Research Article
Solid-Phase Extraction of Pesticides by Using Bioinspired Peptide Receptors

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A virtual development of hexapeptide receptors bioinspired by the acetylcholinesterase enzyme active site is proposed. A semicombinatorial approach was applied to generate a virtual hexapeptides library with different affinity properties towards organophosphate and carbamate pesticides. The virtual screening process was addressed to obtain peptides able to separate pesticide subclasses in the experimental work. Three hexapeptides, two generated by molecular modeling and one having a scrambled sequence, were used as selective sorbent materials for pesticides in preanalytical solid-phase extraction (SPE) method. Selective adsorption and cross-reactivity were tested directly on a mix of four pesticides (carbaryl, chlorpyrifos-ethyl, malathion, and thiabendazole) having different structures and physico-chemical properties, at a total concentration of 120 ppb (each pesticide at concentration of 30 ppb). The results were compared to traditional sorbent material such as C-18 and strata-X. Data showed that only one of the hexapeptides virtually designed had significant differences in competitive absorption between aliphatic pesticide malathion, fungicide thiabendazole chosen as negative control, and aromatic pesticides. These results partially supported the simulated strategy.

1. Introduction

Insecticides, fungicides, herbicides, and rodenticides are chemicals classified below the generic term “pesticide” [1]. They have high toxicity and are widely used in agriculture [2]. Despite the large number of new chemicals that have been introduced as pesticides, the contribution to clinical toxicology is dominated by organophosphate and carbamate classes that have been available for many years [3]. The inhibition of the enzyme acetylcholinesterase (AChE) is the main effect of these molecules resulting in the accumulation of endogenous acetylcholine and continual stimulation of the nervous system [4, 5]. These compounds present different structures and physicochemical properties affecting organisms, vegetation, animals, and humans through absorption, inhalation, or ingestion with serious to fatal health hazards [6].

Different approaches have been proposed to produce specific affinity-based stationary phases, developing selective ligands such as molecularly imprinted polymers, aptamers, or peptides proposed as alternative candidates to antibodies which are expensive and challenging to prepare [7–11]. These synthetic molecular traps are able to selectively interact with analytes, similarly to biological receptors but offering some advantages such as low costs, rapid synthesis, and stability.

In this work, the SPE application of bioinspired peptides was supported by virtual docking that is currently an important tool in drug discovery efforts and a subject of important developments over the last decade [12–14]. The simulated conformation of receptors and their interaction with ligands was demonstrated to have potential predictions for subsequent development of experimental methods [10, 11, 15, 16]. This work confirms that molecular modeling method can be
used as convenient tool for the optimization of SPE sorbent materials.

We propose a virtual development of oligopeptide receptors inspired by the AChE active site and used as traps for organophosphate and carbamate pesticides. This approach has already demonstrated its potentiality in previous works [11, 15]. Here a semicombinatorial approach was applied to generate an 800-hexapeptide library with different affinity properties towards carbamate and organophosphate pesticides. The binding affinity calculated by other works [11, 15, 17, 18] suggested the use of these short peptides as semiselective sorbent materials to be used in preanalytical methods such as SPE.

The SPE procedure setup was optimized using directly four pesticides all together in a mix solution and elution. Two fractions were analyzed via an ultrahigh performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) method for simultaneous screening and quantification of pesticides. The pesticides taken as model analytes came from different chemical classes: the insecticides carbaryl (carbamate), chlorpyrifos-ethyl and malathion (organophosphates) and a fungicide (thiabendazole) as negative control, not belonging to organophosphate or carbamate classes.

2. Materials and Methods

2.1. Virtual Screening. All calculations and molecular modeling experiments were performed using a desktop PC with a 3.4 GHz Intel Core i7-2600 processor having 8 GB double data rate 3 RAM with 1333 MHz bus, running Microsoft Windows 7 Professional 64 Bits. All structures were designed and cleaned up with Hyperchem 8.0.5.

OpenEye Scientific Software package under academic license was used. The energy minimization process was carried out using SZYBKI 1.5.1 in its default parameterization [19]. OMEGA 2.4.3 was used to generate conformers to both pesticides and peptides. The program was used with Merck Molecular as the force field [20–22]. The docking software FRED 2.2.5 was used to conduct the virtual screening applying default parameters [23]. VIDA 4.1.1 was used for visualization, postcalculations analysis, and representation [24].

The binding simulation process was scripted, automated, and executed using AutoIT V3: a freeware BASIC-like scripting language.

Flexibility was considered for both pesticides (maximum conformers set to 200) and peptides (10 conformers) and each peptide conformer was treated as a possible receptor, generating a dedicated box and then running a docking process versus all possible conformers of the 22 pesticides selected and the fungicide thiabendazole.

All structures were visualized and checked to guarantee their accuracy in terms of valence, bond order, bond angles, and geometrical arrangement of atoms. Boxes, defining the docking active site, were generated in order to have the peptide inside, then considering the whole receptor structure as possible binding site for ligands. Each box had a size between 5000 Å³ and 7000 Å³. The time required for each peptide conformer, from the initial design to final docking results, was about 3 minutes. Once the run was completed, the binding scores together with a molecule file containing all docked scoring ligands were generated. The binding score average of each peptide receptor was calculated over 10 peptide conformers. The score values were calculated using the consensus of multiple standard scoring functions where lower values represented higher peptide-pesticide affinity. The scoring function in FRED is given by the sum of 5 terms: shape, hydrogen bond, metal, aromatic, and desolvation [23].

2.2. Competitive Absorption Test Using SPE Procedure. Strat-X 33 μm polymeric reversed phase cartridges (30 mg/mL) were from Phenomenex (USA). SPE Isolute column (Empty 1 mL Reservoir) and Isolute C18 resin were from STEP BIO (Italy). Standard solutions of carbaryl, chlorpyrifos-ethyl, malathion, and thiabendazole were purchased from Dr. Ehrenstorfer GmbH (Germany). Stock solutions of 2000 ppm were prepared for each pesticide in acetonitrile with 0.1% of acetic acid and stored at +4°C. Phosphate buffer reagents and methanol of RS-Plus grade were from Sigma Aldrich (Italy). Ultrapure water was produced by a Milli-Q Plus apparatus from Millipore (USF ELGA LabWater, UK). The peptide sequences were synthesized on the Nova Syn TGA resin via C-terminal by EspiKem (Italy) following the procedure reported in other works with a purity >75% and with a peptide substitution level of 0.17 mmol/g [10, 11]. The SPE sorbent materials were

(i) Glu-Trp-Phe-Gln-Pro-Trp (EWFQPW-resin Nova Syn TGA),
(ii) Glu-His-Trp-Trp-Pro-Ser (EHHWWPS-resin Nova Syn TGA),
(iii) Glu-His-Lys-Met-Pro-Ser (EHKMPS-resin Nova Syn TGA),
(iv) the resin Nova Syn TGA used as blank.

The cartridges (volume 1 mL) were packed with 30 mg of modified peptide or blank resin with a teflon frit at the bottom. A second frit was used to cover the resin. After loading, the cartridges were conditioned and equilibrated by washing with ethanol. During this procedure, the cartridges were continuously shaken in order to obtain a homogeneous packing.

Before use, the cartridges were swelled and dried with 10 conditioning and washing cycles to activate the sorbent material. The experimental test was carried out with the following extraction procedure:

(i) Conditioning of the stationary phase with 1 mL of methanol.
(ii) Equilibrating with 1 mL of 10 mM phosphate buffer pH 7.0/methanol solution (80/20).
(iii) Loading 1 mL of analytes mix in phosphate buffer 10 mM pH 7.0/methanol solution (80/20). The loading solution was a mix of the four pesticides selected, carbaryl, chlorpyrifos-ethyl, malathion, and thiabendazole, at the total concentration of 120 ppb. Every analyte was at concentration of 30 ppb.
(iv) Washing with 1 mL of H$_2$O for three times (fractions were collected in a single vial).

(v) Elution with 500 $\mu$L of methanol for two times (fractions were collected in a single vial).

2.3. LC-MS Analysis. Loading, washing, and elution fractions were collected and then analyzed by an UHPLC-HRMS detection system. The UHPLC equipment consisted of a Dionex UltiMate 3000 RS Pump and Autosampler from Thermo Scientific (San Jose, CA, USA). A High resolution Orbitrap System Q Exactive from Thermo Scientific was used for the mass spectrometric detection. The analytes were separated using an Accucore aQ column (100 mm $\times$ 2.1 mm ID) from Thermo Scientific packed with core solid particles of 2.6 $\mu$m. The column temperature was maintained at 40$^\circ$C. The injected sample volume was 10 $\mu$L. The mobile phases were (A) water and (B) methanol both containing 5 mM ammonium acetate and 0.1% of formic acid. The flow rate was 0.4 mL min$^{-1}$. The following gradient elution scheme was used: phase B was constant for 0.5 minutes then was increased to 98% in 10 min. The latter was maintained for 4 min and then switched back to the initial 20% in 0.5 min and kept constant for 4 min. The complete separation of all substances occurred in 9 min. The UHPLC system was connected to the single stage Orbitrap mass spectrometer through a heated electrospray interface (HESI-II), operating in positive ionization mode using the following operation parameters: electrospray voltage: 3.2 kV; sheath gas: 40 arbitrary units; auxiliary gas: 25 arbitrary units; all other source parameters: electrospray voltage: 3.2 kV; sheath gas: 40 arbitrary units; all other source parameters: electrospray voltage: 3.2 kV; sheath gas: 40 arbitrary units; all other source parameters: electrospray voltage: 3.2 kV; sheath gas: 40 arbitrary units; all other source parameters: electrospray voltage: 3.2 kV; sheath gas: 40 arbitrary units. The acquisition was done in Data Dependent Scan mode with a resolving power of 70,000 FWHM for parent and 17,500 FWHM for daughter ions with mass accuracy $\leq$ 2 ppm. Data processing was done by TraceFinder software (version 3.1).

3. Results and Discussion

3.1. Virtual Screening. The 800-hexapeptide library was built using the amino acids participating in the AChE active site, histidine, glutamic acid, and serine highlighted in Figure 1 [25].

This bioinspired approach has been already demonstrated to be successful in a previous work that used the tetrapeptide His-Glu-Pro-Ser [11] and, later, using a hexapeptide obtained by swapping histidine with glutamic acid and adding two glycine amino acids thus achieving a better simulation of the AChE active site geometry [15]. The hexapeptide Glu-His-Gly-Gly-Pro-Ser obtained was found to have an experimental binding constant of $4.1 \times 10^5$ M$^{-1}$ versus the pesticide dichlorvos. Starting from these preliminary findings, here, we attempted to select a peptide with a certain degree of discriminating properties for a class of pesticides.

![Figure 1: AChE molecule (PDB code 1VXO). In red from left to right, Glu 327, His 440, and Ser 200 positions were highlighted.](image)

![Figure 2: Results obtained in the virtual screening step. Average binding score trend of the 800-hexapeptide library versus the 22 pesticide ligands, carbaryl, chlorpyrifos, and malathion was reported. The cut-off line of 10% top ranked binding scores hexapeptides (80 hexapeptides) is also highlighted.](image)

A semicombinatorial approach was applied to generate the hexapeptides library. In order to increase the degrees of freedom by preserving the verisimilar shape of AChE active site, the hexapeptide Glu-His-Gly-Gly-Pro-Ser was used as a backbone to generate the hexapeptide library changing the two glycine, in third and fourth position, with all the 20 natural amino acids. Positions of histidine and glutamic acid were also swapped, obtaining a library of 800 hexapeptides.

The 800-hexapeptide library was then docked towards 5 carbamates (aldicarb, carbaryl, carbofuran, pirimicarb, and propoxur) and 17 organophosphates (azamethiphos, azinphos, chlorpyrifos, dialifos, diazinon, dichlorvos, isoxathion, malathion, methacrifos, methidathion, paraoxon, parathion, phoxim, pirimiphos, quinalphos, thicrofos, and triazophos) belonging to different pesticide classes. The binding score versus the 22 pesticide ligands (average of binding scores) is reported in Figure 2 and compared with the binding scores obtained for the pesticides used in experimental phase (carbaryl, chlorpyrifos, and malathion).

Binding score data were obtained using an average of ten different peptide conformers, a strategy already proved to represent peptides conformational space with fair speed-accuracy ratio [26].
The score values were calculated using the consensus of multiple standard scoring functions, where lower values represented higher peptide-pesticide affinity. In general, the binding score average trend of the 22 pesticides was very similar to that of malathion, while carbaryl and chlorpyrifos exhibited stronger binding score.

The virtual screening process was then addressed to choose peptides able to separate, in the experimental work, organophosphates from carbamate and possibly organophosphate subclasses.

In order to select peptides with better affinity versus pesticides, a cut-off value was established. Only peptides having binding scores within the better 10% (top 80 ranked hexapeptides) average binding scores were considered for further analysis.

The starting point for selecting possible candidates to be used in experimental trials was the structural analysis (Figure 3) looking at the amino acids occurrence in third and fourth position within the 80 peptides selected.

The third and fourth position of the hexapeptides are crucial, since the hexapeptide library was built using as backbone the AChE amino acids. In fact, hexapeptides had in fifth and sixth position, respectively, proline and serine and in first and second position alternatively histidine and glutamic acid. In the top 10% ranked virtual screening hexapeptides, the occurrences of histidine and glutamic acid in first position were, respectively, 38% and 62% confirming that glutamic acid in first position is important to achieve a good affinity for pesticides as reported also in another work [15].

According to Figure 3 data, tryptophan was the most represented in both third and fourth position followed by arginine and phenylalanine. Both glutamic and aspartic acids have also a good occurrence in fourth position. Looking at the binding score obtained for the pesticides selected for experimental part, no significant difference in amino acids distribution was found in the top 10% ranked hexapeptides. Only lysine in third and methionine in fourth position appeared to have a role in binding malathion compared to carbaryl or chlorpyrifos.

To maximize the differences in binding pesticides classes the hexapeptide EHKMPS with lysine in third and methionine in fourth position, was chosen for SPE experimental test along with the hexapeptide EHWWPS that was in virtual screening the best hexapeptide in binding all kind of pesticides.

In Table 1, the binding score and rank position in the 800 library docking runs obtained by the two hexapeptides (EHWWPS and EHKMPS) versus the 22 pesticides average and the analytes used in experimental SPE test are reported.

### 3.2. Experimental Results.
Peptide based SPE columns were assembled using the two selected hexapeptides EHWWPS and EHKMPS and a third one with a scrambled sequence EWFQPW, chosen as structure control. The aromatic properties of the control hexapeptide were reinforced by using phenylalanine that was also well represented in the structural analysis.
Table 1: The binding score and rank position in the 800 library docking runs obtained by the two hexapeptides versus analytes used in experimental SPE test. AV: average; NC: not calculated.

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Molecular structure</th>
<th>EHWPS AV score</th>
<th>EHWPS Rank</th>
<th>EHKMPS AV score</th>
<th>EHKMPS Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>22-pesticide average</td>
<td></td>
<td>−12.02</td>
<td>1</td>
<td>−5.13</td>
<td>59</td>
</tr>
<tr>
<td>Carbaryl</td>
<td><img src="image" alt="Carbaryl structure" /></td>
<td>−13.45</td>
<td>2</td>
<td>−6.97</td>
<td>165</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td><img src="image" alt="Chlorpyrifos structure" /></td>
<td>−14.20</td>
<td>1</td>
<td>−4.33</td>
<td>321</td>
</tr>
<tr>
<td>Malathion</td>
<td><img src="image" alt="Malathion structure" /></td>
<td>−12.67</td>
<td>1</td>
<td>−9.28</td>
<td>4</td>
</tr>
<tr>
<td>Thiabendazole</td>
<td><img src="image" alt="Thiabendazole structure" /></td>
<td>−11.36 NC</td>
<td>NC</td>
<td>−2.75 NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

The experimental measurements were directed to have a better understanding of the peptides cross-reactivity properties for future applications in real samples where different pesticides and interferences can be present. Competitive adsorption of pesticides versus the hexapeptides was tested by loading on the cartridges a solution of the four pesticides at final concentration of 120 ppb, each pesticide at concentration of 30 ppb in a final volume of 1 mL of 10 mM phosphate buffer pH 7.0/methanol (80/20) solution.

The competitive absorption results of the four analytes bound to the three pesticides sorbent materials are reported in Table 2. The binding properties of resin modified with the peptides were also compared to commercial strata-X and C-18 SPE microcolumns using the same extraction protocol.

The experiments were carried out in triplicate using three different cartridges for each peptide and resins obtaining a reproducibility with a CV < 15% except for the blank.

The data reported in Table 2 demonstrated that for the aliphatic organophosphate malathion the recovery was very high, over 95% for all the SPE columns. Intermediate recovery values ranging from 50 to 81% and from 31 to 81% were found for carbaryl and thiabendazole, respectively. The recovery of chlorpyrifos-ethyl was lower than 61% in all cases.

Table 2: Competitive absorption results using SPE cartridges recovery. The values are reported in percentage and were obtained using four-pesticide solution at the total concentration of 120 ppb (each pesticide at concentration of 30 ppb).

<table>
<thead>
<tr>
<th></th>
<th>Carbaryl</th>
<th>Chlorpyrifos</th>
<th>Malathion</th>
<th>Thiabendazole</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>9 ± 1</td>
<td>20 ± 2</td>
<td>22 ± 3</td>
<td>7 ± 2</td>
<td></td>
</tr>
<tr>
<td>EWFQ PW</td>
<td>60 ± 5</td>
<td>57 ± 3</td>
<td>100 ± 11</td>
<td>66 ± 7</td>
<td></td>
</tr>
<tr>
<td>EHWPS</td>
<td>60 ± 4</td>
<td>60 ± 6</td>
<td>100 ± 9</td>
<td>58 ± 6</td>
<td></td>
</tr>
<tr>
<td>EHKMPS</td>
<td>50 ± 5</td>
<td>49 ± 5</td>
<td>96 ± 8</td>
<td>31 ± 4</td>
<td></td>
</tr>
<tr>
<td>Strata-X</td>
<td>81 ± 4</td>
<td>56 ± 3</td>
<td>100 ± 12</td>
<td>81 ± 4</td>
<td></td>
</tr>
<tr>
<td>C-18</td>
<td>74 ± 5</td>
<td>59 ± 3</td>
<td>95 ± 5</td>
<td>72 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

As reported in Table 2, the unmodified resin (blank) exhibited very low retention with all pesticides demonstrating irrelevant unselective binding of analytes on the resin surface. Only the hexapeptide EHKMPS, without aromatic residue in the primary sequence, showed differences in competitive absorption between thiabendazole (31%) and aliphatic organophosphate malathion, keeping also slightly discriminatory properties between aliphatic and aromatic
pesticides. On the other hand, EWFQPW or EHWWPS modified resins had similar retention versus thiabendazole, carbaryl, and chlorpyrifos probably due to the presence of aromatic rings in molecular structure.

Considering the total recoveries, the C-18 resin and the commercial strata-X showed higher retention than peptide-modified resins but they did not present selective properties between pesticides.

On the basis of these results, it can be stated that peptide-modified resins bind less but more selectively with respect to traditional SPE materials. This could depend on the fact that the chosen concentration of the pesticides mix was lower than the saturation level. The best way to verify this is the calculation of the experimental binding constants but this wanders off the focus of this work which was to obtain a rapid overview of the practical use of the peptide-modified resins by using experimental condition as much as possible close to those expected in real samples.

These preliminary tests demonstrated partial matching between experimental and virtual data. Although the virtual process had only a modest ability to distinguish between peptide-pesticide complexes, it can reliably screen out compounds that have grossly wrong electrostatic properties (e.g., aromatic or aliphatic compounds). Even if there is no direct correspondence between experimental and simulated data, the virtual screening information could be always convenient in practical experimental assays providing useful peptide-pesticide complex proprieties and resulting in a relatively small number of database compounds to be tested.

4. Conclusions

This approach partially confirmed the feasibility of bioinspired hexapeptides as sorbent materials with discriminant properties. Only one hexapeptide had a distinguishable interaction with aliphatic pesticide with respect to the fungicide chosen as negative control and aromatic pesticides. Anyway the preliminary studies using SPE procedure were encouraging for the development of new preconcentration systems with partial semiselective recoveries.

The virtual screening process provided useful information about peptide-pesticide properties in order to avoid in future large screening trial and error work. This approach could be considered as a support method to obtain tailor-made reagents with biological-like binding properties.

Highlights

(i) Peptides biospired by acetylcholinesterase were virtually docked versus pesticides.

(ii) From virtual screening, 2 hexapeptides were selected as new sorbent materials.

(iii) Competitive adsorption was tested on a four-pesticide mix solution.

(iv) Performances of peptide sorbent materials were compared to C-18 and strata-X.

Conflict of Interests

All coauthors do not have a direct financial relation with the trademarks mentioned in the paper that might lead to conflict of interests for any of the coauthors.

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