

A Review on Engineering of Organophosphorus Hydrolase (OPH) Enzyme

Gholamreza Farnoosh¹, Ali Mohammad Latifi*¹

Abstract

Organophosphorus chemicals are compounds which have been used as pesticides and insecticides in agriculture. They're also used as nervous agents and have raised many problems for human and environment. Among the most important methods of decontamination from these compounds are biodegradation methods. Using OPH enzyme in degradation the mentioned compounds is seen as one of the desirable ways, but low activity and specification and low thermostability are among factors significantly decreasing the optimal application of this enzyme. Using methods of protein engineering based on the alteration of specific protein positions in order to improve the activity, specification and thermostability are some common ways used currently. Numerous studies have been done to increase activity and thermostability of OPH enzyme with alteration of some special amino acids the result of which was an increase against different substrates. OPH enzyme active site connected to substrates that consisted of three large, small and releasing packets were one of the goal areas of changing amino acids used by researchers to improve engineered activities. Among other ways of making enzymes more rigid and stable were bending loops by replacing Proline, creating disulfide bonds, ionic bonds by replacing charged amino acids.

Keywords: Organophosphorus, Protein engineering, Thermostability

Introduction

Releasing toxic compounds as pesticides and insecticides, soluble, exploding, cooling, and dyeing materials in industry and agriculture to the environment has increased in recent decades. One of these synthesis compounds are organophosphorus chemicals used in pesticides and insecticides and nervous chemical agents [1]. These compounds have three phosphodiester bonds, so they're called Phosphotriesters. Phosphor is bind by a binary bond either to oxygen (p=O in oxons) or to sulfur (p=S in thion). Some of the pesticides made by Organophosphorus chemicals are Paraoxon, Parathion, Coumaphos, Diazinon as well as Chemical Warfare Agents as G-Type and V-Type. Sudden release or poor management of remainder of pesticides or insecticides results in contamination of soil and underground water in the area [1, 2]. Also, using nervous agents during wars could have a fatal effect on environment and human [2]. These compounds will inactive a large family of Serine Hydrolyze (Lipases and Esterase). Some of the more important enzymes deactivated by some of these compounds are Acetylcholinesterase which exists at the synopsis of central nervous system. Inactivation of this enzyme will lead to accumulation of acetylcholine in nervous synopsis and continuation of nervous system and dysfunction of muscles and impairment in breathing leading to coma and eventually death [1]. Thus, there is a great need in completion of biological filtration technology in order to facilitation of contaminant degradation of organophosphorus chemicals. Today, used methods of decontamination of organophosphorus chemicals are heating, peroxidase, carbon dioxide which have different problems as these methods are often toxic, allergic, corrosive and not specified,

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and they will damage the environment. As a result, to hydrolyze toxic compounds, a safe and healthy technology is required [1,2,3,4].

One of the useful methods of organophosphorus chemicals is using bacterial containing degradation enzymes with a wider range of substrates. Most enzymes used as biocatalysts that can degradation organophosphorus chemicals are organophosphorus hydrolase (OPH) from *Pseudomonas Diminuta* bacteria and *Flavobacterium* species, organophosphorus acid anhydrolase (OPAA) from *Alteromonas* bacteria, and diisopropyl flourophosphatase (DFPase) from a species of *Loligo Vulgaris*. Although using these natural enzymes as vital catalysts is an interesting way of treating organophosphorus chemicals, their inability of being long used and their decrease of activities and low thermostability can cause many problems [1, 2].

One of the most important ways of improving activity and thermostability is using protein engineering methods. Protein engineering methods are done in two ways: rational mutation and evolutionary mutation. Rational mutation method identifies susceptible points by careful investigation of structure and function of protein and using the deigned mutation can apply desirable changes while in evolutionary mutation, random mutation is used to improve activity and stability of proteins. In this review, first we briefly point to some uses of enzymes in degradation organophosphorus chemicals, and then we will review previous studies on increasing activity and stability of this enzyme using protein engineering methods [1, 2]. The serine in active site of the Acetyl cholinesterase enzyme starts a Nucleophilic attack against the carbon in Carbonyl Acetylcholine and an Acetyl enzyme intermediate is



created. At the same time Choline release from the active site.

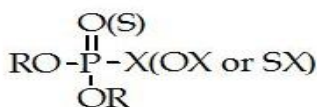


Figure 1. Structure of organophosphorus chemicals [Qureishi, F.M., et al].

In the next phase the enzyme is released with a water hydrolytic attack and the release of acetyl. At the end serine in active site remains as phosphoryl an inactivated, not able to be redegradation [1].

Inactivation of Acetylcholinesterase by Organophosphorus Chemicals

The serine in active site of the Acetylcholinesterase enzyme starts a Nucleophilic attack against the carbon in Carbonyl Acetylcholine and an Acetyl enzyme intermediate is created. At the same time Choline release from the active site. In the next phase the enzyme is released with a water hydrolytic attack and the release of acetyl. At the end serine in active site remains as phosphoryl an inactivated, not able to be redegradation [1].

Biodegradation of Organophosphorus chemicals

Studies show that most mechanisms cannot degradation toxic synthesis compounds. Although using biological methods in degradation toxic and unsafe materials is a healthy and acceptable way, since synthesis compounds do not exist in the nature, microorganisms are not consistent with these compounds an at first this results in slowing down the growth of microorganisms during the degradation of this compounds⁸. Biological method is more significant as it is environment- consistent and the products of its degradation have lower toxicities [1].

Degradation Enzymes of Organophosphorus chemicals

Late in 1980s, American army developed a program called ACES1 to remove Organophosphorus chemicals, in which degradation enzymes of microbial and chemical agents were used. According to this program three detoxicants were selected: DFPase, OPH, OPA. For all three enzymes the genes were cloned and sequenced [5].

Organophosphorus Hydrolase (OPH) (EC. 3. 1. 8. 1)

Organophosphorus chemicals degradation enzyme was first noticed in *Flavobacterium* strain ATCC 27551 and *Pseudomonas diminuta*. Coding genes of OPH on extrachromosomal plasmid of bacteria were identical[1]. OPH acts upon organophosphorus chemicals consisted of acid phosphonic and acid phosphoric esters [1]. After that this enzyme was found only in other organisms such as *Flavobacterium balustinum*, *Chryseobacterium balustinum*,

Deinococcus radiodurans, *Ochrobactrum sp. M231*, *Pseudomonas pseudoalcaligenesC2-1*, *Geobacillus stearothermophilicus*, *Agrobacterium radiobacter* the sequence of which had a different homology compared to the sample in stain *Flavobacterium ATCC 27551* and *Pseudomonasdiminuta*. The most known hydrolase of organophosphorus chemicals existing in soil bacterium *Flavobacterium ATCC 27551* and *P. diminuta* was named Phosphotriesterase (PTE) [3, 4, 5, 6].

Organophosphorus Acid Enhydrolase (EC.3.1.8.2) (OPAA)

OPAA was identified as a hydrolasing enzyme of DFP by Abraham Mazur in rabbit tissue enzyme in 1946, and then was purified from *Alteromonas* species strain JD6.5.19 bacterium. This enzyme is a monomer and a metalloprotease 60kDa which have a Mn in its natural form. High activity of OPAA in P-F bond hydrolyzing in DFP, Sarin, and Soman is admitted but the ability of this enzyme in hydrolyzing P-O and P-CN bond is low and it cannot hydrolyze P-S bond [5].

Diisopropyl-fluorophosphate fluorohydrolase (DFPase)

DFPase was identified by Francis Hoskin in 1966. It needs calcium for its function. The specific substrate of enzyme is (DFP) diisopropyl fluorophosphate and it produces diisopropyl phosphate and fluoride. DFPase is a 35kDa, with 314 amino acid residues, which is able to hydrolyze P-F bond in Fluorophosphate. DFPase is taken from ganglions and brain of *Loligo Vulgaris*squid and its specific substrate is DFP [3].

E. coli Aminopeptidase P (PepP)

Aminopeptidase P is an exopeptidase that cleaves the amino-terminal residue from polypeptides, as long as the following residue is a proline. Aminopeptidase P comprises four PepP monomers, arranged as a dimer of dimers. Kinetic and structural analysis of PepP has identified residues comprising the active site as well as parts of its structure that are required for substrate specificity. This enzyme is a metalloprotein in *E.coli* that needs two Mn^{2+} ions for its performance. The highest speed of degradation of this enzyme is the one which substrate consists of methyl isopropyl and methyl isobutyl group [4].

Phosphotriesterase PON1

PON1, is an Aryldialkylphosphatas or Aromatic esterase from PON family that consists of PON1, PON2, and PON3 subgroups. Sequences of PON2 and PON3 are 60% similar to PON1 but these sequences indicate no paraoxonase activity. PON1 is a mammalian enzyme that is responsible for hydrolyzing oxidized thioate produced by P450 system and is able to hydrolyze P-O, P-F and P-CN [1].

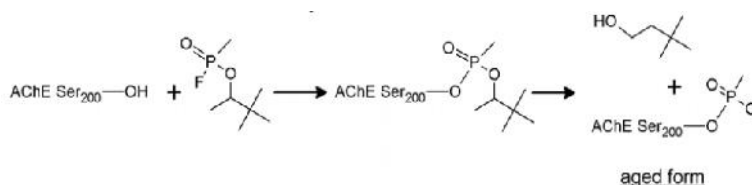


Figure 2. Mechanism of inactivation of Acetylcholinesterase [Adamczak, M., et al].

MPH (*Pseudomonas sp. WBC-3*)

Methyl parathion hydrolase (MPH, E.C.3.1.8.1), isolated from the soil-dwelling bacterium *Pseudomonas sp. WBC-3*, is a Zn(II)-containing enzyme that catalyzes the degradation of the organophosphate pesticide methyl parathion. The enzyme is dimeric and each subunit contains a mixed hybrid binuclear zinc center, in which one of the zinc ions is replaced by cadmium. In both subunits, the more solvent-exposed beta-metal ion is substituted for Cd²⁺ due to high cadmium concentration in the crystallization condition. Both ions are surrounded by ligands in an octahedral arrangement [3].

Protein Engineering Methods

Utilizing these natural enzymes as vital catalysts is a good way of degradation organophosphorus chemicals but their inability to long term usage and declining their activity and their thermostability to environmental agents such as temperature can have some problems [12].

Some of the usages of protein engineering methods are in food industries, environment, medical, pharmaceutical, detergents, biopolymers, and nanobiotechnology [3].

Protein engineering methods are in three ways:

1. Obtaining the desired feature without any change in genome: in this method protein features change without any change in genome that consists of:

- a) Change by chemical materials
- b) Environment engineering
- c) Substrate engineering

2. Obtaining the desired feature with change in genome: in this method the change takes place in the genome, in three different ways: rational mutation, evolutionary mutation and a combination of both Using protein engineering methods is applied to improve activity, stability and substrate specification etc. Increase thermostability of enzyme is one of the important parameters in industry and pharmaceutical. By comparing thermophilic and mesophilic bacteria can identify the differences of these two and apply some methods in order to improve thermostability in mesophiles. In order to change amino acids to obtain the desired sample that is done by taking patterns from thermozyne using protein engineering methods as SDM and Directed evolution[3,4].

Glycine amino acid has the most spatial state compared to other amino acids while proline amino acid has only one

spatial state and it is the most rigid amino acid. Replacement of these two amino acids results in decreasing spatial entropy of the unfolding shape of the protein and causes folding stability of it. As an example, Alcohol Dehydrogenase is from *Thermoanaerobium brockii* bacterium containing 8 proline amino acids compared to its mesozyme homologue enzyme from *Clostridium beijerinckii* bacterium. Comparing samples from homologue thermozyne and mesozyme it was found that in thermozyne samples alanine amino acid replaces glycine and arginine replaces lysine. Alanine is the best amino acid to create alpha helices which results in enzyme stability. Arginine amino acid that contributes to ionic bond is more obvious in thermozyne than in mesozymes. With an investigation on aromatic amino acids including tyrosine, phenylalanine, and tryptophan in thermozyne and mesozymes there was no significant difference. In examining polar amino acids in thermozyne and mesozymes it was found that polar amino acids are less in thermozyne than in mesozymes whereas loaded amino acids are more [3].

Compared to mesozymes, thermozyne has more subunits. Metal ions have an important part in enzyme stability [24].

OPH Enzyme Engineering

OPH (from *pseudomonas* and *flavobacterium* bacterium) is the most important enzyme in degradation organophosphorus chemicals worked on and its range of action is on different substrates. As mentioned above this enzyme was found in two stains from different families which has an identical sequence. In strain *Flavobacterium sp ATCC 27551* the enzyme is coded on 43 kilobase plasmid (pSM55) and in strain *Brevundimonas diminuta* on pCMS1 (40-66 kb) plasmid [3, 4].

Natural substrate of organophosphorus hydrolase has not been known. OPH is a homodimer with molecule weight of 72 kDa. This enzyme is one of the metalloproteins with two cations in its active site. Two divalent ions including Zn²⁺, Co²⁺, Cd²⁺, Ni²⁺, or Mn²⁺ increase catalytic activity of enzymes so that two central Zn²⁺ influence enzyme stability and two central Co²⁺ influence enzyme activity (30%) [3].

Active Site of OPH

In the active site of OPH there exist 6 amino acids that include four histidine amino acids, one acid aspartic and one lysine amino acid.

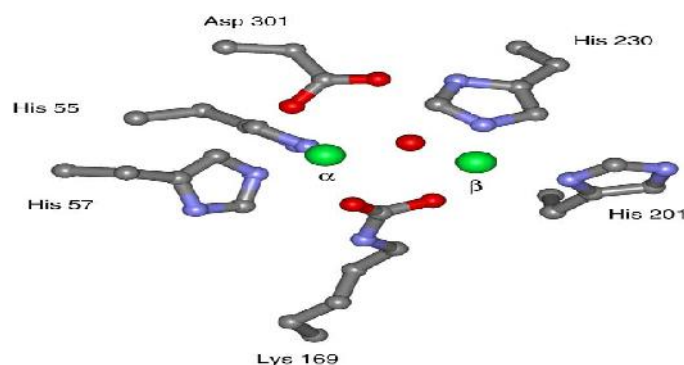


Figure 3. OPH active site [Ghanem, E., et al] OPH, like other members of super family Amidohydrolase, consists of one TIM-()₈ and its shape is such that metallic centre of binucleate is at the end of -barrel domain carboxyl. The name of this enzyme is Aryldialkylphosphatase because the substrate of this enzyme is an arlydialkylphosphate with a water molecule.

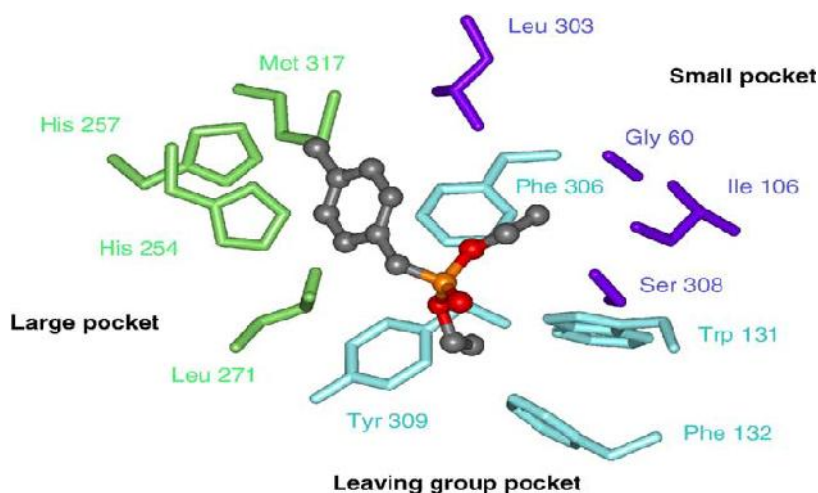


Figure 4. Active site of the substrate bond to the enzyme [Ghanem. E, *et al*]

Each of the two cations in the active site bind to the two histidine amino acids, at the same time aspartic acid binds to alpha cation in active site of the enzyme. Lysine amino acid is carboxylated and a bridge is created between the two cations [29].

Binding Site of OPH enzyme

Binding site of OPH consists of three parts: small packet, large packet, and release group packet that have totally 12 amino acids in this site.

OPH is able to hydrolyze P-O phosphodiester bond in paraoxon, P-N phosphonofluoridate bond in DFP, sarin, and soman and P-S phosphorothioate bond in VX and P-CN phosphoramidocyanide in tabun. Degradation rate of substrate of OPH, paraoxon is $10^8 \text{M}^{-1} \text{sec}^{-1}$ that is near the rate of diffusion ($10^{10} \text{M}^{-1} \text{sec}^{-1}$). However, hydrolyzing rate of P-S and P-F bonds are significantly low. In active site of the enzyme, a Lys-169 and a His-254 take part in hydrolyzing reaction. Two metal divalent ions that are Zn in natural are bond to each other with the help of one hydroxyl molecule that comprise nucleophile part. P=O bond in paraoxon substrate is activated by a tied co ordnance bond with Zn^{2+} and makes a weak complex with binding hydroxyl ion. Binding hydroxyl reaction developed by transmitting proton to a Lys so that nucleophile hydroxyl attack at phosphoric centre starts when the leaving group leaves. One water molecule enters the weak bond site of binuclear then the proton of water molecule is taken by His254 and is released in high pH[3]. OPH enzyme is clone and expression in many differ ent systems such as *Escherichia coli*, *Drosophila melanogaster*, *pichia pastoris*, *Streptomyces lividans*, insect cells *Serratia*, *Arthrobacter*, *Enterobacter*, *Burkholderia*, *Flavobacterium* and *Pseudomonas diminuta* [3].

Natural production of OPH has some problematic consequences so that its produced level is low; as a result some methods of enzyme expression at higher levels using numerous signals have been used. In a Chinese study in 2012, they sought to optimize catalytic activity of enzyme with establishing OPH enzyme on E coli surface by binding IanK anchor to OPH (InaK-N-OPH). After optimization steps, the mentioned bacterium was able to catalytic activity at the low temperature of 20 degrees Celsius. This

process is highly used in huge industries and environmental affairs [3].

In one of the works done by Latifi *et al.* (2011), to surface expression of OPH enzyme use an anchor system taken from N- terminal domain of InaV from *Pseudomonas syringae* called InaV-N. In this study, after they expressed the enzyme at E coli surface they started a successful degradation of the substrate of enzyme (chlorpyrifos). They are also conducting another study in which, by co-display surface expression of OPH and OPAA have shown a significant activity compared to the stain that expression only OPH or OPAA on the surface. Results from examination of newly recombinant stain show that whole cell activity in stain that express OPAA/OPH in degradation organophosphoric chlorpyrifos compounds (P-O bond) is 1.5 times of the whole cell activity that express only OPH. Furthermore, unlike stain that express OPH, stain includes OPAA/OPH grow easily in agar MSM environment complemented with DFP (having a P-F bond). This ability is due to the activity of OPAA enzyme in recombinant stain. These results show that codisplay surface expression of these two enzymes makes the use of recombinant stain in degradation organophosphorus chemicals more efficient [3].

There are at least four definite ways to express enzymes in form of secretion in *E coli* bacterium that consists of Sec, SRP, YidC, and Tat paths. In one of these studies, Korean researchers in 2005 started expression of OPH in the form of secretion using Tat system. Comparing secretion expression with cytosol expression it was confirmed that the secretion form had a more stability [3]. In a study by Latifi *et al.* (2011) 5 strains of bacteria in soil which were able to degradation chlorpyrifos (as one of the enzyme's substrates) were separated using PCR of 16srRNA gene and its genes were extracted and registered in NCBI database. After the extraction of the bacteria they were used as a substrate against chlorpyrifos each one of which could grow and degradation different concentrations of chlorpyrifos. Using HPLC technique, the level of chlorpyrifos degradation by each stain was investigated [3].

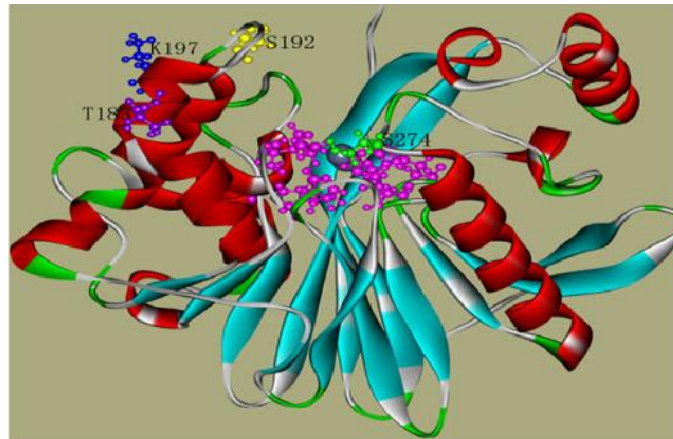


Figure 5. 3-dimensional structure of methyl parathion hydrolyse and the four indicated points of mutation [Luis. B., *et al*]

Increasing the Activity of OPH

Most works done to engineer OPH enzyme has been based on increasing the activity and changing the specification of its substrate and less work has been done on increasing its stability. Some researchers conducted a work in 2011 using SDM method to create a mutation in OPH enzyme in which they could successfully increase the turnover number of it significantly. This was done with mutation of I106L/F132V/S308A/Y309W. In another study by these researchers two mutant samples, each one of which involved 3 point mutations (G60V/I106A/S308G and G60V/I106L/S308G) were resulted. Investigating them showed that after the mentioned mutations their activity against methyl phosphonate substrate increased significantly [3, 4].

In a study in 2009 by researchers of chemistry and pharmaceutical department of University of Chicago, USA, using rational and randoms method, one of the homologue samples of OPH enzyme called Dr-OPH was engineered. The protein had a higher thermal stability than *Pseudomonas diminuta* enzyme OPH so that it can retain activity in 60 degree Celsius for 3 hours, but its activity is lower than the mentioned OPH. Using random and rational methods of protein engineering, Dr-OPH activity against substrates of ethyl and methyl paraoxon increased 126 and 183 times, respectively. Dr-OPH enzyme is extracted from *Deinococcus radiodurans* which is a thermophilic bacterium. With G207D mutation in Dr-OPH they could increase enzyme activity against both substrates which results from prompting proton shuttle through aspartic acid in this situation. Creating Y97W mutation increases the activity and specificity to the substrate. This mutation is patterned according to homologue sample, i.e. *Pseudomonas diminuta* OPH so that the presence of tryptophan in the mentioned situation in OPH causes an increase of 4.2 Å from beta metal and leads to an increase in activity which supports the hypothesis that increasing the peripheral space of the occupied area by this amino acid is useful for catalyzing [3].

Texas University researchers (1999) did two point mutations of H259R/H257L, by which they could increase the substrate kcat/km specificity of OPH from 2 to 30 times. The histidines of 254 and 257 situations are located near the enzyme active site and can affect catalysis by reacting

to active site and the substrate. However, this is useful in bigger substrate which is due to flexibility of structure in this area by replacing arginine and leucine to better access to bigger substrates [3].

In another study in Texas University (2001) three points mutation was created by rational protein engineering method (I106A/F132A/H254Y) to increase the catalytic OPH enzyme which was an increase in enzyme activity [3].

Based on a thesis in University of Texas (2007) different mutations were done using rational protein engineering on *Pseudomonas diminuta* OPH enzyme the change of which is as follows:

1. H254L: increasing large packet size and creating a hydrophobic area for reaction with releasing group of substrate hydrophobic.
2. H254S/H257L: increase hydrophobicity of the large packet to improve kinetic parameters against demeton substrate.

On the other hand, in a study done in 2000 with 8 point mutations on OPH enzyme, only one mutation (L136Y) caused a 33 percent increase in VX degradation and seven other mutations (W131F/F132Y/L140Y/L140Y/L271Y/F306A/F306Y) decreased degradation. This was because of the increase of hydrogen bonds of the mutant sample with VX factors. And only F132Y mutation increased the activity against paraoxon [3].

Increase in OPH Stability

One of the most important things to increase enzymes' effectiveness is increasing their stability against factors as temperature, pH, soluble, etc. one of the most important parameters in stability is temperature which is an important factor in optimal using in industry and has have millions of dollars benefit. Enzymes are often low stability in high temperatures and are inactive in these temperatures. This will lead to their decreasing effectiveness. Today, engineering methods to improve thermostability of enzymes are used including increasing ionic bonds, hydrophobic interactions, enzyme immobilizing, creating disulfide bonds etc. The aim of these changes is increasing enzyme rigidity in order to prevent its unfolding in high temperatures and its inactivation as well as increasing half-life of the enzyme [3, 4, 5, 6]. In 2006 Korean researchers used tat path to import OPH to periplasmic

space in *E.coli* bacterium. This was done to increase effectiveness of the enzyme. The result was that OPH enzyme in samples including tat path was more stability compared to the sample produced in cytosol [34].

In 2010 American researchers started producing OPH enzyme hydrogel using protein engineering methods so that creating domain zip lucine, linker, and poly hystidine started cross link of enzyme and production of hydrogel. This led to produced hydrogels retaining of their activity after 5-month keeping in cold buffers. In this study rational protein engineering methods were used [3].

In 2010 Chinese researchers investigated an enzyme from OPH family called OPHc2 that is extracted from *pseudomonas alcaligenes* and had a homology with *pseudomonas diminuta* OPH and has a higher thermostability compared to other OPHs, they concluded that the mentioned enzyme has a disulfide bridge (Cys110-Cys146) which is one of the major factors in thermostability of this enzyme. This investigation is based on changes in cysteine amino acids which result in creating disulfide bridge resulting in de-

crease in thermostability of the enzyme which was the result of disulfide bridge [3].

Also, chinese (Fig. 10) researchers conducted another research in which they examined thermostability of one of the degradation organophosphoric compound enzymes called methyl parathion hydrolase which is extracted from *Ochrobactrum sp.M231*. They did this by changing glycine 194 and 198 amino acids to proline. They found that changing 194 glycine amino acid in a loop along with proline resulted in loop bending and rigidity of enzyme and eventually its temperature stability [3].

In another study in 2011, these researchers increased thermostability of the mentioned enzyme using ionic bridge. They were able to create an ionic bridge on protein surface using double mutation (P76D/P78K) so that the temperature in enzyme half-life increased from 64 to 68. Replacing aspartic acid and lysine results in ionic bond and increase the electrostatic structural energy and finally the stability of enzyme [3].

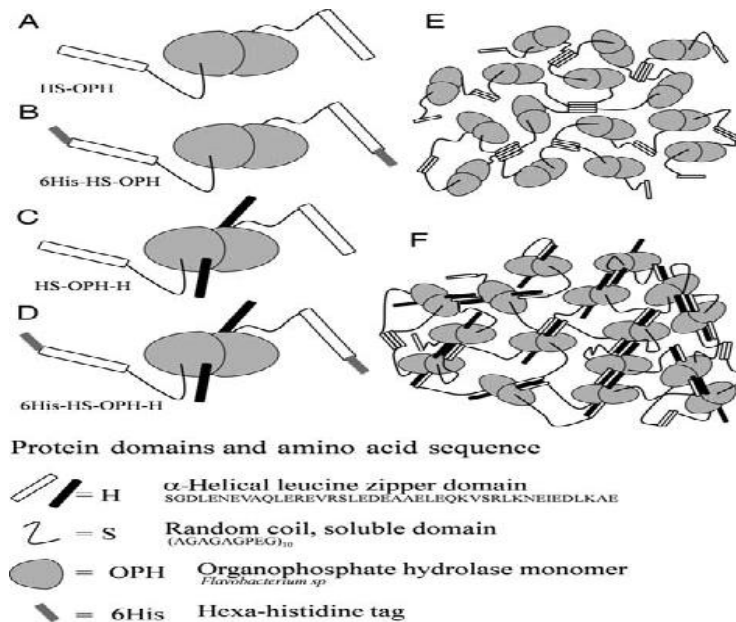


Figure 6. OPH enzyme hydrogel [Hoang. D., et al]

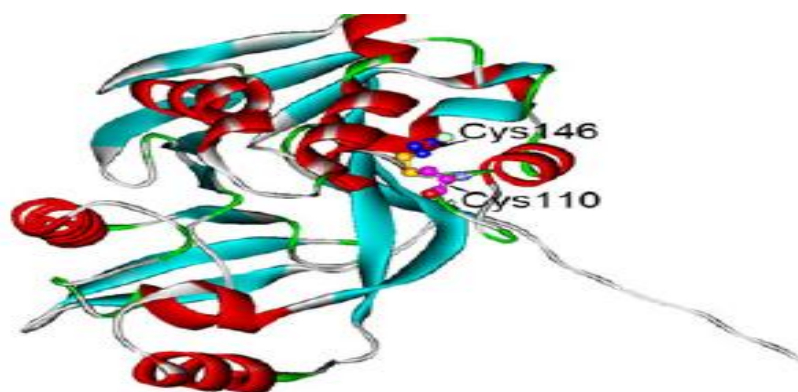


Figure 7. 3-dimensional structure of methyl parathion hydrolase of *Ochrobactrum sp.M231* bacterium and the site of the two glycine amino acids [Jian. T., et al]

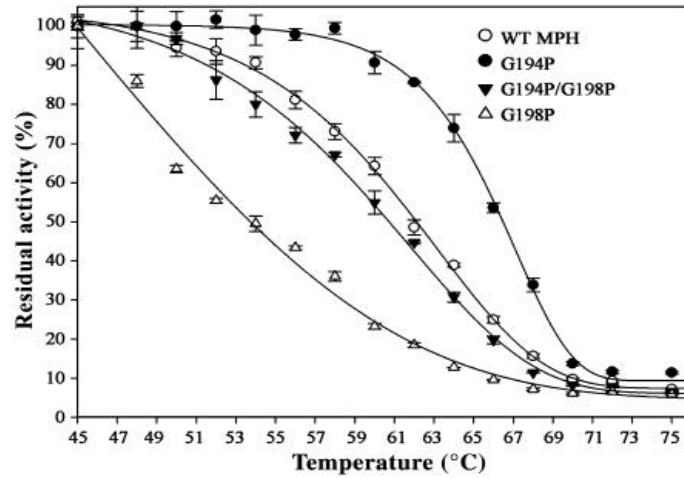


Figure 8. Comparing thermostability of methyl parathion hydrolase and mutant samples [Jian. T., et al].

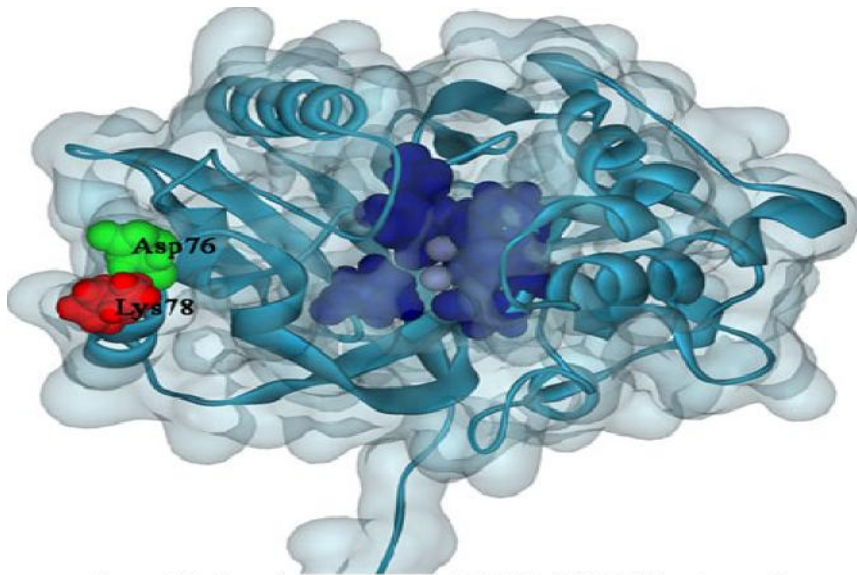


Figure 9. 3-dimentional structure of MPHP76D_P78K [Yidan. Su., et al]

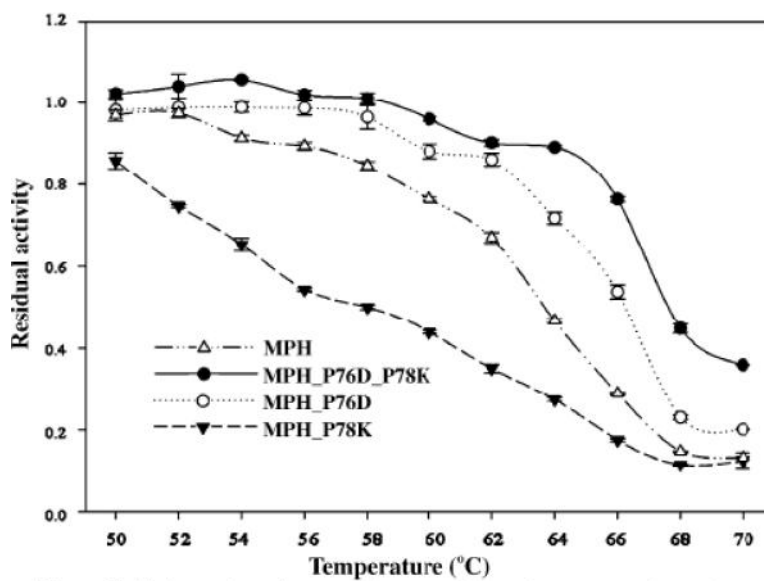


Figure 10. Thermostability of initial sample and mutants (MPHP76K_P78K, MPHP76D, and MPHP78K) [Yidan. Su., et al]

In another study in 2013, these researchers did four other mutations (S274Q/T183E/K197L/S192M) in a semi rational method and using modeling methods they were able to promote T_m and T₅₀, 11.2 and 10.2 degrees Celsius, respectively, compare to the initial sample [3].

In one of the works doing, Latifi *et al* with use of Molecular Dynamics Simulation create nine mutations (Asn4Cys-Arg130Cys, His21Cys-Ser265Cys, Gly40Cys-Ala44Cys, Leu53Cys-Ala86Cys, Phe116Cys-Ala156Cys, Ala154Cys-Leu187Cys, Ala170Cys-Thr200Cys, Gly175Cys-Ile194Cys and Thr94Cys- Glu119Cys) for increasing OPH thermostability that just four mutations confirm by software's (Asn4Cys-Arg130Cys, Gly40Cys-Ala44Cys, Ala170Cys- Thr200Cys and Thr94Cys- Glu119Cys).

Discussion and Conclusion

The most important method of decontamination of organophosphorus chemicals using degradation enzymes. OPH enzyme extracted from *pseudomonas diminuta* and *Flavobacterium* is one of the most important degradation the enzyme of the mentioned compounds on which most studies have been done [8, 17]. This enzyme has signal peptide bind to membrane and natural production of it has some problems and the level of its productivity is very low, so some methods of enzyme expression are used at high level using different signals. Using an anchor method taken from domain N terminal protein InaV from *Pseudomonas syringae* InaV bacterium called to express OPH enzyme on *E.coli* bacterium surface and OPH enzyme expression in a secreted (injected) form using Tat system were among the methods by which researchers can increase production level and effectiveness of the enzyme [33,34]. This enzyme can degradation organophosphorus chemicals, but their low activity, low thermostability, and low specificity against substrates causes a need to protein engineering methods to improve activity and stability. OPH enzyme is one of the metalloproteins and there are naturally two Zn ions in its active site. We can change enzyme activity by replacing divalent ions like cobalt, magnesium and nickel so that by replacing cobalt ion enzyme activity increases significantly. The active site of the enzyme has three packets that can change the activity and specificity level of the enzyme by changing its amino acids. H254 and H257 from the large packet and F360 from the small packet are important points engineered to increase enzyme specificity; histidines of 254 and 257 situations are near the active site of the enzyme and can affect catalysis by reacting to active site and the substrate. Replacing leucine and serine amino acids by these amino acids will cause an increase in the large packet and hydrophobic that will lead to increase kinetic parameters of the enzyme against large substrate like demton, and replacing leucine by phenylalanin 306 causes an increase of the size of the active site for bigger substrates. Researchers were able to change the turnover number of OPH significantly. This was done by mutations I106L/F132V/S308A/Y309W. Amino acids G60/I106/S308/I106 are important and critical points by which researchers were able to increase enzyme activity significantly by protein engineering methods including random and rational. One of the most important

things to increase effectiveness of enzymes is using protein engineering methods for increasing their stability against factors as temperature, pH and solubility. Among these methods we can name increasing ionic bond, hydrophobic reactions, enzyme immobilizing, shortening the loops, creating disulfide bonds, etc. The aim of these changes is increasing enzyme rigidity in order to prevent its unfolding in high temperatures and its inactivation as well as increasing half-life of the enzyme [43, 44, 45, 46]. OPH enzyme has 23 alpha helices and 13 beta boards. Using homologue samples of OPH enzyme that have high thermostability can help create changes and increase stability. OPHc2 enzyme that is extracted from *pseudomonas alcaligenes* and has homology with OPH *pseudomonas diminuta*, has more thermostability compared to other OPHs. Creating different mutations it was found that the presence of a disulfide bridge (Cys110-Cys146) in the mentioned enzyme is one of the basic factors of thermostability of this enzyme. So, creating a disulfide bridge in OPH enzyme to increase its stability can be an important method to be used [8,18, 36, 37, 38, 40, 41, 42, 48]. Changing glycine amino acids 194 and 198 to proline, which was presented in one of the OPH loops, leads to increase in thermostability which has occurred because of loop bending and rigidity of the enzyme by replacing proline [49].

Creating an ionic bridge is one other method to increase thermostability by which researchers have been able to increase OPH thermostability. They created the way to make ionic bonds in OPH enzymes by double mutation (P76D/P78K) and replacing loaded amino acids (lysine and aspartic acid) in the place of proline 76 and 78. This led to increase structural electrostatic energy and eventually stability of OPH enzyme [50].

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