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Monitoring of pesticide amount in fruit and vegetables by a fluorescence-based sensor

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Abstract

Faster, sensitive and real-time methods for detecting organophosphate (OP) pesticides are urged for *in situ* monitoring of these widely spread contaminants. For this reason, several efforts have been addressed for the development of performant biosensors. The thermostable enzyme esterase-2 from *Alicyclobacillus acidocaldarius* (EST2), with a lipase-like Ser-His-Asp catalytic triad with a high affinity to OPs, is a promising candidate as a bioreceptor for biosensor development. Within this EU-FORA fellowship project, two different components of the biosensor were evaluated: (i) the use of the enzymatic bioreceptor in solution or immobilised in a solid membrane; (ii) the measurement of fluorescence quenching by direct measurement of the fluorescence probe intensity signal or by fluorescence resonance energy transfer (FRET) from the tryptophans located in the catalytic site of the enzyme to a binded fluorescence probe. Fluorescence spectroscopy is among the most used techniques in analytical chemistry laboratories, mainly due to its high sensitivity and simplicity. To this aim, the developed IAEDANS-labelled EST2-S35C mutant has been used. Fluorometric measurements with both methods showed linearity with increased EST2-S35C concentrations. No significant interference on FRET measurements was observed due to changes in medium pH or due to the addition of other organic components (glucose, ascorbic acid, yeast extract). Both methods presented similar sensitivity towards detecting OPs, with fluorescence quenching due to the presence of paraoxon at environmentally relevant concentrations from 0.09 μM. The obtained results are of high relevance to further development of biosensors for the pesticide monitoring that: (i) decrease the expenses of the analysis; (ii) simplify the procedures for pesticide detection; (iii) reduce the time of response. Furthermore, the use of biosensors for pesticides real-time and *in situ* detection of pesticides promises to increase the number of samples analysed, providing a larger amount of data for food safety risk assessment.

Keywords: organophosphate pesticides, enzymatic bioreceptor, thermophilic esterase, fluorescence-based methods, environmental monitoring

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1. Introduction

1.1. European Food Risk Assessment Fellowship Programme (EU-FORA)

The present work focusing on the development of fluorescence-based sensors to monitor pesticides in food products was developed in the context of the EFSA EU-FORA fellowship programme. EFSA aims to invest in methodology development, harmonisation activities and capacity building to maintain excellence in performing risk assessment. In this sense, EFSA EU-FORA programme was created to provide practical training to scientists from EU authorities or other Article 36 organisations, increasing and/or updating their knowledge and expertise in food safety risk assessment (Bronzwaer et al., 2016). The fellow was hosted by the Institute of Biochemistry and Cell Biology at the Italian National Research Council (CNR).

1.2. Biosensors as new tools for pesticide monitoring

Synthetic chemical pesticides are essential in modern agriculture and industry practices to increase the overall productivity of crops, cattle farms and other industries by controlling and eradicating pests and related diseases. Nevertheless, the over-application of pesticides in urban and agricultural areas primarily increased pesticides contamination of water bodies and food products, paving the way towards terrestrial and aquatic ecosystems impacts. Thus, pesticides might be present on fruit and vegetables and contaminate their derivates, such as fruit juice and animal meat. Therefore, there is a need for continuous monitoring and report of pesticides residue levels in food. In the European Union, the recent report by EFSA summarised the big picture of pesticides use in 2019, with 96.1% of samples complying with the maximum residue levels (MRLs), but 3.9% exceeded the MRLs, and 2.3% were classified as non-compliant (EFSA, 2021). Although no acute or chronic risk to consumer health through dietary exposure to pesticide residues was estimated, the need to increase and improve the control systems is highlighted (EFSA, 2021). Therefore, establishing a capillary network of sensors to monitor toxic chemicals in food should be considered one of the most critical challenges.

Biosensors appeared as promising tools to develop easy to use, low-cost and rapid-sensing techniques, and increased research has been developed in the last years. Several approaches using enzymes, cell receptors, nanomaterials, among others, have been developed, reaching a good sensitivity, comparable to LS-GC–MS methodology, and reducing the number of false positives (Bhattu et al., 2021; Umapathi et al., 2021).

The research group at the IBBC has been investigating enzymatic-based biosensors for organophosphate pesticides (OP) detection. The carboxylesterase esterase 2 from *Alicyclobacillus acidocaldarius* (EST2) structure has been solved at 2.6 Å resolution (De Simone et al., 2000), allowing the modelling of the structure *in silico* and molecular docking predictions. The EST2 stability, sensitivity and selectivity towards phosphoryl OPs, such as paraoxon and methyl paraoxon, being irreversibly inhibited (Febbraio et al., 2011; Carullo et al., 2015), make it a good candidate to be used as a bioreceptor in biosensors for OPs detection.

Nevertheless, the complexity of food samples can present several challenges to fluorescence-based methods since other organic molecules present in the sample can emit fluorescence at similar wavelengths or interfere with the catalytic site of the enzymatic bioreceptor. Therefore, two fluorescence-based solutions were investigated during this work programme to overcome the mentioned issues: (i) the use of the bioreceptor in liquid solution was evaluated using fluorescence energy transfer (FRET) method; (ii) the immobilisation of the bioreceptor on a solid membrane, and the respective adapter and holder designed and optimised, to be used on different fluorometers, commonly available on research laboratories.

2. Description of work programme

2.1. Aims

The main aim of the present work programme was to develop fast, cheap and user-friendly enzymatic based biosensors to be used in real-time to detect organophosphate pesticides in food samples. Subsequent activities were defined to achieve such goal, starting with the enzyme preparation in free and immobilised form, which was then used as a bioreceptor for two fluorescence-based biosensors. Then, the bioreceptor was validated in the two different operative conditions (in solution and immobilised in a membrane) to detect OP presence in solution, complex mixtures and

fruit washing waters. This work plan allowed the fellow to apply microbiology, molecular biology, biochemistry, biotechnology, and chemical risk assessment knowledge.

2.2. Activities/Methods

2.2.1. Overexpression and purification of EST2-S35C

The fellow performed the over-expression of a mutant of a thermostable carboxylesterase esterase-2 (EST2), to which a serine was replaced with a cysteine near the catalytic site (EST2-S35C), in the mesophilic host *Escherichia coli* strain BL21 (DE3, already available in the laboratory). *E. coli* were grown in an appropriate medium, and the protein overexpression was induced (Carullo et al., 2018). The recovery of the biomass was made by centrifugation, and the protein extract was obtained after a sonication step. The purification of EST2-S35C was achieved by thermoprecipitation followed by ultracentrifugation to remove the host proteins, as described in detail in Rodrigues et al (Rodrigues et al., 2021). Finally, a gel filtration (Se-phadex G-25 column, GE Healthcare Bio-Sciences AB, Sweden) was performed, achieving > 95% purity of the enzyme. The final amount of protein was estimated following the Bradford method (Bradford, 1976).

2.2.2. EST2-S35C labelling

The fellow accomplished the labelling of cysteine in the active site of purified EST2-S35C incubating the protein in the presence of the fluorescent probe 1,5-IAEDANS (5-((((2-iodoacetyl)amino)ethyl) amino) naphthalene-1-sulphonic acid). Different enzyme to probe ratios (1:10 to 1:200) were evaluated to assess the ratio that retrieved the best fluorescence signal. Incubation occurred overnight at room temperature. The excess of the probe was then removed at room temperature in the dark using a QuixSep micro dialyser (Creative Biomart inc., NY, USA). The fluorescence signal intensity was measured on a Jasco FP-8200 (JASCO, Tokyo, Japan) spectrofluorometer.

2.2.3. EST2-S35C as a bioreceptor in FRET-based methods

Fluorescence Resonance Energy Transfer (FRET) method (Medintz and Hildebrandt 2013) was applied, taking advantage of the intrinsic fluorescence of the protein tryptophans (donors) and its transference to the extrinsic IAEDANS (acceptor) (Figure 1). The stability of the labelled EST2-S35C was evaluated under different pH values. Food samples, such as fruit juices, constitute complex matrices rich in various organic molecules that can interfere in the fluorescence measurements by emitting intrinsic fluorescence or interacting with the enzyme's catalytic site. To test the possible interference of such organic molecules, three representative ones were selected (glucose, ascorbic acid, selected yeast extract) and tested in solution with EST2-S35C using FRET. The specificity of EST2-S25C towards phosphoryl OPs was also evaluated using single and complex mixtures of paraoxon and thio-OPs (parathion and diazinon).

2.2.4. EST2-S35C immobilisation and use in a 3D printed system

The fellow accomplished the immobilisation of purified EST2-S35C to a high-quality transfer membrane for biomolecules (Figure 2A,B), a PVDF polyvinylidene difluoride (PVDF) hydrophobic fluoropolymer membrane (pore size 0.20 μm, PORABLOT - MACHEREY-NAGEL GmbH & Co. KG, Germany), as described in detail in Rodrigues et al (Rodrigues et al., 2021). The immobilisation of the enzymatic bioreceptor was optimised. For that, different amounts of protein were spotted and tested. The conditions for adding paraoxon to the membrane (incubation time, spotting *vs* immersion) and the direct fluorescence quenching were evaluated. The vital components required for validating biosensor assays, such as precision, accuracy, linearity, stability of bioreceptor, specificity and sensitivity, were addressed.

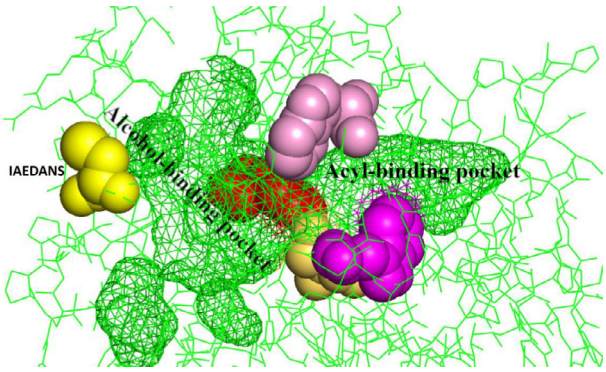

Figure 1: Mesh representation of the cavities inside the EST2-S35C shaping the catalytic site. The acyl- and alcohol-binding pockets and the residues of Cys35 (yellow), Ser155 (orange-yellow), His282 (red), Trp85 (magenta) and Trp 213 (pink) are indicated

2.2.5. Design and optimisation of 3D printed holder for fluorescence measurements

The need to stabilise and uniformise the use of the bioreceptor immobilised in the membrane during the spectrofluorimetric procedures led the fellow to design and develop a 3D printed membrane support (8 × 3 × 36 mm, Figure 2C) and an adapter for solid sample measurement (12 × 12 × 45 mm, Figure 2D), using the software SketchUp Make 2017 (Trimble Inc., USA). Slicing was done using the software Ultimate Cura v4.9.1.1 (Ultimaker B.V., Netherlands), and printing parameters were established on Labslicer 3D Slicing Software for Windows (Labists, Hongyu Zhineng Technology Co., Ltd., China). The bioplastic black polylactic acid (PLA) 1.75-mm filament was used to avoid light refraction. The components were printed in the High Precision Mini 3D printer, X1 entry-level 3D printer DIY kit, from Labists, as detailed in Rodrigues et al (Rodrigues et al., 2021). Different models for the adapter and membrane holder were printed, testing for the optimum angle of light incidence and reflection. The digital design files are available under a creative commons licence, free of charge (https://www.thingiverse.com/febbraio-research-group/designs).

The accuracy and versatility of the 3D adapter were evaluated in different equipment, in collaboration with a group from the Department of Chemistry, University of Naples. Furthermore, the critical factors required for validating biosensor assays, including precision, accuracy, linearity and sensitivity, were again addressed.

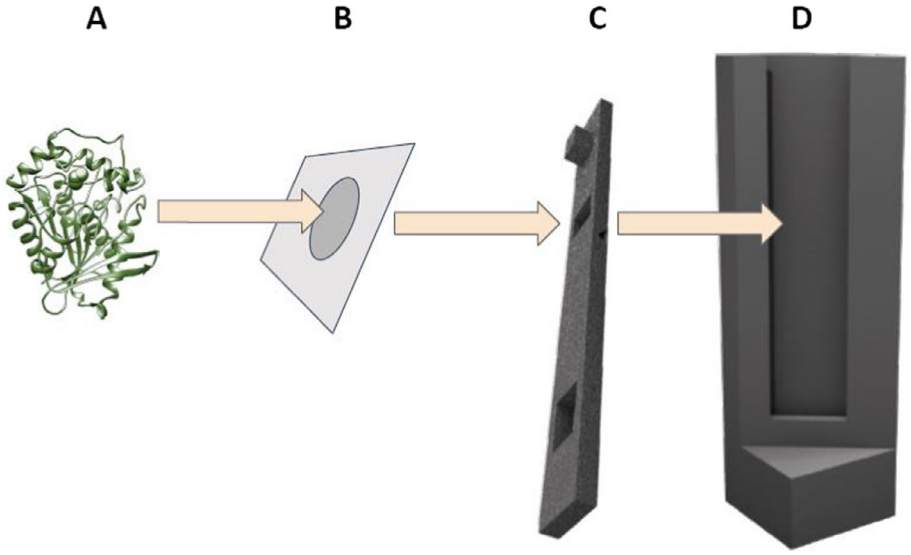

Figure 2: Schematic representation of the assemblage of the immobilised EST2-S35C (A) on the membrane (B) and the 3D printed membrane support (C) and, later, into the 3D printed adapter (D). The icons are not to scale

Part 6. Validation of the bioreceptor using fruit skin

The validation of the bioreceptor in the operative conditions for the detection of OPs concentration in fruit skin was performed. Different conditions were explored, the bioreceptor response on pretreated samples with increasing concentration of OP was evaluated, defining the range of linearity. Both developed methods were evaluated regarding the specificity and reduction of other organic molecules interference in the measurements of fluorescence quenching and consequent OPs detection.

3. Conclusions

3.1. EST2-S35C as a bioreceptor in FRET-based methods

The use of the FRET approach improved the efficiency of paraoxon detection in solution, with a limit of detection (LOD) of 0.09 μM obtained for paraoxon. Furthermore, it was observed that the addition of the fluorescent probes, such as IAEDANS, near the alcohol binding site, does not affect the enzyme's binding and function in the acyl binding site.

Several factors need to be considered when developing a biosensor for field application since the complexity of real samples increases. Therefore, the stability of labelled EST2-S35C at different pH values was tested in the range from 7.0 to 8.5, covering necessary liquid samples, such as drinking water or juice fruits (pH 7.0). The complexity of real samples is also illustrated by complex mixtures of different molecules, such as fruit juices that can contain sugars, vitamins and other proteins. Good stability of the bioreceptor using FRET measurements was also obtained when glucose, ascorbic acid or selected yeast extract were added to the solution.

In summary, two main goals were reached:

i) eliminate the interference in the fluorescence measurements of other organic molecules present in complex solutions;
ii) increase the protein specificity, as the use of FRET measurements allowed to observe changes affecting only the acyl-binding pocket, strongly reducing possible obstructions from nonspecific interactions at the alcohol-binding site.

3.2. EST2-S35C immobilisation and use in a 3D printed system

Similarly to the previous method, a linear relationship between the amount of EST2-S35C and the intensity of the measured fluorescence signal was obtained. A linear increase of fluorescence quenching would be expected until a 1:1 stoichiometric ratio of inhibitor/enzyme. In accordance, the 30 μM EST2-charged membranes presented a dynamic linear range up to 30 μM of paraoxon. A plateau was observed at 60 μM of paraoxon for the 90 μM EST2-charged membranes. So, the amount of bioreceptor should be higher than the expected OPs concentrations to be possible to determine the pesticide concentration in the sample by plotting the fluorescence quenching results, thus, avoiding some altered behaviour due to undesired fluorescence interference from adsorbed paraoxon at very high concentrations.

Regarding the versatility of the 3D printed system, the measurements performed at the different spectrofluorometers with the respective adapted support for the membrane gave comparable results, at similar wavelength (461–462 nm) and fluorescence intensity decrease with increasing paraoxon amount. Thus, these low-cost and straightforward designed 3D printed adapter and membrane supports can be used for fluorescence measurements and applied to detect chemicals in biosensing devices. Furthermore, the adapter's performance was very good, reaching similar results for sensitivity and replicability as a robotic workstation (Cetrangolo et al., 2019, 2020).

In conclusion, the designed 3D adapter has the fluorescence-cuvette dimension and the right angle to be used with minor light scattering. In addition, the developed membrane supports can be easily switched to match the specific heights of the instruments from different manufacturers, being quickly adjusted in different spectrofluorometers. Thus, this work provided an easy and accessible strategy to use low-cost tools for using ad hoc laboratory materials, overcoming the cost of commercial accessories. In fact, 3D printing would be an excellent ally to produce new accessories in different scientific fields, given the opportunity to improve existing methodologies or test new designs and methodologies.

In conclusion, the great advantage of using a 3D adapter for biosensing devices lies in the possibility of using immobilised enzymes on solid membranes, or direct measurement of optically active thin layers. Furthermore, the 3D support with the immobilised enzyme allowed the washing of the

membrane to remove the unreacted substances, including other organic compounds such as pigments, or amino acids, improving the fluorescence measurements and decreasing the background noise.

3.3. Additional scientific activities

In order to maximise knowledge transfer, during the fellowship programme, the fellow took part in internal data clubs, weekly as a participant and twice as a speaker. In addition, the fellow took part in the working meetings with groups at University Federico II (Naples, Italy) and Zewail City for Science and Technology (Cairo, Egypt) to plan collaborations and perform lab work.

The fellow participated in the EUROTOX 2021 meeting as presenting author of the poster presentation 'A FRET approach to detect organophosphate pesticides using a fluorescent biosensor' and as co-author of the poster presentation 'Direct detection of organophosphate pesticides in water by a fluorescence-based biosensor', held on a virtual platform from 27 September to 1 October 2021 (https://www.eurotox2021.com/abstracts/). Two abstracts, one as presenting author and the second as co-author ('Detection of neurotoxic compounds at environmentally relevant concentrations by using a fluorescence-based biosensing device' and 'Application of a fluorescence-based biosensing device for the detection of organophosphate pesticides in water samples') have been accepted as a poster presentation at ONE – Health, Environment, Society – Conference, 21–24 June 2022.

The data resulting from activities 2.2.4 and 2.2.5 are accepted for publication (Rodrigues et al., 2021). In addition, the data resulting from activity 2.2.3 are submitted to another peer-reviewed journal (Sensors), and other publications are planned.

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Abbreviations

EST2	esterase 2 from *Alicyclobacillus acidocaldarius*
EST2-S35C	EST2 where the serine 35 has been replaced by a cysteine residue
EU-FORA	European Food Risk Assessment Fellowship Programme
EUROTOX	Congress of the European Societies of Toxicology
FRET	fluorescence resonance energy transfer
LOD	limit of detection
MRL	maximum residue level