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Vmh2 hydrophobin as a tool for the development of "self-immobilizing" enzymes for biosensing[†]

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Running title: Surface active proteins fused to enzymes

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Abstract

Self-assembling proteins forming amyloid fibrils are promising candidates for the fabrication of biomaterials, due to the chemical and mechanical stability of their structures. Among potential applications, their use as platforms for enzyme immobilization is rapidly gathering attention. In this work we demonstrate that the production of the enzyme glutathione-S-transferase (GST) fused to the class I hydrophobin Vmh2 from *Pleurotus ostreatus* represents an invaluable tool for the development of self-immobilizing enzymes useful for high throughput analyses. The proposed immobilization strategy is versatile since it can be applied, in principle, to every recombinant protein able to refold from *E. coli* inclusion bodies. A GST based biosensor has been developed to quantify toxic compounds, such as the pesticides molinate and captan, in aqueous environmental samples. The main advantages of this sensor include simplicity and speed of preparation, high sensitivity, reusability and accuracy.

Keywords: Amyloid fibrils• biosensors• immobilization• self-assembling proteins

Introduction

In the last few years the demand for valuable scaffolds for the development of promising biomaterials has been rapidly increasing. Biological molecules fully integrated with micro or nano technological platforms are useful tools for the fabrication of hybrid devices. The performances of biomaterials strongly depend on the bio-functionalization of the device surfaces. The design and realization of bio/non-bio interfaces with specific properties, such as chemical stability, wettability, and the immobilization ability of other biomolecules, are key features but at the same time often bottlenecks in bio-device development and optimization (De Stefano et al., 2011). Enzyme immobilization is the basis for the development of various biotechnology tools with applications in industrial biotransformations, diagnostics, and biosensing, and its realization has to address issues such as the loss of the enzyme and the maintenance of its stability. Immobilization techniques in relation to this aim include physical entrapment, microencapsulation, adsorption, and covalent binding, etc. The exploitation of assembled protein layers as platforms for enzyme immobilization is rapidly gathering attention and, in particular, self-assembling amyloid protein fibrils are promising candidates for the fabrication of functional materials and biosensors. Amyloid fibrils self-assemble from soluble precursors and, once formed, are chemically and physically stable, with a uniform architecture (Dehsorkhi et al., 2014; Humenik and Scheibel, 2014). They share a cross–β structure motif and common properties such as a high resistance to protease digestion and denaturation (Fitzpatrick et al., 2013).

Hydrophobins are amphiphilic proteins produced by filamentous fungi, able to spontaneously assemble into amphipathic layers at hydrophobic–hydrophilic interfaces (Linder, 2009). They are very active surface proteins and play a role as a coating/protective agent. All hydrophobins are small proteins with a typical pattern of four disulphide bridges (Khalesi et al., 2015). They are split into two groups, class I and class II, on the basis of some structural differences and properties of the layers they form. Fibrillar structures formed by class I hydrophobins, known as rodlets, share many structural analogies with amyloid fibrils, and are extremely stable, being solubilized only after harsh

acid treatments (Kwan et al., 2006). On the other hand, layers formed by Class II hydrophobins can be easily dissolved in solvents (e.g. 60% ethanol). Hydrophobins have been shown to efficiently adhere to different hydrophobic surfaces, such as teflon (Askolin et al., 2006), polystyrene (Wang et al., 2010), silicon (De Stefano et al., 2007; De Stefano et al., 2009) and graphene (Gravagnuolo et al., 2015).

Hydrophobin assemblies are versatile and simple platforms for the immobilization of proteins on diverse substrates. Two main routes have been taken to immobilize proteins on hydrophobin layers, i.e. deposition on preformed self-assembled films (De Stefano et al., 2009; Longobardi et al., 2015; Wang et al., 2010; Zhao et al., 2007), and genetic fusion to the self-assembling protein (Linder et al., 2002; Takatsuji et al., 2013). This latter strategy combines the ease of obtaining film of the self-assembling protein with the strength of the covalent immobilization, avoiding the need for the chemical reactions used for cross-linking (Pilkington et al., 2010). The binding of proteins on hydrophobin layers depends on the characteristics of the adhering protein and has to be optimized, for instance by varying the pH and ionic strength (Wang et al., 2010), while no optimization is needed to immobilize the protein fused to the hydrophobin. Moreover, when fused, a protein can be anchored onto a surface with a controlled orientation, thus forming a homogeneous biological layer on biodevices (Steen Redeker et al., 2013). However, only class II hydrophobins have so far been fused to other proteins (Linder et al., 2002; Takatsuji et al., 2013), meaning that the formed layer can be easily removed.

The class I hydrophobin Vmh2 from the basidiomycete fungus *Pleurotus ostreatus* (Armenante et al., 2010) is able to spontaneously form a stable and homogeneous layer on hydrophilic or hydrophobic surfaces, changing their wettability (De Stefano et al., 2007). Protein immobilization on the Vmh2 layer has already been assessed (De Stefano et al., 2009; Longobardi et al., 2015). Furthermore, a system for recombinant production in *Escherichia coli* of Vmh2 fused to the enzyme glutathione-S-transferase (GST, EC. 2.5.1.18) has been established with the aim of overproducing the Vmh2 hydrophobin and increasing its solubility (Longobardi et al., 2012).

In this work, the fusion protein Vmh2-GST has been produced and efficiently immobilized via hydrophobin film on the polystyrene surface of multi-well plates. The immobilized enzyme was stable and more active than its soluble counterpart. It was used to develop a fast and easy-to-use biosensor for high throughput analysis, such as the monitoring of xenobiotic compounds. The detection mechanism is based on the inhibition process promoted by the pesticides captan (Choi et al., 2003; Singh et al., 2009) and molinate (Heath et al., 1997) on the GST enzyme. The procedure developed is versatile and straightforward, and thus it is expected to allow the recovery and immobilization of heterologously expressed Vmh2-protein chimera. Since the fusion protein is built by means of an enzyme fused to a self-assembling element, it can be considered as a "self-immobilizing" enzyme.

Materials and Methods

Expression and purification of the recombinant protein

Briefly, the recombinant plasmid pETM 30-GST-Vmh2 (Longobardi et al., 2012) was transformed to *E. coli* BL21 (DE3) and cultured for 16 h in LB broth (Difco) with 100 μg mL^{-1} kanamycin (Sigma-Aldrich). The plasmid allowed the production of Vmh2 fused to *Schistosoma japonicum* GST. The cultures were diluted 1:100 with LB and further incubated at 37 °C. When the culture medium reached an OD_{600} value of 0.6, IPTG (isopropyl-β-D-thiogalacto-pyranoside) (Sigma-Aldrich) at a final concentration of 1 mM was added to induce the recombinant protein expression. After 3 h of induction, the cells were collected by centrifugation at 6,000 rpm at 4°C for 10 min, re-suspended in 100 mM Tris-HCl, 10 mM EDTA, 2 M urea and 2% Triton X-100 pH 8.0 (lysis buffer) and destroyed by French Press.

The inclusion bodies were collected by centrifugation at 10,000 rpm at 4°C for 30 min and then washed four times with lysis buffer to remove any contamination. Next,, the inclusion bodies were dissolved in 40 ml of 100 mM Tris-HCl pH 8 and 10 mM EDTA containing 8 M urea and

incubated for 3 hours at 37°C. The supernatant was collected after centrifugation at 10,000 rpm at 4°C for 10 min.

The recombinant protein obtained from the inclusion bodies was refolded by urea dilution up to a concentration of 0.2 M in 50 mM Tris-HCl buffer, pH 8.0. The sample was then concentrated using a Centricon Centrifugal Filter Unit (Millipore Centricon Ultracel YM-10 membrane). The purity of the recombinant fusion protein was verified by SDS–PAGE (15% acrylamide), and stained with Coomassie brilliant blue R-250. The protein concentration was evaluated using the Pierce 660 nm protein assay kit (Thermo Scientific) using BSA as the standard protein.

Standard enzyme assays

One unit of GST activity is defined as the amount of enzyme that will conjugate 1.0 μmol of glutathione-S-reduced (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) per minute at pH 6.5 and 25°C.

The cuvette assay was performed in 0.1 M phosphate buffered saline (PBS solution, pH 6.5), with 1mM CDNB solution and 1 mM of GSH. The GST activity was monitored at 340 nm for 1 min (ε_{340nm} CDNB-GSH conjugate= 9.6 mM^{-1} cm^{-1}).

The same assay was performed in 96 well plates and the activity was monitored using the microplate spectrophotometer Benchmarch Plus (BioRad) and calculated considering the path length in cm.

The kinetic parameters K_M^{GSH} and k_{cat}^{GSH} were determined for *S. japonicum* GST and for the immobilized and free Vmh2-GST protein. The CDNB concentration used was 1mM, whereas the GSH concentration varied from 0.02 to 6 mM.

GST-Vmh2 immobilization on a multiwell plate

Several tests were carried out pipetting into a 96 well flat plate 100 μL of protein solutions in 50 mMTris-HCl buffer, pH 8.0.

Tests were performed using: GST-Vmh2 (from 0.05 to 1 mg mL^{-1}); a molar mixture of GST-Vmh2: Vmh2 (1:1; 1:3) at 0.1 mg mL^{-1} final concentration; and GST-Vmh2 (0.05 mg mL^{-1}) on a preformed

Vmh2 (0.05 mg mL^{-1}) layer. The proteins used as the controls were: Vmh2 (0.1 mg mL^{-1}); *S. japonicum* GST (0.1 mg mL^{-1}); and *S. japonicum* GST (0.05 mg mL^{-1}) on a preformed Vmh2 (0.05 mg mL^{-1}) layer. The immobilization was evaluated after overnight incubation at different temperatures (20 and 28°C). The effect of the humidity of the chamber was also evaluated. The complete evaporation of the samples was obtained by incubating them in multi-well plates without applying the lids, following which the wells were washed three times with buffer and the protein concentration and enzyme activity of the washes were measured (dried samples). On the other hand, no evaporation of the samples was observed when all the empty wells were filled with buffer and the multi-well plates were closed, following which the protein concentration and enzyme activity were evaluated in the residual liquid (wet samples). Also in this case the wells were washed three times with the same buffer to eliminate unbound proteins. The activity of the immobilized GST was assayed, as described above. The immobilization yield in terms of activity was calculated by considering the immobilized enzymatic IU with respect to that incubated in the well. As for the yield in terms of the protein amount, the immobilized amount was calculated as the difference between the quantity used for immobilization and that measured in the residual liquid.

CD measurements

Far-UV (from 250 to 190 nm) spectra were obtained on a Jasco J715 Spectropolarimeter under constant nitrogen steam. Spectra were collected in a 0.1 cm path length quartz cell with a step size of 1 nm, a bandwidth of 1 nm and an average time of 4 s. The spectra were recorded for Vmh2, Vmh2-GST and GST. For all the spectra, the averages of three scans were obtained.

Thioflavin assay

Vmh2-GST and Vmh2 (from 0.05 to 1 mg mL^{-1}) in 50 mM Tris HCl buffer, pH 8.0, were immobilized in multiwell plates and washed three times with the same buffer (see preparation of the dried samples in par. GST-Vmh2 immobilization on a multiwell plate). The control wells were incubated in the same conditions and with buffer. 100 µL of 30 µM thioflavin (ThT) were added to

the dry wells and incubated at room temperature for 5 mins. The ThT fluorescence intensity of each sample was recorded using a FLUOstar OPTIMA plate reader (BMG Labtechnologies, Melbourne, VIC, Australia) with a 440/490 nm excitation/emission filters set.

Water Contact Angle (WCA) measurement

WCA measurements were performed by using a KSV Instruments LTD CAM 200 Optical Contact Angle Meter. Each contact angle was calculated as the average between the values obtained from three drops (2 µL of a 0.1 mg mL^{-1} protein concentration), spotted on different points of the polystyrene surface, air dried and washed three times with buffer.

Bioinformatic tools

The tango algorithm (Fernandez-Escamilla et al., 2004; tango.crg.es) was used to predict the aggregation propensity of the proteins. The parameters used were: 25 °C, pH 8.0, and ionic strength 50 mM.

Electron Microscopy

Samples were prepared for transmission electron microscopy (TEM) analysis by incubating a 5 µl drop of a 0.001 mg mL^{-1} protein concentration on a standard carbon-coated copper grid (100 mesh) covered with a Formvar film. Then the drop was removed and the grid was air-dried. Images were acquired using a FEI Tecnai 12 transmission electron microscope (FEI Company, Hillsboro, Oregon, USA) equipped with a Veleta CCD digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany) and operating at 120 kV, at magnifications of 21,000X, 30,000X and 68,000X.

Captan quantification

10 µl containing different concentrations of captan (Sigma-Aldrich) from 0.001 to 1 mg L^{-1} were added to the immobilized Vmh2-GST in 96 well plates, and incubated at room temperature for 10 mins and then the GST activity was measured as above described. Up to 5 replicates in two different 96 well plates were performed. The immobilized Vmh2-GST was recycled up to three times for the captan quantification.

Molinate quantification

In the same way as that described above, an assay was developed to detect traces of the herbicide molinate (Sigma Aldrich). 10 µl containing different concentrations of molinate from 0.001 to 4 mg L^{-1} were added to 96 well plates, and incubated at room temperature for 10 mins and then the GST activity was measured as reported above. Up to 5 replicates in two different 96 well plates were performed. The immobilized Vmh2-GSTwas recycled up to three times for the molinate quantification.

Results and Discussion

Production and characterization of the fusion Vmh2-GST protein

The recombinant production of the fusion protein Vmh2-GST was performed as previously reported by Longobardi and coworkers (2012). It has been observed that the protein solubility is highly dependent on the pH of the buffer, since a quick precipitation occurred when the urea concentration was reduced in neutral buffers. Upon urea removal up to 0.2 M in buffer at pH 8, no precipitation occurred and a 100% recovery yield was obtained. The purified fusion protein (see SDS PAGE in Figure S1) displayed GST activity, being able to catalyse the production of the yellow CDNB-GSH conjugate. Far UV CD spectra of Vmh2, Vmh2-GST and GST reported in Figure 1 confirm the refolding of the fusion protein. The fusion protein was characterized by verifying the functionality of both components. The enzymatic activity of Vmh2-GST was analysed by measuring the catalytic parameters K_M and k_{cat} (Table I) which are comparable/better with respect to those obtained for *S. japonicum* GST, suggesting that the kinetic of Vmh2-GST is positively affected by the presence of the Vmh2 moiety. This phenomenon can be explained considering that the propensity of Vmh2 to go to the interfaces could orient the enzyme, and ease its interaction with the substrate molecules.

The ability of the fusion protein to form amyloid fibrils is confirmed by TEM analysis (Figure 2A) showing the formation of long isolated fibrils, similar to those formed by native Vmh2 (Figure 2B) (Gravagnuolo et al., 2016). With the aim of confirming the adhesion ability of the Vmh2-GST

protein on hydrophobic surfaces, changes in the polystyrene wettability upon protein immobilization were monitored by water contact angle measurements (Figure 2C). The dramatic increase in the wettability of the polystyrene surface was even more evident when Vmh2-GST rather than Vmh2 was used, due to the higher hydrophilicity of GST.

Performances of the "self-immobilizing" enzyme

Since the Vmh2-GST fusion protein is an active enzyme endowed with both self-assembling and adhesion properties, it can be considered as a "self-immobilizing" enzyme. In fact the fusion protein was easily immobilized in the polystyrene well and tightly bound to the surface, since it was still active even after several washes. .The immobilization procedure was then optimized by taking into account the time and temperature of incubation, and the degree of humidity in the chamber. A noticeably high immobilization yield of the fusion protein, considering both the activity and the protein amount, was obtained when a complete evaporation of the sample occurred (dried samples) (Table II).

Considering the proven ability of the self-assembled Vmh2 film to bind proteins (De Stefano et al., 2009; Longobardi et al., 2015), the immobilization efficiency of GST and Vmh2-GST on a preformed Vmh2 layer was compared to that of the Vmh2-GST protein alone (Table II). The obtained immobilization yields of both GST and Vmh2-GST were very low with respect to the yield obtained for the direct immobilization of the fusion protein. However, this low yield was comparable with that obtained for the immobilization of other proteins on the same Vmh2 layer (De Stefano et al., 2009). These results demonstrated that gene fusion to the hydrophobin moiety is a much more efficient immobilization procedure than protein deposition on the hydrophobin layer.

In Figure 3 we report the effect of varying the Vmh2-GST concentration on the immobilization yield. High and almost comparable immobilization yields (about 90%) were observed for all tested concentrations, except for the lowest concentration used (0.05 mg mL^{-1}). The trend of the immobilization yield estimated by the activity was consistent with the former. However a drop in activity yield was observed at the highest protein concentration tested. In fact, the same enzyme

units per well were immobilized using either 50 or 100 µg of protein, whereas the actual amount of immobilized protein was doubled. Therefore, steric hindrance preventing enzyme functionality can occur and determine a maximum quantity of protein molecules accessible to the substrate covering the available surface area per well.

Takatsuji and co-workers (2013) reported that the immobilization yield of the fusion protein HFBI-GOx increased when mixtures of hydrophobin HFBI and the fusion protein were used, owing to the presence of the HFBI molecules acting as spacers. With the aim of evaluating the performances of our system in the presence of Vmh2 at different ratios, several trials were carried out. In our case, the best results were obtained through immobilization of the fusion protein alone (Table II), probably because there is no requirement for the spacer due to the lower size of GST compared with that of GOx.

However, it was noticed that the measured yields of Vmh2-GST decreased in the presence of Vmh2 and *vice versa*. It may be inferred that upon protein mixing, Vmh2 and Vmh2-GST were able to reciprocally promote their own self-assembly in bulk solution, thus competing with the surface coating. The concentration of 0.1 mg mL^{-1} was selected as the best condition for the immobilization of Vmh2-GST.

Furthermore, fluorescence analysis in the presence of the Thioflavin T dye was performed on Vmh2 and Vmh2-GST coated wells to verify that the immobilized proteins formed amyloid fibrils. In fact, ThT dye specifically binds the cross-β structure of amyloid aggregates and the increase of its fluorescence intensity confirms the presence of amyloid structures. As shown by the ThT assay results, both Vmh2 and Vmh2-GST spontaneously self-assembled into amyloid structures (Figure 2SA). The ThT assay response of Vmh2-GST is higher than that of Vmh2, and this result was confirmed by *in silico* analysis performed with the Tango algorithm (Fernandez-Escamilla et al., 2004) (Figure 2SB).

The immobilized enzyme displays better performances than its free counterpart both in terms of activity and of stability. In fact a fivefold improvement in the catalytic efficiency is observed (Table

I). This improvement in enzyme activity may be considered more an exception than a rule, as in most instances immobilized enzymes exhibit lower catalytic performances (Rodriguez et al., 2013). As previously suggested, this further increase may be ascribed to an established orientation of the immobilized enzyme, thus increasing the accessibility of substrate molecules to active sites, as reported elsewhere (Longobardi et al., 2015).As far as the stability of the immobilized enzyme is concerned, the enzyme activity was measured every day in different wells of a plate stored dry at room temperature. No significant variation was observed until 60 days, while free GST or GST on the preformed Vmh2 layer exhibited a complete loss of activity in less than three days (Figure 3S). Moreover, when the enzyme activity was measured daily in the same well, a 20% loss of activity was observed up to fifty recycles.

Exploitation of active fibrils in biosensing

The immobilized enzyme was used to develop optical biosensors able to detect traces of pesticides, such as captan (Choi et al., 2003; Singh et al., 2009) and molinate (Heath et al., 1997). Molinate is a thiocarbamate herbicide used primarily in rice production and may have a toxic effect on the local fish population (Heath et al., 1997).

Molinate inhibits GST thus reducing the amount of the yellow CDNB-GSH compound produced, and a lower enzyme activity is measured at the corresponding wavelength. The results are reported in Figure 4A in a logarithmic scale in order to enable an appreciation of the change in the activity occurring in a range of three orders of magnitude. The errors associated with the experimental data are a measure of the whole reproducibility we assessed by repeating the procedure in triplicate and evaluating the standard deviation of the results. The experimental data are well fitted by the following logistic equation (Equation 1):

$$y = \frac{A}{1+\left(\frac{x}{x_0}\right)^p} \quad (1)$$

where A is the value of the activity at [molinate]=0, x_0 is the inflection point i.e. the molinate concentration at which the activity reaches 50% of the initial value (IC50) and p is a fit parameter

which measures the steepness of the curve. The value of A resulting from the best fit procedure (Table III) is compatible with the measured blank (5173± 220 U mL^{-1}) and can be used to estimate the limit of detection (LOD). To achieve this end, we subtracted from the limit value of 5310 U mL^{-1} (A in Table III) three standard deviations (180 U mL^{-1}) and considered the corresponding value (4770 U mL^{-1}) as reliable to assess the presence of molinate. From Eq. (1) the value of the molinate concentration producing such an activity is 5 µg L^{-1} which constitutes our LOD and is about an order of magnitude lower than that reported by Oliveira and coworkers (2013). We highlight that we tested the recycling of the biosensor and the experimental data overlapping those reported in Figure 4A were achieved in up to three recyclings.

Captan is a potential carcinogen and a harmful chemical to the water ecosystem (Choi et al., 2003; Singh et al., 2009). As for molinate, captan caused a reduction in the production of the yellow CDNB-GSH compound and a reduced GST activity was registered upon addition of increasing concentrations of captan (Figure 4B). Thus, the Vmh2-GST activity as a function of inhibitor concentration is fitted by the same logistic equation (Equation 1) as for the molinate inhibition. The IC50 value (x0 in Table III) is about two orders of magnitude lower than that obtained by Di Ilio and coworkers (1996) for human placenta GST, indicating a higher affinity of our system towards captan.

As already observed for molinate, the measured blank (5308± 290 U mL^{-1}) is compatible with the blank of the best fitting (Table III), and this latter was used to calculate a LOD of 1 µg L^{-1} (0.001 ppm) considering an activity value of 4561 U mL^{-1}. It is worth noting that other developed biosensors, based on the human placenta GST, are able to detect up to 1 or 2 ppm of captan (Choi et al., 2003; Singh et al., 2009). The experimental data obtained by recycling the biosensor up to three times overlapped with those reported in Figure 4B.

Thus, the obtained results highlight that this GST-based biosensor can be successfully applied in the quantification of either molinate or captan in aqueous environmental samples. The main advantages

of this sensor include simplicity and speed of preparation, high sensitivity, robustness and recycling.

Conclusions

This work has demonstrated that producing an enzyme (GST) fused to the class I hydrophobin Vmh2 represents an invaluable tool for the development of a robust self-immobilizing enzyme useful for high throughput analyses. Exploiting the self-assembling features and adhesion properties of Vmh2, GST was efficiently immobilized without the need for harsh chemicals or long reaction times. The activity of the immobilized enzyme was maintained during a period of at least 60 days at room temperature.

Active enzymes fused to amyloid polypeptide constructs, spontaneously self-assembling into nanofibrils, have already been described (Zhou et al., 2014). However, the main advantage of our system is the further ability of Vmh2-fused proteins to adhere to surfaces, thus avoiding the additional steps of enzyme separation, which are necessary for enzymes fused to the Ure 2 prion domain (Zhou et al., 2014). Furthermore, as already reported for other amyloid-protein systems (Men et al., 2009), we have observed an enhancement of sensitivity with respect to other reported biosensors (Choi et al., 2003; Oliveira et al., 2013; Singh et al., 2009). The properties of this system have allowed us to set up a robust, sensitive, fast, recyclable and easy-to-use biosensor for the monitoring of toxic compounds, such as captan and molinate. This method is easy and versatile and can be considered as a proof of concept, being potentially suitable for every recombinant protein able to regain activity after refolding from *E. coli* inclusion bodies.

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The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. The molar ellipticity of GST (black dotted line), Vmh2 (black dashed line) and the fusion protein Vmh2-GST (black solid line) are reported.

Figure 2. A. TEM images of Vmh2-GST. **B.** TEM images of nativeVmh2. **C.** Measurement of water contact angle of a water drop on the polystyrene surface after deposition of Vmh2, Vmh2-GST and GST. A polystyrene surface was used as the reference.

Figure 3. Immobilization yield as a function of Vmh2-GST concentration.

Figure 4. Logarithmic plot of the calibration curve of pesticide concentration vs % GST activity. For convenience, a blank is indicated at 1*10-5 mg L-1 **A**. Molinate. **B**. Captan.

Table I. Enzyme catalytic parameters

Enzyme	**K_M^{GSH} (mM)**	**k_{cat}^{GSH} (min^{-1})**	**k_{cat}/K_M (min^{-1} mM^{-1})**
GST	0.43±0.04	$(1.3\pm 0.3)x10^5$	$0.3x10^6$
free Vmh2-GST	0.33±0.06	$(6.5\pm 0.2)x10^5$	$2.0x10^6$
Immobilized Vmh2-GST	0.15± 0.03	$(15.7\pm 0.7)x10^5$	$10.0x10^6$

	Yield (% mg tot)	**Yield (% U tot)**
dried Vmh2-GST	90	89
wet Vmh2-GST	51	57
Vmh2-GST on Vmh2 layer	13	7.5
GST on Vmh2 layer	9.0	1.0
Vmh2-GST:Vmh2, 1:1	78	73
Vmh2-GST:Vmh2, 1:3	75	63
Vmh2	90	--
GST	7.5	0.5

Table II. Immobilization yield of 10 µg of protein

Molinate	**Reduced χ^2**	1.17331
	Parameters	**Value± Standard Error**
	A	5310±180
	x0	0.12±0.02
	p	0.68±0.08
Captan	**Reduced χ^2**	1.12496
	Parameters	**Value± Standard Error**
	A	5595±345
	x0	0.009±0.003
	p	0.57±0.07

Table III. Parameters of logistic fitting

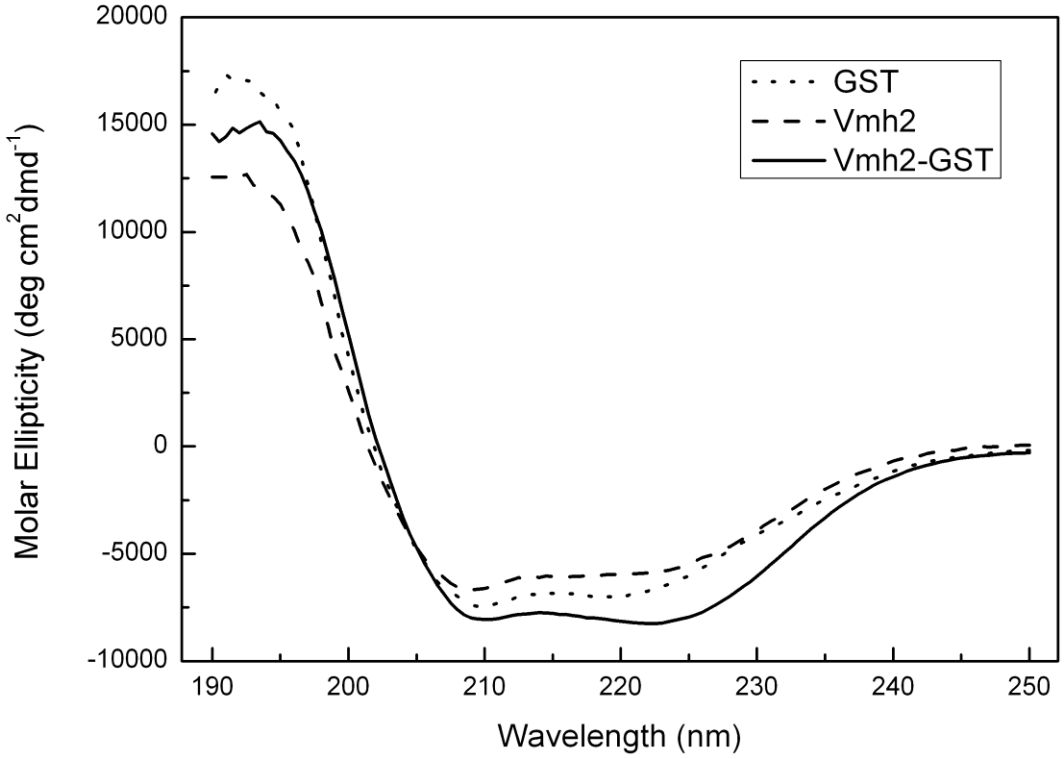

Figure 1

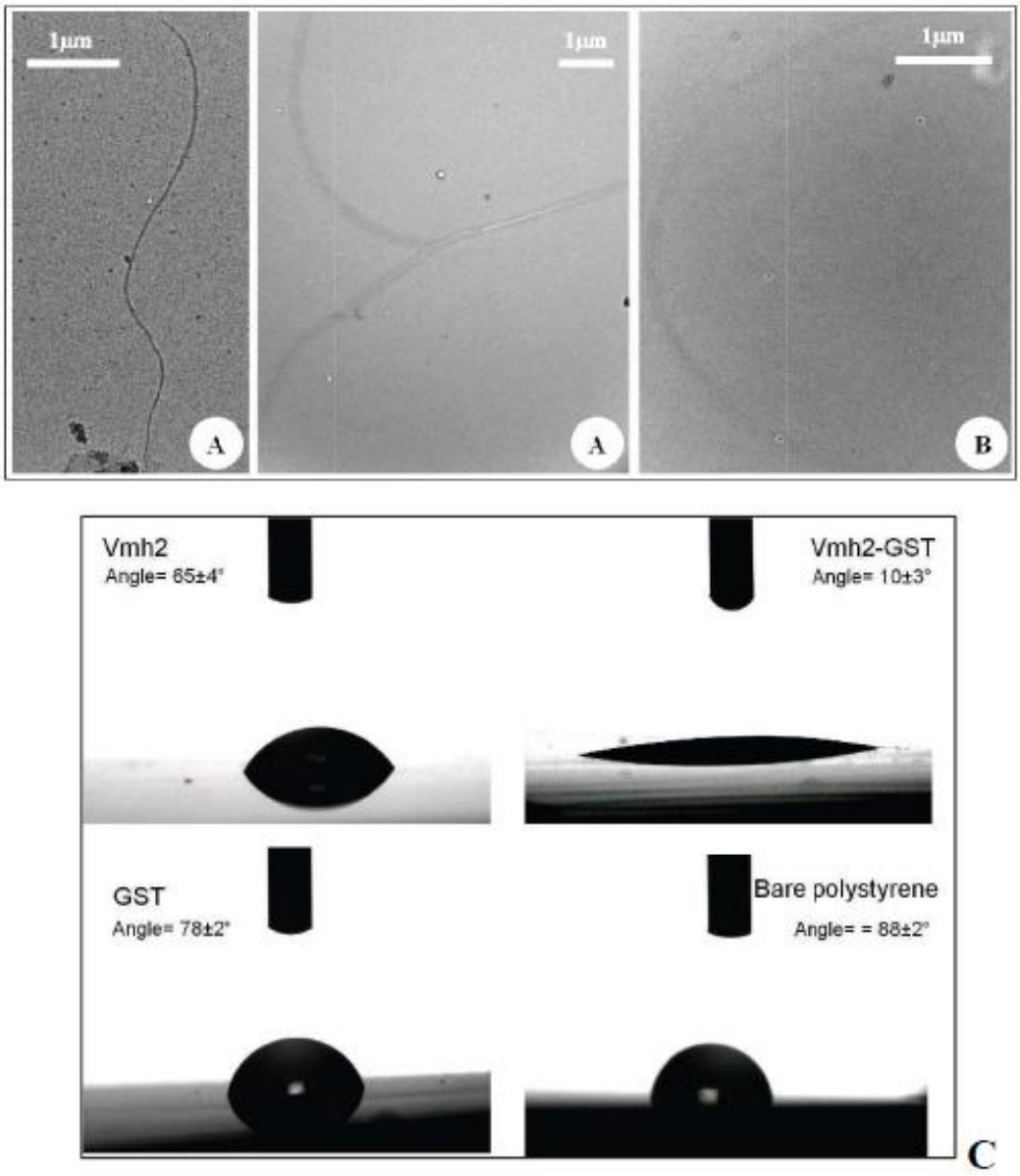

Figure 2

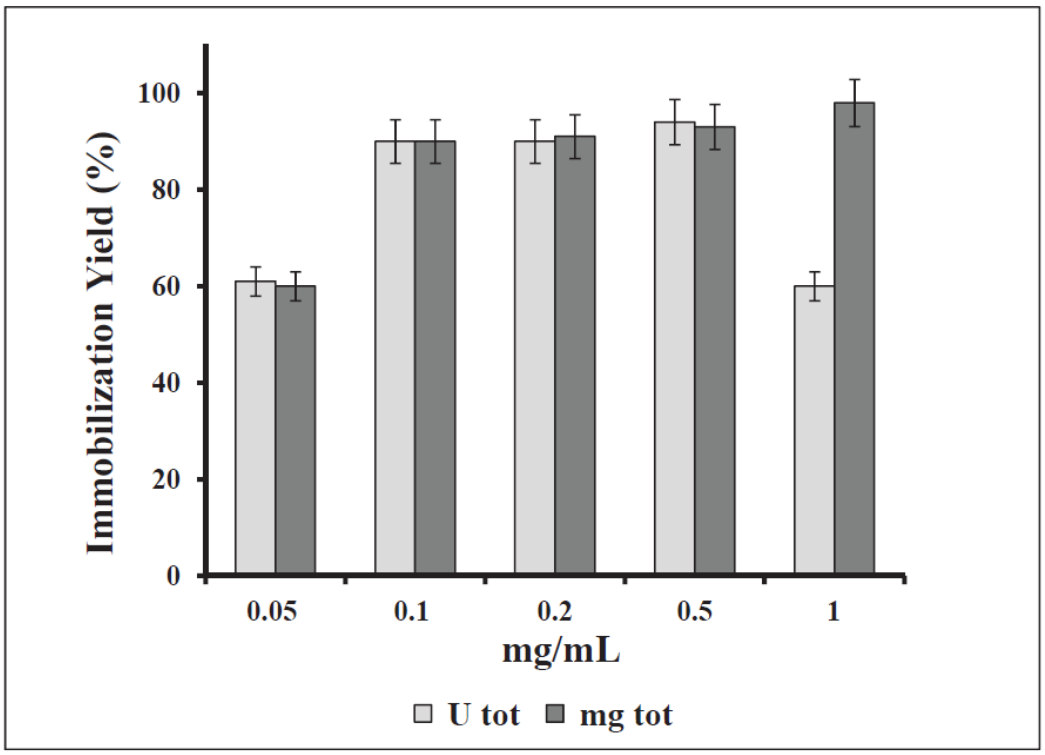

Figure 3

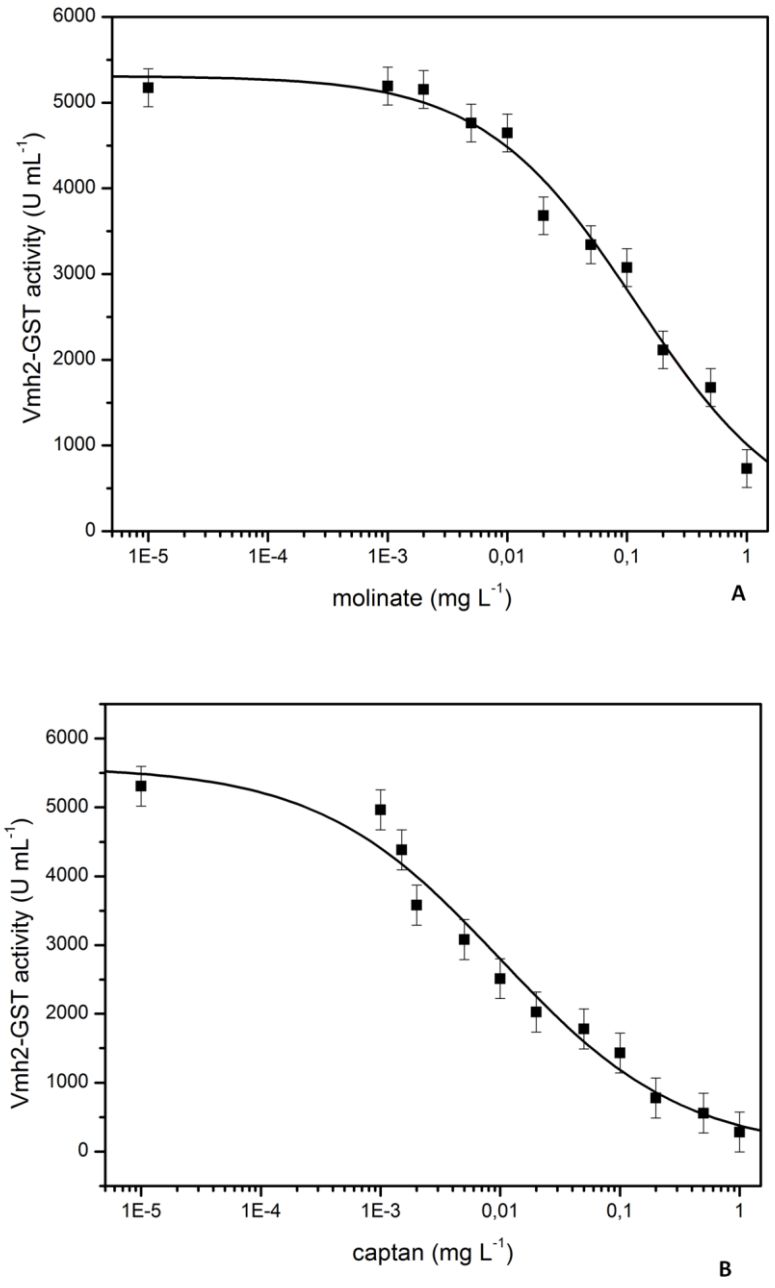

Figure 4