



An immunoassay for atrazine using tunable immunosorbent

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Abstract

A novel, simple, economical, and environmentally friendly tunable immunosorbent-based immunoassay for sensitive and selective determination of atrazine is reported. Tunable immunosorbents consisting of a fusion between an elastin-like polypeptide made up of 77 repeating units of the pentapeptide VPGVG and a single-chain Fv of an anti-atrazine antibody were synthesized biologically and purified by temperature-triggered phase transition. A competitive immunoassay based on the competition of atrazine–horseradish peroxidase and atrazine was established with IC₅₀ and lower detection limit of 0.16 and 0.01 ppb, respectively. Excellent recoveries (mean values ranging between 92 and 104%) were demonstrated in simulated atrazine-contaminated water samples. © 2003 Elsevier Inc. All rights reserved.

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Atrazine is a widely used herbicide for controlling weeds in agricultural areas, especially corn fields. It is a potential endocrine-disrupting agent and is suspected to cause many different types of cancers [1–4]. It functions by interrupting regular hormone function, causing birth defects, reproductive tumors, and weight loss in mothers and embryos [5,6]. Atrazine has a long half-life, due to the exceedingly slow rate of photolysis and hydrolysis [7,8], and traces have been found in water supplies, fruits, vegetables, meat, and dairy products. Because of its toxicity, the US EPA has set the maximum contaminant level at 3 parts per billion (ppb).

Sensitive, selective, rapid, and reliable detection and determination of atrazine is necessary for the protection of our food and water supply, the environment, and workers employed in the agricultural industry. Gas, liquid, and thin-layer chromatography with various detectors and different types of spectroscopy are the most common analytical techniques presently used for its analysis [9]. However, these techniques are time consuming, expensive, in need of highly trained personnel, available only in sophisticated laboratories, and not amenable to field monitoring.

Immunoassays allow quick and inexpensive analysis of pollutants in the laboratory or with high sensitivity and specificity. In addition, they are environmentally friendly because no organic solvents are required. Monoclonal antibodies against atrazine are already available and in use [10,11]. In recent years, a variety of antibody fragment configurations have been developed and shown to retain antigen-binding affinity. The most commonly used configurations are based upon a single-chain Fv (scFv) module and scFv against atrazine has been developed and used in immunoassay [12,13]. Enzyme-linked immunosorbent assay (ELISA)¹ has been widely used in environmental monitoring. Although suitable for screening a large number of samples, ELISA is undesirable for field monitoring. Additionally, ELISA requires extensive handling, a considerable amount of costly plastic trays, and long analysis time because of the slow reaction due to limited mass transport and diffusional resistance across the solid–liquid interface. Consequently, rapid, inexpensive, and effective methods for the detection of atrazine from contaminated water supplies are needed.

¹ Abbreviations used: ELISA, enzyme-linked immunosorbent assay; ELP, elastin-like polypeptide; 2,4-D, 2,4-dichlorophenoxyacetic acid; DCC, dicyclohexyl carbodiimide; NHS, *N*-hydroxysuccinimide; DMF, dimethyl formamide; HRP, horseradish peroxidase; IPTG, isopropyl β -D-thiogalactoside; PBS, phosphate-buffered saline.

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Phase-separation immunoassay is a novel immuno-method developed in recent years [14]. Antigen and antibody react under homogeneous, as opposed to heterogeneous in ELISA, conditions and the immunocomplex is separated from the solution by a simple thermal precipitation. Typically, thermally reversible polymers such as poly-*N*-isopropylacrylamide are chemically conjugated to the antibodies. This is time consuming and harmful to the functionality of the antibodies [15]. One way to circumvent this problem is through the creation of multidomain artificial protein biopolymers in which the interchaining interaction and atrazine-binding functions are engineered independently. The availability of genetic engineering technology now provides the possibility to specifically design immunosorbents with tunable properties that can be used to selectively detect atrazine from dilute solutions.

Elastin-like polypeptide (ELP), consisting of the repeating pentapeptide VPGVG, is structurally similar to the mammalian protein elastin which undergoes a reversible phase transition from water-soluble forms into aggregates as the temperature increases [16]. The temperature at which this transition occurs (T_i) can be manipulated by varying the composition and length of ELP and can be tuned by pH, ionic strength, pressure, and covalent modifications of the amino acid residues [17,18]. This has opened up the exciting possibility of engineering temperature and pH switch sequences of nondisruptive lengths into existing antibody fragments. Moreover, ELP fusions exhibit a similar reversible, soluble–insoluble phase transition with the functionalities of the fusion domain unaffected even after repeated resolubilization [19]. Because both phase transition properties and fusion functionality can be maintained even after repeated phase transition, it is easy to envision that tunable immunosorbents could be similarly designed. Such a property is highly desirable because the antigen–antibody complex can be easily separated from the rest of the solution as precipitate by inducing aggregation of the ELP domain. The goal of this study was to develop a novel immunoassay for atrazine using the tunable immunosorbents. Our results demonstrate that the immunoassay based on tunable immunosorbents is rapid, sensitive, and environmentally affordable and the framework presented here should lay the foundation for the economical and highly efficient detection of diverse pollutants.

Materials and methods

Materials

Atrazine, simazine, and prometryn were purchased from Chem Service Inc. (West Chester, PA). Diethyl-*p*-nitrophenyl phosphate (paraoxon), 1-naphthyl *N*-methylcarbamate (carbaryl), 2,4-dichlorophenoxyacetic

acid (2,4-D), dicyclohexyl carbodiimide (DCC), *N*-hydroxysuccinimide (NHS), dimethyl formamide (DMF), bovine serum albumin, horseradish peroxidase (HRP), and anti-HuCk (human *k* light-chain constant domain)–HRP conjugate were purchased from Sigma Chemical Co. (St. Louis, MO). Primers scFv1 and scFv2 were purchased from Loma Linda University (San Bernardino, CA).

Molecular biology, bacterial strains, and plasmids

DNA manipulations were performed according to standard procedures unless specified otherwise [20]. PCR was performed using the *Pfu* DNA polymerase (Promega, Madison, WI) according to the manufacturer instructions. *Escherichia coli* strains JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F'* [*traD36 proAB⁺ lacI^q lacZ ΔM15*]) and XL1-Blue (*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac⁻ F'* [*proAB⁺ lacI^q lacZ ΔM15 Tn10 (tet^r)*]) were grown on LB agar for solid culture and in terrific broth [20] for liquid culture. All media contained 100 μg/ml ampicillin for selection. Plasmid pET-Ela78h6 [21] carrying ELP78 was used as the source of the ELP gene and plasmid pIMS147 [22] carrying the anti-atrazine scAb was used as the source of the scAb gene.

Construction of pEla77-scAb

The DNA fragment coding for the anti-atrazine scAb from pIMS147 was amplified as a 1096-bp PCR fragment using the primer pair scFv1 (5'-ttt ccc ggg ggg tgg cgg tgg ctc tgg tgg cgg tgg ctc tca ggt gca gct gca gga g-3') and scFv2 (5'-ccc ggc tcc tta gtg gtg gtg gtg gtg tga-3'). The PCR product was digested with *Xma*I and *Bam*HI and inserted into a similarly digested pET-Ela78h6, resulting in pET-Ela77-scAb.

Plasmid pET-Ela77-scAb was digested with *Eco*RI and was made to the blunt-ends by the *Pfu* polymerase. An expression vector backbone was prepared from pIMS147 by *Ppu*MI digestion and *Pfu* polymerase reaction. Following digestion with *Not*I and purification by agarose gel electrophoresis, the ELP77-scAb partial fragment from pET-Ela77-scAb was ligated with previously digested pIMS147 to create pEla77-scAb.

Expression of ELP77-scAb

A 25-ml culture of XL1-Blue cells transformed with pEla77-scAb was grown overnight in terrific broth [20] at 30 °C, transferred into 250 ml of fresh medium, and grown at 37 °C. After incubation with vigorous aeration for 2 h, the samples were transferred to 25 °C. Expression was induced by the addition of IPTG to a final concentration of 0.1 mM after 1 h. Incubation was continued for a further 12 h.

Cell fractionation

Cells were osmotically shocked and fractionated after being normalized to a specific OD₆₅₀. A 500-ml sample (OD₆₅₀ = 10) was spun down and washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.5). The pellet was resuspended in 10 ml of ice-cold 0.75 M sucrose in PBS (pH 8.0) and 1 ml of hen egg white lysozyme (10 mg/ml) was added. The suspension was gently shaken on ice and 20 ml of 1 mM ethylenediaminetetraacetic acid in PBS (pH 7.5) was added dropwise. After 10 min on ice, 2 ml of 0.5 M MgCl₂ was added and incubation continued for an additional 10 min. After centrifugation at 30,000g for 30 min, the supernatant (periplasmic fraction) was collected. The pellet was resuspended in 30 ml of PBS and disrupted by French Press twice. After centrifugation at 30,000g for 30 min, the supernatant was removed (soluble cytoplasmic fraction) and the pellet (insoluble fraction) was resuspended in 28 ml of PBS.

Purification and analysis of ELP77-scAb

The ELP-scAb fusion protein was purified from the periplasmic fraction by temperature cycling after addition of ELP78. The periplasmic fraction with ELP78 was heated to 37 °C for 5 min and centrifuged at 10,000g while maintaining the temperature at 37 °C. The supernatant was discharged and the pellet containing the fusion protein and the added ELP78 was dissolved in cold (4 °C) PBS (pH 7.5) and centrifuged while maintaining the temperature at 4 °C to remove undissolved proteins. The temperature cycling was repeated once more.

To confirm the expression of fusion protein, 5 µl of purified protein was electrophoresed through a 10% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Western blot was performed using an anti-Hu*Ck*-HRP conjugate and signals were detected using the enhanced chemiluminescence kit (Amershampharmacia Biotech, Piscataway, NJ). The intensity of the protein bands was quantified using a Bio-Rad Gel Doc 2000 Gel Documentation System and Quantity One software.

To demonstrate the functionality of the scAb domain, purified proteins were immobilized nonspecifically onto a microtiter plate by 2 h incubation at 37 °C in PBS (pH 7.5) followed by five washings with PBST (0.5% Tween-20 in PBS). The protein-modified plates were incubated with atrazine-HRP conjugate for 1 h at 37 °C, followed by washing five times with PBST. The plates were then treated with 100 µl of the enzyme substrate (18.5 mM *o*-phenylenediamine and 0.04% H₂O₂ in citrate-phosphate buffer (0.1 M, pH 4.6)) and the absorbance of each well was read at 490 nm after 30 min using a microtiter plate reader (Model 3550-UV, Bio-Rad).

Synthesis of HRP-labeled atrazine derivative

The synthesis of HRP-labeled atrazine derivative involved two steps. In the first step an atrazine derivative, 2-chloro-4(6-amino-*n*-hexanoic acid)-6-(2-aminopropane)-*s*-triazine, was synthesized as follows. 0.05 mol of cyanuric chloride, dissolved in 20 ml acetone, was dropped slowly into 40 ml of cold water. To this solution, maintained at -5 °C, 0.05 mol of isopropylamine was slowly added followed by the addition of 0.05 mol of NaOH to neutralize the hydrochloric acid generated. After stirring for 1 h, 0.05 mol of 6-amino-*n*-hexanoic acid was added to the reaction mixture together with an equimolar amount of NaOH. The solution was heated at 45–50 °C and maintained at this temperature for 4–5 h to react. The filtered solid, obtained after the reaction, was washed with cold acetone and then dried. The yield of the reaction was 1.8% and the structure was confirmed by NMR. A freshly prepared solution of NHS (50 µmol) and DCC (100 µmol) in DMF was added to a solution of atrazine derivative (50 µmol) in the same solvent (350 µl) to activate the carboxylic acid group of the derivative. The reaction mixture was stirred at room temperature for 4 h; 10 µl of the product was diluted to 100 µl with DMF and added to a solution of HRP (5 mg/0.9 ml) in 50 mM sodium carbonate (pH 9.5). The mixture was stirred for 2 h at room temperature followed by dialysis overnight at 4 °C in PBS buffer (pH 7.5) and the product stored in freezer with 50% glycerol until use.

Immunoassay for atrazine by tunable immunosorbents

Stock solutions of atrazine were prepared in methanol to a final concentration of 0.1 mg/ml. Ten microliters of serial dilutions of atrazine or other investigated analytes was mixed with 10 µl of atrazine-HRP conjugate (2 µg/ml) and 100 µl of ELP77-scAb fusion protein and incubated at room temperature for 1 h. Then 10 µl of 5 M NaCl was added and the mixture was heated to 37 °C to precipitate the ELP77-scAb-antigen complex. The precipitate was recovered by centrifugation at 10,000g while maintaining the temperature, washed with 100 µl of PBS, resuspended in 50 µl of PBS, and introduced into a microtiter plate. The plates were then treated with 100 µl of the enzyme substrate and the absorbance of each well was read at 490 nm after 30 min.

Results

Expression and purification of ELP77-scAb fusion proteins

Our initial goal was to construct a tunable immunosorbent with high affinity and specificity for atrazine. The synthetic gene encoding for the ELP domain with

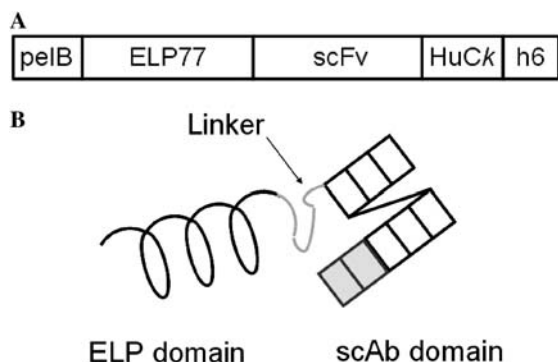


Fig. 1. (A) Expression vectors constructed in this study; pelB, signal sequence from *Erwinia chrysanthemi* pectate lyase; scFv, single-chain antibody fragment; HuCk, human *k* light-chain constant domain; 6 h, six-histidine amino acid tail. (B) Schematic diagram of ELP77-scAb fusion protein; scAb, scFv + HuCk.

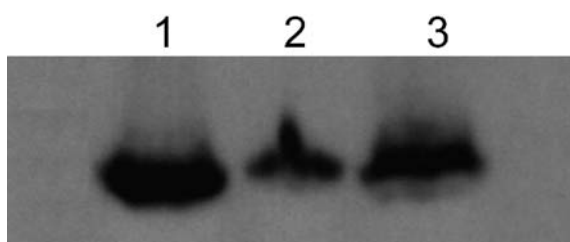


Fig. 2. Western blot of fractionated *E. coli* expressing ELP77-scAb. Lanes: (1) periplasmic fraction; (2) soluble cytoplasmic fraction; (3) total insoluble fraction.

77 repeats was constructed as reported previously [21] and fused to the 5' end of the gene coding for a scAb specific for the herbicide atrazine to create an ELP77-scAb fusion expression vector (Fig. 1). A scAb is simply a scFv bearing a single human *k* light-chain constant domain (HuCk) fused downstream. Addition of the HuCk domain allows facile detection of the fusion protein using the anti-HuCk antisera. A pelB signal sequence was introduced at the N terminus to facilitate the correct translocation of the engineered fusions into the periplasmic space. To improve the yield of soluble fusion, a Skp chaperone was coinduced with the addition of IPTG. Expression of the ELP77-scAb in the different cellular fractions was detected by blotting with the anti-HuCk antisera. As shown in Fig. 2, 40% of the total ELP77-scAb was detected in the periplasmic fraction. Distribution of β -lactamase activity in the different cellular fractions was used to confirm the fractionation efficiency.

Characteristics of ELP77-scAb fusion protein

To demonstrate the functionality of the ELP domain, the periplasmic fraction of XL1-blue/pEla77-scAb was subjected to two repeated cycles of heating and cooling. Because of the small quantity of ELP77-scAb produced, purified ELP78 was used to facilitate precipitation.

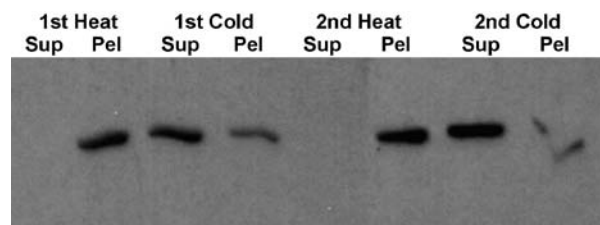


Fig. 3. Recycling of ELP77-scAb. ELP77-scAb fusions were recovered as a pellet by heating to 37°C. The resulting pellet was resolubilized by incubating at 4°C. The soluble and insoluble fractions from each incubation step were probed with an anti-HuCk serum. Heat, incubation at 37°C; cold, incubation at 4°C; Sup, supernatant; Pel, pellet.

Table 1
Atrazine anti-scAb functionality of fusion protein

Sample	Buffer only ^a	ELP78	ELP77-scAb
Atrazine-HRP	0.019	0.020	0.121
HRP ^b	0.023	0.021	0.020

^a Phosphate buffer only.

^b HRP instead of atrazine-HRP conjugate was used.

In the initial heating step, 100% of ELP77-scAb was recovered in the precipitated pellet. However, 90% of the aggregated ELP77-scAb was resolubilized after lowering the temperature to 4°C. After the first heat and cold cycle, the remaining ELP77-scAb could be recycled completely (Fig. 3). This result demonstrates that presence of the scAb has no effect on the transition behavior of the ELP domain.

The antigen-binding characteristics of the scAb domain of the ELP77-scAb fusion were determined in an ELISA format using atrazine-HRP conjugates. As shown in Table 1, ELP78 without the scAb produced almost the same value as that of the background. The absorbance obtained with ELP77-scAb was sixfold higher than those of the other samples. As a control, the same concentration of HRP was used for comparison and only background absorbance was observed.

Phase-separation immunoassay for atrazine

The principle of the competitive phase-separation immunoassay for atrazine is based on thermally triggered precipitation of the antibody-atrazine complex in the presence of HRP-labeled atrazine derivatives and other chemicals. After separation of the immunocomplex from the reagents, the amount of HRP-labeled atrazine conjugated to the antibody was easily quantified. Fig. 4 shows a calibration plot for atrazine generated by phase-separation competitive immunoassay. The observed IC₅₀ was in the order of 0.16 ppb and the lower detection limit was 0.01 ppb (10% inhibition). The assay had a good dynamic range, displayed high linearity between 0.02 and 1 ppb ($y = -19.328\ln(x) + 13.408$; $R^2 = 0.9928$), and had good reproducibility as demonstrated by the low residual standard deviation of less than 10% for four

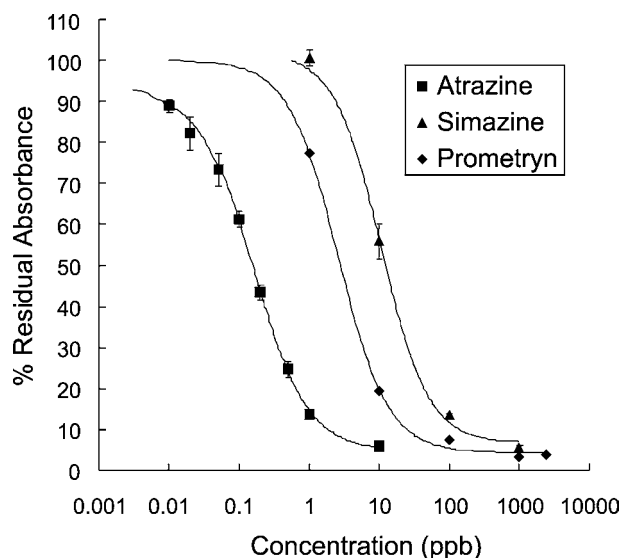


Fig. 4. Calibration plots for atrazine, simazine, and prometryn. IC_{50} represents 50% of the residual absorbance.

replicates. While the IC_{50} of the present assay is similar, the lower detection limit is 10-fold lower than that of the competitive ELISA using the same scAb [13]. Moreover, it is a rapid and simple competitive ELISA method because extensive immobilization, washing, and incubation steps are not necessary.

The developed immunoassay exhibited excellent selectivity against various pesticides such as paraoxon, carbaryl, and 2,4-D (data not shown). As expected, cross-reactivity was observed with the other triazines tested; however, the sensitivity was significantly lower with IC_{50} values of 10.73 ppb for simazine and 2.60 ppb for prometryn (Fig. 4). Because the IC_{50} values for prometryn and simazine are 10- to 100-fold higher than that of atrazine, it may be possible to detect a low concentration of atrazine without interference from other triazines [23,24]. These results are in line with the reported specificity for competitive ELISAs based on the parent anti-atrazine monoclonal antibody and the same scAb, again demonstrating that fusion to the ELP domain has no effect on selectivity [13]. The reported immunoassay demonstrated good accuracy as evident from the greater than 90% recovery in simulated atrazine-contaminated water samples (Table 2).

Table 2
Immunoassay accuracy

Real atrazine (ppb)	Measured atrazine (ppb) ^a	Recovery (%)
0.025	0.026 ± 0.006	104.50
0.25	0.23 ± 0.05	92.46
0.5	0.48 ± 0.06	96.39

^a Values are mean of five determinations ± SD.

Discussion

Thermally triggered phase-separation characteristics have increasingly been exploited for the development of improved immunoassays [25–27]. However, the traditional chemical cross-linking methods to generate antibody conjugates with temperature-sensitive polymers are time consuming and not environmentally friendly. Different from the chemical methods, our tunable immunosorbents are specifically preprogrammed within a synthetic gene template that can be precisely controlled over chain length, composition, sequence, and most important properties. Our results clearly demonstrate that the scAb domain and the ELP domain of ELP77-scAb are fully functional without interfering with each other. One added benefit is the facile purification of the ELP77-scAb fusion by simple reversible phase cycling.

The present immunoassay based on tunable immunosorbent for atrazine enables a simple and sensitive method for the detection of atrazine. It provides the advantages of both fast immunoreaction of homogeneous assay and high sensitivity and selectivity of heterogeneous assay.

In conclusion, a novel phase-separation immunoassay for atrazine has been successfully demonstrated using ELP77-scAb fusion. This thermally triggered method is rapid, economic, and environmentally friendly. In addition, the assay exhibits excellent IC_{50} and detection limit of 0.16 and 0.01 ppb, respectively. Although the results reported here are for the detection of atrazine with anti-atrazine scAb, other scAb domains for other pollutants may be similarly applied and used for detection.

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