Production of a Recombinant Anti-Parathion Antibody (scFv); Stability in Methanolic Food Extracts and Comparison to an Anti-Parathion Monoclonal Antibody

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A recombinant single-chain variable fragment (scFv) antibody, specific for the organophosphate pesticide parathion-ethyl, was produced from genetic material obtained from a hybridoma cell line secreting an anti-parathion monoclonal antibody (Mab). The scFv and parent Mab showed similar, but not identical, characteristics in a microtitration plate enzyme-linked immunosorbent assay (ELISA). Limits of detection for the Mab and scFv-based ELISAs of 1.6 and 2.3 ng/well, respectively, were observed. Cross-reactions of other structurally similar organophosphates were similar in the two assays; highest cross-reactions were observed with parathion-methyl and fenitrothion (both giving 6% and 4% values for the Mab- and scFv-based assays, respectively). Performances of the assays in the presence of methanolic extracts of rice and orange peel were compared; the scFv demonstrated similar stability to the Mab at methanol concentrations up to 5% (v/v). At higher concentrations (above 10%, v/v) performance of both assays deteriorated, though the Mab-based assay was less affected than the scFv-based assay. ScFvs promise to be as useful as Mabs for the analysis of food samples by ELISA.

Keywords: *Recombinant antibody; parathion; ELISA; food; monoclonal antibody*

INTRODUCTION

Immunoassays are now routinely used in the rapid, cost-effective identification and detection of agri-food components and contaminants, ranging from low molecular weight analytes to pathogenic microorganisms. Low molecular weight targets, such as pesticides, test the power of the technology in that they are difficult subjects for antibody production, yet need to be detected with great sensitivity and specificity. The sensitivity and specificity observed in immunoassays is inherent to the properties of the antibody reagents used in the assays.

Over recent years, information on immunoglobulin structure and antibody binding, combined with novel recombinant DNA techniques, has led to the production of antibodies in *Escherichia coli* (Ward *et al.*, 1989) and other organisms (Hiatt *et al.*, 1989; Putlitz *et al.*, 1990; Nyyssonen *et al.*, 1993; Ueda *et al.*, 1993). These recombinant antibodies offer potential advantages such as speed of production, ability to modify properties through mutagenesis, and information on antibody−target interaction. The successful use of such technology would enable the production of novel antibodies that are more (or less) specific or show higher (or lower) affinities to related molecules as desired. Perhaps more significantly, it is technically possible to generate large semisynthetic antibody libraries that offer the potential to derive antibodies *in vitro* which cannot be derived *in vivo* (Marks *et al.*, 1991, 1992). The libraries utilize a phage-display system in which the antibodies are fused to an outer coat protein of an *E. coli*-infecting bacteriophage (McCafferty *et al.*, 1990; Barbas *et al.*, 1991). Successive rounds of phage selection with target ligand and reinfection of bacteria lead to enrichment of phage-bearing target-specific antibodies. Until recently, the antibodies derived from such libraries were of low affinity and, therefore, not that useful for purposes such as immunoassay (Karu *et al.*, 1994). However, the generation of much larger libraries with randomized antibody binding sites may offer new possibilities (Griffiths *et al.*, 1994; Vaughan *et al.*, 1996).

It is because of the difficulty in generating high-affinity antibodies from libraries that we, and others, have decided to use hybridomas secreting relevant monoclonal antibodies (Mab) as the starting point for recombinant antibody work. Recombinant antibody fragments have been produced this way by other groups against the pesticides atrazine (Ward *et al.*, 1993, Byrne *et al.*, 1996), *s*-triazines (Kramer and Hock, 1996), and diuron (Bell *et al.*, 1995).

Parathion belongs to the organophosphorus group of pesticides which deactivate the enzyme acetylcholinesterase. The continued worldwide use of parathion as an insecticide necessitates the development of immunoassays with high sensitivity and specificity for monitoring of agri-food and environmental samples (Ibrahim *et al.*, 1994). As with all small molecules, the pesticide must first be conjugated to a carrier protein to generate an immune response. A sensitive radioimmunoassay has been developed by Ercegovich *et al.* (1981) in which parathion was coupled to protein through the nitro group using diazotization. They produced a polyclonal antiserum that was specific for parathion-ethyl and used it to measure pesticide levels in fortified plasma and plant tissues. Ibrahim *et al.* (1994) produced a monoclonal antibody to parathion using the same conjugation procedure for the immunogen and coating conjugate. The enzyme-linked immunosorbent assay (ELISA) developed using this Mab was capable of detecting parathion-ethyl down to 1 ng/mL in water and milk,

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showing significant cross-reactivity only with parathion-methyl (50%). A commercially available kit for parathion (EnviroGard Parathion Plate Kit; Ensys, Four Marks, U.K.) uses a polyclonal antiserum that recognizes parathion-methyl and parathion-ethyl almost equally well. The assay has limits of detection of 0.03 and 0.05 ng/mL, respectively, for the two pesticides and has been validated for application to water. The antiserum was produced using an immunogen coupled through a methyl group on the phosphate moiety (Ferguson *et al*., 1995; Skerritt and Lee, 1996). In this study we have employed the diazotization conjugation procedure, coupling parathion-ethyl to bovine serum albumin (for the plate coating conjugate and the immunogen). Using this conjugate, both a monoclonal-based and an scFv-based ELISA have been developed and applied to the detection of parathion-ethyl in methanolic extracts of rice and orange peel.

MATERIALS AND METHODS

Preparation of Parathion−Protein Conjugate. Parathion-ethyl was coupled to bovine serum albumin (BSA) by diazotization according to the method of Ercegovich *et al*. (1980). Reduced parathion (70 mg), produced using zinc powder and HCl, was dissolved in HCl (25 mL, 0.1 M), and sodium nitrite (0.1 M) was added dropwise until a positive starch−iodide test was obtained. An aliquot of the reaction mixture (7 mL) was added to BSA (80 mg) dissolved in borate buffer (20 mL, 0.2 M, pH 9.0), and the mixture was stirred on ice for 2 h until a bright orange colour developed. The conjugate was then dialyzed extensively against distilled water, changed daily, for 4 days and freeze-dried prior to storage at −20 °C.

The hapten/protein ratio of the conjugate was determined by measuring the phosphorus content (Chen *et al*., 1956). Conjugate (2 mg) was weighed into a borosilicate glass test tube, ashed by addition of 4 drops of concentrated sulfuric acid, and boiled until the solution developed a blackish appearance and produced white fumes of sulfur trioxide. Subsequently, perchloric acid (3 drops of 60%) was added and the solution reheated until it became clear. After cooling, water was added to a total volume of 25 mL. Aliquots (1 mL) of this solution were then assayed by the addition of 1 mL of a freshly made solution of sulfuric acid (3 M)/H_2O/2.5% ammonium molybdate/10% ascorbic acid in the ratio 1:2:1:1. Blue color formation, the consequence of reduction of the phosphomolybdate, was determined after 1 h by measuring the optical density at 820 nm. The conjugate parathion content, which is proportional to the phosphorus content, was determined by comparison with a range of parathion standards that were treated in the same manner.

Production of Mabs. BALB/c mice were immunized subcutaneously with parathion−BSA conjugate (50 μg) in PBS and Freund's complete adjuvant 1:1 (v/v) initially and subsequently using Freund's incomplete adjuvant at 4-week intervals. A test bleed was taken from the tail to check for antibody activity. Once this was found to be positive, the mouse was given one more booster injection, killed 4 days later, and its spleen was removed. Spleen cells were fused with myeloma cells [X63-Ag8-653, Imperial Laboratories (Europe) Ltd., Andover, U.K.] in the ratio 5:1 in Hepes (75 mM) containing 50% poly(ethylene glycol) (Boehringer Mannheim, Lewes, U.K.) according to the method of Galfré and Milstein (1981). Subsequently, the cells were suspended in OptiMEM 1 (Gibco Ltd., Uxbridge, U.K.) containing fetal calf serum (4%, v/v), hypoxanthine (100 μM), aminopterin (1 μM), and thymidine (16 μM) (HAT medium) and dispensed into three 96-well tissue culture plates (Costar, Northumbria Biologicals Ltd., Cramlington, U.K.). Following 2 weeks of culture, the well supernatants were screened for antibody activity by ELISA on a parathion−BSA-coated plate (10 μg/mL) in the presence and absence of parathion (100 ng) to check for inhibition of binding to the conjugate. Positive wells were expanded and cloned three times by limiting dilution using rabbit blood (1%, v/v) for feeder cells. These operations were performed on a microprocessor-controlled automatic workstation, a Biomek 1000 (Beckman Instruments Inc., High Wycombe, U.K.). To obtain large quantities of Mabs, hybridomas were grown in tissue culture flasks. After the cells were removed by centrifugation, culture supernatant was stored at −20 °C before use. An anti-parathion Mab secreting hybridoma cell line was established (IFRN 1701).

Production of ScFvs. Messenger RNA was prepared from 10^7 hybridoma cells (IFRN 1701) using oligo (dT)-cellulose affinity chromatography (Quick Prep Micro mRNA purification kit, Pharmacia Biotech, St. Albans, U.K.) and first-strand DNA was synthesized using reverse transcriptase and random hexamers (First-Strand cDNA synthesis kit, Pharmacia Biotech). PCR was performed using V_H and V_L primers from the scFv module of the Recombinant Phage Antibody System (RPAS, Pharmacia Biotech) and Ampli*Taq* DNA polymerase (Applied Biosystems, Risley, U.K.) for 30 cycles of polymerization (94 °C for 1 min, 55 °C for 2 min, 72 °C for 2 min) following a hot start of 94 °C for 5 min. After purification of the fragments, using electrophoresis through agarose gel and affinity purification (Qiaquick Kit, Qiagen Ltd., Dorking, U.K.), further amplifications were used to integrate linker DNA between the V_H and V_L fragments and to add the restriction sites *Sfi*I and *Not*I to either end of the scFv fragment according to the RPAS kit instructions. The fragment was then purified using Sephacryl S-400 HR (Pharmacia Biotech) before being digested, extracted with phenol/chloroform/isoamyl alcohol, precipitated, and cloned into the vector pCantab 5E supplied in the RPAS Kit. The ligation product was extracted and precipitated and electroporated into competent *E. coli* TG1-(K12 (lac-pro)supE,thi,hsdD5/F, traD36,proAB,lacIq,lacZ M15) cells. Recombinant bacteria were grown on SOBAG (2% Bactotryptone, 0.5% Bacto-yeast, 0.05% NaCl, 10 mM $MgCl_2$) agar plates containing 100 μg/mL ampicillin and 1% (w/v) glucose.

Growth of Colonies for Screening. Colonies were picked into 96-well tissue culture plates containing 2×YT broth (100 μL) with ampicillin (100 μg/mL) and glucose (0.1%, w/v). Following 4 h of incubation at 37 °C, 2 × tryptone−yeast extract medium, (2×YT; 50 μL) containing IPTG (3 mM) was added to each well and the incubation was continued for 20 h at 25 °C. Finally, PBST (150 μL) was added to each well and the plates were centrifuged at 800*g* for 20 min. The supernatant was then checked for antibody activity in the screening ELISA.

Screening ELISA. Microtitration plates (Nunc Immunoplate Maxisorp, Gibco) were coated with parathion−BSA (1.0 μg/mL) in carbonate−bicarbonate buffer (0.05 M, pH 9.6) for 16 h at 4 °C. Following three washes with distilled water using a Denley Wellwash 5000 plate-washer (Denley Instruments Inc., Billingshurst, U.K.), the plates were blocked with non-fat dried milk in PBST (5%, w/v) at 37 °C for 1 h. Finally, the plates were washed 3 times with PBST, dried, and stored at room temperature. To perform the screening assay for binding to the parathion conjugate, supernatant (100 μL) was added to each well of the coated plates and left at 37 °C for 1 h. To establish a blank value, 2×YT medium (100 μL) was added to two wells. The plates were washed five times with PBST followed by the addition of anti-E tag antibody (100 μL; Pharmacia Biotech), diluted 1:2500 in PBST containing non-fat dried milk (5%, w/v). After 1 h at 37 °C and further washing (five times in PBST), anti-mouse IgG−horseradish peroxidase (HRP) labeled antibody (100 μL; Sigma Chemical Co., Poole, U.K.) diluted 1:2000 in PBST, was added. Again the plates were left at 37 °C for 1 h before being finally washed (five times in PBST). Detection was achieved by the addition of substrate (200 μL of 3,3′,5,5′-tetramethylbenzidine solution; Vetoquinol, Bicester, U.K.). After approximately 10 min at room temperature, the reaction was stopped by adding sulfuric acid (50 μL, 2 M) and the optical densities of each well were read at 450 nm on a plate reader (Dynatech MR5000; Dynatech Laboratories Ltd., Billingshurst, U.K.). Inhibition assays to ascertain binding to the free pesticide in solution were performed as above with the addition of pesticide (100 μL, 100 ng/well) in the initial reaction step.

Large Scale Preparation of Periplasmic ScFv Fragments. A colony of *E. coli* containing pCANTAB 5E with the scFv insert was inoculated into a 1.5 L flask containing 800 mL of 2×YT with ampicillin (100 μg/mL) and glucose (0.1%, w/v) and grown shaking at 37 °C for approximately 5 h. IPTG was added to a final concentration of 1 mM and the flask shaken for a further 5 h at 25 °C. The resulting culture was spun at 5000*g* for 20 min, the supernatant discarded, and the cell pellet resuspended in 4 mL of ice-cold Tris/EDTA/sucrose buffer (TES; 0.2 M Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose). Further dilution was made by adding 6 mL of TES buffer diluted with water (one part TES plus four parts water). The suspension was vortexed and left on ice for 30 min and then centrifuged at 15800*g* in a bench microfuge. The resultant supernatant, containing the periplasmic scFv, was frozen in aliquots at −20 °C.

Parathion ELISA. Microtitration plates were coated as for the screening assay except that the concentration of the parathion−BSA conjugate was reduced to 0.5 μg/mL. For the scFv ELISA, the periplasmic extract was diluted to a final concentration of 1 in 200 with PBST and incubated together with the parathion standards (0.05−50 ng/well) in PBST. The rest of the ELISA was performed in the same manner as the screening assay. For the Mab ELISA, hybridoma culture supernatant was diluted to a final concentration of 1 in 250 with PBST. The assay was identical to the scFv ELISA except that the E-tag antibody step was omitted.

To assess the effect of the food matrices in the ELISA, standard curves were prepared by diluting stock parathion (1 mg/mL in methanol) with extract from the control rice or oranges, previously diluted to 1% (v/v) with PBST. The amount of parathion in the spiked samples was determined by reference to the appropriate standard curve prepared in 1% (w/v) matrix.

To measure the cross-reactivity of other structurally similar pesticides with the Mab and the scFv, standard curves of parathion-methyl, paraoxon-ethyl, paraoxon-methyl, fenitrothion, diazinon, pirimiphos-ethyl, pirimiphos-methyl, chlorpyriphos-ethyl and chlorpyriphos-methyl (Qmx Laboratories, Halstead, U.K.) were constructed in the same manner as for parathion. The level of cross-reactivity was calculated from the ratio of the quantity of parathion required to give 50% displacement of binding from the value in the absence of free parathion to the quantity of the other pesticides required to give 50% displacement. Results are expressed as a percentage value.

Limits of detection for the Mab and scFv ELISAs were calculated by subtracting two standard deviations from the mean zero value and reading this value off the standard curve.

Preparation of Extracts of Rice Spiked with Parathion. American long-grain rice (20 g) was weighed into a beaker, and 10 mL of methanol, containing parathion in solution at 5, 10, 50, or 100 μg/mL was added (Ferguson *et al.*, 1995). Control rice (40 g) received 20 mL of methanol alone. The beakers were left in a fume hood for approximately 24 h, with occasional mixing, until all of the methanol had evaporated. Parathion was extracted from the spiked rice by transferring the contents of each beaker to a round-bottom flask and adding 20 mL of 60% methanol in water; control rice received 40 mL. The flasks were shaken for 2 h at room temperature on a Gallenkamp flask shaker and then left at 4 °C overnight. The supernatant was filtered through Whatman No. 1 filter paper and the resultant solution stored at −20 °C until assay.

Preparation of Parathion-Spiked Orange Peel. Oranges, purchased from a local supermarket, were washed with soap and dried. They were then wiped with an acetone-soaked paper towel to remove any protective coating that may have been applied to the fruit during factory processing to prevent loss of moisture and to remove any parathion on the surface. The surface of the peel was also pricked to improve absorption. Parathion solutions (0.1, 0.2, 0.5, and 1 mg/mL) were prepared in methanol, and 2 mL of each solution was transferred to a watch glass. A prepared orange was slowly turned in the liquid until the whole surface had been coated with pesticide and none remained in the watch glass; one fruit was coated with each parathion concentration. The fruit were dried in a fume hood for 30 min and then kept, individually wrapped in plastic bags, at 4 °C overnight. To prepare extracts, each fruit was peeled and the peel fragmented in a coffee grinder. The total peel from each fruit was then transferred to round-bottom flasks and shaken for 2 h in 50mL of methanol. The supernatant was filtered through Whatman No. 1 filter paper and the resultant clear, orange-colored liquid stored at −20 °C until assay.

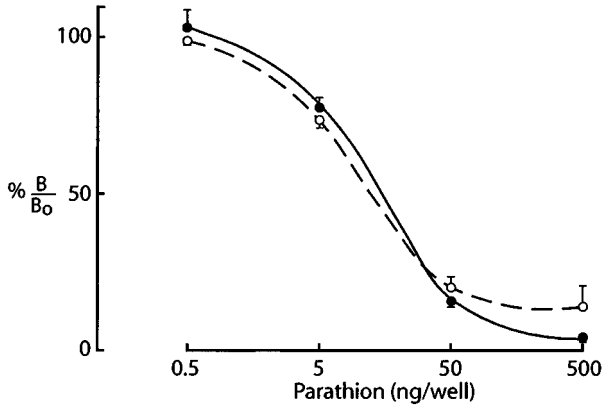

Figure 1. ELISA standard curves for Mab IFRN 1701 (●) and scFv IFRN AA01 (○) in PBST on plates coated with parathion−BSA at 0.5 μg/mL. Binding to the plate is expressed as a percentage of the binding with no free parathion.

RESULTS AND DISCUSSION

The parathion−BSA conjugate used for both immunization and coating microtitration plates had a hapten/protein ratio of 44:1 as determined by measuring phosphorus content.

The hybridoma cell line secreting the anti-parathion monoclonal antibody, IFRN 1701, was characterized in an ELISA system, generating the standard curve seen in Figure 1. The limit of detection was 1.6 ng/well. In 10 assays conducted over a 3 month period, the amount of parathion required to give 50% inhibition of the binding in the absence of parathion was 11.9 ± 5 ng/well. These values compare to 50% inhibition of binding of 25 ng/well as reported by Ibrahim *et al.* (1994) for an ELISA for parathion-ethyl, to 100 ng/well for the radioimmunoassay for parathion-ethyl reported by Ercegovich *et al.* (1981), and to 30 pg/well of parathion-methyl for the EnviroGard parathion plate kit.

We used the hybridoma as the source of functional immunoglobulin genes and chose to produce scFv rather than Fab, in contrast to others seeking antipesticide recombinant antibodies (Ward *et al.*, 1993; Bell *et al.*, 1995). It has been shown that periplasmic yields of Fab fragments can be considerably lower than yields of Fv fragments, possibly due to aggregation of folding intermediates (Pluckthun and Skerra, 1991). A selection scheme avoiding the use of phage display was employed, as phage display of antibody has, in our hands at least, resulted in high background binding and difficulties in recognizing high-affinity antibodies capable of binding to hapten in solution (Lee *et al.*, 1995).

The initial screening program for anti-parathion−BSA scFv antibodies from the bacterial supernatants resulted in the identification of binding significantly above background in 10% of the 120 colonies screened and 4 clones that gave high binding. One of the clones with high binding produced an scFv (IFRN AA01) that could be displaced to a considerable degree in the ELISA by free parathion. The other three clones produced scFv antibodies that demonstrated less or no recognition of

Table 1. Cross-Reactivities (%) of Mab IFRN 1701 and scFv IFRN AA01 with Organophosphate Pesticides Structurally Related to Parathion

Pesticide	scFv	Mab
Parathion	100	100
Parathion-methyl	4	6
Fenitrothion	4	6
Diazinon	0.6	0.6
Paraoxon	0.3	0.3
Paraoxon-methyl	–	0.2
Chlorpyriphos-methyl	<0.08	<0.05
Pirimiphos	<0.04	<0.03
Pirimiphos-methyl	<0.04	<0.06

free parathion. IFRN AA01 was stable for at least 6 months if stored as a periplasmic extract at −20 °C.

IFRN AA01 was used to generate a microtitration plate ELISA. The standard curve is shown in Figure 1, alongside that generated by the Mab. The limit of detection for the scFv-based assay was 2.3 ng/well. In 10 assays conducted over 3 months, the amount of parathion required to give 50% inhibition of the binding observed in the absence of parathion was 11.6 ± 4 ng/well.

Further comparisons in performance were made by determining cross-reactions in the two assays against related organophosphate pesticides (Table 1). The cross-reactions observed in the two assays were very similar. As might be expected, highest cross-reactions were provided by parathion-methyl and fenitrothion (6% and 4% for the Mab and scFv-based assays, respectively). Fenitrothion differs from the immunogen pesticide in that it has methyl groups instead of ethyl groups attached to the phosphate, and it also possesses an extra aromatic methyl group close to the point of hapten conjugation; parathion-methyl and parathion-ethyl are clearly closely related. All other cross-reactions were <1%.

The two assays were very similar (though not identical) in their standard curve shape, detection limits, and specificity. It is tempting to suggest that all amino acid residues within the binding site have been conserved and also that the scFv fragment is behaving in the same manner as the complete, bivalent antibody in the ELISA. However, this is far from certain, especially given the identification of other scFv-secreting clones of different properties. The reason more than one scFv was obtained is far from clear, but other authors have reported similar findings (Navarro-Teulon *et al.*, 1995; Kramer and Hock, 1996; Yuan *et al.*, 1997).

Recombinant antibodies produced from hybridoma mRNA described in the literature have varied widely in their ability to mimic the parent Mab. An anti-2-phenyloxazolone Fv antibody fragment showed the same affinity as the Mab from which it was derived (McManus and Reichmann, 1991). Karu *et al.* (1994) produced four recombinant anti-diuron Fab fragments from a hybridoma cell line which showed identical affinity to the papain-digested Mab but greater affinity than the undigested Mab. Navarro-Teulon *et al.* (1995) showed decreased affinity for both soluble and phage anti-digoxin antibodies originating from a hybridoma cell line. Byrne *et al.* (1996) produced a single-chain antibody from hybridoma mRNA that recognized atrazine significantly better than the Mab. Kramer and Hock (1996) used phage selection with immunomagnetic beads to produce a soluble scFv against *s*-triazines which had lower affinity, by 1 order of magnitude, but similar cross-reactivity characteristics to the parent Mab.

The close similarity of response for the scFv and bivalent Mab described in this paper are slightly surprising if the scFv is univalent. We have previously discussed the contributions of bivalent and monovalent antibody binding in ELISAs (Kemp and Morgan, 1986). We have no evidence of its existence in either form, but it is possible that the univalent scFv molecule is able to dimerize spontaneously, a phenomenon which has been reported by McGregor *et al.* (1993). An increase in affinity was observed for a dimeric single-chain antibody (retaining a light chain constant region) over the monomeric form, though the increased value was not equal to the affinity of the original antibody. It could be, as suggested by McGregor and co-workers, that the light chain constant region was important in the noncovalent association of the two monomers. Dimers can also form from scFv monomers without constant domains, as shown by Griffiths *et al.* (1993) and Kortt *et al.* (1994). In the latter study, a crystal structure of dimerized scFvs showed them binding back-to-back, possibly with the fragments interlocked through the flexible linker. It has also been suggested that the heavy chain of one scFv is associated with the light chain of the other (Griffiths *et al.*, 1993) in dimer formation.

None of the previous literature studies of recombinant antipesticide antibodies (and very few others) have described immunoassay applications. The development and characterization of applications, and an understanding of potential limitations and opportunities for recombinant antibodies as compared to the conventional antibody form, are essential in assessing the likely future role of recombinant antibodies in analysis. We chose to investigate and compare the performance of the two antibodies in application to analysis of parathion in rice and orange peel and, in particular, to focus on performance in extracts of the foods. The effect of methanol (0.6, 1, 5, 10, 20, and 25%, v/v) on the standard curves is shown in Table 2. The presence of 0.6% (v/v) methanol had no effect on either curve. Both curves seemed to be enhanced by 1 and 5% (v/v) methanol, showing higher binding at the B_0 and 50% inhibition points with little detrimental effect on background response. The Mab curve was also enhanced by 10% (v/v) methanol, whereas the scFv curve started to show a drop in binding at this concentration. At greater

Table 2. Effect of Methanol on the Parathion Standard Curve[a]

final MeOH concn (%)	Mab B_0	Mab 50% inhibition	scFv B_0	scFv 50% inhibition
0.0	100	100	100	100
0.6	104	100	103	100
1.0	110	116	109	103
5.0	119	123	115	110
10.0	107	110	80	77
20.0	77	101	29	33
25.0	49	86	9	17

[a] A series of standard curves in increasing concentrations of methanol was set up and compared. For each curve binding at the zero dose (B_0) and the 50% inhibition points were compared and expressed as a percentage of the curve in PBST only.

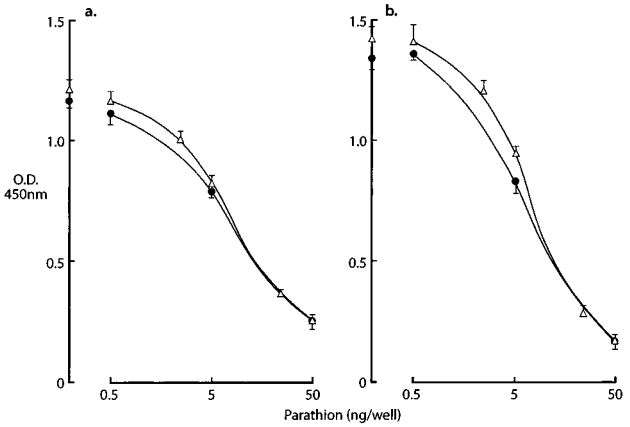

Figure 2. Effect of rice extract at 1% [containing 0.6% (v/v) methanol] (△) on (a) Mab IFRN 1701 and (b) scFv IFRN AA01 standard curves compared with these curves prepared in PBST (●). Figures are ± 1 SD (three replicate wells).

Table 3. Recovery of Parathion from Samples of Rice Spiked before Extraction, As Determined by ELISA

parathion added to rice (μg/g)	concn of parathion recovered as determined by ELISA: Mab μg/g ± 1SD	Mab mean % recovery	scFv μg/g ± 1SD	scFv mean % recovery
2.5	2.5 ± 0.35 (4)[a]	101	2.0 ± 0.5 (5)	80
5.0	3.1 ± 0.65 (5)	62	2.9 ± 0.83 (6)	58
25	13.0 ± 1.3 (6)	52	13.5 ± 1.41 (6)	54
50	26.9 ± 3.6 (6)	54	27.1 ± 3.1 (6)	54

[a] The numbers in parentheses refer to the number of assays from which the results were calculated.

concentrations of methanol, as with most conventional antibodies, the perfomance of the curves showed progressive deterioration. The scFv curve at 20 and 25% (v/v) showed a greater fall in performance than that of the Mab.

Rice and orange peel extracts were assayed after dilution to give a final concentration of methanol in the well of 0.6% or 1% (v/v) for rice and orange, respectively. Blank rice extract at these levels had no effect on the standard curve, whether using Mab or scFv, compared to curves in buffer (Figure 2). Recovery of parathion from rice spiked with the pesticide is shown in Table 3. Similar recoveries were obtained with both the Mab- and scFv-based assays. The values compare with reported recoveries of 36−52% by Ferguson *et al.* (1995) for an ELISA using a polyclonal antiserum.

An extract of orange peel, assayed at 1% (v/v) dilution, slightly depressed the standard curve in both Mab- and scFv-based assays (Figure 3). The effect occurred in the presence of low concentrations of parathion and may

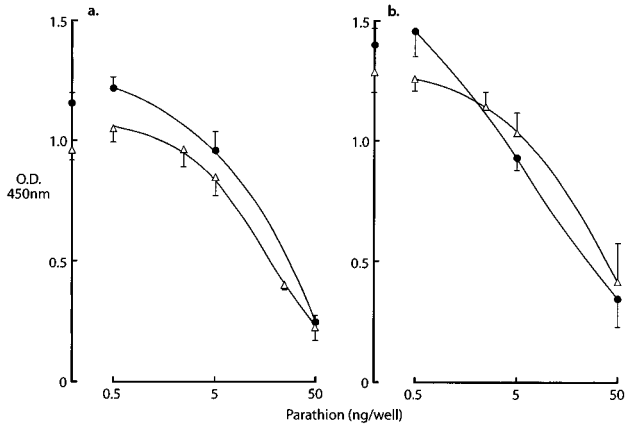

Figure 3. Effect of orange peel extract at 1% (containing 1.0% methanol) (△) on (a) Mab IFRN 1701 and (b) scFv IFRN AA01 standard curves compared with these curves prepared in PBST (●). Figures are ±1 SD (three replicate wells).

Table 4. Recovery of Parathion from Orange Peel Spiked before Extraction, As Determined by ELISA

parathion added to each orange (μg)	concn of parathion recovered as determined by ELISA: Mab μg/orange ± 1SD	Mab mean % recovery	scFv μg/orange ± 1SD	scFv mean % recovery
400	213 ± 76 (4)[a]	53	178 ± 30 (4)	45
1000	299 ± 30 (4)	30	305 ± 32 (4)	31
2000	524 ± 124 (4)	26	548 ± 78 (4)	27

[a] The numbers in parentheses refer to the number of assays from which the results were calculated.

have been a pH-related effect. Recovery of parathion from the spiked peel is shown in Table 4. Recoveries were low and similar in both Mab- and scFv-based ELISAs. This could be due to the breakdown of parathion at low pH (Rigterink and Kenaga, 1966).

It seems clear that the similarity of standard curve performance for the two antibodies is extended to their performance and stability with methanol and with methanolic food extracts. It might have been anticipated that the scFv, being much smaller (28 kDa compared to 150 kDa for an IgG molecule) would be markedly less tolerant of organic solvents, with the precise interaction with hapten being more easily disrupted. Yuan *et al.* (1977) have studied an scFv against the mycotoxin zearalenone, which, while being less tolerant of methanol than the parent Mab, was stable in concentrations of methanol higher than those normally employed in food extracts in an ELISA. The Mab showed tolerance of methanol up to 35% (v/v), and the scFv tolerated a maximum concentration of 15% (v/v). As with the present study, the findings indicate that scFvs may be more tolerant of organic solvents than might have been predicted.

The availability of recombinant antibodies will allow the ready manipulation of various antibody properties. Increased stability to organic solvents and other harsh extraction conditions (extremes of pH, for example) will be a prime target for manipulation for many antibodies. Specificity will be another focus of attention, and the organophosphate pesticides of related structure to parathion provide both a real application and useful model system to increase understanding and provide rational manipulation strategies for future research. The structures illustrated in Table 1 show that a first target might be an increase in recognition of the methyl derivative, while maintaining binding to parathion-ethyl. These moieties are at the part of the molecule

distal to the site of conjugation to protein in immunogen synthesis. Recognition of both P═S and P═O forms, if possible, would be the next target, followed by investigations of substitutions in the ring closest to the conjugation point. Manipulation of the recombinant anti-parathion scFv antibody could, therefore, provide important lessons generally applicable to manipulation strategies for many other haptens. However, the first questions to be asked will be about how to carry out the manipulations—at random or with full knowledge of X-ray crystallographic data? Should we target particular regions of the sequence? It should be borne in mind that knowledge of antibody−hapten interactions is very poor compared with that for antibodies with protein targets.

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