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Engineering sensitive glutathione transferase for the detection of xenobiotics

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ABSTRACT

Cytosolic glutathione transferases (GSTs) are a major reserve of high-capacity ligand binding proteins which recognise a large variety of hydrophobic compounds. In the present study, the binding of non-substrate xenobiotic compounds (herbicides and insecticides) to maize GST I was investigated by employing kinetic inhibition studies, site-directed mutagenesis and molecular modelling studies. The results showed that the xenobiotics bind at the substrate binding site. Based on *in silico* docking analysis, two residues were selected for assessing their contribution to xenobiotic binding. The mutant Gln53Ala of GST I Exhibits 9.2-fold higher inhibition potency for the insecticide malathion, compared to the wild-type enzyme. A potentiometric assay was developed for the determination of malathion using the Gln53Ala mutant enzyme. The assay explores the ability of the xenobiotic to promote inhibition of the GST-catalysing 1-chloro-2,4-dinitrobenzene (CDNB)/glutathione (GSH) conjugation reaction. The sensing scheme is based on the pH change occurring in a low buffer system by the GST reaction, which is measured potentiometrically using a pH electrode. Calibration curve was obtained for malathion, with useful concentration range 0–20 μM. The method's reproducibility was in the order of ±3–5% and malathion recoveries were 96.7 ± 2.8%. Immobilized Gln53Ala mutant GST was used to assemble a biosensor for malathion. The enzyme was immobilized by crosslinking with glutaraldehyde and trapped behind a semipermeable membrane in front of the pH electrode. The results demonstrated that the immobilized enzyme behaved similar to free enzyme.

1. Introduction

The glutathione transferases (GSTs, EC. 2.5.1.18) are a large family of enzymes that catalyse the nucleophilic addition of the thiol of reduced glutathione (GSH) to a wide range of electrophilic compounds (Armstrong, 1998; Sheehan et al., 2001). This conjugation reaction is a critical step in the cellular detoxication of numerous endogenous and exogenous substrates. GSH conjugates are usually more soluble than the original compounds, and are removed from the cell by membrane-associated ATP-binding-cassette transporter (Marrs, 1996).

The cytosolic GSTs are homodimers or heterodimers. Each monomer has two domains, an α/β domain that includes α1–α3 and a large α-helical domain comprised of helices α4–α9. The former contains a GSH binding site (G-site) on top of the α-domain. A hydrophobic pocket (H-site) lies between the domains in which a generally hydrophobic substrate binds and reacts with GSH (Armstrong, 1998; Edwards et al., 2000; Marrs, 1996; Sheehan et al., 2001; Thom et al., 2001).

GSTs were originally termed ligandins because, in addition to their enzymatic roles, they bind to large non-substrate lipophilic molecules (molecular masses > 400 Da) such as bile acids, fatty acids, bilirubin, hemin, and certain drugs, leading to the suggestion that GSTs are involved in the storage and rapid transport of these molecules in the aqueous phase of the cell (Caccuri et al., 1990; Dirr and Wallace, 1999; Habig et al., 1974; Le et al., 2002; Litwack et al., 1971; Tipping and Ketterer, 1981). Non-substrate ligands inhibit in a competitive or noncompetitive manner GST activity which suggest an inter-relationship between binding and catalytic functions (Mosialou and Morgenstern, 1990; Sayed et al., 2000, 2002; Sluis-Cremer et al., 1998). The existence of a distinct ligandin binding site (L-site) has been confirmed by the findings that ligands can bind in the dimer interface of GSTs from *Schistosoma japonica* (McTigue et al., 1995) and squid (Ji et al., 1996). In the case of *Arabidopsis* enzyme the L-site, as identified by crystallography, is located next

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; G-site, glutathione binding site; GSH, glutathione; GST, glutathione transferase; H-site, hydrophobic binding site; L-site, ligandin binding site.

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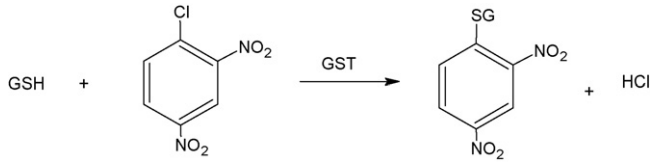

Scheme 1.

to the G-site between the side chains of helices α3″/α3‴ and α5″ (Reinemer et al., 1996). A different picture has emerged in the case of human GST P1-1 isoenzyme and maize GST I where the L-site is located in the H-site (Axarli et al., 2004; Oakley et al., 1999).

In the last two decades, research in the field of enzyme-based bioassays and biosensors has significantly increased as a result of the need for cheap, fast and easy way to use analytical tools that are able to provide real-time qualitative and quantitative information about the composition of a sample with minimum sample preparation (Andreescu and Marty, 2006; Healy et al., 2007). Enzyme-based assays can provide data of superior accuracy and specificity compared to traditional wet chemistry and instrumental methods, with the added advantage of being environmentally benign and safer for the user.

A GST-based inhibition assay was previously developed for the quantification of pyrethroid insecticides. It was based on agGSTD6-catalysed reactions, which was monitored spectrophotometrically or endpoint determined by iodometric titration (Enayati et al., 2001). The latter is a technically demanding analytical chemistry technique, which provides moderate accuracy, as it is relies on the detection of the non-conjugated substrate GSH, only a small fraction of which is utilised in the enzymatic reaction. In the present report we show that the inhibition of a mutant of the isoenzyme GST I from maize (or *Zm*GSTF1, according to the nomenclature of (Edwards et al., 2000)) by several xenobiotics may form the basis for designing a potentiometric biosensor for the determination of xenobiotics. The sensing scheme, in the proposed sensor, is based on the ability of GST to catalyse the conjugation of GSH with CDNB with concomitant proton release (HCl). The concentration of released protons is proportional to the amount of conjugated substrate (Scheme 1) and is measured potentiometric using a pH electrode.

The concentration of xenobiotics may be determined indirectly by measuring the different degree of pH change due to enzyme inhibition by xenobiotics. This pH change can be related to the amounts of xeobiotics using a calibration curve. The proposed assay displays good precision, accuracy, and short response time. The enzyme GST I from maize is of particular value for biosensor applications since it exhibits wide ligand binding specificities, high specific activity and stability (Labrou et al., 2001a,b, 2004).

2. Materials and methods

2.1. Materials

Reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Sigma–Aldrich Co. (USA). Insecticides and herbicides with a minimum certified purity of 97.7% were obtained from Merck and Greyhound (UK). Other reagents and analytical grade chemicals were obtained from Sigma–Aldrich Co. (USA).

2.2. Methods

2.2.1. Cloning, expression and purification of maize GST I and its mutants

The cloning of maize GST I into a pQE70 expression vector to yield the pQEGST expression plasmid was described in (Labrou et al., 2001a,b). Expression and purification of GST I were performed according to Labrou et al. (2001a,b). Site-directed mutagenesis was performed as described in (Labrou et al., 2001a).

2.2.2. Assay of enzyme activity and protein

GST assays were performed by monitoring the formation of the conjugate of CDNB and GSH at 340 nm ($\varepsilon = 9.6\,mM^{-1}\,cm^{-1}$) at 37 °C according to a published method (Labrou et al., 2001a,b). Observed reaction velocities were corrected for spontaneous reaction rates when necessary. All initial velocities were determined in triplicate in buffers equilibrated at constant temperature. One unit of enzyme is defined as the amount of enzyme that gives 1.0 μmole of product per minute at pH 6.5 at 30 °C. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (fraction V) as standard.

2.2.3. Inhibition studies

Initial velocities for the GST-catalysed reaction were measured at 37 °C in a total volume of 1 mL containing 100 mM potassium phosphate buffer, pH 6.5, 1 mM CDNB, 2.5 mM GSH in the presence or absence of 100 μM of a xenobiotic compound.

2.2.4. Kinetic inhibition studies with malathion

Initial velocities for the GST-catalysed reaction with GSH as variable substrate were measured at 37 °C in a total volume of 1 mL containing 100 mM potassium phosphate buffer, pH 6.5, 2.5 mM CDNB, and different concentrations of GSH in the absence or presence of 20 or 30 μM malathion. With CDNB as variable substrate, the reaction mixture contained in a total volume of 1 mL: 100 mM potassium phosphate buffer, pH 6.5, 2.5 mM GSH and different concentrations of CDNB, again in the presence or absence of 20 or 30 μM malathion. The apparent kinetic parameters were determined using the GraFit (Erithacus Software, Ltd.) computer program. The IC_{50} for malathion was determined by fitting the concentration–response data to the Eq. (1):

$$\%\text{inhibition} = \frac{100}{1 + (IC_{50}/[I])} \quad (1)$$

Analysis was carried out through nonlinear curve-fitting methods using the GraFit (Erithacus Software, Ltd.) computer program.

2.2.5. Docking of malathion into the GST I binding site

The high-resolution structure of GST I in complex with the inhibitor atrazine–GSH (PDB identifier 1BYE) (Neuefeind et al., 1997) was used in the docking studies. The protein was prepared for docking by removing the inhibitor and adding hydrogen atoms to fulfil unsatisfied valencies, while retaining crystallographic water molecules. The Molsoft ICM-Docking (Abagyan et al., 1994) suite of programs was employed for predicting the geometry of the ligand bound to the protein. All residues within 6 Å of the inhibitor were included in the definition of the active site used for screening. A distance-dependent dielectric ($\varepsilon = 4r$) and an energy cut-off distance of 10 Å were used in evaluating the interaction energy between protein and ligand. Ligand conformational flexibility was explored through torsion angle sampling and minimization. PYMOL (DeLano, 2002) was used for the preparation of structure figures. WHAT IF (Vriend, 1990) was used for the *in silico* creation of Gln53Ala mutant enzyme. The types of interaction in the wild-type and mutant enzyme were analysed using iMolTalk (http://i.moltalk.org/).

2.2.6. Potentiometric assays of the GST-catalysed reaction

Potentiometric assay of the GST-catalysed reaction was carried out at 25 °C in a total volume of 10 mL containing 2 mM potassium phosphate buffer, pH 7.5, 10 mM NaCl, 1 mM CDNB, 1 mM GSH,

0.5 units enzyme in the presence of 0–50 μM malathion (dissolved in acetone). The initial reaction rate (first 10 min) was calculated by linear regression analysis.

2.2.7. Enzyme stability and stability upon storage

The Gln53Ala mutant enzyme was incubated at different temperatures at a protein concentration of 0.02 mg/mL in 50 mM potassium phosphate buffer pH 7.5. The samples were incubated at different temperatures for 10 min and subsequently assayed for residual activity. T_m values were determined from the plot of relative inactivation (%) versus temperature (°C). The T_m value is the temperature at which 50% of the initial enzyme activity is lost after heat treatment. The stability upon storage of Gln53Ala mutant enzyme at 4 °C was also studied in 50 mM potassium phosphate buffer pH 7.5 in the absence or presence of GSH (0.1 mM) or glycerol (20%, v/v). The course of enzyme inactivation was measured by periodically removing samples for assay of enzymatic activity.

2.2.8. GST biosensor

Immobilized Gln53Ala mutant GST was used to assemble a biosensor for malathion. The enzyme was immobilized by crosslinking with glutaraldehyde (Andreou and Clonis, 2002) as following: 3.0 mg of BSA were dissolved in 50 μL enzyme solution and the resultant solution was rapidly mixed with 5 μL glutaraldehyde solution (8% aqueous, grade I) and deposited on the moist outer surface of the pH electrode. The enzyme–glutaraldehyde mixture allowed to stand at 4 °C for 45 min for crosslinking. The bioactive (enzymatic) layer (typical 20 mg, ~0.2 enzyme units) was placed behind a dialysis membrane (12,000–14,000 MW cut-off) that was held to the bottom of the electrode with a rubber O-ring. The enzyme electrode was then rinsed repeatedly with 2 mM phosphate buffer, pH 7.5, to remove any unreacted crosslinking agent and/or protein. The sensors were placed in a solution of the same phosphate buffer. Malathion solutions (0–20 μM) were used to calibrate the biosensors at 25 °C.

3. Results and discussion

3.1. Inhibition analysis of GST I by xenobiotic compounds

The isozyme GST I from maize has been the major focus of interest as a model enzyme for investigating structure–function relationships in plant GSTs family (Axarli et al., 2004; Labrou et al., 2001a,b; Labrou et al., 2004). The enzyme exhibits wide ligand binding specificities. The hydrophobicity, size, shape and high flexibility of the H-site make it suitable for accommodating a wide variety of large hydrophobic ligands (Neuefeind et al., 1997; Prade et al., 1998).

Several xenobiotic compounds including insecticides (dimethoate, endosulphan, DDT, dieldrin, aldrin, cyhalothrin, β-cypermethrin, α-cypermethrin, permethrin, deltametrin, propoxur, malathion, fenvalerate, diazinon, carbaryl), fungicides (penconazole, aroxystrobin, metalaxyl-M, spiroxamine, tebuconazole) and herbicides (tribenuron methyl, diuron, fluorodifen) were tested at 100 μM concentration for their ability to inhibit GST I activity. Fig. 1 summarizes the %inhibition obtained for the inhibitors mentioned above. All xenobiotic compounds that were tested showed time-independent inhibition, indicating that the inhibitors bind reversibly to the enzyme.

From the results presented in Fig. 1 it is evident that bulky compounds with two aromatic rings (e.g. phenylurea-based insecticides) behaved as strong inhibitors showing inhibition between 75% and 94%. For example, fenvalerate is the strongest inhibitor exhibiting 94% inhibition. On the other hand, aliphatic insecticides such as endosulphan and malathion with aliphatic chains showed in general lower inhibition potency for the wild-type enzyme. Similar, pesticides with one aromatic ring (e.g. diuron, diazinon) exhibited low inhibition potency.

3.2. Inhibition analysis of Ile118Phe and Gln53Ala mutants of GST I by xenobiotic compounds

Site-directed mutagenesis studies were employed to provide structural information and help to locate the xenobiotic ligand binding site on GST I. On the basis of the available X-ray crystal structure of GST I (Neuefeind et al., 1997), two residues (Ile118 and Gln53) were selected to assess their contribution to xenobiotic binding. The H-site of GST I exhibits a unique structure and is built predominantly of hydrophobic residues from helix H_3''' (e.g. Ile118) and helix H_2 (e.g. Gln53). Ile118 is not a conserved residue and has been proposed to modulate ligand recognition by affecting the size and the shape of the H-site (Neuefeind et al., 1997). Gln53 is localised at the end of a type I β-turn formed by Asn49, Pro50, Phe51 and Gly52 and contributes to structural integrity of helix H_2 which build part of the H-site (Labrou et al., 2001a,b).

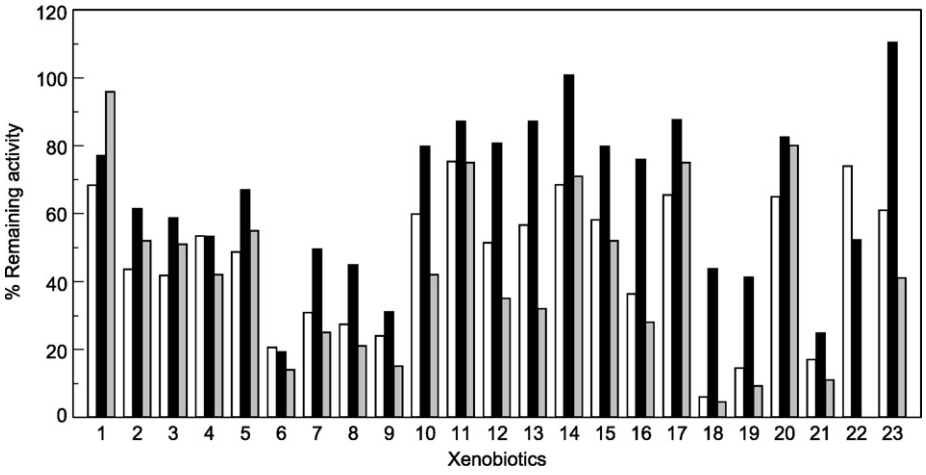

Fig. 1. The inhibition of the wild-type GST I (white bars) and its selected mutants Ile118Phe (black bars) and Gln53Ala (grey bars) by xenobiotics. The xenobiotics that were used are: dimethoate, (1); endosulphan, (2); DDT [(1,1,1-trichloro-2, 2-*bis*(*p*-chlorophenyl)ethane], (3); dieldrin, (4); aldrin, (5); λ-cyhalothrin, (6); β-cypermethrin, (7); α-cypermethrin, (8); deltametrin, (9); penconazole, (10); tribenuron methyl (11); aroxystrobin, (12); metalaxyl-M, (13); spiroxamine, (14); tebuconazole, (15); propoxur, (16); diuron, (17); fenvalerate, (18); fluorodifen, (19); carbaryl, (20); permethrin, (21); malathion, (22); diazinon, (23). In the absence of xenobiotics, enzyme activity was taken as 100%.

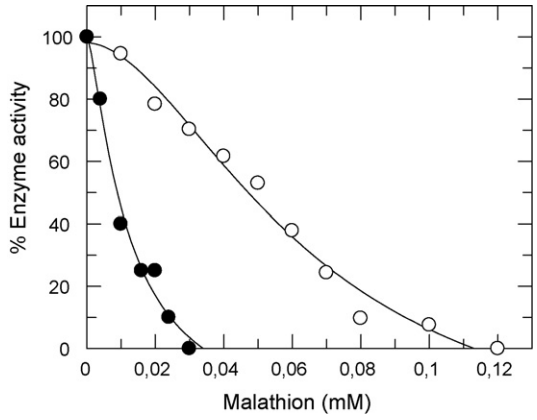

Fig. 2. Concentration–response curve for malathion. IC_{50} for the wild-type enzyme (○) and mutant Gln53Ala (●) was determined by fitting the data to Eq. (1) through nonlinear curve-fitting methods.

Results of inhibition analysis for the mutant enzymes (Gln53Ala and Ile118Phe) are listed in Fig. 1. Both mutants showed differences in %inhibition values when compared to the wild-type enzyme. In particular, the mutant Ile118Phe is less sensitive to xenobiotics, whereas the mutant Gln53Ala is more sensitive than the wild-type enzyme to a broader range of xenobiotics, suggesting higher affinity for the xenobiotics. For example, the mutant Gln53Ala is more sensitive than the wild-type enzyme to 15 out of 23 tested xenobiotics, whereas the mutant Ile118Phe is more sensitive to only one. It is of particular interest that the mutant Gln53Ala exhibited about 100% inhibition by malathion [0,0-dimethyl-*S*-(1,2-dicarbethoxyethyl) dithiophosphate]. Malathion is a broad-spectrum organophosphate parasympathomimetic insecticide which binds irreversibly to cholinesterase. The IC_{50} values of malathion (Fig. 2) for both the wild-type, Gln53Ala and Ile118Phe mutants were determined equal to 77.6 μM, 8.4 μM and 61.2 μM, respectively.

3.3. Mapping of the malathion binding site

Kinetic inhibition studies were carried out to localize the malathion binding site on GST I. Malathion exhibited a competitive-type inhibition with respect to CDNB (K_i = 29.7 μM) and GSH (K_i = 86.1 μM) (data not shown). This finding indicates that malathion binds to the substrate binding site of GST I and provides kinetic evidence that malathion overlaps both the G- and H-site. The only available structures of GST I are those in complex with glutathione conjugates—the atrazine conjugate (Prade et al., 1998) and the lactoylglutathione complex (Neuefeind et al., 1997). Neither of these ligands bears any strong structural resemblance to malathion and therefore molecular docking was employed to characterize its binding site. Malathion was docked to overlap the substrates binding sites of GST I in accordance with the results of the kinetic inhibition study. The predicted mode of interaction of the malathion with GST I is shown in Fig. 3. The results from molecular modelling studies suggest that the binding of malathion to the wild-type GST I may be primarily achieved by hydrophobic interactions that provide the driving force for positioning and recognition of this compound. The bulk of the interactions with the enzyme involve residues Trp12, Asn13, Phe35, Gln53 and Ile118. In addition, two hydrogen bonds may be formed, one by Asn13 and one by Gln53 (Fig. 3).

The model explains well the inhibition data obtained for the mutants. Ile118 is located at the helix H_3''' and is involved in hydrophobic interaction with the xenobiotic substrate. The Ile118Phe mutation may have as a consequence the development of a steric hindrance. In the mutant, the bulk side chain of Phe provides less room, compared to that of Ile side chain and may lead to unfavourable ligand binding. On the other hand, the Gln53Ala mutation may have two semi-independent consequences: the loss of the H-bonds of the Gln53 side chain, and the introduction of a cavity. The cavity could either be filled by new water molecules or by a rearranged local protein structure that gives the ability to malathion to bind to the enzyme with a different conformation (Fig. 3). In this conformation, malathion is developing new additional van der Waals interactions with Met10, Asn110 and Phe114. Moreover, five hydrogen bonds may be formed, two by Ser11 and Asn13 and one by Trp12. These additional interactions may explain the higher affinity of Gln53Ala mutant enzyme for malathion.

3.4. The basis of a potentiometric biosensor for the determination of malathion

The best-suited enzyme to be used in analytic applications should be the most sensitive to xenobiotics to place the threshold of detection as low as possible. Therefore the mutant Gln53Ala

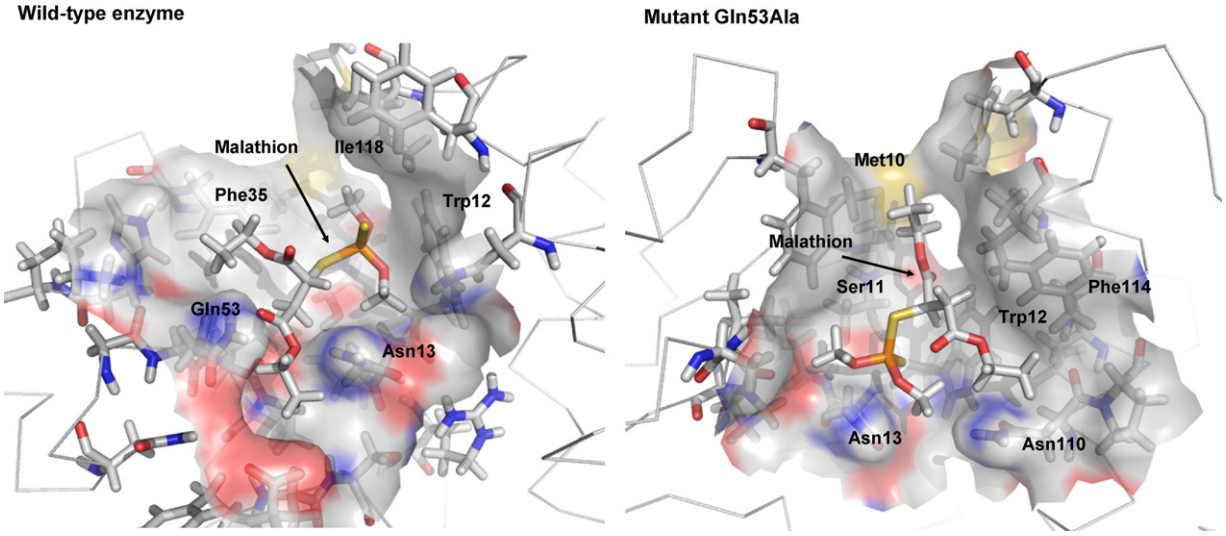

Fig. 3. The predicted mode of interaction of the wild-type enzyme and Gln53Ala mutant with malathion. Malathion and side chains of residues contacting the ligand are shown as sticks and labelled.

was used for the development of a simple potentiometric assay that explores the ability of malathion to act as a strong inhibitor towards this mutant. Potentiometric biosensors make use of ion-selective electrodes (e.g. a pH-meter) in order to transduce the biological reaction into an electrical signal. This type of biosensors offer clear and distinct advantages over standard analytical methods for the direct monitoring of analytes such as low cost, simple installation, direct measurements, and easy applicability for automatic and industrial analysis (Mulchandani et al., 2001).

The sensing scheme, in the proposed sensor, is based on the ability of GST to catalyse the conjugation of GSH with CDNB with concomitant proton release (HCl). The concentration of released protons is proportional to the amount of conjugated substrate. The concentration of malathion is determined indirectly by measuring the different degree of pH change due to enzyme inhibition by malathion. This pH change can be related to the amounts of malathion using a calibration curve.

The sensor signal (pH alteration) was calculated as a difference between reference signal and reaction signal. Fig. 4A shows typical rate curves obtained in the presence of different CDNB concentrations. The concentration of phosphate in the buffer solution was 2 mM. Larger signals were obtained for lower buffer capacities (e.g. 1 mM phosphate) for a given substrate concentration, but compromised sensor response and reproducibility due to the inherent instability of these dilute buffer solutions. As can be seen in Fig. 4A, the pH initially decreases linearly. However, after a certain period of time has elapsed depending on substrate concentration, the rate of decrease gradually becomes smaller. The reproducibility of sensor response to substrate (CDNB), expressed by R.S.D., was in the order of ±3–5% ($N = 5$). The initial reaction rate can be correlated with substrate and malathion concentration. Fig. 4B shows the calibration curve obtained for malathion. The slope was calculated for the first 10 min (Fig. 4A) after initiation of the enzymatic reaction. It can be seen that the percent of enzyme inhibition can be linearly related to malathion concentration in bulk solution within the concentration range of 0–20 μM. Malathion recoveries ranged between 87% and 108%, with an average value of 96.7% ($N = 5$).

The potentiometric assay described above offers clear and distinct advantages over standard analytical methods for the direct monitoring of malathion in the field, such as low cost, real-time detection with minimum sample preparation and handling and wide linear range. For example, the linear range for malathion determination is wider than other methods (e.g. HPLC method: 0.53 to 4.25 μg/g, Gebreegzi et al., 2000; gas chromatography–flame photometric detection: 0.21–3.05 μg/L, Xiong and Hu, 2008; colorimetric assay 0.028–0.28 μg/mL, Mathew et al., 2007 and 0.10–10 μg/mL, Namera et al., 2000). In addition recoveries are higher (e.g. 85.5 ± 6.6%, Gebreegzi et al., 2000; 78.5–117.2%, Xiong and Hu, 2008). The analysis time of the present method (10 min per sample) is shorter than the time reported in other methods (e.g. 30 min, Namera et al., 2000).

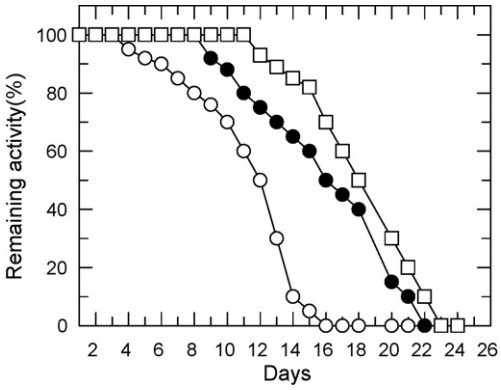

Fig. 5. Stability of Gln53Ala mutant enzyme. Stability of mutant Gln53Ala at 4 °C in 50 mM potassium phosphate in the absence of any stabilizer (○) or in the presence of GSH (0.1 mM, □) or glycerol (20%, v/v, ●).

3.5. Thermal stability and stability upon storage of Gln53Ala mutant enzyme

To determine the structural stability of Gln53Ala, heat inactivation studies were carried out. The enzyme was incubated at different temperatures and subsequently assayed for residual activity. The inactivation profile at pH 7.5 showed one clear transition with inflection point (data not shown) corresponding to T_m value of 57.6 °C. The corresponding T_m value for the wild-type enzyme has been determined to be equal to 58 °C (Labrou et al., 2001a,b), showing that the mutation did not significant affect enzyme's structural stability.

The time course of thermal inactivation of the mutant enzyme was also investigated at 4 °C (Fig. 5). Gln53Ala in 50 mM potassium phosphate buffer, pH 7.5, was inactivated almost completely in 16 days at 4 °C. However, the presence of GSH or glycerol (20%, v/v) exhibited significant stabilizing effects. The strong stabilizing effects observed by GSH and glycerol may be attributed to the stabilization of quaternary enzyme structure. The half-life of enzyme inactivation at 4 °C was 12 d, in the absence of stabilizers, or 18 d and 16 d in the presence of GSH and glycerol, respectively. These findings indicate that the Gln53Ala mutant enzyme exhibits good

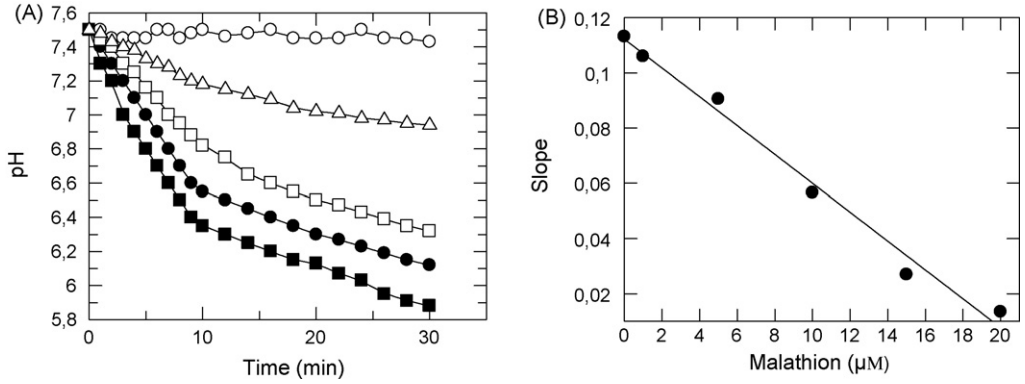

Fig. 4. (A) Reaction rate curves of the free Gln5Ala mutant enzyme, for the following substrate (CDNB) concentrations: blank experiment (0.0 mM, ○); 0.2 mM, (△); 0.6 mM (□); 0.8 mM (●); (iv) 1 mM (■). Experimental conditions were: 2 mM phosphate buffer, pH 7.5 10 mM NaCl, temperature 25 °C. (B) Calibration graph obtained for malathion using Gln53Ala mutant enzyme. The absolute value of the rate of reaction for the first 10 min was used as a signal. Experimental conditions were: 2 mM phosphate buffer, pH 7.5; 10 mM NaCl; temperature 25 °C. Each point represents the average of three determinations.

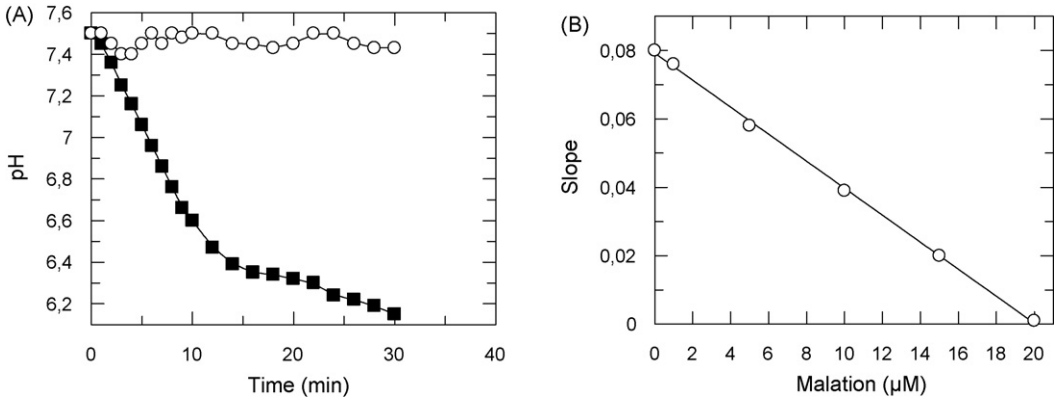

Fig. 6. (A) Reaction rate curves using immobilized Gln53Ala mutant enzyme (20 mg gel), for the following substrate (CDNB) concentrations: blank experiment (0.0 mM, ○); 1 mM, (■). (B) Calibration graph obtained for malathion using immobilized Gln53Ala mutant enzyme. The absolute value of the rate of reaction for the first 15 min was used as a signal. Experimental conditions were: 2 mM phosphate buffer, pH 7.5, 10 mM NaCl, temperature 25 °C. Each point represents the average of three determinations.

stability upon storage at 4 °C which significantly improves the practical viability of this enzyme assay, and provides the basis for the development of a field applicable system for the direct monitoring of malathion.

3.6. The basis of a potentiometric biosensor for malathion

The mutant Gln53Ala was used to develop a biosensor for malathion. In this sensor, the enzyme was immobilized by crosslinking with glutaraldehyde (Andreou and Clonis, 2002) trapped behind a semipermeable membrane in front of the pH electrode. Glutaraldehyde is a bifunctional reagent that binds proteins through their free amino groups, thus forming a three-dimensional network. To stabilize this network mechanically and protect the enzyme activity, bovine serum albumin (BSA) was used. The activity of the bound enzyme was found to be 0.01 units/mg gel, whereas the nominal activity of the added enzyme was 0.05 units/mg gel. The immobilization process itself probably caused significant irreversible enzyme denaturation that led to a subsequent loss of enzyme activity.

The immobilized GST-catalysed CDNB/GSH conjugation reaction results in concomitant release of H^+ protons. The concentration of released protons reflects the progress of the CDNB/GSH conjugation reaction and can be measured by the pH change. After pre-incubation (5 min) of enzyme electrode with malathion solution, the rate of pH decrease declines in a manner similar to that of the free enzyme (Fig. 6A). The percent of enzyme inhibition can be linearly related to malathion concentration in bulk solution. Calibration curve was obtained with useful concentration range 0–20 μM and using the response obtained 15 min after addition of the substrates (Fig. 6B).

In conclusion, the application of GST inhibition test provides a new method for detecting a wide range of xenobiotics. The enzyme system employed (GST) is generally stable and easily reproducible. One representative GST inhibiting pesticide, malathion (organophosphate), has been determined successfully using the proposed method with satisfactory reproducibility and accuracy. Given the current expansion of malathion residual sprayings in many regions, as a key strategic intervention for malaria vector control, the possible application of the assay as a supporting routine control system might have an impact on malaria control.

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References

Abagyan, R.A., Totrov, M.M., Kuznetsov, D.A., 1994. J. Comp. Chem. 5, 488–506.
Andreescu, S., Marty, J.L., 2006. Biomol. Eng. 23, 1–15.
Andreou, V.G., Clonis, Y.D., 2002. Biosens Bioelectron. 17, 61–69.
Armstrong, R.N., 1998. Curr. Opin. Chem. Biol. 2, 618–623.
Axarli, I.A., Rigden, D.J., Labrou, N.E., 2004. Biochem. J. 382, 885–893.
Bradford, M.M., 1976. Anal. Biochem. 72, 248–254.
Caccuri, A.M., Aceto, A., Piemonte, F., Di Ilio, C., Rosato, N., Federici, G., 1990. Eur. J. Biochem. 189, 493–497.
DeLano, W.L., 2002. DeLano Scientific. San Carlos, USA.
Dirr, H.W., Wallace, L.A., 1999. Biochemistry 38, 15631–15640.
Edwards, R., Dixon, D.P., Walbot, V., 2000. Trends Plant Sci. 5, 193–198.
Enayati, A.A., Vontas, J.G., Small, G.J., McCarroll, L., Hemingway, J., 2001. Med. Vet. Entomol. 15, 58–63.
Gebreegzi, Y.T., Foster, G.D., Khan, S.U., 2000. J. Agric. Food Chem. 48, 5165–5168.
Habig, W.H., Pabst, M.J., Fleischner, G., Gatmaitan, Z., Arias, I.M., Jakoby, W.B., 1974. Proc. Natl. Acad. Sci. U.S.A. 71, 3879–3882.
Healy, D.A., Hayes, C.J., Leonard, P., McKenna, L., O'Kennedy, R., 2007. Trends Biotechnol. 25, 125–131.
Ji, X., von Rosenvinge, E.C., Johnson, W.W., Armstrong, R.N., Gilliland, G.L., 1996. Proc. Natl. Acad. Sci. U.S.A. 93, 8208–8213.
Labrou, N.E., Kotzia, G.A., Clonis, Y.D., 2004. Protein Eng. Des. Sel. 17, 741–748.
Labrou, N.E., Mello, L.V., Clonis, Y.D., 2001a. Biochem. J. 358, 101–110.
Labrou, N.E., Mello, L.V., Clonis, Y.D., 2001b. Eur. J. Biochem. 268, 3950–3957.
Le, T., Stenkamp, R.E., Ibarra, C., Atkins, W.M., Adman, E.T., 2002. Proteins 48, 618–627.
Litwack, G., Ketterer, B., Arias, I.M., 1971. Nature 234, 466–467.
Marrs, K.A., 1996. Ann. Rev. Plant Physiol. Plant Mol. Biol. 47, 127–158.
Mathew, S.B., Pillai, A.K., Gupta, V.K., 2007. Spectrochim. Acta A, Mol. Biomol. Spectrosc. 67, 1430–1432.
McTigue, M.A., Williams, D.R., Tainer, J.A., 1995. J. Mol. Biol. 246, 21–27.
Mosialou, E., Morgenstern, R., 1990. Chem. Biol. Interact. 74, 275–280.
Mulchandani, A., Chen, W., Mulchandani, P., Wang, J., Rogers, K.R., 2001. Biosens. Bioelectron. 16, 225–230.
Namera, A., Utsumi, Y., Yashiki, M., Ohtani, M., Imamura, T., Kojima, T., 2000. Clin. Chim. Acta 291, 9–18.
Neuefeind, T., Huber, R., Dasenbrock, H., Prade, L., Bieseler, B., 1997. J. Mol. Biol. 274, 446–453.
Oakley, A.J., Lo, B.M., Nuccetelli, M., Mazzetti, A.P., Parker, M.W., 1999. J. Mol. Biol. 291, 913–926.
Prade, L., Huber, R., Bieseler, B., 1998. Structure 6, 1445–1452.
Reinemer, P., Prade, L., Hof, P., Neuefeind, T., Huber, R., Zettl, R., Palme, K., Schell, J., Koelln, I., Bartunik, H.D., Bieseler, B., 1996. J. Mol. Biol. 255, 289–309.
Sayed, Y., Hornby, J.A., Lopez, M., Dirr, H., 2002. Biochem. J 363, 341–346.
Sayed, Y., Wallace, L.A., Dirr, H.W., 2000. FEBS Lett. 465, 169–172.
Sheehan, D., Meade, G., Foley, V.M., Dowd, C.A., 2001. Biochem. J. 360, 1–16.
Sluis-Cremer, N., Wallace, L., Burke, J., Stevens, J., Dirr, H., 1998. Eur. J Biochem. 257, 434–442.
Thom, R., Dixon, D.P., Edwards, R., Cole, D.J., Lapthorn, A.J., 2001. J. Mol. Biol. 308, 949–962.
Tipping, E., Ketterer, B., 1981. Biochem. J. 195, 441–452.
Xiong, J., Hu, B., 2008. J. Chromatogr. A 1193, 7–18.
Vriend, G., 1990. J. Mol. Graph. 8, 52–56.