

Thermally Triggered Purification and Immobilization of Elastin-OPH Fusions

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Abstract: A bifunctional fusion protein consisting of organophosphorus hydrolase (OPH) and elastin-like polypeptide (ELP) was synthesized for the detoxification of organophosphorus compounds. ELPs undergo a reversible phase transition upon an increase in temperature, forming hydrophobic aggregates. This thermally triggered property of phase transition allows for a simple and rapid means of purifying the fusion protein. Over 1,300-fold purification was achieved after only 2 cycles of inverse phase transition. The purified fusion protein showed identical kinetic properties as the native OPH with only a modest 10% increase in K_m and a 5% decrease of K_{cat} . The ability of the ELP domain to form collapsed aggregates also improved long-term stability of the fusion enzyme. Aggregated ELP-OPH retained nearly 100% activity over a span of three weeks. In addition to facilitating purification and stability, the ELP moiety served as a hydrophobic tag for one-step immobilization of the fusion protein onto hydrophobic surfaces. The ELP-OPH was capable of rapidly degrading paraoxon while immobilized. The protein also retained ELP functionality of reversible phase transition thereby allowing for the regeneration of the treated surface. This technology offers a swift and convenient means for purification, immobilization, and regeneration of OPH onto a variety of hydrophobic surfaces by simple environmental triggers. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* 81: 74–79, 2003.

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INTRODUCTION

Their low cost, widespread availability, and efficacy have made organophosphorus (OP) compounds one of the most widely used pesticides in the world. In the United States alone, over 40 million kilograms of OP compounds are applied annually (Mulchandani et al., 1999). Human exposure to these compounds inhibits acetylcholinesterase activity and can lead to acute and chronic symptoms that include headache, nausea, vomiting, and, in more severe

cases, paralysis and even death. Due to the widespread use of OP compounds, there is a need to treat and dispose of residual pesticide, excess stockpiles, and spray down from agricultural equipment.

With the advent of biotechnology in the past decade, more attention is being focused on enzymatic approaches toward the degradation of OP compounds. An enzyme isolated from *Flavobacterium* sp., organophosphorus hydrolase (OPH), can hydrolyze the P–O and P–S bonds used in commercial pesticides as well as P–F and P–CN bonds found in chemical warfare agents (Karns et al., 1987). Although enzymatic degradation is an efficient and environmentally friendly means to degrade OP compounds, large-scale usage is limited by the high cost of purifying large quantities of OPH. Purification of OPH has been facilitated in recent years by recombinant DNA technologies. Affinity tags, such as the cellulose binding domain, and hexahistidine tags have been used for efficient purification (Richins et al., 2000; Wu et al., 1996). Although these methods allow for simple purification of proteins, they are limited by the use of column chromatography that makes them impractical or too costly in large-scale applications.

Another obstacle in using OPH as biocatalysts is the immobilization of these enzymes onto solid supports. Recombinant and native OPH have been immobilized onto various supports such as nylon, porous glass, and silica beads (Caldwell and Raushel, 1991a,b; Munnecke, 1979). Others have immobilized OPH into polyurethane foam for use as sponges for cleanup of toxic spills (Havens and Rase, 1993; LeJeune and Russell, 1996). Unfortunately, adsorption offers poor and nonspecific binding, while covalent modifications to OPH often results in reductions in enzyme activity and kinetic properties (LeJeune and Russell, 1996). Clearly there is a need for a simple means for direct purification and immobilization without sacrificing enzyme efficiency.

Elastin-like polypeptides (ELPs) consist of repeating sequence of Val-Pro-Gly-Xaa-Gly (Xaa being any amino acid except proline). ELPs undergo a reversible inverse-phase transition from soluble protein into insoluble aggregates

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above their transition temperature (T_i) (Urry, 1992). The overall aggregation is driven by hydrophobic interactions of the side chains of the amino acid residues (Urry, 1997). Various fusion proteins with ELPs have been constructed for the purification of proteins. Meyer and Chilkoti have showed that an N-terminal fusion of ELP to a thioredoxin had no adverse affect on the functionality of the enzyme (Meyer and Chilkoti, 1999). More recently, a metal binding domain was shown to be functional as a C-terminal fusion to the ELP moiety for the treatment of contaminated waste streams (Kostal et al., 2001).

The devised ELP-OPH fusion construct offers a very simple and rapid means for OPH purification. In addition, the thermally triggered hydrophobic nature of the ELP moiety enables the self-assembly of OPH fusions onto hydrophobic surfaces or supports. The ability of the ELP-OPH fusions to reversibly self-assemble onto a hydrophobic surface was demonstrated.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Escherichia coli strain XL1-Blue (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (r_k^- , m_k^+), *supE44*, *relA1*, *lac* [F' , *proAB*, *lacI^q*ΔM15, Tn10 (Tet^r)] was used for all cloning steps. Plasmid pET78UF (Kostal et al., 2001) containing 78 VPGVG repeats was used for OPH integration. The *opd* gene (with native signal sequence) was PCR amplified with primers 5'-GGGCCCGGGTGGCGGTGGCGGTGGCATTGAGGGTCGCTCGATCGGCACAGGCGATCG and 3'-GGGGAATTCAAGCTTCCAAAAAAGCCCCGCTCATTAGGCGGGCTGCGTCATGACGCCCGCAAGGTCGGTGACAG from pOP131 (Richins et al., 1997) and partially digested with *Xma*I and *Hind*III to obtain a 1.1-kb fragment. Plasmid pET78UF was digested with same enzymes and ligated with the *opd* gene, resulting in pKEG01. A polyglycine linker was used to provide flexibility between the elastin and the OPH domains.

Production and Purification of Fusion Protein

E. coli strain BLR(DE3) (Novagen, Madison, WI) containing plasmid pKEG01 supplemented with 100 μg/mL ampicillin was inoculated from a single colony and grown at 20°C and 300 rpm in 500 mL of Terrific Broth (Guda et al., 1995). After 72 h, the culture was harvested and resuspended in 30 mL of 50 mM citrate-phosphate buffer, pH 8.0. Cells were then passed through a French Press (SLM Instruments, Inc.) five times at 20,000 psi to disrupt the cells. Cell debris was removed by centrifugation at 20,000g (Beckman). To induce inverse temperature transition, NaCl was added to a final concentration of 2.5 M to the crude extract, which was then incubated at room temperature for 30 min. Centrifuging at 30,000g for 15 min at room temperature resulted in a pellet containing the fusion protein

and a small amount of nonspecifically precipitated proteins. The pellet was retained, and supernatant was discarded. To remove nonessential proteins, the sample was resuspended and incubated for 30 min in ice-cold 50 mM citrate-phosphate buffer. The solution was then centrifuged at 4°C at 30,000g, and the supernatant containing the fusion protein was retained. The temperature transition cycle was then repeated an additional time to increase purity of the protein. Wild-type OPH was also purified for kinetic comparison (Mulchandani et al., 1999). All protein concentrations were estimated with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Inverse Phase Transition

The inverse-phase transition of the ELP-OPH was determined spectrophotometrically in a 96-well microplate reader (Bio-Rad 3550-UV). Two-hundred microliters of 50 mM citrate-phosphate buffer containing 1.0 M NaCl with 200 μM of ELP and ELP-OPH was added to the wells. Temperature was increased (in 2°C increments) from 20 to 40°C and the absorbance at 655 nm was measured.

SDS-PAGE and Western Blotting Analysis

Purified ELP-OPH fusion protein (30 μL) was analyzed on a 10% SDS-PAGE gel (Laemmli, 1970). For immunoblotting, proteins from SDS-PAGE gels were transferred onto a nitrocellulose support. The membrane was incubated for 2 h with anti-OPH primary antiserum (Richins et al., 2000) diluted to 1:3,000. Protein visualization was performed using a Bio-Rad Immun-Blot GAR-AP kit (Bio-Rad).

Kinetics Characterization of ELP-OPH

Assays were conducted in 1.5-mL disposable methacrylate cuvettes (Fisher, Tustin, CA). Paraoxon concentration ranging from 10 to 320 μM were used as a substrate and the formation of *p*-nitrophenol ($\epsilon = 16,500 M^{-1} cm^{-1}$) was measured at 412 nm using a Beckman Model DU 640 spectrophotometer in a 2-min assay at 37°C; 2 μg of both OPH and ELP-OPH suspended in 50 mM citrate-phosphate buffer, pH 8.0, 50 μM CoCl₂ were used in the kinetic assays.

Storage Stability

ELP-OPH (200 units) was stored as aliquots over a period of 3 weeks in the following conditions, all resuspended in 50 mM citrate-phosphate buffer: room temperature, 4°C, -20°C (in 50% glycerol), in the presence of 5% BSA, and as ELP-OPH aggregates. The ELP-OPH aggregates were prepared by precipitation above the transition temperature, followed by centrifugation and then discarding of all supernatant. Each aliquot was resuspended in 50 mM citrate-phosphate buffer before assay. Enzyme activity was measured as previously described (Shimazu et al., 2001).

Thermally Triggered Immobilization of ELP-OPH

For all hydrophobic binding experiments, polystyrene microtiter plates (Corning) were used. In each experiment, 5 mU of enzyme (OPH or ELP-OPH) suspended in 50 mM citrate-phosphate buffer, 2.5 M NaCl, pH 8.0, was added to a single well and incubated at 37°C for 1 h. Plates were washed 3 times by the same buffer at 37°C to minimize any nonspecific binding. The amount of immobilized OPH or ELP-OPH was determined by measuring the rate of paraoxon hydrolysis. Two hundred microliters of 1.0 mM paraoxon in 50 mM citrate-phosphate buffer (pH 8.0), and 10% methanol was added to the wells. The release of *p*-nitrophenol from paraoxon hydrolysis was measured by the microplate reader at 405 nm. To test the reversibility of binding, the wells were washed five times with ice-cold 50 mM citrate-phosphate buffer. Each well was then assayed as described above.

RESULTS AND DISCUSSION

Production and Purification of ELP-OPH

Since the chain length of elastin repeats has been shown to affect the transition properties, initial efforts were focused on achieving a T_i of approximately 37°C at moderate NaCl concentrations, a condition that could be used for practical purification and to provide optimum activity for the immobilized ELP-OPH. Based on our previous findings with elastin biopolymers (Kostal et al., 2001), a bifunctional protein consisting of 78 repeats of (VPGVG), a flexible glycine linker, and the OPH domain was constructed.

Plasmid pKEG01 was introduced into *E. coli* strain BLR(DE3) and maximum OPH activity was achieved by incubating cells in Terrific Broth at 20°C for 72 h. The reversible phase transition properties of the ELP domain allowed for the simple and rapid purification of the ELP-OPH fusion proteins by inverse temperature cycling. Phase transition was induced by adding 2.5 M NaCl to the cell lysate, and aggregation was immediately visible at room temperature. A pellet containing primarily the ELP-OPH fusions was obtained after centrifugation. After solubilization at 4°C, the supernatant was subject to an additional round of inverse temperature cycling resulting in highly purified ELP-OPH. The purity of the protein was determined by a 10% SDS-PAGE gel (Fig. 1A) and only a single protein band was detected. The apparent molecular mass of 80 kDa was slightly higher than the expected size, a phenomenon observed with other elastin-based proteins (McPherson et al., 1996). Presence of the OPH domain was confirmed by Western blot analysis using OPH antisera (Fig. 1B) and by activity assays using paraoxon as a substrate. Approximately 15 mg/L of purified ELP-OPH fusion was obtained using the inverse transition cycling.

The overall efficiency of precipitation and recovery based on the inverse temperature transition is summarized in Table I. Close to 70% of the initial OPH activity was re-

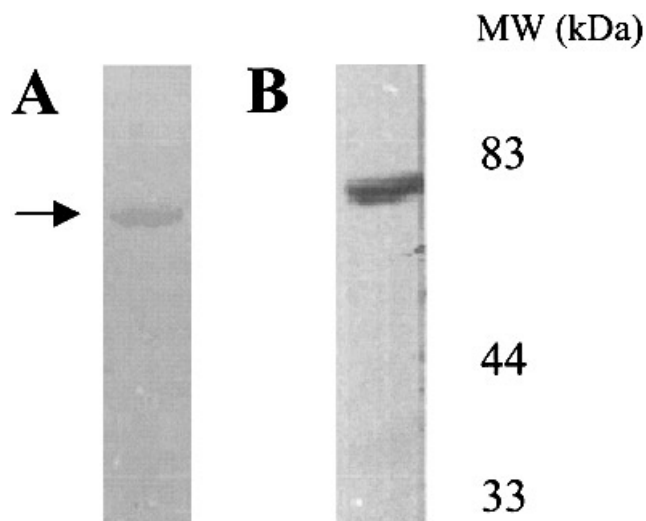


Figure 1. Purification of ELP-OPH fusion by 2 cycles of inverse phase transition. Purity of the fusion proteins was analyzed by (A) 10% SDS-PAGE gel and (B) Western-blot analysis using OPH antisera.

covered after 2 cycles and more than 1,300-fold purification was achieved. These results clearly demonstrated the utility of the ELP tag as a simple and efficient technique for the purification of biocatalysts suitable for organophosphate detoxification and detection.

Inverse Temperature Transition

The functionality of the elastin domain was demonstrated by the ability to obtain highly purified fusion protein with the inverse temperature cycling. The transition profiles of purified ELP-OPH fusions were further characterized to determine whether the T_i was within the desired range for our application. Turbidity measurements were used to determine the onset of folding and aggregation. The value of T_i , which is defined as the temperature at which 50% turbidity occurred, was used to indicate the phase transition properties (McPherson et al., 1996). The transition profiles of both the ELP and the ELP-OPH are shown in Figure 2. The ELP-OPH fusion showed an expected T_i around 37°C at 1.0 M NaCl concentration. The free ELP without the OPH domain showed a slight 2°C shift in the T_i , indicating that the transition property is only minimally affected by fusion to OPH. In contrast, purified OPH exhibited no changes in absorbance with increasing temperature (data not shown), again demonstrating that ELP was solely responsible for the

Table I. Purification of recombinant ELP-OPH fusions.

	Protein (mg)	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$)	Total activity (units)	Yield (%)	Purification factor
Crude extract	10,550	4.65	49,058	100	–
ELP-OPH	8	4212	32,848	67	1,300

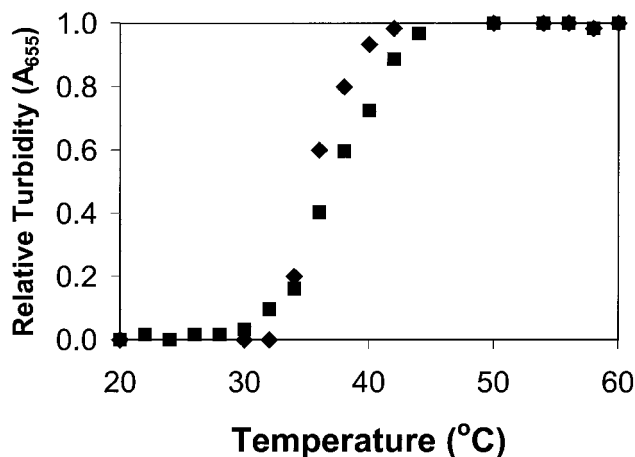


Figure 2. Turbidity profile of (◆) ELP78 and (■) ELP-OPH undergoing inverse temperature transition.

inverse phase transition. The temperature transition was fully reversible above and below the transition temperature.

Characterization of Kinetic Properties

The kinetic characteristics of ELP-OPH were compared to that of the wild-type OPH to determine whether the N-terminal ELP moiety had any detrimental effect on OPH activity. Table II shows a summary of kinetic properties of both the ELP-OPH fusion and wild-type OPH. Paraoxon was used as a substrate and the formation of *p*-nitrophenol was monitored at 412 nm. No significant impact on the catalytic performance was observed; the value of K_m for ELP-OPH was increased by 10%, while K_{cat} was decreased by 5%. These modest effects on OPH activity are in line with results reported for ELP fusions with thioredoxin and tendamistat (Meyer and Chilkoti, 1999). The specific activity of the ELP-OPH fusion of 4,212 U/mg is also significantly higher than the 1,976 U/mg reported for an OPH fusion using a different repetitive peptide [(AG)₃EG]₁₆ (Dong et al., 1994). These results suggest that the ELP domain may be useful as a general tag for the rapid purification of enzymes without any effect on catalytic efficiency.

Stability of ELP-OPH Aggregates

The stability of the ELP-OPH fusion both in the soluble and aggregated forms was determined. Soluble ELP-OPH fusions were stored in 50 mM citrate-phosphate buffer at different temperatures and in the presence or absence of

Table II. Kinetic properties of recombinant ELP-OPH and OPH.

	K_m (mM)	K_{cat} (s ⁻¹)	K_{cat}/K_m
OPH	0.057 ± 0.002	3,214	5.6 × 10 ⁴
ELP-OPH	0.064 ± 0.003	3,055	4.7 × 10 ⁴

BSA as a stabilizer. Similar conditions were used to assess the storage stability of aggregated forms of ELP-OPH fusions.

ELP-OPH stored in buffer showed a significant decrease in activity after 1 week of storage independent of storage temperature (Fig. 3). In the case of storage at room temperature and 4°C, all activity was lost after 5 days. This is in line with soluble OPHs, which have been shown to lose more than 80% activity at 25°C even after 3 days incubation (LeJeune and Russell, 1996). Addition of a stabilizing agent (5% BSA) helped in maintaining close to 70% of the initial activity at 4°C over a period of 21 days (Fig. 3).

Since the ELP inverse transition properties allowed the fusions to be stored as an aggregated pellet, it can be exploited as a possible means for enhancing storage stability. ELP-OPH was aggregated above the transition temperature and all supernatant was decanted from the resulting pellet. The pellet was then stored at 4°C and resolubilized before activity was measured over the course of 21 days. The aggregated ELP-OPH offered superior stability over that of the soluble fusion protein and maintained nearly 100% of activity over 3 weeks (Fig. 3). The long-term storage stability was even better than that of a stabilizing agent BSA. It is possible that the tight packing of the aggregated ELP-OPH into a highly rigid and stable conformation may help stabilize OPH under these conditions. Owing to the high activity, stability, and the ease of purification, these ELP-OPH fusions are expected to be ideal for a wide range of detoxification and monitoring applications.

Reversible Self Assembly onto Hydrophobic Surfaces

Biological molecules can be attached non-covalently to a support surface using the specific adhesion based on hydrophobic interaction (Catimel et al., 1998). This technique is

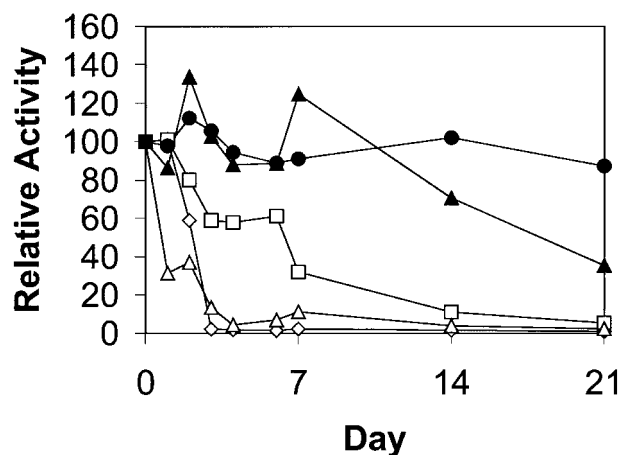


Figure 3. Stability of ELP-OPH stored in 50 mM citrate-phosphate buffer, pH 8.0 at (△) 25°C, (◇) 4°C, (□) -20°C, (▲) 5% BSA, and (●) as aggregated pellet. Normalized activities for paraoxon degradation are shown.

simple and rapid, and it results in an extremely stable surface. Unlike covalent modifications, the surface can be readily reconditioned and reused. Because the ELP can undergo a hydrophilic to hydrophobic phase transition above T_i , this thermally triggered hydrophobic property is ideal in providing selective and reversible self-assembly of enzyme fusions onto a variety of hydrophobic surfaces.

The initial question was whether the aggregated fusions retained OPH functionality. This was investigated by first inducing aggregation by salt addition and comparing the hydrolysis of paraoxon between the aggregated form and the soluble form of the fusion. The aggregated form retained over 90% of OPH activity of the soluble form (results not shown) and therefore aggregation did not significantly affect the efficiency of the enzyme.

To demonstrate the thermally triggered self-assembly of the ELP-OPH fusions onto a hydrophobic surface, hydrophobic polystyrene microtiter plates were used for immobilization and regeneration of the ELP-OPH fusions. A solution consisting of ELP-OPH fusions was added to a single well, and the plate was heated to 37°C for 1 h to induce aggregation and self-assembled adhesion. After the supernatant was removed and the pellet was washed with buffer three times, the amount of immobilized ELP-OPH was assayed by measuring rate of paraoxon hydrolysis at 405 nm. To determine nonspecific binding to the hydrophobic surface, purified OPH was used in similar experiments. As shown in Figure 4, virtually no purified OPH immobilized onto the surface under these conditions. On the other hand, presence of the elastomeric moiety on the ELP-OPH fusions allowed a 20-fold increase in the immobilization efficiency. The self-assembly process is very specific via hydrophobic interactions since no immobilization was ob-

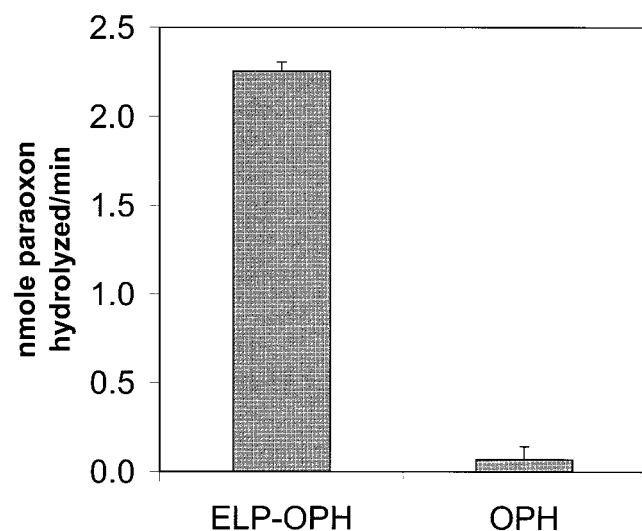


Figure 4. Hydrophobic binding efficiency of ELP-OPH versus OPH. Binding experiments were conducted in polystyrene microtiter plates. Five milliunits of enzyme were added to each well and washed three times with buffer after 1 h of incubation. Degradation of paraoxon was used as a measure for the extent of enzyme binding.

served when the ELP-OPH fusions were incubated at a temperature (4°C) below the T_i (data not shown).

The direct relationship between triggered control of hydrophobicity and immobilization efficiency onto the surface is shown in Figure 5. Increasing salt concentrations while maintaining temperature at 37°C resulted in a greater immobilization efficiency, an observation consistent with the dependency of aggregate sizes on salt concentration (Meyer et al., 2001). Larger size aggregates may become more hydrophobic and promote better self-assembly onto the polystyrene surface.

The practical use of the thermally triggered self-assembly onto hydrophobic surfaces for biocatalysis or biosensor fabrication will depend, to a large extent, on the ability to easily regenerate the surfaces. It was predicted that the nature of the ELP domain to undergo a reversible phase transition between its hydrophilic and hydrophobic states could be exploited for the regeneration of the surfaces. Simple changes in temperature or salt concentration should allow for the transition back into the hydrophilic state, enabling the detachment of the ELP-OPH fusions from the hydrophobic surface.

ELP-OPH was first immobilized under conditions described above and the binding efficiency was determined by immobilized enzyme activity. The well was then washed with ice-cold citrate-phosphate buffer. The activity of the remaining ELP-OPH fusions was determined. As shown in Figure 6, the surface was capable of regeneration by a simple temperature transition. The washing step resulted in the effective stripping of over 70% of the immobilized ELP-OPH fusions. A small fraction of the immobilized fusions remained probably due to very tight adsorption onto the polystyrene surface. This could be minimized by using a different type of hydrophobic surface with lower background adsorption. The possibility to regenerate the surface with fresh ELP-OPH was also investigated. The regener-

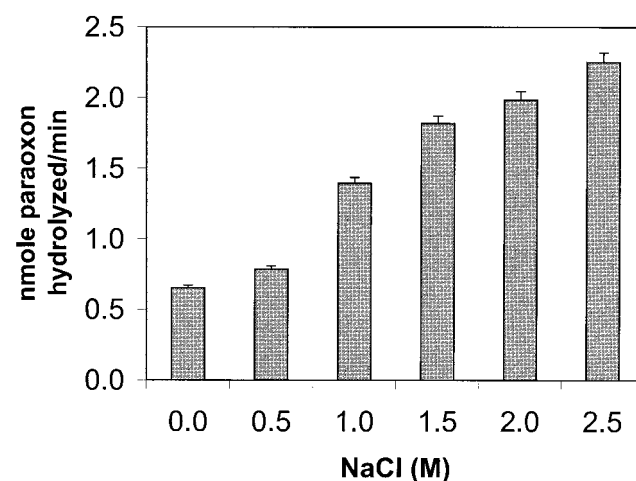


Figure 5. Relationship between increased salt-induced hydrophobic folding of ELP-OPH and hydrophobic binding to the surface. Binding was determined by paraoxon degradation.

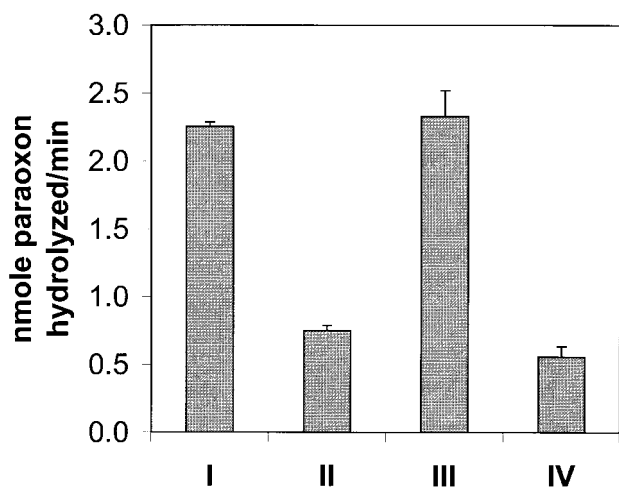


Figure 6. Reversibility of hydrophobic binding by the ELP-OPH fusion. Immobilization of ELP-OPH fusion was monitored as the temperature was cycled between 37 (I, III) and 4°C (II, IV). Amount of ELP-OPH immobilized was determined by paraoxon degradation.

ated surface was subject to a second round of immobilization and washing, and the same efficiencies were obtained (Fig. 6).

CONCLUSIONS

ELP tag offers a simple and rapid means for the purification and immobilization of OPH. Environmentally triggered changes in temperature or salt concentration overcome the complex immobilization and washing steps required with chromatographic purification. The ELP-OPH fusion showed almost identical kinetics when compared to wild-type OPH. The ability of the ELP moiety to form aggregates also dramatically prolonged storage stability. No change in activity was observed over a period of 3 weeks. The temperature triggered hydrophobic nature of the ELP-OPH fusions enabled the simple and reversible immobilization onto hydrophobic surfaces. The ELP-OPH fusion immobilized quickly onto a polystyrene surface and remained bound even after extensive washing. A simple change in temperature or salt concentration triggered the release of fusions from the hydrophobic surface, allowing for regeneration of the surface with fresh ELP-OPH.

The flexibility of the ELP tag and the ability to tune the fusion to a desired aggregation temperature and the reversible binding of the fusion to hydrophobic surfaces make this system ideal in offering simple purification and reversible immobilization. This central framework presented here should be applicable for the immobilization of a wide variety of proteins onto different hydrophobic surfaces and materials.

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