Available online at http://pbr.mazums.ac.ir

PBR

Original Article

Pharmaceutical and Biomedical Research

Evaluation of the organophosphorus hydrolase enzyme activity in creams and investigation of its stability

Mariye Rajaie[1], Seyed Morteza Robatjazi*[1], Hamid Akbari[2], Sanaz Mahboudi[1], Jafar Mohammadian Mosaabadi[1]

[1]Department of Bioscience and Biotechnology, Malek Ashtar University of Technology, Tehran, Iran
[2]Department of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

Received: Jun 26, 2016, Revised: Jul 16, 2016, Accepted: Aug 7, 2016

Abstract

The main purpose of this project is investigation of the organophosphorus hydrolase (OPH) enzyme activity in water in oil (w/o) and oil in water (o/w) creams and investigation of the OPH enzyme stability in formulated creams**.** OPH enzyme was extracted and purified from strain flavobacterium. The w/o and o/w creams were prepared using different formulations. In order to achieve an emulsion with maximum stability, appropriate percentage of the cream components was selected by studying different formulations and the physical and chemical stability of the produced cream were considered. $5U_{enzyme}/90g_{cream}$ enzyme was used for each formulation. To measure the enzyme activity in creams, extraction method was used and enzyme activity was determined based on parathion hydrolysis. The thermal stability of OPH in both types of w/o and o/w creams was studied at 4 and 30 °C for various time periods. The average enzyme activity was about 0.0065 U/g_{cream} and 0.018 U/g_{cream} for w/o and o/w creams respectivly. According to the results, the relative activity at 4 °C was reduced to 50% after 26 and 45 days in w/o and o/w creams, respectivly. The results showed that the OPH enzyme activity in o/w cream was 2.6 times more than that of w/o cream, because of the higher hydrophobicity of o/w cream compared to w/o. The OPH enzyme stability in o/w cream was greater in comparison to w/o cream. The OPH enzyme was active for nearly 2 months on o/w creams at 4 °C.

Keywords: Creams, organophosphorus hydrolase, enzyme stability

Pharm Biomed Res 2016; 2(2): 75-83 DOI: 10.18869/acadpub.pbr.2.2.75

Introduction

Organophosphate compounds (OPs) are esters of phosphoric acid that are extremely toxic compounds that act as acetylcholinesterase (AChE) inhibitors in the external and central nervous system (1, 2). These compounds are widely used in agriculture and are toxic insecticides, pesticides and chemical nerve agents and military applications (3-5). OP insecticides are degraded when exposed to sunlight, water and microbial hydrolysis in the soil (6). Most OPs are nearly lipophilic (7). Organophosphate pesticide poisoning of humans and animals is caused by skin contact, inhalation and body ingestion. The toxic compounds in the form of vapor or liquid are easily absorbed through transcutaneous contact (8). With

* E-mail: **s_m_robatjazi@mut.ac.ir**

developments in biotechnology, calculable methods for the detoxification and modifications of these compounds in order to prevent percutaneous penetration of OPs can be employed (9). In this perspective, personal protective equipment, including specific clothing, gloves, face masks and overboots, lead to efficient conservation against OP toxins (10). Organophosphorus hydrolase (EC: 3.1.8.1) enzyme, commonly named OPH is listed in phosphoric triester hydrolases group which was initially derived from *Pseudomonas diminuta MG* and *Flavobacterium sp* in the 1980s (11,12), and is capable of hydrolyzing a wide range of organophosphorus esters bonds such as P-O, P-S, P-F and P-CN bonds (13-14). OPH is a 72-KDa homodimeric metalloenzyme contains two Zn^{2+} ions at the active site in the native enzyme. A number of different divalent metal ions (Mn^{2+}, Cd^{2+}, Co^{2+}, or Ni^{2+}) can be used as substitute for Zn^{2+} ions. When the enzyme substituted with Co^{2+}ions, it showed the highest activity on organophosphorus compounds used as substrate that contain P-F and P-S bonds (15). OPH enzyme can catalyze the hydrolysis reaction of OPs such as paraoxon, parathion and methyl parathion to produce phosphate esters and p-nitrophenol (PNP). The sample cream formulations could be used to protect the skin against any of the toxic chemicals that are rapidly absorbed in the skin. Cream is an emulsion of two immiscible liquids, oil and water. The organic phase is composed of a hydrocarbon solvent, emulsifiers, and various additives. Stable emulsions can be prepared by using emulsifiers with a hydrophilic-lipophilic balance (HLB) value, this value varies from 0 to 20, lower than 10 in w/o emulsion but higher than 12 in o/w emulsion (5). The o/w and w/o creams were made on the basis of chemical composition. To investigate the stability, viscosity and best physical shape, cream formulation was selected for both types of cream (9, 10). In this research, two different sample formulations, oil-in-water and water-in-oil emulsions were evaluated to determine whether they contain OPH enzyme for hydrolyzed OPs such as paraoxon and parathion. The activity and stability of OPH enzyme were evaluated in both o/w and w/o creams.

Materials and methods

Bacterial strain and chemicals

Flavobacterium ATCC 27551 was obtained from Microbial Type Culture Collection (MTCC, Chandigarh, India) and cultivated in Wakimoto medium on slant (16). Organophosphorus hydrolase enzyme was extracted and purified from *Flavobacterium ATCC 27551* using chromatography methods with at least 80–90% purity (17, 18). OPH stock solution (200 U/ml) was prepared in 50mM phosphate buffer at pH 7.8, containing 10 µM $CoCl_2$. The stock solution was kept at 4 °C. Parathion ([O, O-diethyl]-O-4-nitrophenyl phosphorothioate) was obtained from Sigma-Aldrich Company (USA).

Cream preparation

The oil and the aqueous phases of cream were prepared separately, the oil phase contained liquid paraffin, Bees wax, estearil alcohol, Span 60 and Petrolatum gel (Sigma Co. USA) which was heated at 75 °C and the

aqueous phase which included deionized water, glycerol, polyethylene glycol 6000, Methyl paraben and Tween 60 (Sigma Co. USA) heated at 75 °C. The formulas were prepared by adding the oily phase to the heated aqueous phase and mixing with mechanical stirrer. The cream was then stored in the refrigerator at 4 °C. Emulsions containing enzyme were prepared using enzyme solutions as the inner phase. Combination of the main membrane phase should be based two initial requirements: it must capable of forming a stable emulsion, and it must have a trivial impression on enzyme activity (19). The cream containing OPH was prepared by dispersing the desired amount (0.1-0.5 wt%) of enzyme into buffer solution (50 mM phosphate buffer, pH 6.2, 0.02 wt% sodium azide as an antimicrobial agent) (20), then the required amount of OPH (5 U / ml) was added to emulsion and was mixed to ensure distribution of the enzyme as much as possible over the cream for 1 h using stirrer at 40 °C.

Measurement of OPH activity

Enzyme activity was determined by hydrolyzing parathion which produces p-nithrophenol (PNP). One unit of phosphotriesterase activity was defined as the amount of enzyme required to hydrolyze 1 µmol of ethyl-parathion per minute at 30 °C (21, 22)**.** The reaction mixture consisted of 485 µl of extracted sample (pH 8) and 5 µL of 50 mM $CoCl_2$ placed in a 1mL microfuge tube and agitated at 200 rpm in a shaker incubator. Tubes were equilibrated at 30 °C for 30 min and the reaction was started by the addition of 10 µL of 40 mM ethyl-parathion. The increasing absorbance rate of samples was measured at 410 nm by a spectrophotometer (Jenway 6310, UK). The cream without OPH was used as blank.

Measurement of OPH activity on the w/o cream

The following methods were used in order to extract OPH enzyme from w/o cream and measurement of the enzyme activity (22):

1. The first method; the prepared cream was mixed with PBS buffer in a 1:1 (w/w) ratio and the cream was homogenized by sonication for 1 min in a microfuge at 4 °C. The microfuges were centrifuged at 13000 rpm for 15 min, the clear solution was separated as the sample containing OPH enzyme and then the enzyme activity was determined.
2. The second method; the OPH activity was measured in cream directly, 0.1 g from the prepared cream was mixed with 485 µL PBS buffer containing 50 mM $CoCl_2$ in a 1-mL microfuge tube and the mixture was homogenized by sonication for 1 min at 4 °C. The microfuge tubes were equilibrated at 30 °C for 30 min and the reaction was started by the addition of 10 µL of 40 mM ethyl-parathion. The microfuges tubes were incubated for 5-10 min at 30 °C and then the microfuges were centrifuged at 13000 rpm for 3 min. the supernatant was separated and the absorbance samples were measured at 410 nm by a spectrophotometer.

The enzyme stability in the creams

The thermal stability of OPH enzyme in both types of w/o and o/w creams was studied at 4 and 30 °C for various time periods. 0.5 g of cream with known enzyme activity (~5 U/g_{cream}) was added to 1 mL microfuge and the microfuge was incubated at 4 and 30 °C. OPH enzyme was extracted from cream and enzyme activity was determined as described before. The relative activity was calculated by the following equation (22):

$$\text{Relative activity}(\%) = \frac{Activity_{time=t}}{Activity_{time=0}}$$

Results

Evaluation the o/w and w/o creams formulations

The optimum formulations were determined by investigating the stability and physical form of o/w and w/o creams. According to the results of cream physical appearance evaluation, penetration properties of w/o cream on skin were lower than o/w cream because of higher oily phase percentage. Warmth and oily were felt when using the w/o and coolness was felt when using o/w creams on the skin. The optimized formulations are shown in Table 1.

The OPH enzyme activity on the creams

The average of OPH enzyme activity was measured approximately 0.0065 and 0.005 U/ g_{cream} (Fig. 1) in w/o cream by the first and second methods, respectively. The OPH activity was measured in more levels by using the first method as compared with the second one. The hydrophilicity of OPH and higher water percentage of o/w cream in comparison with w/o cream were caused by using the first method for determination of the enzyme activity on the o/w cream. The average levels of the enzyme activity were approximately 0.0065 and 0.018 U/ g_{cream} for w/o and o/w creams, respectively (Table 2 and 3). With regard to the amount of used enzyme, it was the same in both types of creams, measurement of higher enzyme activity showed availability of the OPH enzyme on the o/w cream was better compared with w/o cream.

The OPH enzyme stability on the creams

OPH stability on the o/w and w/o creams was evaluated at two temperatures (4 and 30 °C) during 2 months and the results are shown in Fig 2. According to the results, the enzyme stability was decreased with increasing temperature and OPH enzyme was more stable at 4 °C than 30 °C. The required time for the w/o cream enzyme activity to reach 50% of initial level was 26 days at 4 °C and 21 days at 30 °C, while it was 45 days at 4 °C and 35 days at 30 °C for o/w cream. Therefore, it was shown that the enzyme stability on o/w cream was higher than w/o cream.

Discussion

In formulation studies of cream preparation and optimization, the amount of each formulation compound was examined by HLB system, oily substances and surfactants required HLB calculations and investigation of the cream's appearance and physical forms, viscosity and stability. Water and poly ethylene glycol 6000 content were higher in o/w cream than in w/o cream. These differences in composition could

Table 1 The optimized cream formulations

No.	Ingredients	W/O[1] (%w/w)	O/W[2] (%w/w)
1	Liquid paraffin	15	10
2	Poly ethylene glycol 6000	3	8
3	Bees wax	5	1
4	Sterile alcohol	2	2
5	Petrolatum gel	10	5
6	glycerol	2	5
7	Span 60	4	0.86
8	Tween 60	1	2.13
9	Methyl parabon	0.1	0.2
10	water	57.9	65.81
11	OPH[3] enzyme	$5U/90g_{cream}$	$5U/90g_{cream}$

1:Water in oil, 2:Oil in water, 3:Organophosphorus hydrolase

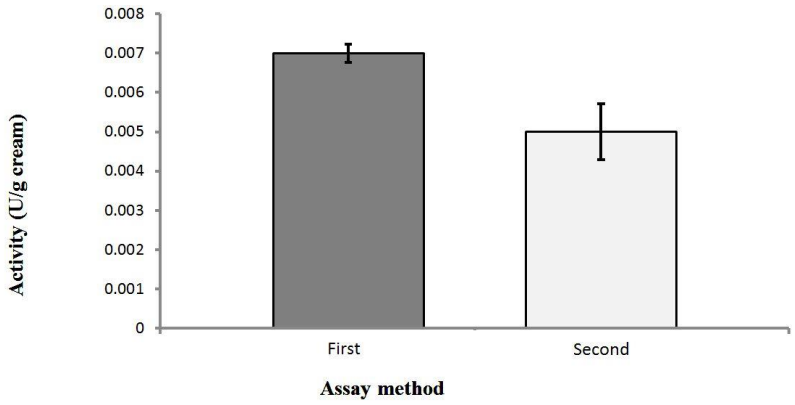

Figure 1 The activity of OPH enzyme in water in oil cream
■:The activity was measured by first method, ■:The activity was measured by second method

Table 2 The OPH activity in w/o cream by the first method

No.	Absorbance	Enzyme unit (U/mL)	U/g_{cream}	Mean ± SD[1]
1	1.47	0.009	0.009	
2	1.56	0.007	0.007	
3	2.1	0.006	0.006	0.0065 ± 0.0021
4	2.4	0.004	0.004	
5	Average activity		0.0065	

SD: Standard deviation

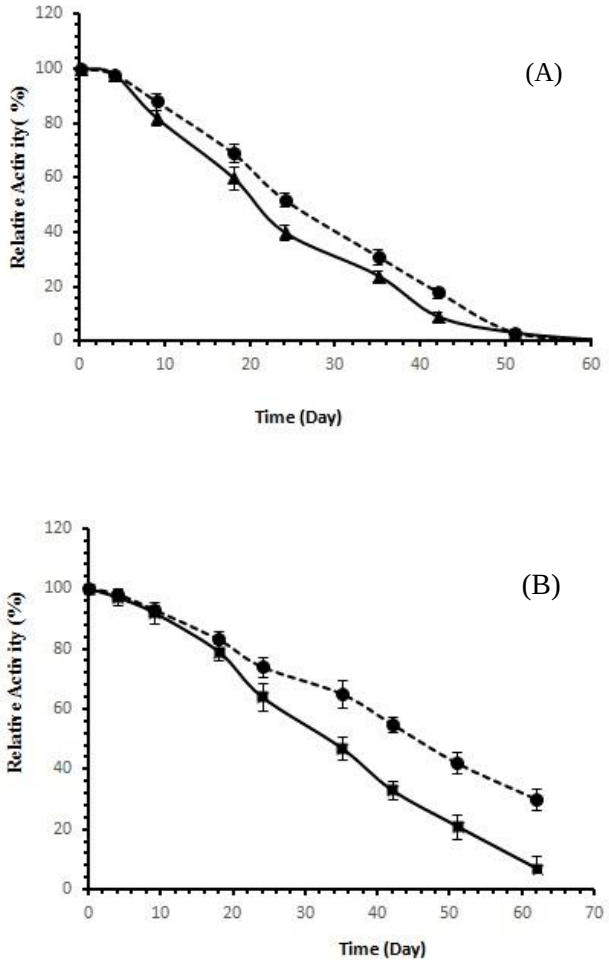

Figure 2 The thermal stability of OPH enzyme in the creams.
A: water in oil cream, B: oil in water cream, (---●---):4 °C (——►): 30 °C

Table 3 The OPH activity in o/w cream by the first method

No.	Absorbance	Enzyme unit (U/mL)	U/g_{cream}	Mean ± SD[1]
1	0.696	0.022	0.022	0.018 ± 0.0038
2	0.72	0.017	0.017	
3	1.16	0.019	0.019	
4	1.586	0.013	0.013	
5	Average activity		0.018	

SD: Standard deviation

partly account for differences in observed emulsifying properties and dispersing of OPH enzyme on creams. Proteins are dissolved in aqueous buffers, according this fact, OPH is well dispersed in o/w cream and extraction of enzyme from o/w cream was exactly carried out. According to the high o/w cream HLB (hydrophilic), low w/o cream HLB (lipophilic) and OPH hydrophilicity, enzyme extraction in o/w cream is easier than w/o and enzyme activity was measured higher in o/w cream. A lack of uniformity was observed on dispersion of OPH in w/o cream. The results showed that the OPH enzyme activity in o/w cream was 2.6 times more than that of w/o cream. Using buffers and organic solvents, such as chloroform and diethyl ether was reported for protein extraction from semi solids formulation. Regarding the cream's lipophilicity using oily solvents, also chloroform and diethyl ether for extraction is better than polar solvents, but oily solvents might cause enzyme inactivation (23). The majority of enzymes are fairly unstable and industrial application is often hampered by a lack of long-term operational stability (24). In this study, the results showed that OPH enzyme has been unstable in w/o and o/w creams. The results indicated the required time for decreasing the enzyme activity to 50% of initial level was 26 and 45 days at 4 °C for w/o and o/w creams, respectively. The OPH enzyme was active for nearly 2 months in o/w creams. This stability is not enough for a cream fabrication based on enzyme activity. The use of stable enzyme forms such as immobilized OPH enzyme and capsulated OPH enzyme propose for increasing of enzyme stability in cream. According to results of enzyme activity and cream properties, the o/w cream containing of OPH enzyme can be used for preventing or reducing the penetration and absorption of organophosphate toxins into the skin.

Conclusions

The thermal stability of OPH in both types of w/o and o/w creams was studied at 4 and 30 °C. The average enzyme activity was about 0.0065 U/g_{cream} and 0.018 U/g_{cream} for w/o and o/w creams respectability. According to the results, the relative activity at 4 °C was reduced to 50% after 26 and 45 days in w/o and o/w creams, respectability. The results showed that the OPH enzyme

activity in o/w cream was 2.6 times more than that of w/o cream, because of the higher hydrophobicity of o/w cream compared to w/o. The OPH enzyme stability in o/w cream was greater in comparison to w/o cream. The OPH enzyme was active for nearly 2 months on o/w creams at 4 °C .

Acknowledgement

The authors gratefully acknowledge the technical support from the Laboratory Pharmacy of Tehran University**.**

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Munneck DM. Enzymatic hydrolysis of organophosphate insecticides, A possible pesticide disposal method. Appl Environ Microbiol 1976;32:7-13.
2. Rao S, Venkateswarlu V, Surender T, Eddleston M, Buckley NA. Pesticide poisoning in south India: opportunities for prevention and improved medical management. Trop Med Int Health 2005;10:581-8.
3. Raushel F M. Bacterial detoxification of organophosphate nerve agents. Curr Opin Microbiol 2002;5:288-95.
4. Allard AS, Neilson AH**.** Bioremediation of organic waste sites: a critical review of microbiological aspects. Int Biodeterior Biodegradation 1997;39:253-85.
5. Singh BK. Organophosphorus-degrading bacteria: ecology and industrial applications. Nat Rev Microbiol 2009;**7**:156-64.
6. Ragnarsdottir KV. Environmental fate and toxicology of organophosphate pesticides. J Geol Soc London 2000;157:859-76.
7. Millerioux J, Cruz C, Bazire A, Polly V, Lallement G, Lefeuvre L, et al. Evaluation of in vitro tests to assess the efficacy of formulations as topical skin protectants against organophosphorus compounds. Toxicol in Vitro 2009;23:127-33.
8. Archibald BA, Solomon KR, Stephenson GR. Estimating pirimicarb exposure to greenhouse workers using video imaging. Arch Environ Contam Toxicol 1994;27:126-9.
9. Barel AO, Paye M, Maibach HI,. Handbook of Cosmetic Science and Technology. 3rd. ed London: Informa Healthcare; 2009.
10. Ballantyne B, Marrs TC. Clinical and experimental toxicology of organophosphates and carbamates. Oxford; Butterworth-Heinemann; 1992
11. Mulbry WW, Karns JS. Parathion hydrolase specified by the *Flavobacterium* opd gene: relationship between the gene and protein. J Bacteriol 1989;171:6740-6.
12. Serdar CM. Murdock DC, Rohde MF. Parathion hydrolase gene from *Pseudomonas diminuta MG*: subcloning, complete nucleotide sequence, and expression of the mature portion of the enzyme in Escherichia coli. Nat Biotechnol 1989;**7**:1151-5.
13. Lei Y, Mulchandani P, Chen W, Mulchandani A. Biosensor for direct determination of fenitrothion and EPN using recombinant *Pseudomonas putida JS444* with surface-expressed organophosphorous hydrolase. 2. Modified carbon paste electrode. Appl Biochem Biotechnol 2007;136:243-50.
14. Singh BK,Walker A. Microbial degradation of organophosphorus compounds. FEMS Microbiol Rev 2006;30:428-71.
15. Omburo GA, Kuo JM, Mullins LS, Raushel FM. Characterization of the zinc binding site of bacterial phosphotriesterase. J Biol Chem 1992;267:13278-83.
16. Sethunathan N,Yoshida T. A *Flavobacterium sp.* that degrades diazinon and parathion. Can J Microbiol 1973;19:873-5.
17. Kapoor M, Rajagopal R. Enzymatic bioremediation of organophosphorus insecticides by recombinant organophosphorous hydrolase. Int Biodeterior Biodegradation 2011;65:896-901.
18. Lewis VE, Donarski WJ, Wild JR, Raushel FM Mechanism and stereochemical course at phosphorus of the reaction catalyzed by a bacterial phosphotriesterase. Biochemistry 1988; 27:1591-7.
19. Scheper T. Enzyme immobilization in liquid surfactant membrane emulsions. Adv Drug Deliv Rev 1989;4:209-31.
20. Onsaard E, Vittayanont M, Srigam S, McClements DJ. Comparison of properties of oil-in-water emulsions stabilized by coconut cream proteins with those stabilized by whey protein isolate. Food Res Int 2006;39:78-86.

21. Brown KA. Phosphotriesterases of *Flavobacterium sp.* Soil Biol Biochem 1980;12:105-12.
22. Robatjazi SM, Shojaosadati SA, Khalilzadeh R, Farahani EV. Optimization of the covalent coupling and ionic adsorption of magnetic nanoparticles on *Flavobacterium ATCC 27551* using the Taguchi method. Biocatal Biotransformation 2010;28:304-12.
23. Bennett NT, Schultz GS. Growth factors and wound healing: Part II. Role in normal and chronic wound healing. Am J Surg 1993;166:74-81.
24. Mohamad NR, Marzuki NHC, Buang NA, Huyop F, Wahab AR . An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotechnol Biotechnol Equip 2015;29:205–20.