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Analytical approaches to investigate protein–pesticide adducts☆,☆☆

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ABSTRACT

Organophosphorus pesticides primarily elicit toxicity via their common covalent adduction of acetylcholinesterase (AChE), but pesticide binding to additional sensitive secondary targets may also compromise health. We have utilised tritiated-diisopropylfluorophosphate (^{3}H-DFP) binding to quantify the levels of active immune and brain tissue serine hydrolases, and visualise them using autoradiography after protein separation by one-dimensional and two-dimensional techniques. Preincubation of protein extracts with pesticide *in vitro* or dosing of rats with pesticide *in vivo* was followed by ^{3}H-DFP radiolabelling. Pesticide targets were identified by a reduction in ^{3}H-DFP radiolabelling relative to controls, and characterised by their tissue presence, molecular weight, and isoelectric point. Conventional column chromatography was employed to enrich pesticide targets to enable their further characterisation, and/or identification by mass spectrometry. The major *in vivo* pesticide targets characterised were 66 kDa, serum albumin, and 60 kDa, likely carboxylesterase 1, both of which displayed differential pesticide binding character under conditions producing approximately 30% tissue AChE inhibition. The characterisation and identification of sensitive pesticide secondary targets will enable an evaluation of their potential contribution to the ill health that may arise from chronic low-dose pesticide exposures. Additionally, secondary targets may provide useful biomonitors and/or bioscavengers of pesticide exposures.

1. Introduction

Organophosphorus (OP) compounds have been utilised as commercial pesticides for over 50 years. OP pesticides are distinguished by a central phosphorus atom double bonded to either sulphur (thion) or oxygen (oxon), an R1 group, R2 group, and a leaving group. Pesticides in common commercial use possess R1 and R2 groups that are ester linked to dimethyl or diethyl groups (see Fig. 1). Pesticides covalently bind protein targets directly as oxons, or require liver bioactivation from phosphorothionates (thions) to their corresponding oxons.

OPs form covalent adducts with protein nucleophilic sites, such as the carbonyl group of the hydroxy amino acids serine or tyrosine, thereby producing a phosphorylated derivative. Hydrolysis and removal of this phosphate moiety may be relatively slow, or essentially irreversible if adduct side chain dealkylation (ageing) occurs. The serine hydrolase family of enzymes possess an active site serine that is susceptible to OP adduction, and this has been exploited in the use of pesticides to covalently bind and inactivate acetylcholinesterase (AChE) within synapses. AChE hydrolyses the neurotransmitter ACh resulting in a termination of nerve signal conduction, hence its inactivation by OP adduction results in ACh signal persistence and cholinergic toxicity.

Additionally, inadvertent OP protein adduction of other serine hydrolases may arise, whose enzymatic inactivation may also contribute to ill health [1–3]. Although OP pesticides constitute a generic compound class by virtue of their binding and inactivation of AChE, disparate binding and structure–activity relationships may well exist for secondary protein targets. Moreover, OP secondary targets may impact upon health status at OP exposures at or below the 30% inhibition of brain AChE that can trigger signs of cholinergic toxicity [2,4–9]. Additionally, secondary (blood) protein targets may provide useful adjuncts to measurements of cholinesterase inhibitions or analysis of urinary metabolites for pesticide exposure biomonitoring [10], and also provide bioscavengers capable of depleting toxic OPs [11].

Toxicologically relevant pesticide secondary targets can be evaluated systematically by analysis of pesticide structure–activity profiles, but this can only be accomplished if the tissue activity of a given serine hydrolase is known. An alternative approach has been to interrogate tissues directly for active serine hydrolases using compounds retaining an OP chemical signature, for example, affinity ligands that possess a fluorophosphonate (FP) probe [12–15]. As an alternative, we and others have utilised the OP compound diisopropylfluorophosphate (DFP) for which a tritiated version is commercially available, to provide a means to track active serine hydrolases by radiolabelling and autoradiography [16–20].

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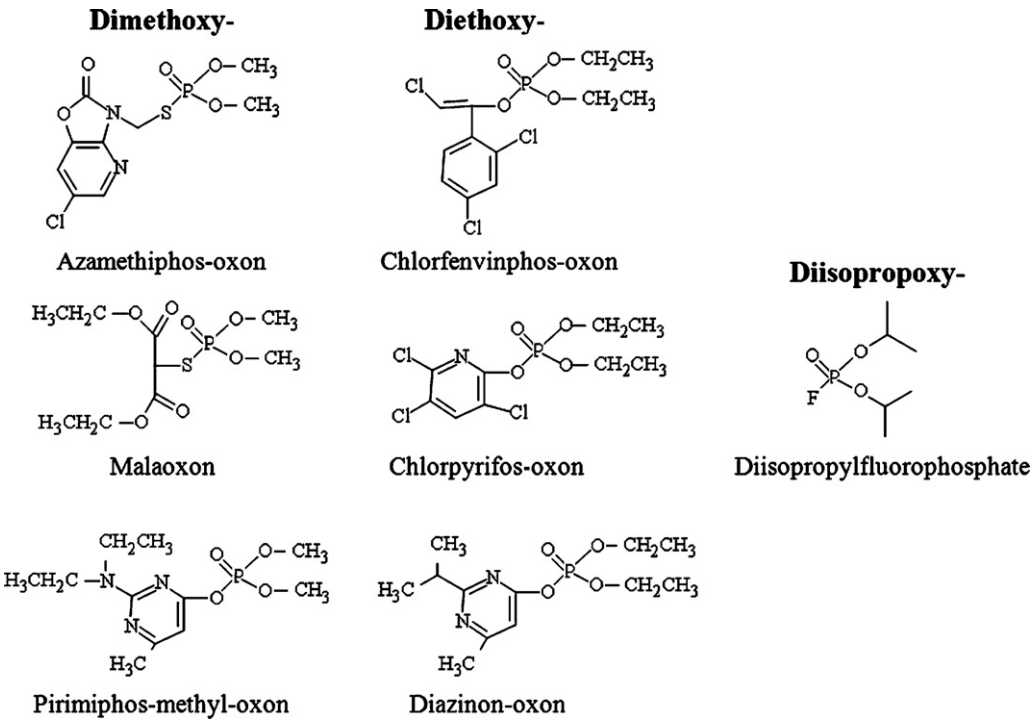

Fig. 1. Structures of the pesticides examined, drawn as their active oxons, and divided into dimethoxy-, diethoxy-, and diisopropoxy-forms.

Our studies have focussed upon brain tissue targets of pesticides (neurotoxicity), and tissues of the immune system and blood (immunotoxicity), since accumulating evidence suggests that the immune and neuro-immune system also represent important targets of OP toxicity [21,22]. In this paper we describe the use of ^{3}H-DFP to quantify active serine hydrolases within immune and brain tissues, and a strategy for detecting which of these active hydrolases is also a target of pesticide binding. This strategy also provided a means of purification, characterisation, and ultimately identification of pesticide targets.

2. Experimental

2.1. Materials

The organophosphorus pesticides: azamethiphos-oxon (*S*-6-chloro-2,3-dihydro-2-oxo-1,3-oxazolo[4,5-*b*]pyridin-3-ylmethyl *O*,*O*-dimethyl phosphorothioate), chlorfenvinphos-oxon (2-chloro-1-(2,4-dichlorophenyl)vinyl diethyl phosphate), and malathion (diethyl (dimethoxyphosphinothioylthio)succinate) and its corresponding oxon (malaoxon) were purchased from QMX Laboratories Ltd., Thaxted, UK, and were at 95–99.5% purity. Chlorpyrifos (*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate) and diazinon (*O*,*O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate) as their corresponding oxons, and pirimiphos-methyl (*O*-2-diethylamino-6-methylpyrimidin-4-yl *O*,*O*-dimethyl phosphorothioate) were purchased from Greyhound Laboratories, Birkenhead, UK, at 97.2–99.4% purity. For *in vitro* assays, pesticides were prepared as 100 mM stock solutions in ethanol (Sigma, HPLC grade, <0.10% water), except azamethiphos-oxon which was at 50 mM, and were stored at 4 °C for up to 2 weeks. Pesticides were diluted in phosphate buffered saline (PBS) to required concentrations just prior to use. Tritiated-diisopropylfluorophosphate (^{3}H-DFP) at a specific activity of 150 GBq/mmol was purchased from Perkin Elmer, Boston, USA. NuPAGE Novex pre-cast gels (4–12% Bis–Tris gels for 1D SDS–PAGE and 4–12% Bis–Tris Zoom gels for 2D-PAGE), MOPS–SDS running buffer, transfer buffer, SeeBlue Plus2 prestained gel standards, and Safe stain were all obtained from the Invitrogen Corporation. Precision plus prestained protein standards, and IPG strips (pH 3–10, 7 cm length) were purchased from BioRad, with all isoelectric focussing performed using a BioRad Protean isoelectric focussing cell. Dithiothreitol (DTT), wide range molecular weight markers, ρ-nitrophenyl acetate, gel filtration molecular weight standards, and recombinant human AChE (C1682) were all purchased from Sigma.

2.2. Blood and tissue preparations

Male F344 strain rats weighing between 200 and 230 g were used for experiments. Rats were maintained in cages (four/cage) under controlled temperature (21 ± 1 °C) and light (16 h light/8 h dark cycle) with *ad libitum* access to food intake and water. All animal procedures were approved by the University of Nottingham Local Ethical Review Committee and were carried out in accordance with the Animals Scientific Procedures Act (UK) 1986. Rats were dosed orally by gavage with OPs in arachis oil. Rat blood, and whole tissue and cytosolic extract were prepared according to previous publications [19,20]. Control human blood (taken with University of Nottingham Ethical Review Committee approval) from one of the authors (male, 41 years of age) was collected into heparin and erythrocytes and plasma prepared as described previously [20]. Protein concentrations in homogenates were measured using the DC Protein assay (Biorad) using bovine serum albumin as a protein standard.

2.3. Acetylcholinesterase measurements

Blood or tissue AChE activity measurements were based upon the spectrophotometric method described by Ellman et al. [23]. Spectrophotometric measurements were performed at 412 nm in a Perkin Elmer Lambda 2S spectrophotometer operated using UV KinLab software as described previously [20].

2.4. ^{3}H-DFP radiolabelling of tissues and quantitation

Rat tissue homogenate or cytosolic extract was incubated with 24 μM ^{3}H-DFP for 60 min at 37 °C. Radiolabelled protein was precipitated with acetone:diethyl ether (2:1) (v/v) on ice and the precipitate retained by centrifugation at 5000 rpm for 3 min. The precipitate was washed with 1 ml of ether:industrial methylated spirit:water (10:7:2) (v/v/v), and recovered by similar centrifugation. Four further washes were used to remove extraneous ^{3}H-DFP. The precipitate was air-dried and then dissolved in 100 μl of solubilisation buffer: 40 mM Tris, 9.8 M urea, 4% CHAPS (w/v), 50 mM DTT, pH 8.0. Ten microliters of solubilised extract was added to 10 ml of scintillation fluid, and the radioactivity incorporated quantified by liquid scintillation counting using a Packard Tri-Carb 2100 counter.

2.5. Protein separation

For one-dimensional SDS–PAGE, whole tissue homogenate, cytosolic extracts, or recombinant human AChE were incubated with either pesticide or PBS as solvent for 20 min at room temperature. Proteins were then radiolabelled by incubation with 24 μM ^{3}H-DFP (final concentration) for 1 h at 37 °C. Proteins were heated for 10 min at 70 °C in sample buffer (Novex) containing 100 mM DTT, and then resolved by gel electrophoresis through NuPAGE Novex 4–12% Bis–Tris gels run with 3-(N-morpholino)propanesulfonic acid (MOPS) buffer in an X Cell surelock gel tank (Invitrogen). Proteins were either stained with colloidal Coomassie blue overnight (Invitrogen) or stained with silver (Amersham PlusOne kit) according to the manufacturer's instructions, and then photographed using a Fugi E900 digital camera. Alternatively, resolved proteins were electroblotted at 80 V for 2 h onto a polyvinylidene difluoride (PVDF) membrane (Millipore) using NuPage Transfer buffer. The PVDF membrane was air-dried overnight at room temperature to fix the proteins, and then stained for 30 min with Coomassie Blue (Safestain) to visualise the proteins and ensure even protein transfer across the blot. PVDF membranes were then destained with 50% methanol (v/v), 10% acetic acid (v/v), washed with PBS, and proteins cross-linked as described previously [19]. Protein cross-linking was performed to limit protein loss and radioactive contamination of the microchannel detector under the high vacuum conditions used for autoradiography.

For two-dimensional (2D)-PAGE, 600 μg of protein was used for each individual 2D-PAGE analysis, radiolabelled with 24 μM ^{3}H-DFP (final concentration), and then proteins resolved in two dimensions according to a previous publication [24]. 2D-PAGE separated proteins were transferred to a PVDF membrane and autoradiographed.

2.6. Autoradiography

Approximately 0.25 μl of 100 KBq ^{14}C-amino acid mixture (1.85 MBq/ml, Amersham) was applied to the PVDF blot at the positions of the molecular weight markers to provide an estimation of the molecular weight of ^{3}H-DFP radiolabelled proteins after autoradiography. Blots were subjected to 24 h of autoradiography within a microchannel plate detector, an autoradiographic device that provides high sensitivity (detection capability of 6 dpm/mm^{2}), good XY spatial resolution (70 μm), and signal production in real time which is linear over six orders of magnitude [25]. Radiolabelled protein bands in autoradiographic images were quantified using Quant scan software (Beta autoradiographic image acquisition software), with band intensities (in pixels) plotted using excel to determine relative radiolabel incorporations.

2.7. Column chromatography

Column chromatography was performed using an FPLC system (Amersham). A MONO Q anion exchange column (HR 5/5) was equilibrated with 50 mM Tris/HCl, pH 8.0 (Buffer A) at a flow rate of 1 ml/min. Thymus cytosol was diluted 1:1 with Buffer A and then loaded onto the column at this flow rate. Protein was eluted with a linear gradient of 0–1 Molar sodium chloride in Buffer A collecting twenty 1 ml fractions.

A Superose 12 size exclusion column was equilibrated with 50 mM Tris/HCl, pH 8.0, 100 mM sodium chloride, 1 mM DTT at a flow rate of 0.4 ml/min. MONO Q concentrate was loaded onto the column at this flow rate and 280 μl fractions collected. The protein standards, β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa), were passed through the Superose 12 column to calibrate it for molecular weight determination.

2.8. Matrix assisted laser-desorption ionization-time of flight (MALDI-TOF) mass spectrometry

Silver stained protein bands from 1D PAGE of Superose 12 column fractions were excised and transferred into a 96-well plate using an automated MassPrep robotic system (ProteomeWorks, Bio-Rad). Gel pieces were destained, reduced with DTT, alkylated with iodoacetamide, and then proteins digested *in situ* with trypsin. Tryptic peptides were desalted by binding and then elution from a C18 Zip-tip (Millipore), and then mixed with α-cyano-4-hydroxycinnamic acid (Sigma, C-2020) matrix, and peptides analysed by MALDI-TOF mass spectrometry, using a Micromass MALDI (Waters, UK). The masses of intact, singularly charged peptides were used in a search algorithm (MASCOT peptide mass fingerprint) to screen protein databases, such as SwissProt to determine mass matches and enable protein identification [26].

2.9. Esterase assays

Twenty microliters from a MONO Q column fraction was diluted to a final volume of 190 μl with 50 mM Tris–HCl, pH 8.0. Ten microliters of ρ-nitrophenyl acetate was added (final concentration 1 mM) and the liberation of ρ-nitrophenol monitored at 400 nm for 5 min at 37 °C using a Molecular Devices Spectramax plate reader run with SoftmaxPro software. Esterase activity rates were all linear over the 5 min assay period, and were quantified as rate of formation of ρ-nitrophenol/minute.

3. Results and discussion

Our studies have focussed upon pesticides commonly deployed within the UK: azamethiphos, chlorfenvinphos, diazinon, malathion, and pirimiphos-methyl, and also chlorpyrifos which has widespread use within the USA. Pesticides as thions undergo *in vivo* liver bioactivation to their corresponding active oxons, thus for our *in vitro* studies we have used pesticides directly as their oxon forms (Fig. 1). Studies with pirimiphos-methyl were always undertaken *in vivo* since a commercial oxon for *in vitro* assays was not available.

In the absence of commercially available radiolabelled pesticides, definitive pesticide–protein targets are difficult to assign. We have employed the OP compound DFP to investigate protein adducts, since tritiated DFP is available for purchase. Although DFP is susceptible to hydrolysis after prolonged (24 h) incubation in aqueous solution [27], short-term incubation (1–4 h) with ^{3}H-DFP produces stable ^{3}H-DFP-protein binding. Furthermore, the leaving group of DFP is a fluoride ion which confers relatively high reactivity

Table 1
Summary of the major pesticide targets in rat thymus and brain tissues. Proteins radiolabelled with ^{3}H-DFP were resolved by 1D PAGE and characterised by their denatured molecular weight. *In vitro* radiolabelled proteins specific for thymus tissue (t) or brain tissue (b) are indicated. The symbol (+) denotes a significant pesticide adduction when the corresponding level of tissue AChE inhibition was approximately 30%; a (−) denotes no significant adduction; (+/−) constitutes equivocal adduction due to protein band heterogeneity. Pesticide adduction results were from *in vitro* studies except for pirmiphos-methyl, for which all data was from *in vivo* rat dosing. The number of experiments performed with each pesticide was at least eleven.

Pesticide	Pesticide adduct target (kDa)						
	90	82	74	66^t	60^t	34, 32, 30^b	28
Azamethiphos-oxon	–	–	–	–	–	–	+/–
Chlorfenvinphos-oxon	–	–	–	+	+	–	+/–
Chlorpyrifos-oxon	–	–	–	+	+	–	+/–
Diazinon-oxon	–	–	–	+	+	–	+/–
Malaoxon	–	–	–	–	–	–	+/–
Pirimiphos-methyl	–	–	–	+	+	–	+/–

potential, and DFP also exploits the relative resistance to hydrolysis of a diisopropoxy adduct [27–30].

We have used ^{3}H-DFP binding to tissue homogenates to quantitate the level of active serine hydrolases, and also a means to visualise protein targets by autoradiography. Both thymus and brain cytosolic protein extracts bound ^{3}H-DFP to approximately 1800 and 1000 pmol of DFP/gram respectively; radiolabel incorporation that reflected an overlap of active serine hydrolases when visualised by autoradiography after protein separation by one-dimensional SDS–PAGE (Fig. 2A, left panel).

In order to determine which ^{3}H-DFP protein targets were also specific pesticide targets *in vitro* or *in vivo*, tissue extracts were preincubated with unlabelled test pesticide or rats were dosed with individual pesticides to bind some (or all) of the available active site molecules, and then tissue extracts were radiolabelled with an excess of ^{3}H-DFP. Pesticide adduction resulted in a lowering of subsequent ^{3}H-DFP radiolabelling, and this was visualised and quantified by autoradiography (Fig. 2A, right panel). Additionally, the level of tissue AChE inhibition was also simultaneously measured for each *in vitro* or *in vivo* analysis. This provided a correlation of the relevance of specific protein adductions to the dose used, which enabled us to focus upon significant (sensitive) protein adductions evident at relatively low or moderate dose exposures; at approximately 30% inhibition of AChE, which is sub-symptomatic of cholinergic toxicity [4]. In addition to their tissue-specific expression, active serine hydrolases also displayed differential pesticide binding character, with some pesticides able to adduct certain proteins but not others. For example, dosing of rats with pirimiphos-methyl resulted in the adduction of thymus proteins at molecular weights of 66, 60, and 28 kDa (marked with arrowheads in Fig. 2A, right panel). A summary of our *in vitro* and *in vivo* protein targets for thymus and brain pesticide adductions are included as Table 1.

Our strategy for identifying pesticide targets did have limitations since only protein targets that are sensitive to both ^{3}H-DFP binding and the assayed pesticide will be detected. There may be proteins that are pesticide specific targets that react relatively slowly (or not at all) with ^{3}H-DFP. However, since ^{3}H-DFP possesses a relatively small (lacking steric constraints) and reactive fluoride leaving group it would be expected to react rapidly and promiscuously with serine hydrolase target proteins.

Additionally, this approach to pesticide target characterisation requires quantitative autoradiography to track hydrolase activity. Since serine hydrolases such as AChE bind OPs stoichiometrically, the binding of OP and hence reduced subsequent ^{3}H-DFP adduction should linearly correlate with the autoradiographic signal, and concomitant inhibition of hydrolase activity. We validated this sup-

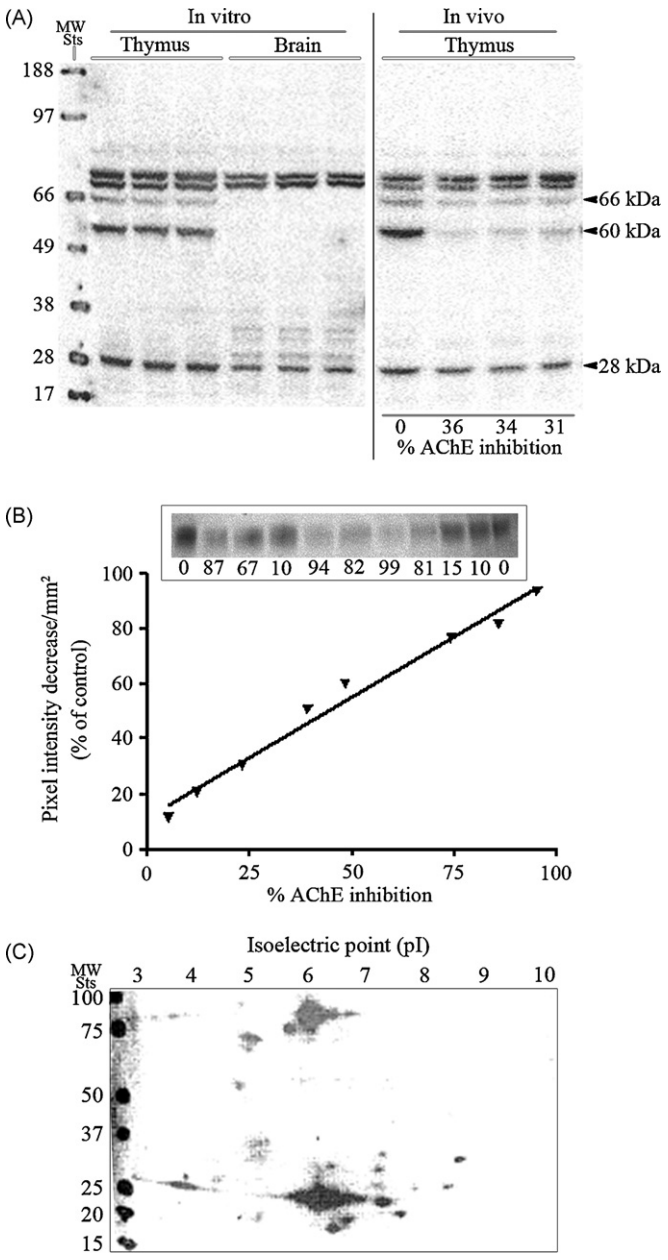

Fig. 2. (A) ^{3}H-DFP incorporation into rat thymus and rat brain cytosolic protein extracts: protein resolution by 1D PAGE. Rat thymus or brain cytosolic protein extracts were radiolabelled with ^{3}H-DFP *in vitro*, proteins resolved by 1D PAGE, and visualised by autoradiography (left panel). Male F344 rats were dosed with arachis oil only (controls) or dosed with pirimiphos-methyl at 354 mg/kg (25% of LD_{50}). Thymus cytosolic proteins were radiolabelled with ^{3}H-DFP, resolved by 1D PAGE, and autoradiographed. The tissue AChE inhibition of each dosed rat was also measured (zero for control rats). Thymus protein pesticide targets at 66, 60, and 28 kDa are marked with arrowheads (right panel). (B) Relationship between the percentage of AChE inhibition after incubation with OP, and corresponding decrease in ^{3}H-DFP binding. Recombinant human AChE was preincubated with OP prior to radiolabelling with ^{3}H-DFP. The level of OP-induced AChE inhibition was monitored using the Ellman assay. The ^{3}H-DFP radiolabelled AChE was resolved by 1D PAGE and the level of radioactivity incorporated determined by quantitative autoradiography. The reduction in ^{3}H-DFP incorporation after OP preincubation is plotted against the percentage of AChE inhibition. The insert panel shows an example of a 1D PAGE autoradiograph of AChE after preincubation with test pesticides and subsequent radiolabelling with ^{3}H-DFP. The level of AChE inhibition generated from OP preincubation is listed under its corresponding gel lane. All pesticides used for tissue *in vitro* assays were demonstrated to inhibit recombinant AChE. Measurements of recombinant AChE inhibitions and quantitative autoradiography were performed for a total of forty-four analyses, from which representative values across a range of AChE inhibitions are shown. (C) ^{3}H-DFP incorporation into rat thymus cytosolic protein extracts: protein resolution by 2D-PAGE. Rat thymus cytosolic proteins were radiolabelled with ^{3}H-DFP, resolved by 2D-PAGE, and autoradiographed.

position using recombinant human AChE. Recombinant AChE was preincubated with a range of OP concentrations prior to ^{3}H-DFP radiolabelling and autoradiography. Simultaneous determination of the level of AChE inhibition was performed. Fig. 2B shows that the level of OP binding to AChE (measured via a reduction in subsequent ^{3}H-DFP incorporation) increased proportionately to AChE inhibition. The slope of the linear relationship was 0.88 ± 0.5, with an R^2 value of 0.98. The graph insert shows an example of recombinant AChE resolved by 1D PAGE after preincubation with test pesticides, with the level of AChE inhibition listed under each gel lane. The level of AChE inhibition increased in proportion to a decline in ^{3}H-DFP radiolabelling.

Another shortcoming in characterisation of protein–pesticide adducts by 1D protein separation is the potential of protein bands to be heterogeneous in nature. If a protein band is comprised of both OP adducted and ^{3}H-DFP radiolabelled but non-adducted protein(s), pesticide binding may be masked by the presence of the unbound yet still radiolabelled protein(s). To reduce the likelihood of this possibility, we have also resolved pesticide protein adducts by 2D-PAGE. Proteins were initially separated in the first dimension by isoelectric focussing within immobilised pH gradient strips, and then resolved in the second dimension by denaturing SDS–PAGE. Isoelectric focussing was performed using the pI range of 3–10 to provide broad isoelectric point coverage. Fig. 2C shows thymus cytosolic protein radiolabelled with ^{3}H-DFP and then resolved by 2D-PAGE, and autoradiographed. The 26–28 kDa protein band was typically resolved into 15 reproducible protein spots after 2D-PAGE, but only some of these protein spots were responsive to pesticide adduction. Current studies are underway to characterise all of the pesticide sensitive protein spots, and ultimately identify these protein(s).

An alternative method of detecting active serine hydrolases within cells and tissues has utilised a fluorophosphonate (FP) probe whose biotinylated tag also affords a means of purification of associated proteins [12–15]. This approach has also proved fruitful for detecting and subsequently purifying pesticide and nerve agent targets, some of which overlap with the proteins we have characterised or identified using our methodology [12–20]. However, FP-probes also have limitations arising from steric constraints, non-specific protein binding, limited lipophilic character, and also the functional OP motif may require modification to provide a suitable OP chemical signature. Nevertheless, it is likely that a range of probes, and radiolabelling techniques, will collectively provide useful and overlapping protein adduction information in future studies.

Protein radiolabelling with ^{3}H-DFP is a sensitive method for characterising pesticide targets when coupled to protein resolution by either 1D- or 2D-PAGE. However, radiolabel incorporation is more sensitive than the nanogram levels of protein detected with protein Coomassie or silver staining for subsequent mass spectrometry protein identification. Hence a method of further protein enrichment is generally required to enable protein identification.

To concentrate pesticide targets to provide further protein characterisation and/or identification by mass spectrometry, conventional column chromatography was employed. Thymus cytosolic protein extract was pumped onto a MONO Q anion exchange column, and bound protein eluted with a linear gradient between 0 and 1 M sodium chloride, collecting twenty 1 ml fractions. An anion exchanger was used for protein concentration since cytosolic proteins are generally predicted to have a pI that is negative at physiological pH [31], and hence retained by interaction with a positively charged matrix. Twenty microlitres from each column fraction was either resolved by SDS–PAGE and proteins visualised by staining with colloidal Coomassie blue (Fig. 3, upper left panel), or radiolabelled with ^{3}H-DFP, and radiolabelled proteins visualised by autoradiography (Fig. 3, upper right panel). The major ^{3}H-DFP radiolabelled proteins of interest were recovered from column elution fractions 4 to 10, hence elution fractions 1, and 11–20 have not been included.

Peak elution of the 60 kDa pesticide target was fraction 5 (250 mM sodium chloride), fractions 6 and 7 (300–350 mM sodium chloride) for the 66 kDa pesticide target, and relatively dispersed from fractions 5 to 10 (300–500 mM sodium chloride) for the 26–28 kDa pesticide target band. Noteworthy was that chromatographic enrichment also revealed additional ^{3}H-DFP binding proteins (and/or posttranslational modified forms of the aforementioned pesticide targets), such as the ^{3}H-DFP protein targets at approximate molecular weights of 20 kDa (visible in fraction 5), 32 kDa (fraction 6), 50 kDa (fractions 6–8), 58 kDa (fraction 8), and 45, 90, and 100 kDa (fraction 10). Future work will be directed toward further purification of these ^{3}H-DFP radiolabelled proteins, and an evaluation of their pesticide binding characteristics undertaken.

To validate that the 60 and 66 kDa ^{3}H-DFP radiolabelled proteins still retained an active site responsive to pesticide adduction, column fractions were incubated with an adducting pesticide, chlorfenvinphos-oxon, at a concentration/time known to produce approximately 30% inhibition of tissue AChE activity; a level of inhibition which was simultaneously validated using recombinant AChE (results not included). Chlorfenvinphos-oxon produced clear adduction of both the 60 and 66 kDa proteins, whereas the proteins recovered within the molecular range of 26–30 kDa were not adducted by this pesticide (Fig. 3 lower left panel). Additional *in vitro* analysis of these column fractions reiterated previous tissue findings that chlorpyrifos-oxon, and diazinon-oxon also adducted both proteins at AChE IC_{30} concentrations, whereas azamethiphos-oxon and malaoxon did not elicit significant adduction (results not included).

We also examined the MONO Q fractions for esterase activity using ρ-nitrophenyl acetate as a substrate. Esterase activity followed the ^{3}H-DFP radiolabelling distribution of the 60 kDa protein; such that it was first detected in fraction 4, peaked in fraction 5, and then declined over fractions 6–12. Esterase peak shoulders between fractions 6 and 7 and to a lesser extent 8 and 9 also indicated the presence of additional esterase activity within these fractions (Fig. 3 lower right panel).

To further purify the 60 and 66 kDa pesticide targets, and to characterise their native molecular weights, the major MONO Q eluting fractions containing each protein were concentrated and subjected to size exclusion chromatography on a Superose 12 column. MONO Q fraction 5 retained the majority of the 60 kDa protein. This fraction from five MONO Q runs was pooled and concentrated to 200 μl using a microconcentrator (Amicon), and loaded onto a Superose 12 column that had been previously calibrated with the passage of proteins of known molecular weight. The 60 kDa protein began to elute from the Superose 12 column in fraction 36 corresponding to a molecular weight of approximately 180 kDa; indicative of a native trimer. By overlaying the autoradiograph of this protein with the silver staining of column fractions, a co-distribution of protein stain with ^{3}H-DFP radiolabelling was apparent, and has been marked with arrowheads in Fig. 4 upper panels.

Collectively, a protein of denatured molecular weight of 60 kDa, having a pI in the range of 5–6 (Fig. 2C), and displaying native trimeric binding, and esterase activity sensitive to DFP adduction are all recognised characteristic properties of carboxylesterase 1 (CES1) [32]. We therefore propose that the 60 kDa pesticide target that we have purified is CES 1, and this concurs with studies that have identified an FP-probe binding carboxylesterase in other immune cells and tissues [14,15,33].

Carboxylesterases can act prophylatically to counter OP toxicity by stoichiometric binding to OPs and/or actively hydrolysing them. The low level of protein visualised using silver stain-

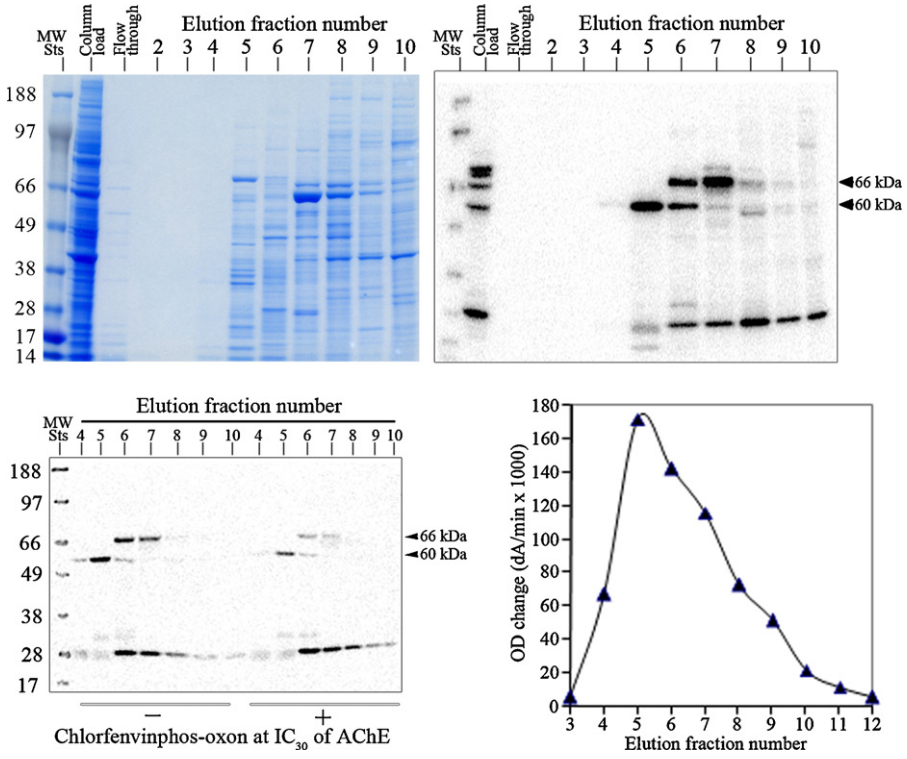

Fig. 3. Purification and characterisation of pesticide targets using MONO Q anion exchange chromatography. Thymus cytosolic proteins were passed onto a MONO Q column and then eluted with an increasing salt gradient between 0 and 1 M sodium chloride collecting twenty 1 ml fractions. Twenty microliters from fractions 2 to 10 were removed and proteins stained with Coomassie blue (upper left panel) or radiolabelled with ^{3}H-DFP and then autoradiographed (upper right panel). Peak column elution fractions 4–10 were either preincubated with PBS or chlorfenvinphos-oxon at a concentration/time producing approximately 30% inhibition of AChE, prior to radiolabelling with ^{3}H-DFP. Radiolabelled proteins were resolved by 1D PAGE and autoradiographed. Chlorfenvinphos-oxon adduction of 66 and 60 kDa proteins are marked with arrowheads (lower left panel). Esterase activity within MONO Q fractions 3–12 was measured using ρ-nitrophenol acetate as substrate (lower right panel) (error bars were below the levels of tick-marks). All images are representative of 4–5 experiments.

ing despite relatively significant ^{3}H-DFP incorporation indeed suggest stoichiometric binding of OPs to this carboxylesterase. Recently, Crow et al. [33] reported that human CES1 is probably cholesteryl ester hydrolase (CEH), an enzyme responsible for cholesterol ester hydrolysis to yield free cholesterol for export out of macrophages. These authors show that CES1 is a resident protein of human immune cells and can be inhibited with chlorpyrifos-oxon, paraoxon, or methyl paraoxon, at nanomolar IC_{50} values. Our novel results suggest CES1 (or a highly homologous enzyme) also displays a high sensitivity of inhibition by chlorfenvinphos-oxon, and diazinon-oxon, but not azamethiphos-oxon or malaoxon *in vitro*, and that this enzyme can be inhibited by pirimiphos-methyl *in vivo* under conditions producing approximately 30% inhibition of tissue AChE. The inferred functional significance of pesticide inhibition of CES1 activity is to interfere with cholesterol export, a possible contributory factor in the development of atherosclerosis [33]. Further investigation is warranted to evaluate whether OP inhibition of CES1 activity could constitute a mechanism of ill health following chronic low-dose pesticide exposure.

The MONO Q peak fractions of the 66 kDa pesticide target (fractions 6 and 7) were concentrated and then subjected to size exclusion chromatography using the Superose 12 column (Fig. 4, lower panels). Radiolabelled protein began to elute at fraction 40 corresponding to a protein molecular weight of 66 kDa, indicative of a monomeric protein. A relatively abundant silver stained protein that co-distributed with ^{3}H-DFP radiolabelling through fractions 40–44 was visible and has been marked with arrowheads in Fig. 4. This protein was excised and mass analysed, and identified as serum albumin – refer to Supplemental Data [19,20]. Interestingly, unlike the 60 kDa carboxylesterase, the level of protein staining was relatively high to that of ^{3}H-DFP radiolabelling, indicative of low adduction stoichiometry.

To evaluate the functional significance of protein–pesticide adduction the stoichiometry of binding needs to be determined since it will govern what proportion of active proteins are affected. With radiolabelling methods this requires separation of incorporated from unincorporated radiolabelling agent. For albumin within rat or human plasma we have used retention of albumin on charged filters, with a filtration volume of 50 ml used to remove extraneous ^{3}H-DFP. Pesticide adduction of plasma albumin molecules was quantified as approximately 0.5% of molecules in rat and 1% in humans [20].

The stoichiometry of pesticide adduction may also reflect more than one binding site. Historically, the methodology used for identification of a site(s) of phosphorylation has involved protein *in vitro* or *in vivo* radiolabelling, followed by enzymatic digestion, chromatographic separation of the radiolabelled peptide fragments, and posttranslational modification site assignment via localisation of radioactivity to an identified peptide fragment, or residue after radiosequenation [34,35]. However, with the advent of sensitive mass spectrometry techniques, if the adduction binding site is known, mass spectrometry can be focussed upon a region that encompasses the modified amino acid, and used to detect the mass change arising from a specific OP adduction [36–40].

The influence of pesticide binding on protein activity will also be governed by the stability of the pesticide adduct which may persist as a bound inhibitor for the lifetime of the protein until it is either turned over or actively degraded. This 'permanent' inhibition may arise if the adduct undergoes side chain dealkylation (ageing). Alternatively, the protein may recover from inhibition after spontaneously hydrolysis of the adduct bond. For a pesticide adduct this

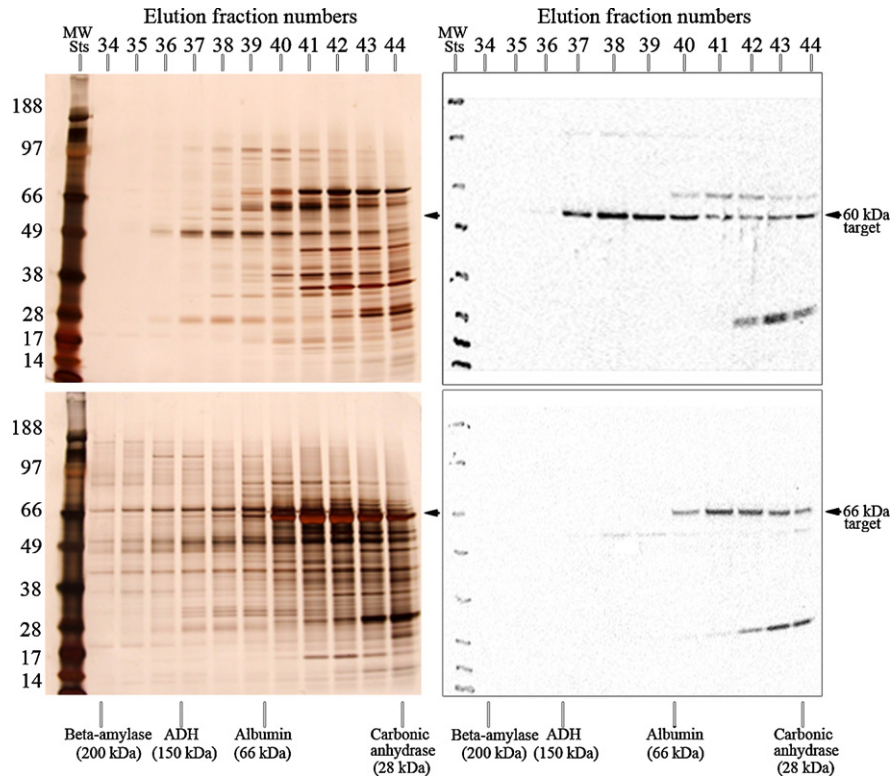

Fig. 4. Purification and characterisation of pesticide targets using Superose 12 size exclusion chromatography. Thymus cytosol MONO Q peak fractions were pooled, concentrated, and then resolved by size exclusion chromatography on a Superose 12 column. The column was run at a flow rate of 0.4 μl/min collecting 280 μl fractions. Twenty microliters from each fraction was resolved by 1D PAGE and proteins stained with silver (left panels), or radiolabelled with ^{3}H-DFP and autoradiographed (right panels). Co-distribution of protein staining with ^{3}H-DFP radiolabelling for the 60 and 66 kDa pesticide targets are marked with arrowheads. The elution points of proteins of known molecular weight that were used to calibrate the column are also included.

warrants study since it not only affects the influence of the pesticide on the active site of the protein, but also its potential usefulness as a biomarker of pesticide exposure [20].

The relatively low stoichiometry of adduction of albumin with OPs suggest that adduction would only have a limited influence on the circulatory drug or fatty acid binding that occurs at the same region of albumin, however, the lack of ageing and persistence of an intact adduct suggest albumin retains usefulness as a biomonitor of certain pesticide exposures [14,19,20]. Furthermore, given the relatively high albumin content within blood, albumin binding may provide protective OP detoxification via OP binding and/or OP hydrolysis [41].

4. Summary and conclusions

OPs covalently adduct AChE, a member of the serine hydrolase family of enzymes, but also other members of this hydrolase family are susceptible to adduction, potentially altering their function. Here we describe methodology for detecting and resolving these secondary protein–pesticide targets, their purification and characterisation, and evaluation of the stoichiometry and site of pesticide binding. Our results detail that secondary targets display differential pesticide binding character, and therefore suggest their toxicity evaluation should be undertaken on a compound-by-compound basis. Secondary targets may provide useful adjuncts to cholinesterases as biomonitors of pesticide exposures, particularly if significant protein adduction occurs at exposure levels close to the threshold of cholinesterase monitoring. By providing a panel of biomonitors of exposure, secondary targets could provide an insight into exposure to a particular pesticide or mixture of pesticides. This could assist clinical and epidemiological analyses, particularly those associated with longitudinal low-dose pesticide exposures. Furthermore, secondary targets also have potential to provide novel means to counter OP toxicity by their use as surrogate targets (bioscavengers) of OP binding and/or OP hydrolysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.10.018.

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