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# Development of a Fluorescence-based Biosensor for Coumaphos

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**Abstract:** A screening procedure has been implemented based on the inverse virtual screening technique to identify new bioreceptors for the set-up of pesticide biosensors. Different pesticides of interest were docked on a limited database of proteins with known 3D structures. Some compounds of interest were chosen among the following classes: organophosphates, organo-clorurates, carbamates, anilides, neonicotinoids, aromatic and heterocyclic azoto-organics. Hundreds of molecules were automatically docked on hundreds of structures and results analysed. We identified many interesting binders among which we focused on an *E. coli* helicase that was demonstrated to be able to bind the organophosphate Coumaphos on multiple sites. In addition we report on the purification of the binder and testing with Coumaphos and other compounds in a fluorescence-based assay.

**Keywords:** Helicase, Pesticides, Docking analysis, Bioreceptor, Biosensor, Fluorescence-based assay.

## 1. Introduction

Frequently, in the last decades, the community has been facing the problems arising from the transfer of potentially harmful substances to the environment, altering the ecosystem, and to the human, causing pathological symptoms, and sometimes, death [1]. Recently, the European Union has banned the use of some compounds thought to be carcinogenic [2]. Nevertheless, some of these compounds, as pesticides, are necessary and useful to the human well-being. Pesticides represent an example of toxic molecules intentionally released into the environment with the aim of preventing, destroying and removing pests.

Their diffusion is not limited only to the agricultural sector, but also to houses and public areas of cities, representing a great risk to the environment and human health [3].

Pesticides can be classified on the basis of their function (insecticides, fungicides, acaricides, herbicides, plant growth regulators) and/or their chemical structure in: Organophosphates (OP), Organochlorines (OC), Carbamates (CMs), Neonicotinoids, Anilides, Aromatic and Heterocyclic AzoOrganics. In Table 1 the general chemical structures for the main pesticide families are shown.

Some actions have been undertaken; for example, this century has marked the disappearance in the environment of the highly persistent organochlorine pesticides, gradually replaced by organophosphates and carbamates that, in spite of a lower resistance to degradation, have become the most diffuse neurotoxic chemical compounds. The mechanism of action of these neurotoxic compounds concerns mainly the irreversible inhibition of acetylcholinesterase [4], a key enzyme for the correct activity of the nervous

system. Other pesticides have different mechanisms of action. Because of toxicity to humans, the removal of excess of these compounds from the environment is mandatory, but a preliminary action of detection and monitoring is also required. Over the last decades, different biosensors have emerged as ultra-sensitive and rapid techniques for environmental monitoring and food quality control [2, 5]. These tools have the potential to complement or replace the classical analytical methods by simplifying or eliminating sample preparation protocols and making the testing in the field easier and faster with a significant decrease of the analysis costs. Unfortunately, most of the available biosensors are not su□ciently robust to deal with raw samples and do not o□er adequate selectivity. For these reasons we started a project for the identification and testing of new bioreceptors for different classes of pesticides, in particular for the organophosphate Coumaphos [6].

**Table 1.** Pesticides classification based on chemical structure.

| | |
|---|---|
| ORGANOPHOSPHATES |  |
| ORGANOCLORINATED |  |
| CARBAMATES |  |
| ANILIDES |  |
| NEONICOTINOIDS |  |
| HETEROCYCLIC AZOTORGANICS |  |
| AROMATIC AZOTORGANICS |  |

Coumaphos is one of the main pesticides of concern for the health of bees. It is a widely available organophosphate-based acaricide formulated by Bayer into CheckMite strips and Perizin®. Beekeepers introduce these coumaphos products into colonies to control parasitic varroa mites (*Varroa destructor*). Coumaphos acts systemically when small quantities of it are ingested by bees, with consequent trophic diffusion.

However, most of the acaricide is distributed colony-wide within 3 h through dermal contact among nestmates [7].

In fact, acaricides like Coumaphos are non-pathogenic external agents widely used by beekeepers, so Coumaphos residues are often found in products like bee bread, propolis, wax, comb and royal jelly, the latter being a vital foodstuff for brood and queen rearing [8, 9]. The acute lethal dose (LD50) of Coumaphos for individual bees varies from 3 to 6 μg, with lower doses proving more toxic to older bees [7]. Chronic exposure to Coumaphos can result in reduced foraging activity, affect the size of hypopharyngeal glands, and increase the level of programmed cell death within bee tissues [8]. Although Coumaphos is regarded as being weakly toxic to honey bees, more study is needed to determine whether this acaricide decreases individual bee survival and hence the health of the pollinator workforce. Coumaphos is classified as an extremely hazardous substance in the United States and is subject to strict reporting requirements by facilities which produce, store, or use it in significant quantities [10].

## 2. Identification of Coumaphos as a Binder

### 2.1. Docking Analysis

A screening procedure has been implemented based on the “inverse virtual screening” technique to identify new bioreceptors for the set-up of pesticide biosensors. In particular, a docking analysis was conducted through the use of scripts in the Linux bash shell, via the command line, which invoked the AutoDock Vina software, with a series of parameters defined in a configuration file. Specifically, the script accessed the workbook relating to a specific family of pesticides, and for each file "ligand_name.pdbqt" associated a "protein_name.pdbqt" file, transferring the files generated with the configuration parameters of the number of models to be created to AutoDock Vina software. For each analysis an output of the possible protein-ligand conformations was generated, with the respective binding energies, and a file containing the corresponding ligand structures in space. The output files were always analyzed with the help of scripts in the Linux bash shell, in order to identify the conformations with the greatest affinity for binding.

A cut-off of -6.0 kcal/mol for the binding energies was established with reference to the minimum free energy values obtained from the in silico measurements of EST2 with the pesticides that experimentally showed greater affinity [11].

Different pesticides of interest were docked on a limited database of proteins with known 3D structures. We have set up automatic procedures for docking and analysis of hundreds of molecules on hundreds of structures (Fig. 1) (manuscript in preparation).

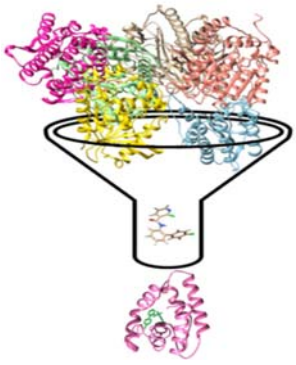
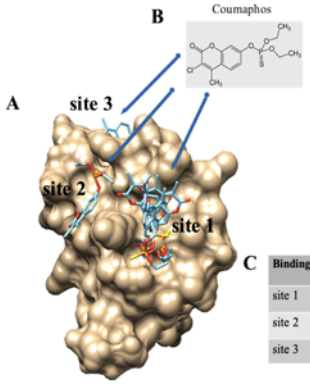


**Fig. 1.** Computational docking analysis "inverse virtual screening" approach.

Coumaphos emerged as a good binder of the protein Helicase RecQ from *E. coli* with a binding energy of about -6.4 kcal/mol.

From the data analysis, the C-terminal domain of helicase RecQ (HRDC domain) (ID PDB: 1WUD) was demonstrated to bind Coumaphos on three sites (Fig. 2 A, B). The energy interaction (-6.4 kcal/mol; site 1 Fig. 2 C) was the best we detected among all the organophosphate compounds.




| Binding sites | Energy interaction (kcal/mol) |
|---|---|
| site 1 | -6,4 |
| site 2 | -6,0 |
| site 3 | -5,8 |

**Fig. 2.** Results of the docking analysis of the HRDC domain with Coumaphos. (A) Coumaphos binding sites on the HRDC domain. (B) Chemical structure of Coumaphos. (C) Table of the interaction energies in kcal/mol of the three binding sites.

## 3. Cloning, Expression, and Purification of the HRDC-RecQ Domain in *E.Coli*

The plasmid vector used for the *in vitro* expression of the HRDC-RecQ domain of *E. coli* is the plasmid pET28b (+), which was provided by Prof. James L. Keck from the Department of Biomolecular Chemistry (BMC) of the University Wisconsin School of Medicine and Public Health - Madison, WI - United States. For cloning, *E. coli* (Invitrogen) competent Top10 cells were used, with high replication efficiency, while for gene expression, the recombinant plasmid was hosted by *E. coli* BL21 (DE3) cells.

### 3.1. Protein Purification

The recombinant protein was purified by affinity chromatography with immobilized metal ions (IMAC, Immobilized Metal Affinity Chromatography) (manuscript in preparation). The highly selective technique is based on the formation of specific bonds between the biological macromolecule of interest and a metal ion (ligand), immobilized on the stationary phase. In the specific case, a nickel resin (Ni-NTA) (Quiagen) was used as the stationary phase, i.e. functionalized with chelating groups loaded with divalent Ni2+ ions, with which the histidines, through their imidazole ring, can establish coordination bonds. The protein was eluted from the column with 200 mM Imidazole, which competes with the protein in binding to the resin.

Purification of the recombinant protein was completed by removing residual impurities by molecular exclusion chromatography. Gel filtration chromatography was conducted using an ÄKTA-FPLC (GE Amersham Pharmacia) system, using a HiLoad ™ 16/600 Superdex ™ 75 PG column (GE Healthcare) with a capacity of 120 mL. The column has a highly cross-linked dextran and agarose matrix. The strong selectivity of dextran and the high chemical and physical stability of the agarose allows high-resolution separations. The chromatography was performed by equilibrating the column in 20 mM Tris-HCl pH 8.0 buffer, the sample was loaded into the same running buffer by applying a flow of 0.5 mL/min, and fractions of 1 ml volume were collected (Fig. 3).

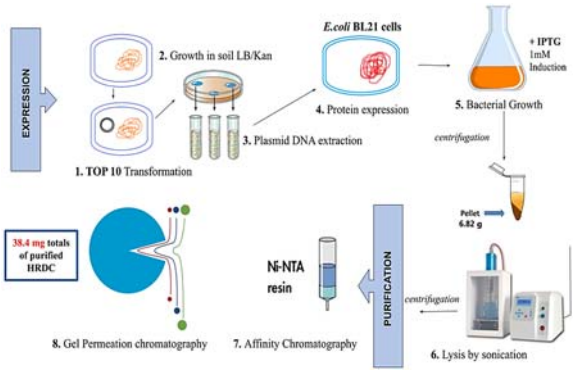



**Fig. 3.** Expression and Purification of the HRDC domain. 1-4. Transformation of E. Coli TOP10 and BL21 cells for cloning and expression of the HRDC-RecQ domain gene, respectively. 5. Induction of protein expression by Isopropyl-β-D-1-thiogalact-pyranoside (IPTG). 6-8. Cell lysis and purification of the protein domain.

## 4. HRDC Domain Labeling and Fluorescence Analysis

The domain sequence was modified by site-directed mutagenesis to change the exposed serine 575 into a cysteine for the binding of the IAEDANS fluorescent probe [12]. The main site-directed mutagenesis technique uses the experimental implantation of Polymerase Chain Reaction (PCR). In fact, oligonucleotides are produced, which act as primers, containing the desired mutation. Therefore, we designed the forward and reverse oligonucleotides in the wild-type sequence of the HRDC domain. The mutant is obtained by replacing a serine residue with a cysteine residue at position 575 of the amino acid sequence. The oligos were ordered and supplied by the Eurofin/Genomics Company and used as primers for PCR. Then, I ran various rounds of PCR based on the characteristics of the Wonder Taq HOT Start (Hot Start Thermostable DNA polymerase) enzyme. Subsequently, I performed the enzymatic digestion with the restriction enzyme Dpn1, specific for

methylated DNA, in order to digest the parental DNA. The mutant sequence was controlled for any additional mutations introduced accidentally.

We expressed and purified the two proteins with the procedure described above.

The wild-type protein was quite soluble and stable. In contrast, the mutant had some stability problems because of a tendency of the protein to aggregate. After appropriate dilution below 0.15 mg/ml, the protein domain was proven to be much more stable.

Therefore we proceeded with the optimization of the IAEDANS binding under the following conditions: 1 mg of the protein was dialyzed with buffer 20 mM Tris/HCl pH 8.0 containing 0.5 mM Tri-n-butyl phosphine (TBP) and incubated with IAEDANS under the conditions indicated in Table 2.

**Table 2.** Optimization of the IAEDANS binding.

| Condition No. | Incubation Time | Temperature | Molar Ratio (protein:probe) | AUF/ug |
|---|---|---|---|---|
| 1. | Over night | 4 °C | 1:50 | |
| 2. | Over night | 4 °C | 1:10 | |
| 3. | 3h | 37 °C | 1:10 | |
| In the presence of Imidazole | 3h | 37 °C | 1:10 | 380 |
| In the absence of Imidazole | 3h | 37 °C | 1:10 | 95 |

Condition No. 3 was found to be the best in terms of specific fluorescence intensity and responsiveness to quenching by using a Jasco FP-8200 spectrofluorometer (Jasco, Tokyo, Japan) at room temperature; in particular, the labeling carried out in the presence of Imidazole (6 mM) gave an increase of specific fluorescence intensity (Fig. 4). The labeled protein was stable for at least 30 days at 4 °C and the specific fluorescence activity was around 380 AUF (Arbitrary Unit of Fluorescence)/µg. The graph shows a biphasic trend of fluorescence quenching for Coumaphos in a semilogarithmic plot suggesting the presence of heterogeneous binding sites in agreement with the docking analysis. The biphasic behavior was independent of the Imidazole thus suggesting different interaction sites.

After optimization, we tested the labeled domain by quenching of fluorescence with Coumaphos and Paraoxon as a control.

As reported in Fig. 5, Coumaphos at 300 nM gave a 30 % quenching of fluorescence whereas under the same conditions paraoxon was almost ineffective.

## 5. Conclusions

Pesticides are among the most common contaminants in the environment. Although their use is necessary to protect crops, their constant presence in the various matrices represents one of the main environmental problems, as they can compromise the health of humans and animals.

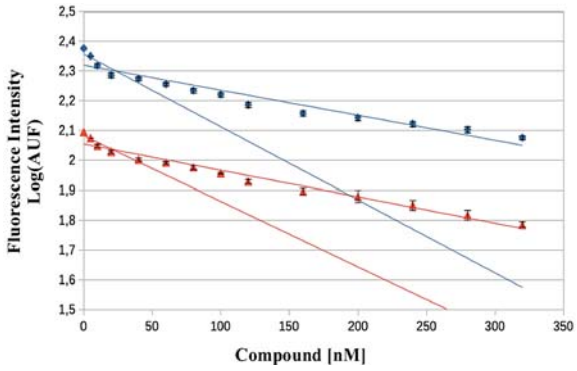



**Fig. 4.** Quenching of fluorescence by Coumaphos of protein labeled with IAEDANS in the presence (blue line) or in the absence (red line) of Imidazole. The fluorescence signal of the labeled protein significantly improved in presence of Imidazole.

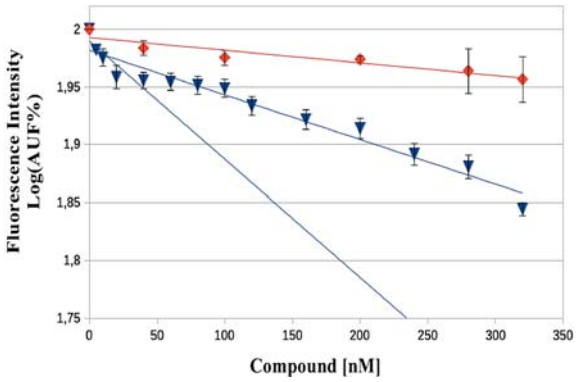



**Fig. 5.** Quenching of fluorescence intensity by Paraoxon (red point) or by Coumaphos (blue point). The interfering effect of acetonitrile (in which pesticides were dissolved) has been subtracted.

To Protect the environment and public health, each year the European Food Safety Association (EFSA) prepares reports to be able to verify the maximum residue limits (MRLs) on food products, intended for human and animal consumption. Due to the presence of various pesticide residues not approved by the European Union and the persistence of their potentially toxic metabolites in the various environmental matrices, EFSA has motivated research on toxicological studies and the development of new detection systems. An example is Coumaphos, a pesticide to which EFSA turned its attention due to the persistence of its metabolites in water and soil. To optimize risk management throughout the country, however, it is necessary to develop systems that are efficient, fast, cheap, and easy to use, such as biosensors. As a consequence, it is necessary to identify new bioreceptors capable of binding potentially toxic substances, belonging to the various pesticide families, which can then be used for the design of a multi-biosensor system. The study of the *in vitro* interactions of the HRDC domain with toxic molecules supports the results obtained from bioinformatics analyses, opening up new perspectives. Although these studies are still preliminary the data

stimulate further analysis, intending to be able to consider this domain in the future as a possible bioreceptor in a biosensory system.

Our results indicate that the methodology used was good for identifying new bioreceptors to be exploited for the development of specific biosensors. Furthermore, the improvement of the specific fluorescence intensity of the labeled protein in the presence of imidazole suggests a conformational change induced by imidazole, which we will have to evaluate. We are currently testing other pesticides to confirm the specificity of the bioreceptor.

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