Bacterial Expression and Characterization of a Picloram-Specific Recombinant Fab for Residue Analysis

Kerrm Y. F. Yau, Nancy L. Tout, Jack T. Trevors, Hung Lee, and J. Christopher Hall*

Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Complete κ-light chain and V_H-C_{H1} (Fd) genes were cloned by PCR from cDNA synthesized from RNA isolated from a picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid)-specific hybridoma cell line. Both genes were cloned into the phagemid vector pComb3 for expression of soluble Fab. Extracts from the periplasmic space of recombinant *Escherichia coli* expressing the Fab exhibited specificity to picloram with an IC_{50} of 50 ng/mL, as determined by competitive indirect ELISA. This value was comparable to that obtained when using the parent monoclonal antibody (IC_{50} = 20 ng/mL). Two different matrices, river water and soil extract, did not interfere with the sensitivity and specificity of the assay. Cross-reactivity was detected to the pyridine herbicide clopyralid (IC_{50} > 30 μg/mL) but not to the pyridine herbicides triclopyr and fluoroxypyr. A single-chain variable fragment was constructed with the same variable chain sequences, but no specific activity to picloram was detected. The soluble Fab was found to be a suitable recombinant antibody fragment for the purpose of quantifying picloram in environmental samples.

Keywords: *Competitive indirect ELISA; monoclonal antibody; recombinant Fab; herbicides; picloram*

INTRODUCTION

Functional expression of antibody fragments from bacteria was first reported in the late 1980s (Better et al., 1988; Bird et al., 1988). Since then, recombinant antibody (rAb) technology has been applied to many different areas of scientific research. Before the discovery of recombinant DNA technology, antibodies (Abs) with predetermined affinity and specificity could be obtained only from animals (polyclonal Abs) or tissue culture supernatant of hybridoma cells (monoclonal Abs). Development of polyclonal Abs with high affinity and specificity is time-consuming, with results varying from one animal to another. Synthesizing monoclonal Abs (mAbs) requires large-scale screening strategies and expensive production costs. Recombinant Ab technology and phage display technology (Smith, 1985) may provide an alternative means of synthesizing various Abs in vitro. Recombinant Abs have been synthesized with higher affinity and specificity than parent mAbs (Ward, 1995; Vaughan et al., 1996), as well as novel binding characteristics (Blondelle and Houghten, 1996).

Immunoassay has proven to be accurate and sensitive for the purpose of monitoring pesticides used in agriculture to comply with registration and environmental stewardship requirements (Kaufman and Clower, 1995). High-quality antibodies are required to develop these assays. Antibodies produced from recombinant bacteria not only provide antibody fragments with high affinity and specificity suitable for immunoassays, but they are also useful for detection, quantification, purification, and extraction purposes (Hall et al., 1997).

To date, a limited number of pesticides can be detected by pesticide-specific rAb. More information on the structure of these rAbs is essential before a general strategy for synthesizing rAbs with desired specificity and affinity can be achieved. Different formats of immunoassays to detect and quantify picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) using both polyclonal and monoclonal antibodies have been developed (Hall et al., 1989; Deschamps et al., 1990; Deschamps and Hall, 1991). In this paper, we describe the strategy involved in the cloning and production of a recombinant Fab from a picloram-specific mAb cell line. Soluble Fab was expressed into the periplasm of recombinant *Escherichia coli* cells. The affinity and specificity of the Fab were determined and compared to those of the parent monospecific antibody against picloram. Cross-reactivity of the rAb with herbicides that are structurally similar and the effects of two matrices (river water and soil extracts) on the rAb were also determined.

MATERIALS AND METHODS

Cloning of Heavy and Light Chain Genes. A mAb-secreting cell line specific to picloram was synthesized and propagated as described previously (Deschamps et al., 1990). Total RNA was isolated from 10^7 hybridoma cells using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Purity and concentration of the RNA were determined by UV spectrophotometry and electrophoresis (Sambrook et al., 1989). First-strand complementary DNA (cDNA) was synthesized using the First Strand cDNA synthesis kit from Pharmacia Biotech (Baie D'Urfé, PQ). Total RNA (5 μg in 20 μL) was mixed with 11 μL of bulk first-strand reaction mix, 1 μL (0.2 μg/mL) of oligo-dT primer, and 1 μL of 200 mM dithiothreitol. The mixture was incubated at 37 °C for 1 h. Five microliters of the product was used as a template for the amplification of heavy (H) and light (L) genes by Polymerase Chain Reaction (PCR) using the conditions and universal primers described previously (Tout and Lam, 1997). By incorporating the *Spe*I and *Xho*I sites on the H chain

* Author to whom correspondence should be addressed [telephone (519) 824-4120, ext. 2740; fax (519) 837-0442; e-mail jchall@evbhort.uoguelph.ca].

10.1021/jf980643m CCC: $15.00
Published on Web 09/30/1998

primers and the *Xba*I and *Sst*I sites on the L chain primers, the amplified products were cloned separately into the phagemid pBluescript KS II (±) (Stratagene Corp., La Jolla, CA). Inserts from three representative clones containing H and L genes of the correct sizes were analyzed by fluorescent automated sequencing at the Laboratory Services Division, University of Guelph (Guelph, ON).

Construction of Soluble Fab (sFab) Gene. Previous isotyping experiments confirmed that the mAb belongs to the isotype IgG subclass 1 (IgG1) (data not shown). Sequence-specific primers were designed to amplify the entire C_{H1} region for Fab construction. The 3′ primer annealed to the last 23 bp of the mouse $C_{\gamma 1}$ gene (Kabat et al., 1991) and includes a *Spe*I restriction site for cloning purposes. The 5′ primer, which contains the *Xho*I site, annealed to the 5′ end of framework region 1 (FR1). Using the two primers, complete V_H-$C_{\gamma 1}$ (Fd) was amplified from cDNA by PCR. The H chain product was cloned into the expression vector pComb3, provided by the Scripps Research Institute (La Jolla, CA), forming pComb3:Fd. The L gene was subcloned from pBluescript KS II into the pComb3:Fd construct. After both genes were inserted, the soluble Fab (sFab) expressing phagemid (pComb3:sFab) was constructed through excision of the *gIIIp* gene using a *Nhe*I/*Spe*I digest and religation of the compatible ends (Barbas and Lerner, 1991). The construct was electroporated into *E. coli* XL-1 Blue for sFab expression. Internal primers for both chains were synthesized for bidirectional sequencing to confirm positive clones.

Expression of sFab and Extraction from Periplasmic Space. Starter cultures for sFab expression were prepared by growing a single colony in 5 mL of LB medium containing 50 μg/mL of carbenicillin and 1% (w/v) glucose at 37 °C with shaking at 250 rpm for 16 h. One milliliter of the starter culture was transferred to 1 L of SB medium (30 g of Tryptone, 20 g of yeast extract, 10 g of MOPS buffer, pH 7.0) containing 20 mM $MgCl_2$ and 50 μg/mL carbenicillin. The cells were grown at 37 °C with shaking at 250 rpm until absorbance at 600 nm reached 0.2. IPTG was added to a final concentration of 1 mM, and the cells were allowed to grow at 30 °C with shaking at 250 rpm for 12 h.

With the leader sequence *pelB* included in the expression vector pComb3, the H and L chains were secreted and assembled in the periplasmic space (Barbas and Lerner, 1991). To extract periplasmic proteins, bacterial cells were pelleted by centrifuging at 5000*g* for 20 min at 22 °C and washed once with 400 mL of wash solution [10 mM Tris, pH 8.0, 0.9% (w/v) NaCl]. Cells were pelleted again as described above, resuspended in 40 mL of sucrose solution (25% w/v sucrose, 1 mM EDTA, 10 mM Tris, pH 8.0), and incubated at 22 °C for 10 min before centrifugation. The pellet was resuspended in 40 mL of ice-cold shock solution (10 mM Tris, pH 8.0, 0.5 mM $MgCl_2$). The suspension was incubated on ice for 10 min and pelleted at 4 °C by centrifuging at 5000*g* for 20 min. The supernatant containing the sFab was stored at 4 °C.

Competitive Indirect Enzyme-Linked Immunosorbent Assay (CI-ELISA). *Synthesis of Coating Conjugate.* Picloram was conjugated to ovalbumin (OVA) using mixed anhydride (Vallejo et al., 1982). Picloram (100 μmol) was dissolved in 1 mL of dioxane. Triethylamine (15 μL) and isobutyl chloroformate (15 μL) were added to the solution and mixed by vortexing. After incubation at 22 °C for 30 min, the mixture was filtered through glass wool. The filtrate was added dropwise to 3 mL of 0.2 M $NaHCO_3$ (pH 9.5) containing 44 mg of OVA and stirred continuously for 6 h at 22 °C. The final solution was dialyzed against four changes of ultrapure water during the next 24 h to remove unconjugated hapten. Final volume after dialysis was ∼5 mL.

Picloram Standards. A 10 mg/mL (10000 ppm) stock solution, prepared by dissolving picloram in 100% acetone, was stored in the dark at 22 °C. A 100 ppm working standard was prepared by diluting 1 mL of stock in 99 mL of phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per liter of ultrapure water, adjusted to pH 7.4 using NaOH). Working standards containing 10 to 0.001 ppm of picloram were serially diluted from the 100 ppm solution into PBS, river water, and soil extract containing 1% (v/v) acetone.

Sample Preparation. Surface water was collected from the Speed River, Guelph, ON, and stored at 4 °C. A sandy loam soil (76.42% sand, 16.64% silt, and 6.94% clay) containing 2.24 g/cm^3 organic matter, not previously treated with picloram, was obtained from a University of Guelph Research Station at Elora, ON. The soil (4 g wet weight) was shaken for 15 min with 20 mL of a water/methanol (1:1 v/v) solution. The mixture was filtered through a glass fiber filter, and methanol was removed under vacuum at 50 °C. The volume of the resulting aqueous solution was returned to 10 mL with ultrapure water (Deschamps et al., 1990).

CI-ELISA Protocol. Coating conjugate (5 μg in 100 μL) diluted in PBS was added to wells of microplates and incubated at 4 °C overnight. Wells were emptied and washed twice with PBS containing 0.05% (v/v) Tween 20 (PBST). PBS (200 μL) containing 3% (w/v) nonfat milk powder (Bio-Rad Laboratory Ltd., Hercules, CA) was added to each well to block any unoccupied sites on the plate. Plates were incubated at 22 °C for 1 h. At the same time, mAb or sFab was preincubated for 1 h at 22 °C with the same volume of standard solutions containing a known concentration of picloram. This mixture (100 μL) was added to individual wells of the microtiter plate after the blocking solution was removed. The plate was incubated at 22 °C for 2 h, followed by five washings with PBST. Different secondary Abs, obtained from Pierce Chemical Co. (Rockford, IL), were used in different assays. When the mAb was used as a first Ab, goat anti-mouse IgG (Fc-specific) conjugated to horseradish peroxidase was diluted 5000-fold in PBS and 100 μL was used as secondary Ab. When sFab was used as a first Ab, 100 μL of 5000-fold-diluted goat anti-mouse IgG (Fab-specific) conjugated to horseradish peroxidase was used. The secondary antibody was incubated in the well for 1 h at 22 °C and washed five times with PBST. One hundred microliters (0.4 mg/mL) of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium (Sigma-Aldrich Chemicals Ltd., St. Louis, MO) dissolved in 50 mM citrate buffer (pH 4.0) containing 0.005% H_2O_2 was added and incubated for 15 min before absorbance (A) was measured at 405 nm using a microplate reader (model 3550-UV, Bio-Rad). Percent inhibition was determined by the formula $[(1 - A/A_0) \times 100\%]$, where A_0 is the absorbance of the well in which the antibody was not challenged with free picloram. Percent inhibition was plotted against log concentration of free picloram to generate an inhibition curve. The affinity of each antibody was compared in terms of its IC_{50} (the concentration of free picloram that inhibited 50% of the binding) determined from the inhibition curve.

Purification of sFab by Affinity Chromatography. *Construction of Affinity Beads.* An affinity column was built to purify sFab from the crude cell extract using a previously described method (Harlow and Lane, 1988). Briefly, 2 mg of goat anti-mouse IgG (Fab-specific) Abs (Pierce Chemical Co., Rockford, IL) was incubated with 1 mL of protein G-conjugated Sepharose 4B Fast Flow matrix, obtained from Pharmacia Biotech, for 1 h at 22 °C with gentle rocking. The beads were washed with 10 mL of 0.2 M borate buffer (pH 9.0) and centrifuged at 3000*g* for 5 min. After a second wash, the beads were resuspended in 10 mL of 0.2 M borate buffer (pH 9.0), and dimethylpimelimidate was added to a final concentration of 20 mM to covalently link the Abs to the protein G. The mixture was incubated with gentle mixing for 30 min at 22 °C. The beads were centrifuged and resuspended in 0.2 M ethanolamine (pH 8.0) to stop the coupling reaction. After 2 h of incubation at 22 °C with gentle shaking, the beads were washed in PBS containing 0.01% (w/v) merthiolate and stored at 4 °C.

Purification of sFab. Periplasmic cell extract of sFab was diluted to 10 mL in PBS and added to the beads described above. After incubation at 4 °C for 2 h with gentle rocking, the beads were washed three times with 10 mL of PBS. Phosphate buffer (5 mL, 10 mM, pH 6.8) was used to pre-elute the column. Bound sFab was eluted by adding 0.5 mL of 0.1 M glycine buffer (pH 2.5). Eluant was collected in eight

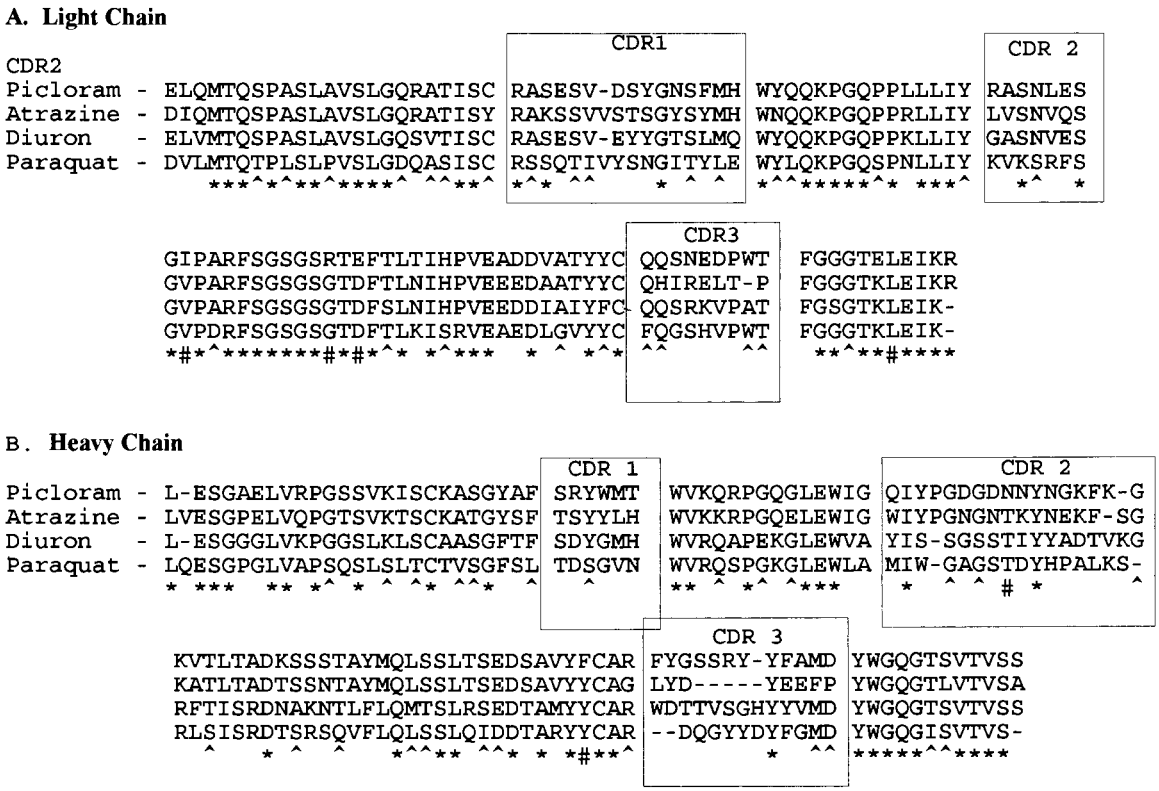

Figure 1. Comparison of the deduced amino acid sequences of variable regions of three herbicide-specific immunoglobulins, atrazine (Ward et al., 1993), diuron (Bell et al., 1995), and paraquat (Graham et al., 1995), with anti-picloram antibody. An asterisk (*) indicates perfect match among the four sequences, a circumflex (∧) indicates a picloram amino acid match with two other sequences, and a number sign (#) indicates all except picloram have the same residue.

microfuge tubes containing 25 μL of 1 M phosphate buffer (pH 8.0). The protein content of each tube was analyzed using polyacrylamide gel electrophoresis (Laemmli, 1970).

RESULTS AND DISCUSSION

Immunoassays are widely accepted as a screening method in pesticide residue analysis. However, to date only 70 of 300 pesticides are detectable by immunoassays (Kramer and Hock, 1996). Recombinant antibody technology may improve the development of immunoassays in residue analysis. Furthermore, rAb produced in bacteria is more economical and easier to maintain than hybridoma tissue cultures. In addition, the genes encoding the antibody are readily available for further manipulation to improve the assay sensitivity through improvement of Ab affinity and specificity. The results presented here agree with those of other researchers (Ward et al., 1993; Kramer and Hock, 1996; Scholthof et al., 1997; Webb et al., 1997), who demonstrate that rAbs can be used in traditional immunoassay for residue analysis without compromising accuracy and sensitivity.

Cloning of H and L Genes. The H gene was isolated by PCR from cDNA, cloned into pBluescript, and transformed into *E. coli*. Inserts from three clones were sequenced in both directions and found to be identical. The cloned H chain gene sequence, which was composed of the complete V_H and partial $C_{\gamma 1}$ genes, contained 411 bp encoding 137 amino acids with no immature stop codon. These cloning and sequencing steps confirmed that the universal primer sets used in the PCR were able to amplify the functional V_H gene. With another round of PCR using sequence-specific primers, the complete V_H-$C_{\gamma 1}$ (Fd) gene (669 bp long encoding 223 amino acids) was amplified and cloned into pComb3, forming pComb3:Fd. The L chain gene cloned into pBluescript contained the complete κ-light chain (663 bp) encoding 221 amino acids. Again, sequences of inserts from three different clones were found to be identical. The final construct pComb3:sFab for sFab expression in *E. coli* XL1-Blue was sequenced with internal primers of both genes. The results confirmed that there was no discrepancy between the product and the original cloned gene. The absence of immature stop codons and nonfunctional transcripts and the homogeneity of the cloned Ab genes revealed that the isolation of the picloram-specific Ab genes from this hybridoma cell line did not require extensive screening from phage display libraries. The major advantage of phage panning is the isolation and enrichment of a clone of interest from a large population (McCafferty et al., 1990; Barbas and Lerner, 1991) such as spleen or hybridoma cells containing multiple transcripts (Frohman et al., 1988; Ruberti et al., 1993; Ruberti et al., 1994; Bradbury et al., 1995). The variable regions of the H and L chain genes were submitted to GenBank under Accession No. AF045183 and AF045463, respectively.

Three herbicide-specific Ab genes were retrieved from the Genbank Database, namely anti-atrazine (Accession No. S69212 and S69214), anti-diuron (Accession No. U04352−3), and anti-paraquat (Accession No. S82427 and S82434) antibodies. When the variable regions of the deduced amino acid sequences of these antibody fragments were aligned (using PC/GENE) with the picloram-specific antibody genes, homologies were found only in the framework regions (FRs) in both H and L chains (Figure 1). No similarity was found within the complementarity determining regions (CDRs) in any of the anti-herbicide antibodies. Tyr and Ser, however,

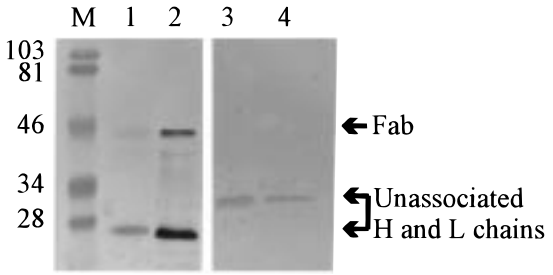

Figure 2. Western blot analysis of periplasmic expression of Fab in *E. coli* XL1-Blue containing pComb3:sFab phagemid. Osmotic extraction (lanes 1 and 3) and affinity chromatography purified Fab (lanes 2 and 4) were separated by SDS−PAGE and electrotransferred to PVDF membrane. Under nonreducing (lanes 1 and 2) conditions, both intact Fab and unassociated H and L chains were detected using goat anti-mouse IgG (H+L) conjugated to alkaline phosphatase. Under reducing conditions (lanes 3 and 4), the Fab dissociated into its components. Sizes of MW standards (M) are labeled on the left.

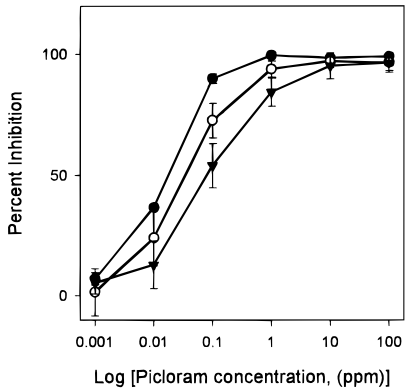

Figure 3. Inhibition curves of crude and purified sFab compared to the parent mAb obtained from CI-ELISA. The binding of sFab and mAb to OVA−picloram was challenged with different concentrations of free picloram. The IC_{50} for mAb (●), crude (○), and purified Fab (▼) were determined to be 20, 50, and 80 ppb, respectively. Values are the averages of four independent experiments, and error bars are ±1 SD.

were found to be the most abundant amino acid residues in different locations of the H and L chain CDRs, respectively. Padlan (1990) showed that Tyr, Trp, Ser, and Asn are the most common residues present in the antigen binding sites of antibodies and interact with antigen. Computational modeling also indicated that three Tyr and one Trp residue in the H chain of a diuron specific-antibody form a pocket with an Ala residue in the L chain to accommodate the diuron molecule (Bell et al., 1995). Understanding the role of these differently located Tyr and Ser residues in CDRs of H and L chains may assist in predicting the structure of the paratope on the anti-picloram Ab.

Soluble Fab Expression. Correctly folded H and L chains were secreted separately into the periplasmic space of *E. coli*, where they assembled to form the functional Fab. The oxidizing environment in the periplasmic space allows the formation of a disulfide bond between the two chains to stabilize the final product (Plückthun and Skerra, 1989). Periplasmic extract of recombinant *E. coli* containing pComb3:sFab was separated by SDS−PAGE. Goat anti-mouse IgG (H+L) conjugated to alkaline phosphatase was used in Western blot analysis. Under nonreducing conditions, two bands were detected with relative mobility (M_r) estimated to be 46 and 24 kDa, respectively (Figure 2). Because the MW of the H and L chains deduced from the amino acid sequences were 24.1 and 23.8 kDa, respectively, the upper band on the Western blot corresponds to the Fab and the lower band corresponds to unassociated H and L chains. Under reducing conditions, the disulfide bond of the Fab was broken, thereby separating the H and L chains. The MW of the products dissociated from the sFab were found to be 31 kDa, which was higher than that seen under nonreducing conditions. In a separate experiment, it was confirmed that 2-mercaptoethanol bound to the proteins and lowered their mobility in the gel (results not shown).

Activity of sFab. Crude extract of sFab from the periplasm of recombinant *E. coli* was used directly in ELISA to test for specificity to picloram. In the absence of free picloram, the sFab bound to OVA−picloram with an absorbance 5 times higher than background, but not to microplates coated with the same concentration of OVA (data not shown). Affinity-purified sFab displayed greater activity to OVA−picloram with an absorbance >10 times higher than that of the background control.

The specificity and the affinity of the sFab toward picloram were further examined by CI-ELISA. Crude periplasmic extracts and affinity-purified sFab were compared to the parent mAb. Both sFab preparations were inhibited by free picloram at a concentration as low as 10 ng/mL (Figure 3). At concentrations of 1 ng/mL, no inhibition was observed ($A_{405} = 0.523 \pm 0.0248$), whereas at a concentration of 100 μg/mL, free picloram completely inhibited sFab in crude periplamsic extracts ($A_{405} = 0.111 \pm 0.0146$) (averages from four individual experiments each with three replicates ± 1 SD). The IC_{50} of the sFab from crude extract was estimated to be 50 ng/mL, whereas the IC_{50} of the affinity-purified sFab was 80 ng/mL. Both values are higher than that of mAb (20 ng/mL). The bivalency in the parent mAb, which displays higher avidity than the monovalent sFab, may account for the difference in the level of inhibition.

There was no difference between results using unpurified versus purified sFab; therefore, crude periplasmic extract was used in ELISA. From an application perspective, it will also save both time and resources. However, after 72 h of storage at 4 °C, the IC_{50} of the crude sFab decreased by 1 order of magnitude (data not shown). The instability observed may be due to the actions of proteases present in the crude periplasmic extract. Addition of protease inhibitors such as 4-(2-aminoethyl)benzenesulfonyl fluoride may improve the shelf life of the recombinant protein (Baker and Cory, 1971).

A single-chain variable fragment (scFv), an alternate type of rAb fragment, was also constructed from the picloram-specific H and L genes. Although expression of the correct size scFv was confirmed using Western immunoblots, weak to no binding to picloram was seen (data not shown). A number of problems with scFvs have been reported in the literature, including incorrectly folded fragments (Somerville et al., 1994; Le Calvez et al., 1995). In some cases, the weak noncovalent interactions that rely solely on the amino acid sequences of the two variable regions are not always strong enough to hold the two variable chains together to form a complete paratope (Somerville et al., 1994), resulting in dissociation of the two domains. It appears that the constant regions of Fab proteins play an important role in their stabilization through the covalent interchain disulfide bond between the two domains

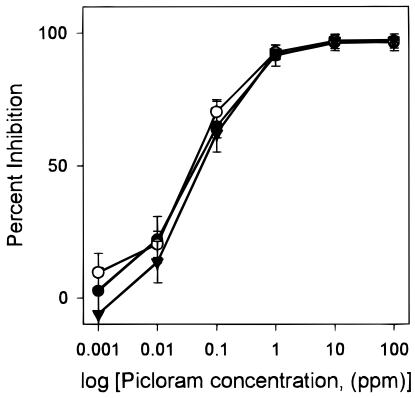

Figure 4. Matrix effect on the binding of recombinant Fab to OVA−picloram. Standard picloram solutions were prepared in PBS (●), soil extract (○), or river water (▼). Each solution was incubated with crude sFab preparation prior to CI-ELISA. Values are the averages of four independent experiments, and error bars are ±1 SD.

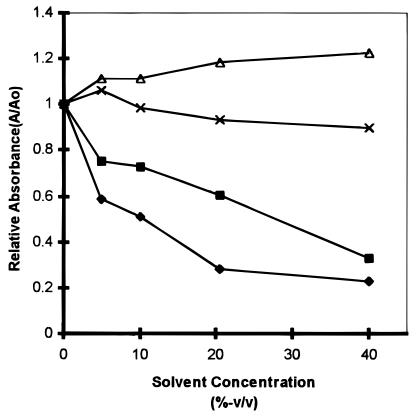

Figure 5. Effect of acetone and methanol on the binding of sFab and its parent mAb to the coating conjugate OVA−picloram: (■) sFab−methanol; (◆) sFab−acetone; (×) mAb−methanol; (△) mAb−acetone).

C_κ and $C_{\gamma 1}$ (Carter et al., 1992). On the basis of extensive modeling information, this added stability has also been engineered into scFvs to help restore activity in certain cases (Webber et al., 1995; Young et al., 1995).

Matrix Effects. One of the concerns of using immunoassays in the analysis of contaminated environmental samples is matrix effects. Co-contaminants present in different environmental matrices may randomly interfere with the analysis, independent of the type of antibodies or the format of the assay used (Weiler and Wieczoek, 1981; Deschamps and Hall, 1991; Del Valle and Nelson, 1994). Picloram has been reported to be very mobile in the soil (Hamaker et al., 1963), and analysis of picloram residue in soil and water samples is therefore essential. Soil extract and river water were spiked with picloram standards and analyzed by CI-ELISA using sFab from crude periplasmic extract. Inhibition curves were generated and compared to those generated using PBS (Figure 4). Similar inhibition curves with the same IC_{50} were obtained, and the amounts of free picloram in these matrices were accurately determined. Results showed that the methanol-extractable matrices in both environmental samples did not affect the activity of the sFab.

Organic solvents are commonly used in the extraction of herbicide residues from environmental samples. Their effect on binding of the sFab to OVA−picloram was also investigated (Figure 5). The sFab tolerated up to 20% methanol, maintaining its activity above 50%. Less tolerance was seen with 10% acetone, which reduced the binding activity by 50% or more. In contrast, the mAb was not affected by these organic solvents at concentration up to 40%. Destabilizing effects of organic solvents on two different recombinant Ab fragments (Scholthof et al., 1997; Yuan et al., 1997) and a proteolytic Fab from IgG (Scholthof et al., 1997) were reported by other researchers. The reason for differences in properties between intact Ab and fragments of an Ab is not completely understood. Intact Ab molecules may have higher solubility in different organic solvents than recombinant fragments. After expression from eukaryotic cells, the C_{H2} domains of IgG1 Abs are glycosylated. The carbohydrate residue may maintain the intact Ab in organic solvent without loss of activity.

Specificity. Cross-reactivity of the picloram-specific sFab to three pyridine herbicides with similar molecular structures, clopyralid (3,6-dichloro-2-pyridinecarboxylic acid), fluoroxypyr [[(4 amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid)], and triclopyr [(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid], was examined. At 10 μg/mL, none of the analogues inhibited binding of the parent mAb to OVA−picloram using the CI-ELISA. Similarly, fluoroxypyr and triclopyr did not inhibit sFab binding at 10 μg/mL. However, clopyralid at 10 μg/mL inhibited the binding of sFab by 34%. The low cross-reactivity to clopyralid will not affect the accuracy of applying the assay to analyze picloram-contaminated samples. In addition, picloram is rarely used on field crops and is seldom applied as a mixture with clopyralid for general vegetation control (Worthing and Walker, 1987). Therefore, there is little likelihood of false positive results arising from the presence of clopyralid in environmental samples.

Conclusions. Picloram-specific immunoglobulin genes were cloned from picloram-specific hybridoma cells into the expression vector pComb3, resulting in the expression of a functional sFab from recombinant *E. coli*. There was little difference between the mAb- and sFab-based CI-ELISAs. The sFab, like the parent mAb, did not cross-react with other auxinic herbicides of the pyridine family including triclopyr and fluoroxypyr. However, unlike the mAb, there was some cross-reactivity with clopyralid (34% at 10 μg/mL). On the basis of the low cross-reactivity to that compound, the accuracy of the sFab-based CI-ELISA should not be affected. Our results show that sFab from bacterial extract, with no prior purification, can be used to quantify picloram levels in water and soil samples by CI-ELISA.

ACKNOWLEDGMENT

We thank the Scripps Research Institute for supplying the pComb3 phagemid vector.

LITERATURE CITED

Baker, B. R.; Cory, M. Irreversible inhibitors of the C′1a component of complement derived from m-(phenoxypropoxy) benzamidine and pnenoxyacetamide. *J. Med. Chem.* **1971**, *14* (2), 119−125.

Barbas, D. F.; Lerner, R. A. Combinatorial immunoglobulin libraries on the surface of phage (phabs): rapid selection of antigen-specific Fabs. *Methods: Companion Methods Enzymol.* **1991**, *2*, 119−124.

Bell, C. W.; Scholthof, K. G.; Zhang, G.; Karu, A. E. Sequences of the cDNAs encoding the heavy- and light-chain Fab region of an antibody to the phenylurea herbicide diuron. *Gene* **1995**, *165*, 323−324.

Better, M.; Chang, C. P.; Robinson, R. R.; Horwitz, A. H. *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* **1988**, *240*, 1041−1043.

Bird, R. E.; Hardman, K. D.; Jacobson, J. W.; Johnson, S.; Kaufman, B. M.; Lee, S.; Lee, T.; Pope, S. H.; Riordan, G. S.; Whitlow, M. Single-chain antigen-binding proteins. *Science* **1988**, *242*, 423−426.

Blondelle, S. E.; Houghten, R. A. Novel antimicrobial compounds identified using synthetic combinatorial library technology. *Trends Biotechnol.* **1996**, *14*, 60−65.

Bradbury, A.; Ruberti, F.; Werge, T.; Amati, V.; Di Luzio, V.; Gonfloni, S.; Hoogenboom, H.; Piccioli, O.; Biocca, S.; Cattaneo, A. The cloning of hybridoma V regions for their ectopic expression in intracellular and intercellular immunization. In *Antibody Engineering*; Borrebaeck, C. A. K., Ed.; Oxford University Press: New York, 1995; pp 295−361.

Carter, P.; Kelly, R. F.; Rodrigues, M. L.; Snedecor, B.; Covarrubias, M.; Velligan, M. D.; Wong, W. L. T.; Rowland, A. M.; Kotts, C. E.; Carver, M. E.; Yang, M.; Bourell, J. H.; Shepard, H. M.; Henner, D. High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. *Bio/Technology* **1992**, *10*, 163−167.

Del Valle, P. L.; Nelson, J. O. Evaluation of atrazine soil extraction methods for the determination by enzyme immunoassay and gas chromatography. *Arch. Environ. Contam. Toxicol.* **1994**, *27*, 375−383.

Deng, S.; MacKenzie, C. R.; Sadowksa, J.; Michniewicz, J.; Young, N. M.; Bundle, D. R.; Narang, S. A. Selection of antibody single-chain variable fragments with improved carbohydrate binding by phage display. *J. Biol. Chem.* **1994**, *269*, 9533−9538.

Deschamps, R. J.; Hall, J. C. Validation of a monoclonal antibody-based indirect enzyme immunoassay method for picloram detection in soil and plants. *Food Agric. Immunol.* **1991**, *3*, 127−134.

Deschamps, R. J.; Hall, J. C.; McDermott, M. R. Polyclonal and monoclonal enzyme immunoassays for picloram detection in water, soil, plants, and urine. *J. Agric. Food Chem.* **1990**, *38*, 1881−1886.

Frohman, M. A.; Dush, M. K.; Martin, G. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 8998−9002.

Graham, B. M.; Porter, A. J. R.; Harris, W. J. Cloning, expression and characterisation of a single-chain antibody fragment to the herbicide paraquat. *J. Chem. Technol. Biotechnol.* **1995**, *63*, 279−289.

Hall, J. C.; Deschamps, R. J.; Krieg, K. K. Immunoassyas for the detection of 2,4-D and picloram in river water and urine. *J. Agric. Food Chem.* **1989**, *37*, 981−984.

Hall, J. C.; O'Brien, G. M.; Webb, S. R. Phage-display technology for environmental analysis. In *Immunochemical Technology for Environmental Applications*; Aga, D. S., Thurman, E. M., Eds.; ACS Symposium Series 657; American Chemical Society: Washington, DC, 1997.

Hamaker, J. W.; Johnston, H.; Martin, R. T.; Redemann, C. T. A picolinic acid derivative: a plant growth regulator. *Science* **1963**, *141*, 363−366.

Harlow, E.; Lane, D. *Antibodies: a Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1988.

Kabat, E. A.; Wu, T. T.; Perry, H. M.; Gottesmann, K. S.; Foeller, C. *Sequences of Proteins of Immunological Interest*; U.S. Department of Health and Human Services, U.S. Government Printing Office: Washington, DC, 1991.

Kaufman, B. M.; Clower, M. Immunoassay of pesticides: an update. *J. AOAC Int.* **1995**, *78*, 1079−1090.

Kramer, K.; Hock, B. Recombinant single-chain antibodies against s-triazines. *Food Agric. Immunol.* **1996**, *8*, 97−109.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970,** *227*, 680−685.

Le Calvez, H.; Fieschi, J.; Green, J. M.; Marchesi, N.; Chauveau, J.; Baty, D. Paratope characterization by structural modelling of two anti-cortisol single-chain variable fragments produced in *E. coli*. *Mol. Immunol.* **1995**, *32*, 185−198.

McCafferty, J.; Griffiths, A. D.; Winter, G.; Chiswell, D. J. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **1990**, *348*, 552−554.

Padlan, E. A. On the nature of antibody combining sites: unusual structural features that may confer on these sites an enhanced capacity for binding ligands. *Proteins* **1990**, *7*, 112−124.

Plückthun, A.; Skerra, A. Expression of functional antibody Fv and Fab fragments in *Escherichia coli*. *Methods Enzymol.* **1989**, *178*, 497−515.

Ruberti, F.; Bradbury, A.; Cattaneo, A. Cloning and expression of an anti-nerve growth factor (NGF) antibody for studies using the neuroantibody approach. *Cell Mol. Neurobiol.* **1993**, *13*, 559−568.

Ruberti, F.; Cattaneo, A.; Bradbury, A. The use of the RACE method to clone hybridoma cDNA when V region primers fail. *J. Immunol. Methods* **1994**, *173*, 33−39.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

Scholthof, K.-B. G.; Zhang, G.; Karu, A. E. Derivation and properties of recombinant Fab antibodies to the phenylurea herbicide diuron. *J. Agric. Food Chem.* **1997**, *45*, 1509−1517.

Smith, G. P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **1985**, *228*, 1315−1317.

Somerville, J. E., Jr.; Goshorn, S. C.; Fell, H. P.; Darveau, R. P. Bacterial aspects associated with the expression of a single-chain antibody fragment in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **1994**, *42*, 595−603.

Tout, N. L.; Lam, J. S. Phage display and bacterial expression of a recombinant Fab specific for *Pseudomonas aeruginosa* serotype O6 lipopolysaccharide. *Clin. Diag. Lab. Immunol.* **1997**, *4*, 147−155.

Vallejo, R. P.; Bogus, E. R.; Mumma, R. O. Effects of hapten structure and bridging groups on antisera specificity in parathion immunoassay development. *J. Agric. Food Chem.* **1982**, *30*, 572−580.

Vaughan, T. J.; Williams, A. J.; Pritchard, K.; Osbourn, J. K.; Pope, A. R.; Earnshaw, J. C.; McCafferty, J.; Hodits, R. A.; Wilton, J.; Johnson, K. S. Human antibodies with subnanomolar affinities isolated from a large nonimmunized phage display library. *Nat. Biotechol.* **1996**, *14*, 309−314.

Ward, E. S. VH shuffling can be used to convert an Fv fragment of anti-hen egg lysozyme specificity to one that recognizes a T cell receptor Vα. *Mol. Immunol.* **1995**, *32*, 147−156.

Ward, V. K.; Schneider, P. G.; Kreissig, S. B.; Hammock, B. D.; Choudary, P. V. Cloning, sequencing and expressing of the Fab fragment of a monoclonal antibody to the herbicide atrazine. *Protein Eng.* **1993**, *6*, 981−988.

Webb, S. R.; Lee, H.; Hall, J. C. Cloning and expression in *Escherichia coli* of an anti-cyclohexanedione single-chain variable antibody fragment and comparison to the parent monoclonal antibody. *J. Agric. Food Chem.* **1997**, *45*, 535−541.

Webber, K. O.; Reiter, Y.; Brinkmann, U.; Kreitman, R.; Pastan, I. Preparation and characterization of a disulfide-stabilized Fv fragment of the anti-tac antibody: comparison with its single-chain analog. *Mol. Immunol.* **1995**, *32*, 249−258.

Weiler, E. W.; Wieczorek, U. Determination of femtomol quantities of gibberellic acid by radioimmunoassay. *Planta* **1981**, *152*, 159−167.

Worthing, C. R.; Walker, S. B. *The Pesticide Manual: A World Compendium*, 8th ed.; British Crop Protection Council: Thornton Heath, U.K., 1987.

Young, N. M.; MacKenzie, C. R.; Narang, S. A.; Oomen, R. P.; Baenziger, J. E. Thermal stabilization of a single-chain Fv antibody fragment by introduction of a disulfide bond. *FEBS Lett.* **1995**, *377*, 135−139.

Yuan, Q.; Clarke, J. R.; Zhou, H.; Linz, J. E.; Pestka, J. J.; Hart, L. P. Molecular cloning, expression, and characterization of a functional single-chain Fv antibody to the mycotoxin zearalenone. *Appl. Environ. Microbiol.* **1997,** *63*, 263−269.

Received for review June 15, 1998. Revised manuscript received August 19, 1998. Accepted August 20, 1998. This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), and Environmental Science and Technology Alliance Canada (ESTAC) to J.C.H., H.L., and J.T.T.

JF980643M