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Table of Contents

I Background

The acute toxicity of organophosphate (OP) compounds in mammals in general, and in humans in particular, is generally ascribed to their irreversible inhibition of acetylcholinesterase (AChE), the enzyme that terminates the action of acetylcholine (ACh) at cholinergic synapses by its rapid hydrolysis (1). Current medical protection against OPs involves anticholinergic drugs to counteract the accumulation of ACh (2), and quaternary oximes to reactivate OP-inhibited AChE (3). Since some OP compounds, such as soman, inhibit AChE and then rapidly "age" (4), carbamate pretreatment was developed (5). Although both oximes and carbamylation regimens are partially effective, they have substantial side effects, and do not provide complete protection (6). This led to an interest in non-pharmacological approaches to protection, such as administration of enzyme scavengers (7), or OP hydrolases (8). The feasibility of using bioscavengers, such as AChE or BChE, has been demonstrated in rodents as well as in non-human primates (7). Animal trials have shown that injection of purified human serum paraoxonase (PON1) can protect against OP toxicity (9), and PON1 knockout mice display heightened sensitivity to chlorpyrifos oxon relative to wild-type mice (9, 10). The hydrolytic capacity of PON1 might render it even more effective relative to the stoichiometric capacity of AChE and BChE. Since wild-type PON does not display high turnover in hydrolysing nerve agents (11), site-directed mutagenesis might also be used to enhance its catalytic activity (12).

ChE scavengers and OP hydrolases also have considerable potential for medical, surgical and skin decontamination, as well as for decontamination of materials, equipment and the environment ((13) and Abstracts International Symposium on Applications of Enzymes in Chemical and Biological Defense, Orlando, FL, May 13-18, 2001). Currently, methods of decontamination use such agents as sodium hydroxide and bleaching compounds, substances which are both harmful and an environmental hazard.

Use of enzymes to treat OP intoxication obviously requires substantial amounts of enzyme protein, and their use as decontaminants even larger amounts, especially for environmental contamination. Moreover, although enzymes of any species origin may serve for decontamination, human enzymes will be required to treat OP intoxication. Large amounts of

AChE and PON are not available from natural sources, and large-scale expression will be required to achieve the necessary amounts. Although a large number of expression systems are currently available, only a few have the capacity to generate large amounts of proteins in a cost-effective manner. Two such systems, both of which have the potential for such cost-effective production, are the *E. coli* prokaryotic system (14), and the *Pichia pastoris* eukaryotic system (15, 16).

E. coli can generate very large amounts of protein, but is not uniformly applicable to eukaryotic proteins because they do not always fold correctly (especially large proteins), nor do they undergo the post-translational modifications conferred by the eukaryotic intracellular environment. A highly relevant case is that of expression of human AChE (hAChE) in *E. coli* (17). These investigators expressed large amounts of hAChE (~50 g) in *E. coli*. But the enzyme was sequestered in inclusion bodies, and refolding yielded pure and catalytically active enzyme in only 1% yield. The pure enzyme possessed the catalytic characteristics of authentic hAChE, but physicochemical characterization showed that, although chemically homogeneous, it displayed a tendency to aggregate not displayed by hAChE expressed in eukaryotic systems (K. Giles, L. Toker, J. L. Sussman & I. Silman, unpublished results). This severely impeded our attempts to obtain crystals from this preparation.

In recent years, *Pichia pastoris* has been used increasingly as a cheap and reliable vehicle for large-scale expression of eukaryotic proteins. Expression at levels of the order of 100 mg/L is very common, and amounts greater that 10 g/L have been achieved (18). A relevant case is the recent work of Rüterjans and colleagues, who expressed the diisopropylfluorophosphatase from *Loligo vulgaris* in *Pichia* at levels of 3-5 g/L (Abstracts International Symposium on Applications of Enzymes in Chemical and Biological Defense, Orlando, FL, May 13-18, 2001, pp 25-27), as compared to their earlier expression of the same enzyme in *E. coli* at levels of ~100 mg/L (19).

Thus, it is our feeling that a two-pronged approach, utilizing the bacterial and the yeast expression systems, should serve as a sensible strategy for exploring scaling up production of scavengers and decontaminases of human origin, with AChE and PON being the enzymes in

question. Obviously, such systems could also serve to provide enzyme for structural studies as well as for large-scale production of engineered mutants.

Under Results, we will describe our progress in the expression of PON1. Attempts to express soluble and catalytically active PON1 in *E. coli* have been unsuccessful due to sequestration of the expressed protein in inclusion bodies (20, 21). Expression in *Baculovirus* ((20) , and Ben-Yohanan, Toker, Sussman & Silman, unpublished results presented in our request for renewal and extension of US Army funding under contract DAMD17-97-2-7022, 31-Aug-2003) and mammalian cells (22, 23) yield only limited amounts of active protein. Our colleague in the Dept. of Biological Chemistry at the Weizmann Institute, Dr. Dan Tawfik, is an expert in using the powerful technique of directed evolution (24, 25) for expressing and modifying enzymes. In particular, he has used this approach to generate an extremely fast *Flavobacterium* phosphotriesterase with a k_{cat} 63-fold higher than that of the already very active wild-type enzyme (26). As will be apparent in the Results section, his initial attempts at expression of PON1 in *E. coli* yielded insoluble and inactive enzyme as previously. Accordingly, directed evolution was combined with another novel technique, namely gene shuffling (27), in which shuffling of homologous DNAs from a family of genes taken from related species is used to accelerate directed evolution.

II Results

Evolution, expression and purification

Dr. Tawfik has utilized the human, mouse, rat and rabbit PON1 genes, which display 79-93% nucleotide sequence identity, to perform gene shuffling. The gene mixtures were digested and shuffled using established protocols (28-30). The expression libraries utilized were cloned into an expression vector in which the PON genes were fused to thioredoxin via a peptide linker containing a His-tag and an S-tag, and expressed in *E. coli*. Constructs in which the wild type PONs were fused to thioredoxin yielded small amounts of soluble enzyme when expressed in *E. coli*. Colonies were screened on agar plates for esterase activity, using naphthyl acetate and Fast Red (31, 32). Colonies that turned red first were picked directly from the original plates. Three

rounds of evolution were performed in this fashion with the 200-500 clones exhibiting the highest esterase activity being selected in each round (Fig. 1).

Single colonies obtained after fresh transformation with plasmid DNA were used to inoculate 5 ml of LB media and the resulting cultures were grown O/N at 30°C (starter). Typically, 1 L of LB media was inoculated 1:200 with O/N starter and grown O/N at 30°C. For purification, the cells were disrupted by sonication, centrifuged and ammonium sulfate added to 50% (w/v). The precipitate was dissolved in 1 mM $CaCl_2$/50 mM Tris, pH 8.0, and dialyzed against the same buffer. Initial purification involved adsorption on an Ni-NTA resin followed by elution with imidazole. Fractions with the highest paraoxonase activity were pooled, dialyzed and chromatographed on a Hitrap Q column (Pharmacia) with a linear NaCl gradient. Fractions with the highest paraoxonase activity were analyzed for purity by SDS/PAGE (Fig. 2), pooled, dialyzed and concentrated to ca. 10 mg/ml for crystallization trials. For purification of preparations lacking the His-tag, the dialyzed ammonium sulfate precipitate was chromatographed on Hitrap Q as above. Active fractions were pooled, dialyzed, loaded onto a ceramic hydroxyapatite column, and eluted with a 0-200 mM potassium phosphate buffer gradient. All purified rePONs could be stored for over 2 months at 4°C without apparent loss in activity.

The first round of evolution already yielded substantial amounts of soluble enzyme, which allowed us to obtain >2mg/L culture of purified PON. The levels of purified enzyme obtained increased to a range of 10-20mg/L of purified enzyme for rounds two and three. No significant difference was observed in kinetic parameters for hydrolysis of either phenyl acetate or paraoxon. Sequences of the selected variants revealed conversion to the rabbit PON1 gene, with the other species making smaller contributions. This was not entirely surprising because the product of expression of the wild-type PON1/thioredoxin fusion construct for the rabbit enzyme itself displayed some limited solubility (Fig. 1A). Preliminary analysis reveals that evolution involves a small number of quite subtle conserved mutations, mostly of one hydrophobic amino acid to another.

Crystallization and X-ray analysis

In the course of the last six months, we have carried out extensive screening of crystallization conditions for 11 different batches of PON1 obtained from the three rounds of directed evolution

outlined above. These preparations differ not only in their primary sequences but also in their attached tags. Furthermore, some preparations were expressed in the presence of selenomethione (Se-Met) to facilitate Multiple Anomalous Dispersion (MAD) (33, 34) data collection at synchrotron sources. The preparations screened are listed in Table 1.

Screening was carried using the standard vapor diffusion hanging-drop method (35) as described previously in our work on acetylcholinesterase (36-38). In parallel, we have taken advantage of the facilities at the newly established Weizmann Structural Proteomics Center (Directed by one of us, *viz* JLS; http://www.weizmann.ac.il/~wspc). The center has a state-of-the-art crystallization robot (Douglas Instruments IMPAX 1-5 Robot) in which sitting drops are covered with a viscous oil and allowed to grow without vapor diffusion (See Fig. 3). Several advantages of this system are:

- The drop size is significantly smaller than in the hanging-drop method, viz ~.25-.5 μL as compared to 1-5 μL, which permits extensive screening with quite small amounts of protein.
- The robot works quickly, i.e. can set up 72 drops in less than 5 min.
- The robot generates highly reproducible drops.
- The robot has also been programmed for refinement of initially promising conditions
- The oil immersion system appears, in our hands, to have a much higher success rate in producing diffracting crystals from a broad variety of proteins than either the hanging-drop method or other conventional techniques.

It should also be noted that our experience shows for many proteins that conditions which yield crystals in hanging drops do not do so under oil and vice versa.

Accordingly, we have utilized both the manual hanging drop technology and the oil immersion robotic technology to screen a wide variety of crystallization conditions. The results of this screen to date are summarized in Table 1. As can be seen, we have examined almost 3,000 conditions using one or the other of the two techniques. Unless otherwise stated, crystallization was at 19° C. Fig. 4v shows that crystals were obtained from several different preparations of PON1, with and without Se-Met in the construct. For four preparations, crystals were obtained which diffracted to 3-4 Å resolution. With the exception of P9T, for which X-ray data were collected at the ESRF synchrotron in Grenoble, all diffraction data reported were obtained 'in house' at the Weizmann Institute.

Although we collected a full native data set at 3-Å resolution for a purified P5 preparation obtained from construct G2-P2E6 (which did not contain Se-Met), we were not able to use molecular replacement to determine its structure, despite the high quality of the data, since there are no structures in the PDB of high enough homology to PON1. All the remaining preps for which diffracting crystals were obtained did not yield data sets of high enough resolution and/or completeness to permit structure solution, including those containing Se-Met. We are currently taking two approaches. One is to persist in screening various preparations, both with and without tags, in order to obtain Se-Met-containing crystals of improved quality. The other is to repeat preparation P5, and to determine the phases by either of two alternative methods, *i.e.* multiple isomorphous replacement (MIR) or multiple anomalous dispersion (MAD). The latter method necessitates data collection at a synchrotron, but should have a much higher possibility of success, especially since the free Cys- of PON1 provides an ideal site of attachment for mercury salts or organomercurials, and Hg is a very suitable anomalous scatterer for MAD data collection.

IV Tables

Purified Prep	Name	Construct	No. hanging drops	No. robot drops	Crystals	Diffraction
P1	G2-P1D6	full length[1]	220		none	
P2	G1-C4	lack 20 N-term AAs[1]	200		none	
P3	G1-P1D6	full length[2]	96		none	
P4	G2-P2D4	full length[1]	96		none	
P5	G2-P2E6	full length[1]	250		Several	3.0Å
P6	G3-P3H8	No tags, full length	264		Several	
P8	G2-P2E6	Se-Met[1]	244		Several	3.8Å
P8R	G2-P2E6	Same repurified	24		Several	
P9	G2-P2E6	Se-Met[1], spontaneous hydrolysis	72 48 at 4°C	48	Several	
P9T	G2-P2E6	Se-Met[1], thrombin hydrolysis	96 48 at 4°C		Several	3.1Å[3]
P10	G2-P2E6	Same as P9T	72	192	Several	4.0Å
P10I	G2-P2E6	Same + inhibitor[4]	127	192	Several	
P11	G2-P2E6	No tags, Se-Met	212	240	Several	

[1] 3 tags – thioredoxin, S-tag, His-tag
[2] 2 tags – S-tag, His-tag
[3] ESRF – diffraction taken at ESRF synchrotron
[4] Inhibitor – 2-hydroxyquinoline

Table 1 – Crystallization trials on purified PON1 preparations obtained from the three rounds of directed evolution.

V Figures

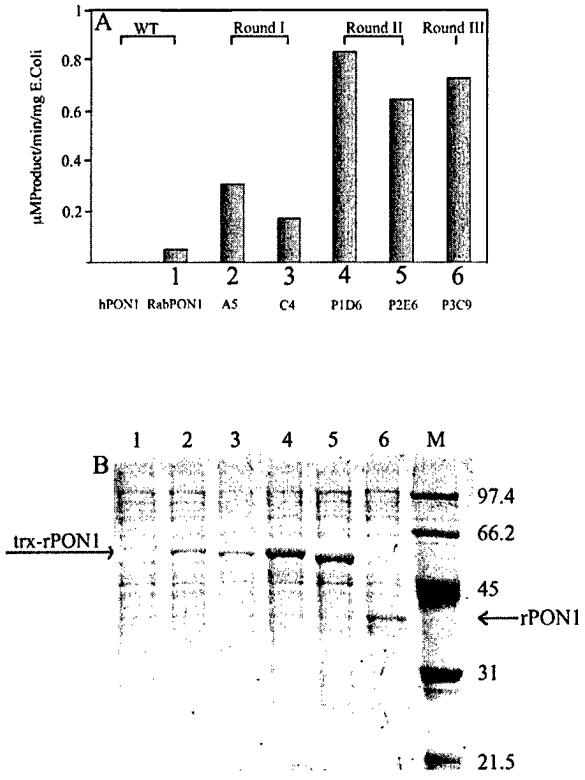

Figure 1. Directed evolution of PON1 for soluble expression in *E. coli*. A. Paraoxonase activity of PON1 variants in the crude *E. coli* lysate was calculated per mg of *E. coli* cells. Activity is shown for the wild-type trx-HuPON1 (nil), for trx-RabPON1, for trx-rePON1 variants A5 and C4 from first round and P1D6 and P2E6 from the second round, and for rePON1 variant P3C9 from the third round of evolution (devoid of tags). B. SDS-PAGE of the crude cell lysates as numbered in panel A above.

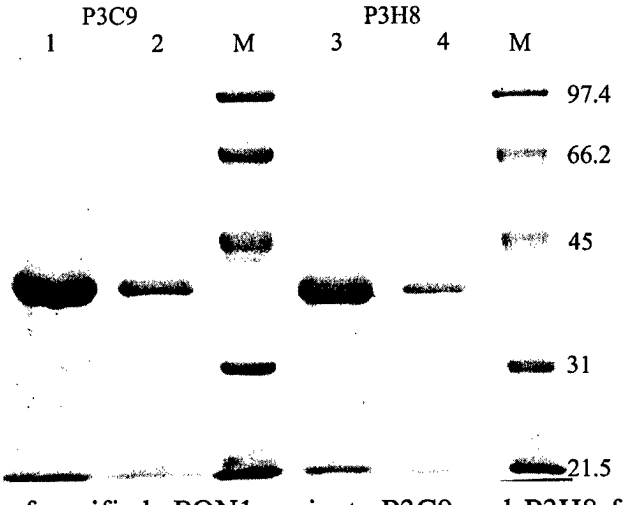

Fig. 2. SDS-PAGE gel of purified rPON1 variants P3C9 and P3H8 from the third round of evolution. The protein is expressed with no tags in a modified pET32b vector. Lanes 1 and 2 are purified P3C9; lanes 3 and 4 are purified P3H8.

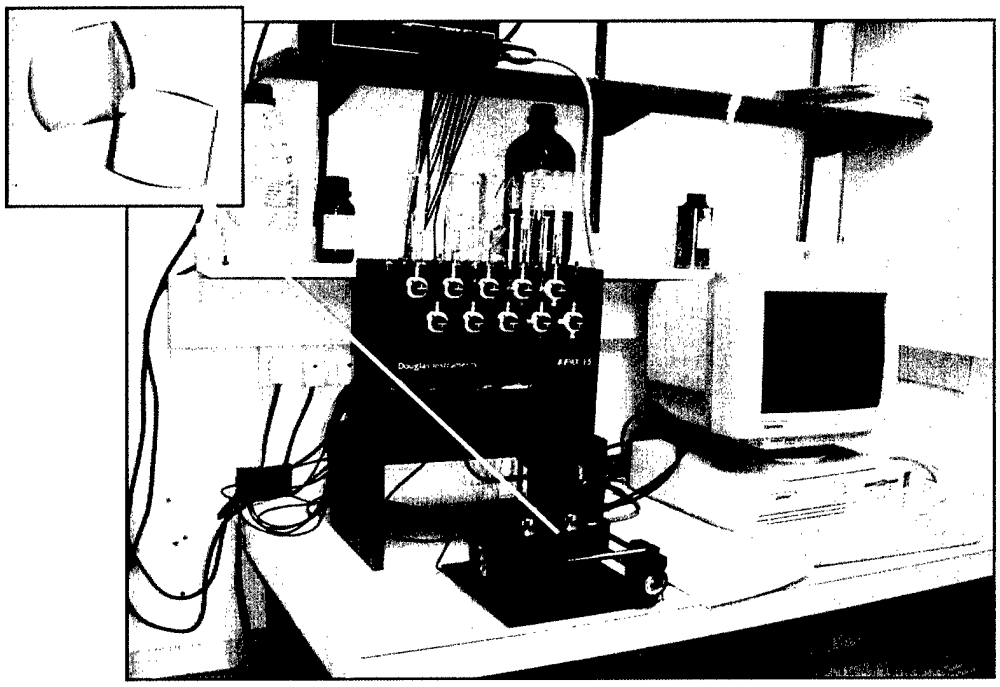

Fig 3. IMPAX 1-5 Crystallization Robot. Insert shows a typical protein crystal grown using it.

Fig 4, Crystals obtained from purified preparations P10 (left) and P11 (right). Details concerning these preparations are presented in Table 1.

V *References*

1. Anticholinesterases: medical applications of neurochemical principles (1995), Millard, C. B. and Broomfield, C. A., *J. Neurochem.*, **64**, 1909-1918.

2. Anticholinesterase agents (1996), Taylor, P., In The Pharmacological Basis of Therapeutics, 9th Ed. (J.G. Hardman, L.E. Limbird, P.B. Molinoff, R.W. Ruddon and A.G. Gilman, Eds.), pp. 161-176, McGraw-Hill, New York.

3. A comparison of the efficacy of HI6 and 2-PAM against soman, tabun, sarin, and VX in the rabbit (1994), Koplovitz, I. and Stewart, J. R., *Toxicol. Lett.*, **70**, 269-279.

4. Crystal structure of "aged" phosphylated *Torpedo* acetylcholinesterase: nerve agent reaction products at the atomic level (1999), Millard, C. B., Kryger, G., Ordentlich, A., Harel, M., Raves, M., Greenblatt, H. M., Segall, Y., Barak, D., Shafferman, A., Silman, I. and Sussman, J. L., *Biochemistry*, **38**, 7032-7039.

5. The protection of primates against soman poisoning by pretreatment with pyridostigmine. (1979), Dirnhuber, P., French, M. C., Green, D. M., Leadbeater, L. and Stratton, J. A., *J. Pharm. Pharmacol.*, **31**, 295-299.

6. Improvements in scavenger protection against organophosphorus agents by modification of cholinesterases (1999), Maxwell, D. M., Saxena, A., Gordon, R. K. and Doctor, B. P., *Chem. Biol. Interactions*, **119-120**, 419-428.

7. Enzymes as pretreatment drugs for organophosphate toxicity (1991), Doctor, B. P., Raveh, L., Wolfe, A. D., Maxwell, D. M. and Ashani, Y., *Neurosci. Biobehav. Rev.*, **15**, 123-128.

8. Rational design of organophosphorus hydrolase for altered substrate specificities (1999), Di Sioudi, B. D., Miller, C. E., Lai, K., Grimsley, J. K. and Wild, J. R., *Chem. Biol. Interactions*, **119-120**, 211-223.

9. The role of paraoxonase (PON1) in the detoxication of organophosphates and its human polymorphism (1999), Costa, L. G., Li, W. F., Richter, R. J., Shih, D. M., Lusis, A. and Furlong, C. E., *Chem. Biol. Interactions*, **119-120**, 429-438.

10. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis (1998), Shih, D. M., Gu, L., Xia, Y. R., Navab, M., Li, W. F., Hama, S., Castellani, L. W., Furlong, C. E., Costa, L. G., Fogelman, A. M. and Lusis, A. J., *Nature*, **394**, 284-287.

11. On the physiological role(s) of the paraoxonases (1999), La Du, B. N., Aviram, M., Billecke, S., Navab, M., Primo-Parmo, S., Sorenson, R. C. and Standiford, T. J., *Chem. Biol. Interactions*, **119-120**, 379-388.

12. Protein engineering of a human enzyme that hydrolyzes V and G nerve agents: design, construction and characterization (1999), Broomfield, C. A., Lockridge, O. and Millard, C. B., *Chem. Biol. Interactions*, **119-120**, 413-418.

13. Organophosphate skin decontamination using immobilized enzymes (1999), Gordon, R. K., Feaster, S. R., Russell, A. J., LeJeune, K. E., Maxwell, D. M., Lenz, D. E., Ross, M. and Doctor, B. P., *Chem. Biol. Interactions*, **119-120**, 463-470.

14. Recombinant protein expression in Escherichia coli (1999), Baneyx, F., *Curr. Opin. Biotechn.*, **10**, 411-421.

15. *Pichia protocols* (1998), Higgins, D. R. and Cregg, J. M., Eds., Humana Press, Totowa.

16. Production of recombinant proteins by methylotrophic yeasts (1997), Hollenberg, C. and Gellissen, G., *Curr. Opin. Biotechn.*, **8**, 554-560.

17. Recombinant human acetylcholinesterase expressed in *Escherichia coli*: refolding, purification and characterization (1995), Fischer, M., Ittah, A., Gorecki, M. and Werber, M. M., *Biotechnol. Appl. Biochem.*, **21**, 295-311.

18. High-level expression of Tetanus toxin fragment c in *Pichia pastoris* strains containing multiple tandem integrations of the gene (1991), Clare, J. J., Rayment, F. B., Ballantine, S. P., Sreekrishna, K. and Romanos, M. A., *Bio/Technology*, **9**, 455-460.

19. High-yield expression, purification, and characterization of the recombinant diisopropylfluorophosphatase from Loligo vulgaris (2001), Hartleib, J. and Rüterjans, H., *Protein Expr. Purif.*, **21**, 210-219.

20. Baculovirus-mediated expression and purification of human serum paraoxonase 1A (2001), Brushia, R. J., Forte, T. M., Oda, M. N., La Du, B. N. and Bielicki, J. K., *J. Lipid Res.*, **42**, 951-958.

21. Oligomeric states of the detergent-solubilized human serum paraoxonase (PON1) (2002), Josse, D., Ebel, C., Stroebel, D., Fontaine, A., Borges, F., Echalier, A., Baud, D., Renault, F., Le Maire, M., Chabrieres, E. and Masson, P., *J. Biol. Chem.*, **277**, 33386-33397.

22. Enzymatically active paraoxonase-1 is located at the external membrane of producing cells and released by a high affinity, saturable, desorption mechanism (2002), Deakin, S., Leviev, I., Gomaraschi, M., Calabresi, L., Franceschini, G. and James, R. W., *J. Biol. Chem.*, **277**, 4301-4308.

23. Identification of residues essential for human paraoxonase (PON1) arylesterase/organophosphatase activities (1999), Josse, D., Xie, W., Renault, F., Rochu, D., Schopfer, L. M., Masson, P. and Lockridge, O., *Biochemistry*, **38**, 2816-2825.

24. Man-made enzymes--from design to in vitro compartmentalisation (2000), Griffiths, A. D. and Tawfik, D. S., *Curr. Opin. Biotechn.*, **11:**, 338-353.

25. In vitro selection and evolution of proteins (2000), Pluckthun, A., Schaffitzel, C., Hanes, J. and Jermutus, L., *Adv. Protein Chem.*, **55**, 367-403.

26. Directed evolution of an extremely fast phosphotriesterase by in vitro compartmentalization (2003), Griffiths, A. D. and Tawfik, D. S., *EMBO J.*, **22**, 24-35.

27. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution (1994), Stemmer, W. P., *Proc. Natl. Acad. Sci. USA*, **91**, 10747-10751.

28. High efficiency family shuffling based on multi-step PCR and in vivo DNA recombination in yeast: statistical and functional analysis of a combinatorial library between human cytochrome P450 1A1 and 1A2 (2000), Abecassis, V., Pompon, D. and Truan, G., *Nucl. Acids Res.*, **28**, E88.

29. DNA shuffling of a family of genes from diverse species accelerates directed evolution (1998), Crameri, A., Raillard, S. A., Bermudez, E. and Stemmer, W. P., *Nature*, **391**, 288-291.

30. Analysis of shuffled gene libraries (2002), Joern, J. M., Meinhold, P. and Arnold, F. H., *J. Mol. Biol.*, **316**, 643-656.

31. A highly fluorescent simultaneous azo dye technique for demonstration of nonspecific alkaline phosphatase activity (1990), Ziomek, C. A., Lepire, M. L. and Torres, I., *J. Histochem. Cytochem.*, **38**, 437-442.

32. Screening, nucleotide sequence, and biochemical characterization of an esterase from Pseudomonas fluorescens with high activity towards lactones (1999), Khalameyzer, V., Fischer, I., Bornscheuer, U. T. and Altenbuchner, J., *Appl. Environ. Microbiol.*, **65**, 477-482.

33. Determination of macromolecular structures from anomalous diffraction of synchrotron radiation (1991), Hendrickson, W. A., *Science*, **254**, 51-58.

34. Phases determination by multiple-wavelength X-ray diffraction: crystal structure of a basic 'blue' copper protein from cucumbers (1988), Guss, J. M., Merritt, E. A., Phizackerley, R. P., Hedman, B., Murata, M., Hodgson, K. O. and Freeman, H. C., *Science*, **241**, 806-811.

35. Crystallization of macromolecules: General principles (1985), McPherson, A., In *Diffraction Methods for Biological Macromolecules* (H.W. Wyckoff, C.H.W. Hirs and S.N. Timasheff, Eds.), **114**, pp. 112-120, Academic Press, New York.

36. Purification and crystallization of a dimeric form of acetylcholinesterase from *Torpedo californica* subsequent to solubilization with phosphatidylinositol-specific phospholipase C

(1988), Sussman, J. L., Harel, M., Frolow, F., Varon, L., Toker, L., Futerman, A. H. and Silman, I., *J. Mol. Biol.*, **203**, 821-823.

37. Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein (1991), Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L. and Silman, I., *Science*, **253**, 872-879.

38. Structures of recombinant native and E202Q mutant human acetylcholinesterase complexed with the snake-venom toxin fasciculin-II (2000), Kryger, G., Harel, M., Giles, K., Toker, L., Velan, B., Lazar, A., Kronman, C., Barak, D., Ariel, N., Shafferman, A., Silman, I. and Sussman, J. L., *Acta Crystallogr. D Biol. Crystallogr.*, **56**, 1385-1394.