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Dried–Reswollen Immobilized Biocatalysts for Detoxification of Organophosphorous Compounds in the Flow Systems

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Abstract New immobilized biocatalysts based on polypeptides containing N- or C-terminal polyhistidine sequences and possessing organophosphorus hydrolase activity were investigated for detoxification of organophosphorous neurotoxic compounds in the flow systems. The biocatalysts were revealed to have a high catalytic activity within wide pH and temperature ranges 7.5–12.5 °C and 15–65 °C, respectively. The immobilized biocatalysts can be dried and reswollen before use with 92–93% catalytic activity remaining after drying and rehydration procedures. The half-lives of the biocatalysts under wet and dry storage conditions were 420 and 540 days, respectively.

Keywords Organophosphorus hydrolase · Polyhistidine sequence · Dried/reswollen immobilized biocatalyst · Organophosphorous compound

Abbreviations

OPH	Organophosphorus hydrolase
polyHis	Polyhistidine sequence
OPC	Organophosphorous compound
IDA-cryoPAAG	Polyacrylamide cryogel modified by iminodiacetic acid residues
N-His_6-OPH/OPH-His_6-C	OPH with N-/C-terminal hexahistidine tag

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N-His_{12}-OPH	OPH with N-terminal dodecahistidine tag
i-N-His_6-OPH/i-OPH-His_6-C/i-N-His_{12}-OPH	Immobilized N-His_6-OPH/OPH-His_6-C/N-His_{12}-OPH

Introduction

Organophosphorous compounds (OPCs) are widely used in agriculture (primarily as pesticides) and other aspects of technology [1]. The most toxic chemical warfare agents such as Soman, Sarin, and Vx, which were outlawed under the "Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on Their Destruction" [2], are also among OPCs of the same group.

The low rate of environmental detoxification of OPCs results in their accumulation, presumably in river and ground water. It's not surprising therefore that OPCs are found in foods, tobacco products, cotton wares, and even household dust [3–4].

The OPCs are characterized by neurotoxic and mutagenic activity that causes various disorders of the nervous system, multiple chromosomal aberrations, and carcinogenesis [5–6]. Thus, development of effective methods for detoxification of OPCs present in water sources is quite important. An enzymatic approach to solving this problem looks very attractive.

Organophosphorus hydrolase (OPH; EC 3.1.8.1) is a key enzyme in the hydrolysis of a wide variety of derivatives of phosphoric and alkylphosphonic acids [2]. Application of this enzyme in biotechnological OPC destruction is considered to be the basis for advanced bioremediation processes.

A stable form of OPH for repeated application in OPC hydrolysis is usually obtained via immobilization of the enzyme [7–10], and there have been a number of various approaches to enzyme immobilization in the development of effective biocatalysts with OPH activity suitable for flow systems [11–13]. The use of various amino acid sequences genetically introduced into OPH structure as ligands for enzyme immobilization proved to be an advanced and very effective method for biocatalyst production [14–15]. The use of specific amino acid sequences allows for combining the isolation, purification, and immobilization of the target protein on a carrier into a single step.

In flow systems, the effective activity of an immobilized biocatalyst depends on the catalytic activity of the enzyme as well as the properties of the carrier, such as porosity, reusability, sorption capacity in relation to substrates and products, resistance to mechanical deformation, etc. These aspects were taken into account when a newly immobilized biocatalyst for the treatment of OPC-polluted water was developed [16] using an OPH-active polypeptide with a polyhistidine (polyHis) tag (polyHis-OPH) [17]. Macroporous cryogel produced by cross-linking radical polymerization of acrylamide at subzero temperatures [18–19] and modified by metal-chelating ligands charged with divalent metal ions was used as a carrier.

The freezing of polymer or monomer solutions with the addition of cross-linking agents allows the synthesis of the monolith with three-dimensional structure containing numerous pores of 10–100 μm size [20]. At the same time, the carrier is the integral monolith [19] allowing a notable decrease in diffusional limitations when used in a column reactor. The large pores size allows liquid to flow through the cryogel monolith with high enough flow rates, while sizeable pore surface provides sufficient area for its modification by metal-chelating ligands and further effective enzyme immobilization. The large pores size in the cryogel structure enables direct loading of cell extracts containing target proteins onto the carrier without additional purification steps and with no risk of blocking the pores with cell debris [21–22].

The main catalytic and chemical–physical characteristics of the new immobilized biocatalysts produced from OPH with N- or C-terminal hexahistidine (His_6) tags (N-His_6-OPH, OPH-His_6-C, respectively) [23–24] and N-terminal dodecahistidine (His_{12}) tag (N-His_{12}-OPH) [25] immobilized on polyacrylamide cryogel bearing Cu (II)-loaded iminodiacetate groups (i-N-His_6-OPH, i-OPH-His_6-C, and i-N-His_{12}-OPH, respectively) were studied in this work.

Materials and Methods

Chemicals

The following chemicals were purchased from Sigma (St. Louis, MO, USA): *O*,*O*-diethyl *O*-(4-nitrophenyl) phosphate (paraoxon), *O*,*O*-diethyl *O*-(4-nitrophenyl) phosphorothioate (parathion), *O*,*O*-dimethyl *O*-(4-nitrophenyl) phosphorothioate (methyl parathion), 2-(cyclohexylamino)ethanesulfonic acid (CHES), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), imidazole; isopropyl-β-D-thiogalactoside (IPTG), chicken egg albumin (protein standard for Bradford assay), and cobalt chloride hexahydrate. Middle range molecular weight protein markers for electrophoresis were purchased from Fermentas (Vilnius, Lithuania). Tryptone and yeast extract were bought from Difco (Detroit, MI, USA). The monolithic polyacrylamide cryogels bearing iminodiacetate groups as metal-chelating ligands were provided by Protista Biotechnology AB (Lund, Sweden). All other chemicals were of analytical grade and purchased from Reachim (Moscow, Russia).

Cell Culture

The previously patented expression system [17] was used to obtain the following polypeptides: N-His_6-OPH, OPH-His_6-C, and N-His_{12}-OPH. Overnight cultures of *E. coli* SG13009[pREP4] strain as a producer of N-His_6-OPH or N-His_{12}-OPH and *E. coli* DH5α strain as a producer of OPH-His_6-C were grown at 30 °C in Luria–Bertani medium containing 100 mg/L ampicillin and 25 mg/L kanamycin. The 2% inoculum of each culture was seeded in rich medium containing 12 g/L tryptone, 24 g/L yeast extract, 4 g/L glycerin, 6.95 g/L KH_2PO_4, 12.54 g/L $K_2HPO_4 \times 3H_2O$. Protein expression was induced via addition of IPTG up to 0.25 mM. The cells were incubated for 19 h in thermostatically controlled shaker Adolf Kuhner AG (Basel, Switzerland) with constant agitation (30 °C, 180 rpm) and harvested using Beckman J2-21 centrifuge (Fullerton, CA, USA) at 8,000×*g* for 20 min.

Biocatalyst Production

Cell biomass (10 g) was suspended in 50 mL of 50 mM K-phosphate buffer containing 0.3 M NaCl (PBS; pH 7.5) and homogenized by sonication on ice. The cell debris was separated by centrifugation (30 min, 15,000×*g*). Cell supernatant was loaded onto a Tricorn™ column (5 mL, 1×10 cm, GE Healthcare, Germany) filled with Co^{2+}-IDA-cryoPAAG [22] and equilibrated with PBS buffer, at a flow rate of 6 mL/min using Complete L/S® Variable-Speed Digital Pump System 1 (Cole-Parmer, IL, USA). The system was washed with 6 mL/min PBS buffer containing 10 mM imidazole until OD_{280} became less than 0.01. The wash was repeated with the same buffer without imidazole. Wet immobilized biocatalysts [16] were stored at 8 °C in the 50 mM K-phosphate buffer (pH 7.5).

Determination of Catalytic Characteristics

Accumulation of 4-nitrophenolate anion, an enzymatic hydrolysis product, was monitored (25 °C, 405 nm) using 8300 Uvicord II Spectroscopy System (Pharmacia, Sweden). Aqueous solutions of paraoxon, parathion, and methyl parathion (15 mM) were used as stock solutions to investigate the substrate specificity of the biocatalysts. In all cases, the catalytic reaction was initiated via pumping of substrate-buffer solution through the column filled by immobilized biocatalyst. One unit of enzymatic activity was defined as the quantity of the enzyme necessary to hydrolyze 1 μmol of substrate per 1 min at 25 °C.

The amount of the immobilized target polypeptide was determined after its elution in a linear imidazole gradient (0–500 mM) via both Bradford assay and electrophoretic analysis as described previously [22]. The concentrations of N-His_6-OPH, OPH-His_6-C, and N-His_{12}-OPH in the IDA-cryoPAAG carrier were 1.2, 1.0, and 0.9 mg/mL, respectively.

To determine the effectiveness of catalytic activity of immobilized enzymes, the kinetics of substrate conversion at various initial substrate concentrations at pH 10.5 (50 mM Na-carbonate buffer) were analyzed with the assumption that the columns with immobilized biocatalysts worked as ideal plug-flow reactors.

To derive an equation describing the kinetic properties of plug-flow reactor, the material balance equation for elementary volume dV was used [26]. The equation was integrated over the full reactor volume. At the same time:

$$-d[S] = v\frac{dV}{U} \tag{1}$$

where v—rate of catalytic reaction, $[S]$—substrate concentration, and U—flow rate. Since at a stationary flow $dV=Udt$, the rate of catalytic reaction was expressed as follows:

$$v = \frac{V_{\max}[S]}{K_M + [S]}. \tag{2}$$

The integration of Eq. 1 over full reactor volume gave the next equation:

$$-\int_{[S]_0}^{[S]} \frac{d[S]}{v} = \int_0^V \frac{dV}{U} \tag{3}$$

that was reduced to another one:

$$[S]_0 - [S] - K_M \cdot \ln\frac{[S]}{[S]_0} = \frac{V_{\max}}{U/V}. \tag{4}$$

An Eq. 4 was transformed to more suitable form:

$$\frac{U}{V} \cdot \ln\frac{[S]_0}{[S]} = \frac{V_{\max}}{K_M} - \frac{1}{K_M} \cdot \left([S]_0 - [S]\right) \cdot \frac{U}{V}. \tag{5}$$

Therefore, to determine both K_M and $V_{\max}$, the initial substrate concentration was varied at the constant flow rate (200 mL/h). According to Eq. 5, the experimental data were treated in coordinates $\ln([S]_0/[S])\times U/V$ versus $([S]_0-[S])\times U/V$. The values of catalytic constants were calculated from the coefficients of linear dependences ($y=b-ax$) established from the plots: $K_M=1/a$, $V_{\max}=b/a$. Further, the catalytic constants were used to estimate the effectiveness of activity of the developed immobilized biocatalysts (Table 1). Every

Table 1 The effectiveness of catalytic action of biocatalysts based on the immobilized OPH and its derivatives.

Immobilized enzyme	$V_{max}/(K_m \times [E]_0)$ (M^{-1} s^{-1})			Reference
	Paraoxon	Parathion	Methyl parathion	
i-N-His_6-OPH[a]	$(2.7\pm0.2)\times10^6$	$(2.5\pm0.2)\times10^5$	$(6.9\pm0.2)\times10^4$	This work
i-OPH-His_6-C[a]	$(4.3\pm0.3)\times10^5$	$(1.9\pm0.2)\times10^5$	$(6.0\pm0.3)\times10^4$	
i-N-His_{12}-OPH[a]	$(9.1\pm0.5)\times10^5$	$(2.1\pm0.2)\times10^5$	$(6.2\pm0.3)\times10^4$	
OPH-chitosane	$(15.7\pm0.1)\times10^3$	$(1.9\pm0.3)\times10^3$	n/d	[7]
OPH-nylon	$(3.2\pm0.2)\times10^3$	n/d	n/d	[11]
OPH-trityl agarose	13.5±0.3	n/d	n/d	[12]

n/d the parameter was not determined

[a] The initial concentration of each type of immobilized enzyme was $(1–1.5)\times10^{-8}$ M in reaction mixture

experimental point was the average of three to four measurements, and the background substrate hydrolysis was taken into account as a reference point.

Chemical–Physical Characteristics of Biocatalysts

To investigate the pH influence on the activity of immobilized biocatalysts, various 50 mM buffers with overlapping pH values (K-phosphate, pH 7.0–8.0 and 11.0–12.0; HEPES, pH 7.5–8.5; KCl-NaOH, pH 12.0–13.0; CHES, pH 8.5–10.0; Na-carbonate, pH 9.5–11.0) were used. To determine the temperature profile of the immobilized biocatalyst activity, a reservoir with 50 mM Na-carbonate buffer (pH 10.5) and paraoxon (1 mM) was thermostatted for 10 min at a specific temperature in the range of 20–65 °C. The substrate solution was then pumped through the thermostatted column at the flow rate of 200 mL/h. Activities of the immobilized biocatalysts revealed under various pH and temperature conditions were expressed as a percentage of corresponding maximal values established for each biocatalyst.

To investigate the thermostability of tested samples, the immobilized biocatalysts were exposed to specific temperatures from the range of 20–65 °C for 15 min in 50 mM Na-carbonate buffer (pH 10.5) and then cooled down to room temperature. The residual activity of immobilized biocatalysts was determined spectrophotometrically as mentioned above.

The drying of biocatalysts was carried out at 25 °C under vacuum (100 Pa) using Laborota 4000 (Heidolph Instruments GmbH & Co. KG, Germany), up to the 4–5% residual humidity of samples. The dried biocatalysts were stored at 8 °C in a hermetically sealed container. To conduct the rehydration of dried samples, the dry monolithic immobilized biocatalysts were immersed in the 50 mM K-phosphate buffer (pH 7.5) at 8 °C for 24 h.

Results and Discussion

pH profiles for all immobilized preparations showed significant similarity independent of the length of the tag or the tag attachment site (Fig. 1a). Interestingly, a notable difference in pH-optimum values of the soluble proteins N-His_6-OPH, OPH-His_6-C, and N-His_{12}-OPH

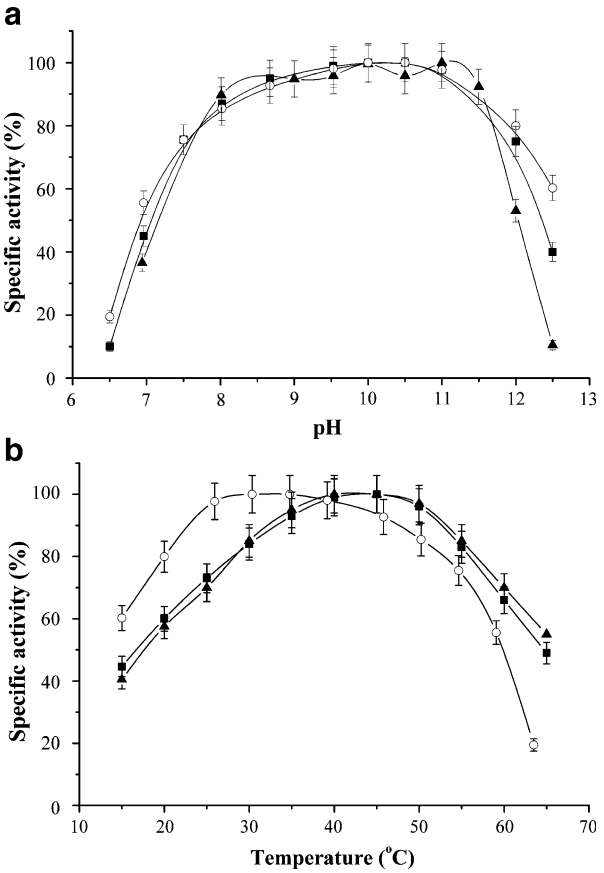

Fig. 1 pH (**a**) and temperature (**b**) profiles of action of immobilized biocatalysts based on the N-His_6-OPH (*closed triangle*), OPH-His_6-C (*closed square*) and N-His_{12}-OPH (*open circle*)

[23–25, 27] became negligible during carrier binding via the polyHis-tags on each of the immobilized biocatalysts. All biocatalysts possessed up to 80% of the maximal activity in a wide pH range from 7.5 to 12. This fact suggests that the biocatalysts can be used effectively for elimination of residual concentrations of OPCs generated by alkaline hydrolysis of pesticides [28] and chemical warfare agents [29] from the media.

The temperature profiles of i-N-His_6-OPH and i-OPH-His_6-C were similar to each other (Fig. 1b), whereas the temperature profile of i-N-His_{12}-OPH was characterized by a weak (about 5 °C) shift toward lower temperatures as compared to the temperature profile of i-OPH-His_6-C. Significant difference between temperature characteristics of immobilized and soluble forms of N-His_{12}-OPH [25] was observed, while there was no significant difference between temperature characteristics of immobilized forms of OPH with differently localized His_6-tags [24, 27].

A high enough thermostability was revealed for all immobilized biocatalysts tested (Fig. 2). A negligible but statistically proven difference was observed in the thermostability of immobilized biocatalysts prepared with i-N-His_6-OPH and i-N-His_{12}-OPH.

Thus, the thermostability and temperature profiles of immobilized biocatalysts activities were very similar to each other as well as to the same characteristics of soluble unmodified OPH, whereas the ranges of pH-optimum activity of immobilized biocatalysts were much wider as compared to soluble OPH [24]. A minor discrepancy between the properties of

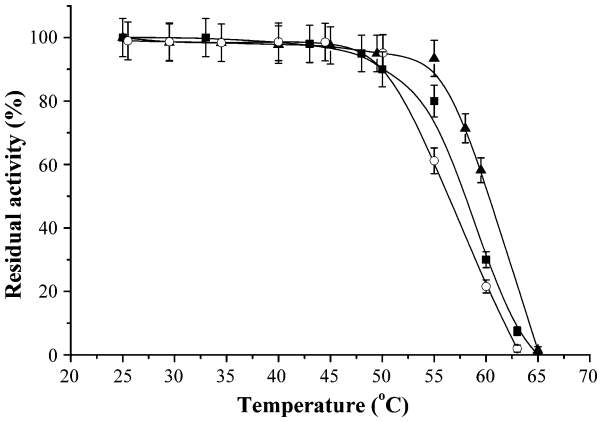

Fig. 2 The residual activity of immobilized biocatalysts based on the N-His_6-OPH (*closed triangle*), OPH-His_6-C (*closed square*), and N-His_{12}-OPH (*open circle*) after their exposition at various temperatures for 15 min (50 mM Na-carbonate buffer, pH 10.5)

immobilized biocatalysts obtained via immobilization of enzymes with polyHis-tags of varying length and localization within the protein molecule was also revealed. Considering the known effects of polyHis-tags on the properties of soluble forms of OPH [27], it is necessary to note that immobilization of polyHis-analogs of OPH via affinity sequences

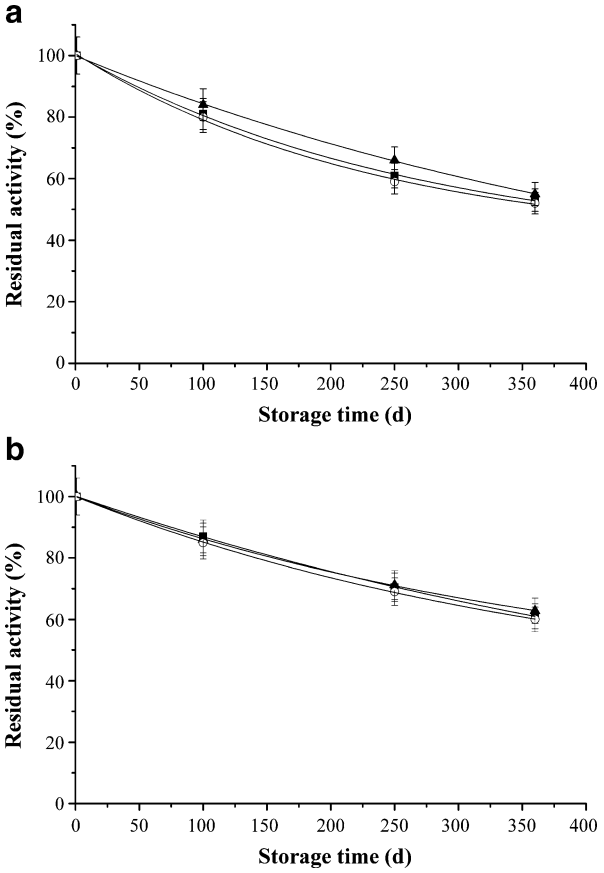

Fig. 3 The residual activity of biocatalysts based on the N-His_6-OPH (*closed triangle*), OPH-His_6-C (*closed square*), and N-His_{12}-OPH (*open circle*) after **a** storage in wet form and **b** storage in dry form

significantly reduces the influence of tag presence on enzyme characteristics, but does not completely eliminate it.

Immobilized biocatalysts developed in this work catalyzed the hydrolysis of various OPCs with high efficiency (Table 1). The specificities of immobilized preparations appeared to be similar to those of soluble forms with substrate preferences as follows: paraoxon > parathion > methyl parathion [23–25, 27]. Thus, substrate specificity of polypeptides was not affected by chosen type of immobilization. The comparison of catalytic efficiency of the biocatalysts obtained via our method to the known analogs produced by other methods [7, 11–12] revealed the obvious advantages of our chosen immobilization technique and its components (Table 1). The comparison of catalytic activities of known immobilized biocatalysts, based on OPH with genetically introduced affinity tags (440 U for OPH/GFP-OPH [14] and 0.0025 U for ELP-OPH [15]) to the same parameters of newly developed preparation (660 U) revealed the latter one's advantage.

It was known that polyacrylamide cryogels can be dried and rehydrated without any changes in their properties [30–32]. This ability of the carrier to be reswollen within seconds while retaining the original pore structure was so attractive that drying–rehydration of the immobilized enzymes was tested. The i-N-His_6-OPH, i-OPH-His_6-C, and i-N-His_{12}-OPH retained 93.8%, 93.3%, and 92% of the original catalytic activity after drying and rehydration, respectively. This property constitutes a unique advantage of these new biocatalysts for OPC hydrolysis, as compared to the already known biocatalysts used for this function.

The developed immobilized biocatalysts demonstrated good long-term operational and storage stability (Fig. 3a, b). The absence of enzyme elution from the carrier was evident following the storage and operation procedures. Mathematic analysis of obtained data revealed that the most appropriate fit was the first order exponential decay function. Therefore, estimated half-lives of biocatalysts for storage in wet and in dry forms were 390–420 and 515–540 days, respectively, at temperatures below 8 °C. There was no significant difference between the storage stability of immobilized enzymes with various poly His-tags.

Conclusion

We have shown that biocatalysts produced by immobilization of polypeptides with OPH-activity and N- or C- terminal polyHis-tags onto supermacroporous cryoPAAG via genetically introduced fusion sequences had more advantages compared to samples derived from other known techniques. The method for the immobilization of polyHis-OPH offered herein can be used to produce various immobilized biocatalysts on the basis of different polyHis-containing enzymes.

The carrier and technique we have described provides significant stabilization of the immobilized enzymes and drying–reswolling of the biocatalysts without significant losses of their initial catalytic activity. This new capacity of biocatalysts to be dried and rehydrated constitutes the essential difference between the biocatalysts studied in this work and other known biocatalysts, which is a result of the combination of the components such as polyHis-OPH and cryoPAAG.

The storage of the biocatalyst in dry form is more convenient than wet storage, since dry materials are more stable to microbial contamination. Since the developed immobilized biocatalysts were characterized by catalytic activity in relation to various substrates and possess long-term storage and operational stability even after the drying and rehydration

procedure, their application for biotechnological treatment of various water sources polluted by OPCs looks quite promising.

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