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SITE-DIRECTED MUTAGENESIS AND CHARACTERIZATION OF RECOMBINANT PHOSPHOTRIESTERASE HOMOLOGY PROTEIN FROM *GEOBACİLLUS CALDOXYLOSİLYTİCUS* TK4

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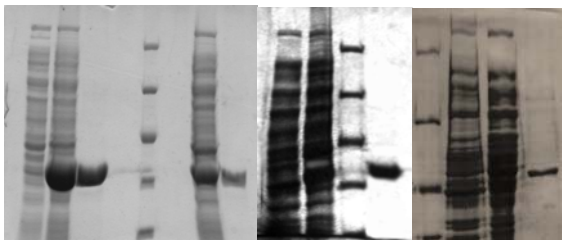
Many toxic insecticides used worldwide are organophosphates (OPs) derivatives. Phosphotriesterase (PTE) has been rewarding to protect against OP poisoning *in vivo* or *in vitro,* associated with advanced catalytic efficiency and stereoselectivity toward the hydrolysis of OPs. Phosphotriesterase homology protein (PHP) exhibits high sequence identity and similarity to PTE. In this study, site-directed mutagenesis on recombinant *Geobacillus caldoxylosilyticus* TK4 PHP (TK4PHP) was performed for improving the existing esterase activity, even gaining a new PTE activity. After eliminating the deficiencies in the recombinant TK4PHP gene, mutant proteins were purified and characterized biochemically. Considering all the data obtained, it was determined that the major sequence differences between PTE and TK4PHP were removed by three site-directed mutations. However the mutant TK4PHPs did not have PTE activity, it was informed that mutant esterases were more resistance to some metal ions and organic solvents and more thermal stable when it was compared with recombinant type.



INTRODUCTION

Organophosphates (OPs) are well-known toxic compounds that inhibit a key enzyme of the central nervous system, acetylcholinesterase. OP compounds have been synthesised since the late 1940s and commonly used as insecticides and chemical warfare agents due to their hazardous properties. Late studies report that OP inquination in ground and drinking water, as well as grains, vegetables and fruits is at the alarming rate.[1] Also, human exposure to OPs causes approximately 3 millon cases of severe poisoning and 200.000 deaths each year worldwide.[2] Owing to their high acute toxicity and risk towards the environment and health, detoxifcation of OP compounds has become the subject of numerous studies. Early detection of OPs is thereby momentous not only for preserving water resources and food supplies, but also for defense against terrorist activity.[3] During the past decade, enzymatic degradation of OPs has attracted an overwhelming interest, because contemporary methods of removing them, such as treatment with bleach and incineration, are impractical, expensive and cause environmental concerns.[4] Hence, there is an instant need for progress of eco-friendly, efficient and reliable methods for hydrolyzation and neutralization of OPs.

The enzyme that catalyses the hydrolysis of the phosphoester bonds in OPs is termed phosphotriesterase (PTE; E.C.3.1.8.1), has been

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detected in many bacteria as well as from squid and mammals.[5,6] PTE belongs to the amidohydrolase superfamily, as such, it acquires a TIM(β/α)-barrel fold, a hydrophobic active site with three asunder binding pockets to correlate the substrate ester groups and two other divalent metals interact to locate the phosphorus center for catalysis.[7,8] Both metals are required for full catalytic activity and are bridged by a hydroxide and a carboxylated lysine (K169) from β-strand. The native metal found in PTE is Zn^{2+}, but substantial catalytic activity is observed with the Co-, Cd-, Mn- or Ni-substituted forms of the enzyme.[9]

The closest sequence homolog known to date is phosphotriesterase homology protein (PHP), which is either a member of amidohydrolase superfamily with recondite function, showing approximately 30% sequence identity and 66% sequence similarity to PTE. Unlike PTE, PHP does not exhibit an activity of catalyzing the hydrolysis of nonspecific phosphotriesters. Studies reported a weak esterase and a weak paraoxonase activity in an *Escherichia coli* PHP (ECPHP) mutant.[10] Whole 3D structures of both enzymes superpose quite well, and they also have a superposable binuclear (Zn^{2+}) metal center. Besides, analysis of an array of the primary sequence of PHP and PTE displays that the four histidine residues and the aspartate related in coordination of zinc in PTE are maintained in PHP. PHP differs from PTE in three active site loops which connect the first, seventh and eighth β/α modules. These modules, most often involved determining substrate specificity in the amidohydrolase superfamily members, are shorter in PHP.[11,12] In addition, K169, zinc coordinator of PTE, substituted with E125 and shift in position in ECPHP.[13]

When the structural differences between diverging enzymes are minimized by laboratory evolutionary pathway, an enzyme with weak promiscuous function can exhibit a dramatically higher proficiency, even a novel activity.[10] Our group identified and cloned a PHP, from the thermophile *Geobacillus caldoxylosilyticus* TK4 (TK4PHP).[12] The enzyme has esterase activity, but not PTE activity. The residues coordinating the two catalytic metals are completely conserved (corresponding to H55, H57, H201, H230 and D301 in *Pseudomonas diminuta* PTE (PDPTE) (most widely studied OP-degrading enzyme) in TK4PHP (Figure 1). The sixth ligating residue corresponding to K159 in PTE, is conserved too, but is translocated with an E residue just like with ECPHP. Moreover, the 1, 7 and 8 loops are shorter (4, 14 and 9 amino acids, respectively) than PDPTE.[12] Because TK4PHP is associated with PDPTE in terms of amino acid sequence and the structure of the bimetal active site, we focused in this study on obtaining a thermostable novel OP-degrading enzyme variety by resolving the incomplete loops by site-directed mutagenesis.

The studies related to obtaining a PHP having PTE activity scarce. Formerly, several mutant forms of bacterial PTE were developed, in order to increase the promiscuous weak hydrolyzing activity. In addition, the presence of PHP was only reported for a few organisms and there are few studies regarding the mutation of PHP genes from different organisms, their expressions and characterizations. In this study, site-directed mutagenesis was performed on recombinant TK4PHP for improving the existing activity or stability. To achieve this, three loops were mutated in the recombinant TK4PHP gene. After the mutant proteins were purified, they were biochemically characterized. At the end of these studies, it was observed that pH- and thermal stable mutant esterases were obtained. It is well known that esterases (EC 3.1.1.1) are a class of hydrolases responsible for catalyzing the hydrolysis and formation of ester bonds.[14] Since they catalyze reactions such as esterification, interesterification and *trans*-esterification in organic media,[15] esterases, especially extracted from bacteria, have become one of the worldwide important industrial enzymes used in organic synthesis and various industrial processes (food, detergent, pharmaceutical, chemical and agricultural industries).[16] Bacterial esterases have some advantages in terms of producibility in high amount, performing genetic manipulation easily and having good thermostability.[17] Nowadays, even the interest in mutations of the esterases have increased greatly, the number of the mutant enzymes is still low.[18,19] In the present study, pH- and thermal stable esterases were obtained by conducting gene engineering methods.

RESULTS AND DISCUSSION

Site-directed mutagenesis

Across the amidohydrolase family, loops 1, 7 and 8 of the protein fold are the most diverse in structure and are thought to be responsible for differential activity between enzymes in this family.[20] Gene sequence comparison revealed that loops 1, 7 and 8 differ greatly between TK4PHP and PTE.[12] Therefore, the first attempt at

improving the catalytic activity of TK4PHP was to switch these loops in TK4PHP to the corresponding loop sequences in PTE. Because loop 8 includes F306 and Y309 substrate binding units, the first mutation was applied on this loop to fulfill the 9 amino acids shortage. After that, the 14 amino acids shortening in loop 7 and 4 amino acids shortening in loop 1 was applied, respectively.

```
TK4PHPM1-M2-M3 ----------------------------------MYIQTVLGRIQPEELGVCACHEHLYI 26
TK4PHPM1-M2    ----------------------------------MYIQTVLGRIQPEELGVCACHEHLYI 26
TK4PHPM1       ----------------------------------MYIQTVLGRIQPEELGVCACHEHLYI 26
TK4PHP         ----------------------------------MYIQTVLGRIQPEELGVCACHEHLYI 26
PDPTE          MQTRRVVLKSAAAAGTLLGGLAGCASVAGSIGTGDRINTVRGPITISEAGFTLTHEHICG 60
                                                                * *

TK4PHPM1-M2-M3 DLSRVKKNTDTCLQNRKALLDLVVEDLKVFLQYGGKAIVEMTNDGMGRNVKKLVEISKLL 86
TK4PHPM1-M2    DLSRVKKNTDTCLQN----LDLVVEDLKVFLQYGGKAIVEMTNDGMGRNVKKLVEISKLL 82
TK4PHPM1       DLSRVKKNTDTCLQN----LDLVVEDLKVFLQYGGKAIVEMTNDGMGRNVKKLVEISKLL 82
TK4PHP         DLSRVKKNTDTCLQN----LDLVVEDLKVFLQYGGKAIVEMTNDGMGRNVKKLVEISKLL 82
PDPTE          SSAGFLRAWPEFFGSRKALAEKAVRGLRRARAAGVRTIVDVSTFDIGRDVSLLAEVSRAA 120
                      Loop 1

TK4PHPM1-M2-M3 DLHIIASTGCYKDPFIPQEKINWDRDEFAKWMIDEIENGIDGTNIKPGVIGEIGSSFNEF 146
TK4PHPM1-M2    DLHIIASTGCYKDPFIPQEKINWDRDEFAKWMIDEIENGIDGTNIKPGVIGEIGSSFNEF 142
TK4PHPM1       DLHIIASTGCYKDPFIPQEKINWDRDEFAKWMIDEIENGIDGTNIKPGVIGEIGSSFNEF 142
TK4PHP         DLHIIASTGCYKDPFIPQEKINWDRDEFAKWMIDEIENGIDGTNIKPGVIGEIGSSFNEF 142
PDPTE          DVHIVAATGLWFDPPLSMRLR--SVEELTQFFLREIQYGIEDTGIRAGIIK-VATTG-KA 176

TK4PHPM1-M2-M3 KPVELELFYGAIEAAKTTKLPLSTHTTLG-TLALEQVELFIRENLPLHQVVIGHQDLNED 205
TK4PHPM1-M2    KPVELELFYGAIEAAKTTKLPLSTHTTLG-TLALEQVELFIRENLPLHQVVIGHQDLNED 201
TK4PHPM1       KPVELELFYGAIEAAKTTKLPLSTHTTLG-TLALEQVELFIRENLPLHQVVIGHQDLNED 201
TK4PHP         KPVELELFYGAIEAAKTTKLPLSTHTTLG-TLALEQVELFIRENLPLHQVVIGHQDLNED 201
PDPTE          TPFQELVLKAAARASLATGVPVTTHTAASQRDGEQQAAIFESEGLSPSRVCIGHSDDTDD 236
                                      *                       *

TK4PHPM1-M2-M3 DEVVLEVLSSGVYIALDTIGKENYRLEDNASASALLGIRSDMSRMKSLLYFLERGYEDQI 265
TK4PHPM1-M2    DEVVLEVLSSGVYIALDTIGKENYRLEDNASASALLGIRSDMSRMKSLLYFLERGYEDQI 261
TK4PHPM1       DEVVLEVLSSGVYIALDTIGKENYR--------------SDMSRMKSLLYFLERGYEDQI 247
TK4PHP         DEVVLEVLSSGVYIALDTIGKENYR--------------SDMSRMKSLLYFLERGYEDQI 247
PDPTE          LSYLTALAARGYLIGLDHIPHSAIGLEDNASASALLGIRSWQTRALLIKALIDQGYMKQI 296
                                            Loop 7

TK4PHPM1-M2-M3 LLSSDVTRGFSSYVTNIQSHLLSRGGQGYSVVLRKFIPALREMGVLETTIEKLLVKNPQR 325
TK4PHPM1-M2    LLSSDVTRGFSSYVTNIQSHLLSRGGQGYSVVLRKFIPALREMGVLETTIEKLLVKNPQR 321
TK4PHPM1       LLSSDVTRGFSSYVTNIQSHLLSRGGQGYSVVLRKFIPALREMGVLETTIEKLLVKNPQR 307
TK4PHP         LLSSDVTR---------QSHLLSRGGQGYSVVLRKFIPALREMGVLETTIEKLLVKNPQR 298
PDPTE          LVSNDWLFGFSSYVTNIMDVMDRVNPDGMAFIPLRVIPFLREKGVPQETLAGITVTNPAR 356
                    *     Loop 8
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Fig. 1 – Sequence alignment of *Pseudomonas diminuta* PTE (PDPTE), *Geobacillus caldoxylosilyticus* TK4 PHP (TK4PHP) and the mutant variants of TK4PHP. The loops of the first, seventh and eighth β/α modules, the length of which distinguishes PTE and PHP, were stated with italic letters. The four histidine residues and the aspartate related in coordination of zinc in PTE are maintained in PHP are marked with an asterisk. 9 amino acids shortage in loop 8, 14 amino acids shortage in loop 7 and 4 amino acids shortage in loop 1 (stated with dark letters) were eliminated, respectively.

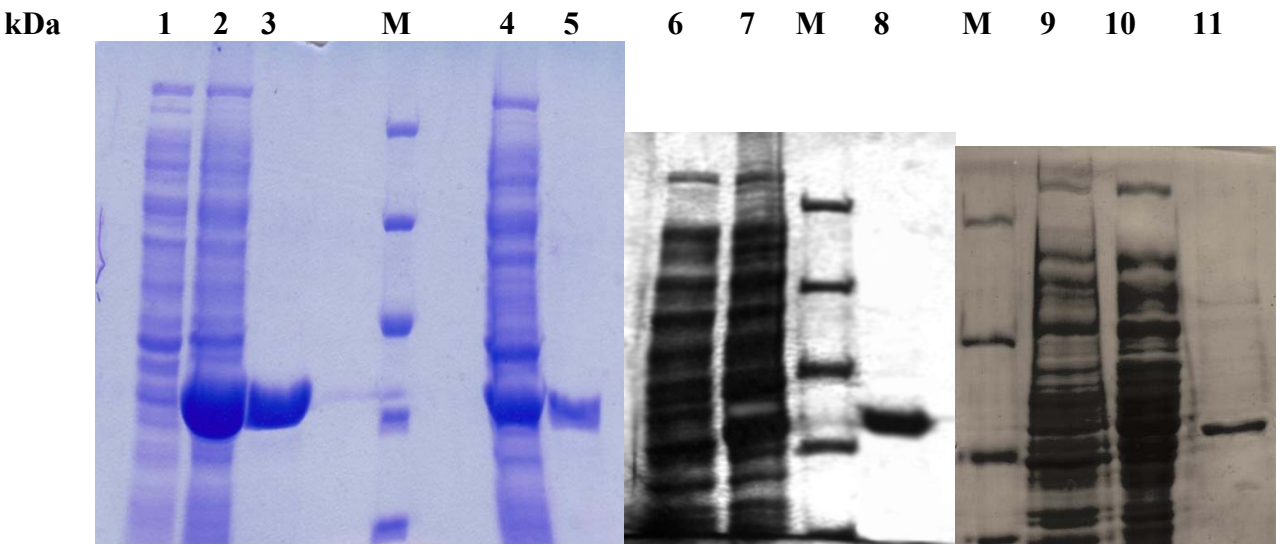

Fig. 2 – SDS polyacrylamide gel electrophoresis. Lane M, molecular weight markers; lane 1, before IPTG induction of lysate *E. coli* BL21(DE3)pLysS including TK4PHP; lane 2, after 3 h of IPTG induction of lysate *E. coli* BL21(DE3)pLysS including TK4PHP; lane 3, purified TK4PHP; lane 4, after 3 h of IPTG induction of lysate *E. coli* BL21(DE3)pLysS including TK4PHPM1; lane 5, purified TK4PHPM1; lane 6, before IPTG induction of lysate *E. coli* BL21(DE3)pLysS including TK4PHPM1-M2; lane 7, after 3 h of IPTG induction of lysate *E. coli* BL21(DE3)pLysS including TK4PHPM1-M2; lane 8, purified TK4PHPM1-M2; lane 9, before IPTG induction of lysate *E. coli* BL21(DE3)pLysS including TK4PHPM1-M2-M3; lane 10, after 3 h of IPTG induction of lysate *E. coli* BL21(DE3)pLysS including TK4PHPM1-M2-M3; lane 11, purified TK4PHPM1-M2-M3.

The three loops directed mutations were performed through the overlap extension PCR. The mutant TK4PHPM1 was generated using the TK4PHP as the template with the forward primer M1F and the reverse primer M1R. Then, TK4PHPM1 was used as the template to obtain the mutant TK4PHPM1-M2 with the forward primer M2F and the reverse primer M2R, so did TK4PHPM1-M2 was used as the template for the mutant TK4PHPM1-M2-M3 with the forward primer M3F and the reverse primer M3R. In an attempt to eleminate the template plasmids, the purified PCR products were processed with *Dpn*I (10 U/ μL) and transformed into DH5α competent cells. The desired mutants were selected and sequenced. In comparison with the multiple amino acid sequences alignments from Macrogen Inc. (Seoul, Korea) and (by Clustal W Method), it was confirmed that the gaps in loop 1, 7 and 8 were fulfilled successfully (Figure 1).

Protein expression and purification

The recombinant and mutant proteins were produced under the control of T7 RNA polymerase promoter and 6×His tagged. Expression of the proteins was successfully achieved in *E. coli* BL21 (DE3)pLysS by the induction with 1 mM IPTG, at 37 °C for 3 h. Then, expressed proteins were efficiently purified to homogeneity with one-step purification procedure using nickel affinity chromatography. The molecular weight of the monomeric polypeptides determined to be approximately 36.50–38.50 kDa by SDS polyacrylamide gel electrophoresis (Fig. 2), were also consistent with those calculated (by ProtParam) 35.57 kDa for TK4PHPM1, 37.00 kDa for TK4PHPM1-M2 and 37.45 kDa for TK4PHPM1-M2-M3. These findings promoted the report of Buchbinder in which the authors reported a monomer, 32.9 kDa ECPHP.[13]

Determination of enzymatic activity

To determine whether the mutant types of TK4PHP evolved for PTE activity, enzymes were evaluated for PTE efficiency with paraoxon, parathion and malathion substrates. However, no phosphotriesterase activity was detectable for the mutant enzymes. A previous study was conducted in which all loop regions in a *Deinococcus radiodurans* PTE homolog were switched to those in PTE, but no PTE activity was obtained due to the construct was found to be insoluble.[21] Indeed with T210A and T245A mutations in loop 7 (located in the active site) and loop 8, an unexpectedly low level of paraoxonase activity

appeared in the evolved ECPHP variants despite its absence in the wild-type enzyme.[10]

In order to examine the changes in esterase activity, mutant enzymes were studied using different *p*-nitrophenyl esters; acetate (C2), butirate (C4), laurate (C12) and palmitate (C16). While the enzymes had little or no activity for substrates with longer chain lengths, the highest activities were detected against *p*NPA. As shown in Table 2, when the acyl chain length increased, the enzyme activities declined. The results showed a preference of the mutant enzymes for short chain fatty acids. The reported esterases from *Bacillus subtilis* DR8806,[22] *Pelagibacterium halotolerans* B2^{T23} and *Mycobacterium tuberculosiss* [24] had shown substrate preference for the hydrolysis of *p*NPA.

Effect of pH and temperature on the esterase activities

The enzyme activity assays at different pHs in the presence of *p*NPA as a substrate revealed that the optimum pH was 8.0 for TK4PHP, TK4PHPM1-M2 and TK4PHPM1-M2-M3 and 8.5 for TK4PHPM1. The optimum pH value of 8.0–8.5 is similar to many other esterases. It was reported that the esterases from *Geobacillus thermodenitrificans* T2 and *Bacillus cereus* had maximum activity at pH 8.0 and 8.5, with pNPB, respectively.[25,26] Moreover, the esterases from chicken with the optimum activity at pH 8.5 [27] and *Geobacillus thermoleovorans* YN with optimal pH 8.0 have been reported.[28]

Temperature influences not only the stability of proteins but also the maximum reaction rate of enzymes by influencing the enzyme and the substrate.[29] The effect of temperature on the enzyme activities was assayed from 10 to 90 ºC with an interval of 10 ºC. Maximum activity of TK4PHPM1 was occurred at 60 ºC, while TK4PHPM1-M2, TK4PHPM1-M2-M3 and the recombinant TK4PHP have an optimum at 50 ºC. Results also exhibited that the enzymes have proper activities between 20 and 80 ºC. Similar results were also reported for esterases from *Picrophilus torridus,*[30] *Bacillus cereus* AGP-03,[26] *Pyrococcus furiosus,*[31] *G. thermodenitrificans* T2[25] and *Thermomyces lanuginosus*.[32]

Both pH and temperature optima studies easily showed that mutations performed on TK4PHP templates enhanced these properties as compared with the recombinant enzyme.

Enzyme kinetics

To compare the catalytic activities between the recombinant TK4PHP and the mutants, the kinetic values were estimated at different *p*NPA substrate concentrations ranging from 5–800 μM, by Lineweaver-Burk plot (Table 3). As indicated from the results, mutant TK4PHPM1 increase its V_{max} to 14.53 U/mg proteins about a 2-fold improvement and mutant TK4PHPM1-M2-M3 showed a lower (2.8- fold) K_m value of 0.46 mM, compared to the recombinant type. The resultant K_m values were lower than the any other previously reported esterases from *Bacillus subtilis* DR8806 having 4.2 mM K_m value when *p*NPA was used as substrate[22] and *Sulfolobus solfataricus* P1 having 24.0 mM K_m value against *p*-nitrophenyl caprate.[33]

Table 2

Substrate specificities of recombinant and mutant TK4PHPs

	Specific activity (U/mg protein)			
Substrate	**TK4PHP**	**TK4PHPM1**	**TK4PHPM1-M2**	**TK4PHPM1-M2-M3**
Paraoxon	0	0	0	0
Parathion	0	0	0	0
Malathion	0	0	0	0
*p*NPA	0.423±0.005	0.616±0.005	0.453±0.004	0.468±0.004
*p*NPB	0.423±0.003	0.597±0.002	0.418±0.003	0.427±0.002
*p*NPL	0.004±0.001	0.005±0.001	0.014±0.001	0.012±0.002
*p*NPP	0	0	0.003±0.001	0.003±0.001

Table 3

Kinetic parameters of the recombinant and mutant TK4PHPs. Enzyme kinetic assays were done using *p*-nitrophenyl acetate (*p*NPA) (5–800 μM); K_m and V_{max} values were determined by Lineweaver–Burk plot

	TK4PHP	TK4PHPM1	TK4PHPM1-M2	TK4PHPM1-M2-M3
K_m(mM)	1.27±0.02	1.43±0.03	0.59±0.03	0.46±0.02
V_{maks}(U/mg protein)	7.59±0.03	14.53±0.04	3.34±0.02	4.04±0.01

pH- and thermal-stability of the enzyme

To evaluate the improvement in pH-stability, the residual activity of TK4PHP and the mutants were determined over a pH range from 3.0 to 9.0, after pre-incubation for 2, 5 and 7 days, at 4 ºC. In accordance with pH stability studies, the mutant enzymes showed stability at a wide range of pH. As depicted in Figure 3B, the stability of mutant TK4PHPM1 has increased by 10% in all examined pHs and it is indicated from Figure 3D that mutant TK4PHPM1-M2-M3 retained all of its original activity for up to 7 days. As compared to literature, mutant types of TK4PHP were pH-stable and the enzymes may be utilized in various industrial applications.[26,34,35]

The thermostability of mutant esterases was investigated in the range of 50–90 ºC, with increasing incubation time up to 7 days (Figure 4). However, no significant change in TK4PHPM1 stability was observed, the stability of TK4PHPM1-M2 and TK4PHPM1-M2-M3 increased after incubation for 7 days, compared to the recombinant enzyme. It could be suggested that the stabilities against high temperatures were improved by the mutations on recombinant TK4PHP. While there were several reports on thermostable esterases [36-38] there were few reports considering to TK4PHP mutants.

Effect of some metal ions and organic solvents

The effect of metal ions on enzyme activity was also studied, at final concentrations of 1 mM and 5 mM, with Na^+, Li^+, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+} and Cu^{2+} ions. Our findings indicated that the activity of mutant enzymes was not affected significantly in the presence of Mg^{2+}, Mn^{2+}, Co^{2+} and Ca^{2+}. Whereas 5 mM Cu^{2+} completely inhibited the activity of recombinant TK4PHP, almost 50% of activity was found in mutant TK4PHPM1at this concentration, while the mutant TK4PHPM1-M2 was totally active. The activities of the recombinant esterase from *Bacillus brevis* [39], thermophilic alkaline esterase from *Bacillus subtilis* DR8806 [22], extremely thermostable *Geobacillus* sp. HBB-4 and *Picrophilus torridus* esterases were also inhibited by Cu^{2+}.[30,40]

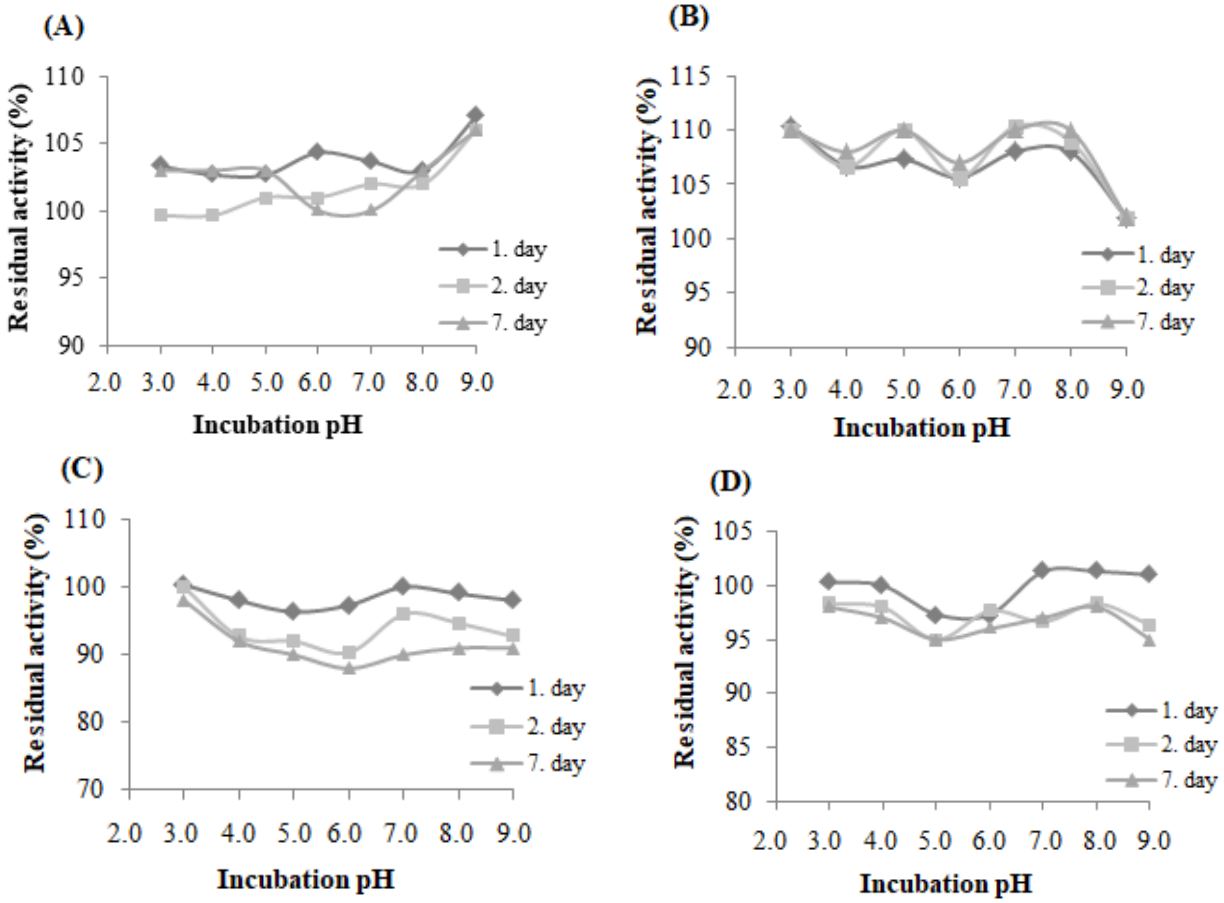

Fig. 3 – pH stability profiles of TK4PHP (A), TK4PHPM1 (B), TK4PHPM1-M2 (C), and TK4PHPM1-M2-M3 (D). Enzyme assays were done by using *p*-nitrophenyl acetate (*p*NPA) as the substrate. The enzyme activities without treatment were taken as 100% (The standard deviation values were within ±5%).

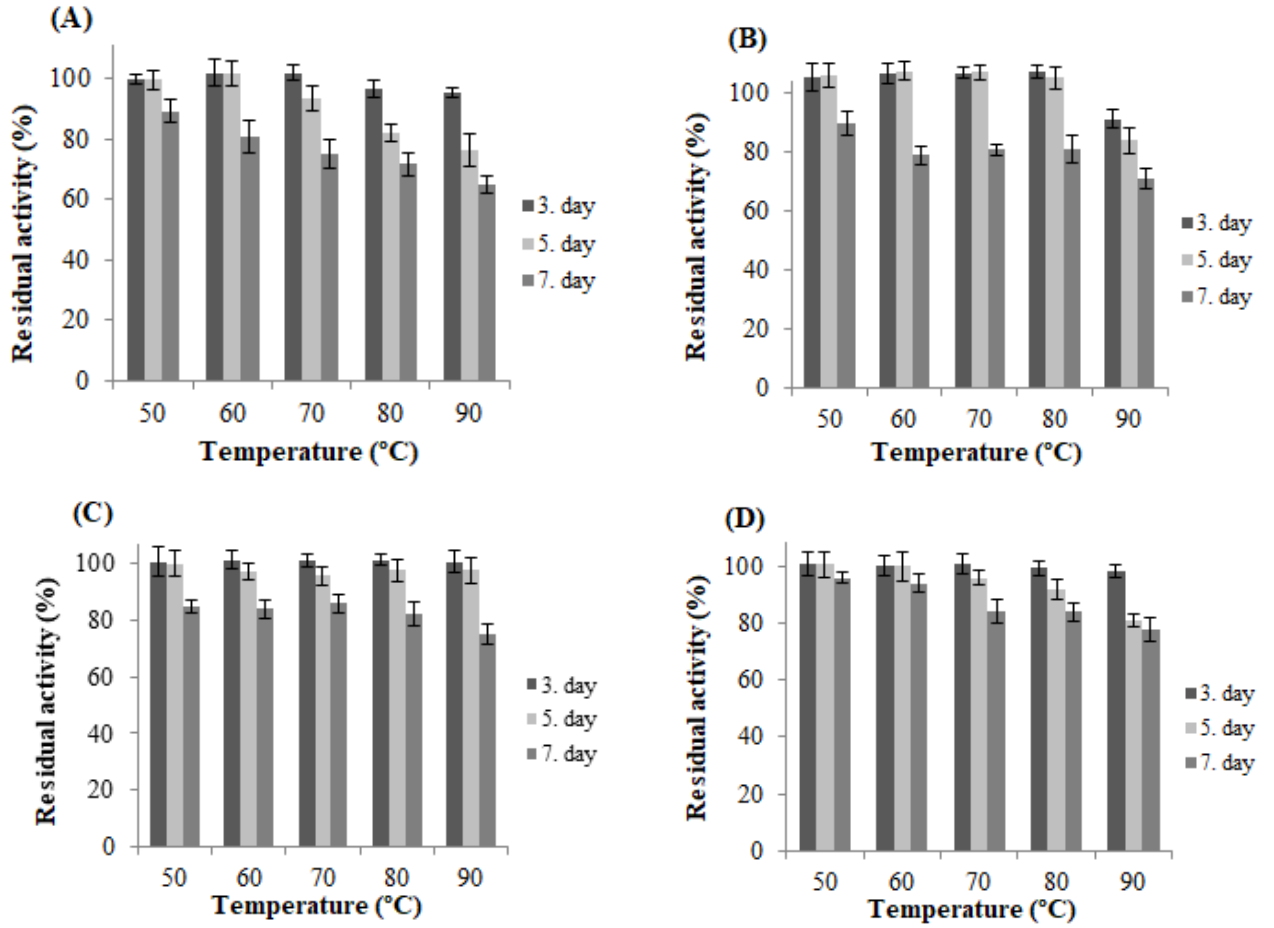

Fig. 4 – The effects of temperature on the stabilities of recombinant and mutant TK4PHPs. Enzyme assays were done by using *p*-nitrophenyl acetate (*p*NPA) as the substrate. The thermostabilities of enzymes were determined by after incubation of purified esterases at different temperatures ranging from 50 ºC to 90 ºC for 3, 5 and 7 days.

Organic solvents can be advantageous in various industrial enzymatic processes as they increase the solubility of non-polar substrates and the thermal stability of enzymes, decrease the water-dependent side reactions and eliminate the microbial contamination. In this study, activities of recombinant and mutant enzymes were analyzed in the presence of acetone, acetonitrile, dimethylsulphoxide (DMSO), ethanol, isopropanol and methanol at final concentrations of 10% and 30%. Although the activity of mutant TK4PHPM1 esterase was inhibited with 10% acetonitrile and acetone, an increase was observed with 30% ethanol, isopropanol, acetonitrile, acetone compared to TK4PHP. Also, TK4PHPM1-M2-M3 showed higher activities toward acetonitrile and DMSO in 30% concentration. In the presence of 10% DMSO, all mutants retained 100% relative activity as similar to a thermoalkaliphilic halotolerant esterase from *Rhodococcus* sp. LKE-028.[34] In addition, 30% final concentration of acetone inhibited the mutants in different ratios, likewise a thermoactive uropygial esterase from chicken.[27]

EXPERIMENTAL

All the chemicals in this study were of analytical grade and were purchased from Sigma (St. Louis, MO, USA), Merck A.G. (Darmstadt, Germany) or Fluka Chemie A.G. (Buchs, Switzerland). Wizard Plus SV Minipreps DNA Purification System, Wizard SV Gel and PCR Clean-Up System, MagneHis Protein Purification System were obtained from Promega (Madison, USA). Broad Range Protein Molecular Weight Markers for SDS-PAGE and 1 kb DNA Ladder were supplied from Fermentas (Vilnius, Lithuania). Fast PCR enzyme mix and the restriction enzyme (*Dpn*I) were purchased from Thermo Scientific (Waltham, ABD).

The recombinant plasmid harboring TK4PHP (GenBank accession number: FJ788931) in pET-28a(+) was previously described by our group.[12] The DNA sequence analyses were carried out by Macrogen Inc. (Seoul, Korea). *E. coli* DH5α was used as the host for cloning whereas *E. coli* BL21(DE3)pLysS harbored the mutant plasmids for gene expression. Both of the strains were cultured in LB medium at 37 °C supplemented with kanamycin (50 μg/mL) if required.

The multiple amino acid sequences alignments of TK4PHP, PDPTE (known as the most active OP-degrading enzyme), *Mycobacterium tuberculosis* PHP (MTPHP) and ECPHP were examined [12] in order to decide which mutations should be performed to give TK4PHP a novel PTE activity. So, the differences between PHP and PTE in the length of three active site loops (the loops connecting the first, seventh, and eighth α/β modules) were typically the first target in mutagenesis studies aimed at generating catalytic rate enhancements. To this end, the primers comprising the mutation locus were intended (Table 1).

The site-directed mutations were done by a one-step overlap extension PCR. TK4PHP, TK4PHPM1 and, TK4PHPM1-M2 plasmids were used as templates for PCR practices, respectively. All fragments were amplified by the same PCR run (denaturation, 94 °C, 1 min; annealing, 56 °C, 1 min; and extension, 68 °C, 5 min; 30 cycles). The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System kit and used as templates for the next mutations. Mutated vectors were treated with the restriction enzyme *Dpn*I for 3 h at 37 °C to remove the methylated template from mutant types. After transformation in *E. coli* DH5α by $CaCl_2$ method,[41] cells transformed with mutant plasmids were grown in LB media with 50 mg/mL kanamycin. Plasmid isolation was performed with Wizard Plus SV Minipreps DNA Purification System. Sequence analyses of mutant plasmids were made by Macrogen Inc. (Seoul, Korea).

The mutated plasmids were transformed into *E. coli* BL21(DE3)pLysS and transformed cells were grown in Luria Bertani medium (LB) agar (NaCl 5 g/L, tryptone 10 g/L, yeast extract 5 g/L, agar 15 g/L) comprising 50 μg/mL kanamycin. When the A_{600} reached to 0.6-0.8, the gene expression was induced by supplementing isopropyl thio-β-d-galactoside (IPTG) at a conclusive concentration of 1 mM and incubating for 3 h at 37 °C. The induced cells were harvested by centrifugation (10000 rpm, 4 °C and 10 min), resuspended in cold 100 mM Tris-HCl buffer (pH 7.4) containing 0.5 mg/mL lysozyme. In the wake of crashed by sonication with cooling on ice, the sonicate was fend off by centrifugation and supernatant was incubated at 70 °C for 5 min for pre-purification. Finally, the mixture was centrifuged (10000 rpm, 4 °C, and 20 min) for removing the denatured proteins. As they contained 6 × histidine tags at their N-termini, the mutant proteins were purified by MagneHis Protein Purification System (nickel affinity chromatography system) including paramagnetic precharged nickel particles by using a manual procedure based on the manufacturer's protocol.

Protein concentrations were determined according to the Lowry method using a calibration curve obtained with bovine serum albumin (BSA).[42]

SDS-PAGE (12% acrylamide) was carried out according to Sambrook. [43] Molecular weight markers were run along with samples to determine the purity of the enzymes. Protein bands were visualized by Coomassie Brilliant Blue staining R-250 staining.

PTE activity assays were performed spectrophotometrically in the presence of paraoxon, parathion and malathion substrates, with a PerkinElmer spectrophotometer, as described previously.[44-46] One unit of enzymatic activity was the releasing of 1μM of *p*-nitrophenol (*p*NP) per minute.

General substrates of esterases were chosen to determine the esterolytic activity of recombinant and mutant enzymes. Enzyme activities against different *p*-nitrophenyl esters were detected spectrophotometrically by using *p*-nitrophenyl acetate (*p*NPA), *p*-nitrophenyl butyrate (*p*NPB), *p*-nitrophenyl laurate (*p*NPL) and *p*-nitrophenyl palmitate (*p*NPP). 1 mM of stock substrate solution (10 mM) was added to a final composition of 4:95 (v/v) of ethanol/buffer (50 mM Tris-HCl, pH 8.0). The reaction was started by the addition of 10 μL of enzyme and followed by incubation at 50 °C for 15 min. The activity was surveyed by observing the absorbance at 405 nm. One unit of enzymatic activity was the release of 1 μM *p*NP per minute in the above assay condition.[47]

Esterase activity was investigated at different pH (3.0-9.0) values using 50 mM of the following buffers: Mcilvaine (pH 3.0-7.5) and Tris-HCl (pH 7.5-9.0) at the optimal temperatures of the mutant enzymes. Overlapping pH values were used to verify that there were no buffer effects on substrate hydrolysis. Reactions were performed under standard assay protocol, using *p*NPA as substrate. The activity was expressed as percent relative activity with respect to maximum activity, which was accepted as 100%.[47]

The enzyme activities were assayed at varied temperature ranging between 10 ºC and 90 ºC with 10 ºC intervals, at optimum pH values by using *p*NPA as substrate, so as to detect the optimum temperature values. The activity was expressed as percent relative activity with respect to maximum activity, which was considered as 100%.[47]

Kinetic parameters, Michaelis–Menten constants (K_m) and the maximum reaction rates, (V_{max}) were calculated at optimal conditions, using different concentrations (5-800 μM) of the *p*NPA substrate. Each reaction at varied substrate concentration was surveyed spectrophotometrically at 405 nm. The K_m and V_{max} values were calculated from the substrate saturation plots using Microsoft Excel software.[48]

Table 1

Primers for site-directed mutagenesis

Primer	Sequence (5'–3')
M1F	gtggctttagcagctatgtgaccaacattcaatcacatttactttctcgcggcgg
M1R	gaatgttggtcacatagctgctaaagccacgcgttacatcgctagaaagcaag
M2F	gcctggaagataacgcgagcgcgagcgcgctgctgggcattcgcagtgacatgagcc gaatgaaatcgttgc
M2R	ctgcgaatgcccagcagcgcgctcgcgctcgcgttatcttccaggcgataattctcttttcc tatcgtatctagcgcgatgtatacgcc

M3F	atcgcaaagcgctgctggatcttgtagtagaagatttaaaag
M3R	gcagcgctttgcgattttgtagacacgtatcggtatttttttttaac

To examine the pH stabilities, the purified enzyme solutions were pre-incubated with different pH values using Mcilvaine (pH 3.0-7.5) and Tris-HCl (pH 7.5-9.0) buffers, at 4 °C, for 1, 2 and 7 days. After incubation, the remaining activities of the treated samples were measured in optimal conditions, according to the standard assay. The percentage residual enzyme activities were determined by comparing with the unincubated enzyme.[12]

The thermal stabilities of recombinant enzymes were investigated by measuring the residual enzyme activities at varied temperatures ranging from 50 ºC to 90 ºC for 3, 5 and 7 days. The percent residual activities were detected under standard assay conditions using *p*NPA as substrate by comparing with unincubated enzyme.[12]

The effects of metal ions on enzyme activities were studied by adding various metal salts (NaCl, LiCl, $MgCl_2$, $MnCl_2$, $ZnSO_4$, $CaCl_2$, $CoCl_2$ and $CuCl_2$) at final concentrations of 1 mM and 5 mM directly to the substrate mixtures, individually. After reactions were initiated by adding the purified enzymes to the substrate mixtures, they were carried out for 15 min. The residual activities were measured by comparison with the assay mixture without any metal ion.[12]

To determine the effects of some organic solvents on purified esterases, methanol, ethanol, isopropanol, acetonitrile, acetone, and dimethylsulphoxide (DMSO) were added to the reaction mixtures at final concentrations of 10% and 30%. Residual activities were assayed under standard conditions with *p*NPA as substrate and calculated considering with no organic solvent inclusion.[12]

All experiments were performed in triplicate and the results presented are the mean of three values. The MINITAB version 16 statistical software was used for all analysis [49] and the standard deviation was within ± 5%.

CONCLUSIONS

Industrial processes often require different pHs, as well as high temperatures and the majority of known enzymes, need to be stabilized under these conditions; therefore, there is a great interest in enzymes that are derived from thermophiles and they are stable without pretreatment. Limited thermostability and pH stability in operating industrial conditions are the common problems for the esterases. The mutant esterases obtained in this study on recombinant TK4PHP were more resistant to metal ions and organic solvents. Besides, the mutant enzymes exhibited significant pH and thermo-stability indicating that the mutant esterases described the potential to be used in harsh industrial/biotechnological processes.

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