, the cloned and functionally expressed chromosome-based *opd*D gene from the *L. sakei* WCP904 increases the diversity of the hosts of OP hydrolase.

Phosphotriesterase from different microorganisms has been found to hydrolyze OP insecticides, specifically hydrolyze phosphoester bonds, such as P–O, P–E, P–NC, and P–S [22]. The OpdD enzyme hydrolyzed a range of OP insecticides containing P–O and P–S bonds (Table 1), indicating that the recombinant OpdD has broad substrate specificity. Comparable results were obtained according to previous reports [11, 21, 23, 24]. However, relative activity of the OpdD enzyme against the P–O bond insecticides was much higher than against P–S bond insecticides (Table 1), which is consistent with the previously reported OpdB enzymic character [11], while the *opd*E gene of *Enterobacter* sp. had shown only the breakdown ability of P–S bond of some organophosphate pesticides instead of P–O bond [22]. However, minor variations of relative substrate activities were observed for the OpdD enzyme versus those reported for the OpdB enzyme. Thus, the OpdD hydrolysis activity dependence on the molecular structure of insecticides was investigated in this study.

Temperature was found to influence OpdD activity. The optimum pH of OpdD was consistent with OpdB from *L. brevis* WCP902 (6.0) [11] and was lower than that of OpdB from *Pseudomonas* sp. BF1-3 (8.0) [25]. During *yeolmu-mulkimchi* fermentation with CP, the LAB such as *L. brevis*, *L. plantarum*, *L. sakei*, and *Weissella koreensis* were at the rancid stage where pH was low [7]. Therefore, the observation that OpdD maximum activity was obtained at acidic pH 5–6 was logically consistent. However, OPs are stable in mild acidic to neutral pH and easily degraded in alkaline pH [26]. As a consequence, the degradation rate of CP in acidic soils was slower than in neutral and alkaline soils [23, 24, 27, 28]. Additionally, the optimum temperature of OpdD protein was found to be 30 °C which is similar with OP hydrolase (Mpd protein) [29], but a higher temperature than that was recorded for the OP hydrolase of *L. brevis* WCP904 (40 °C) [11].

The OpdD protein has a Gly-X-Ser-X-Gly sequence motif and catalytic active site of serine residues. Significantly, this motif and active site are found in most bacterial and eukaryotic serine hydrolases, such as esterase, lipase, and serine proteinase as well as in β-lactamase [30–32]. However, the phylogenetic tree analysis of the OpdD protein has shown that it does not belong to the known families of esterolytic and lipolytic protein groups I, II, III, and IV or even a new group of soil metagenome. Importantly, previous studies reported that the OpdB enzyme had the Gly-X-Ser-X-Gly motif and catalytic active site of

serine residues [11]. In addition, the *opd*D gene had shown only 40.74% homology with other related OP hydrolase genes from four different bacteria. As a consequence, we propose a new esterase group (LAB-opd esterase) that is based on the isolation of OP hydrolase genes from LAB cultured in *kimchi* during its fermentation.

In previous reports, CP-degrading *L. sakei* WCP904 was isolated from kimchi fermentation in the presence of 200 mg/L CP [7]. In fact, the CP was degraded rapidly until day 3 (83.3%) and degraded completely by day 9 [7]. Besides CP, the OpdD protein also degraded other OP pesticides, which is a remarkable achievement of the study. Although the chemical structure of CP, PT, and DZ is slightly different, all the three pesticides contain phosphodiester bond that is targeted by OpdD protein. As the results, the slight differences of residual CP, DZ, and PT concentration were observed. Since the research was started from CP-impregnated *kimchi* fermentation with *L. sakei* WCP904, OP degradation, optimum temperature, and optimum pH of the OpdD protein were observed and optimized with CP as the representative organophosphorus pesticides in this study. Regarding the safety against insecticides in *kimchi*, we had concluded that the fermented *kimchi* met the minimal residue criteria for food safety due to the degradation of OP insecticides by *L. mesenteroides*, *L. brevis*, *L. plantarum*, and *L. sakei* [7]. The present study suggests that the *opd*D gene in *L. sakei* WCP904 along with the *opd*B gene in *L. bevis* WCP902 plays roles in degrading OP insecticides during *kimchi* fermentation.

In conclusion, the isolate *L. sakei* WCP904 degraded nine OP insecticides in M9 media and utilized the OP insecticides as the sole source of carbon and phosphorus. Of interest, the recombinant *E. coli* harboring the *opd*D gene degraded approximately 73% initial CP by 6 days of incubation. Moreover, the product of the *opd*D gene showed strong degradation ability against P–O bond insecticides such as CM, CP, DZ, PT, and MPT. The catalytic conserved amino acid sequence of OpdD is different from the known families of esterolytic and lipolytic proteins. Importantly, the catalytic esterase action of the protein OpdD is demonstrated by the amino acid serine at position 116. This is the first study that revealed OP insecticide-degrading gene from *L. sakei* WCP904, which therefore increased the diversity of the hosts of OP hydrolase.

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