Original Research

Engineering Novel Aptameric Fluorescent Biosensors for Analysis of the Neurotoxic Environmental Contaminant Insecticide Diazinon from Real Vegetable and Fruit Samples

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Abstract

Background: Diazinon is a widely used organophosphorus neurotoxic insecticide. It is a common environmental contaminant and a hazardous agri-waste. Its detection is critical to control entry into food systems and protect the environment. **Methods**: In this study, three single-stranded DNA aptamers specific for diazinon were discovered using the systematic evolution of ligands by the exponential enrichment (SELEX) process. Since aptamer-based sensors are quick and straightforward to analyze, they could potentially replace the time-consuming and labor-intensive traditional methods used for diazinon detection. **Results**: Here, we show the engineering of novel sensors for diazinon detection with a high affinity (Kd), specificity, and high sensitivity at the ppb level. Moreover, the aptamers were helpful in the simultaneous detection of two other structurally relevant insecticides, fenthion, and fenitrothion. Furthermore, the real vegetable and fruit samples confirmed the specific detection of diazinon using DIAZ-02. **Conclusions**: We developed novel biosensors and optimized the assay conditions for the detection of diazinon from food samples, such as vegetables and fruit. The biosensor could be adopted to analyze toxicants and contaminants in food, water, and nature as point-of-care technology.

Keywords: food contaminant; SELEX; insecticide detection; ssDNA aptamer; fluorescence; biosensor; binding affinity; dissociation constant

1. Introduction

Diazinon is a broad-spectrum organophosphorus insecticide and a neurotoxic compound. Since its registration (1956) for commercial use in the US, it has become a top-ranked insecticide in consumption [1]. The diazinon and its products are available under various formulations and trade names, including Alfatox, Basudin, Cekuzinon, Diazol, Gardentox, and Knoxout [1], used to kill several classes of insects. In agriculture, it has been helpful to control the pests of the soil, field crops, vegetables, and livestock. In residential areas, it is used to protect houses, gardens, and pets from aggressive insects and insect-borne diseases. The mode of action of diazinon targets the central nervous system via the blocking of acetylcholinesterase. The enzyme is involved in neurotransmission; loss in signaling due to diazinon causes insect muscle to fail to function, eventually leading to death [2].

Uncontrolled use spreads of insecticide chemical contaminants into soil, water, and food and produces deleterious consequences on biodiversity, the environment, and human health. Diazinon is highly poisonous to beneficial insects (bees), birds [3], aquatic species [4], and other wildlife [5]. According to the International Agency for Research on Cancer (IARC) report, diazinon may cause cancer in humans [6]. In 2004, although the USA banned diazinon in residential areas, it is still legally available for agricultural applications. This presents a danger of contaminating soils [7], food [8], and water [3,4] with diazinon, and it is an eminent element of threat to human health and biodiversity. Depending on the time and amount of exposure to diazinon, humans can have several neurological problems, such as dizziness, headache, weakness, difficulty breathing, or death [9]. Recent studies have shown that diazinon inhibits acetylcholinesterase [10], induces oxidative stress [11], promotes inflammation, and causes DNA damage [12]. Therefore, monitoring diazinon residue levels is essential for food and biosafety, human health, and environmental protection [5].

Early detection of toxic environmental and food contaminants such as insecticides is a challenge due to the small molecule nature of these chemicals [13–15]. At present, the

most commonly used methods for insecticide analysis demand highly sophisticated machinery, heavy investment (or high maintenance cost), and the need for skilled workers to analyze the samples. Moreover, these methods are challenging to deploy in remote areas due to a lack of funds, bulkiness, and mobility issues. Examples of these methods include high-performance liquid chromatography (HPLC), mass spectrometry (MS), and gas chromatography, which demand laborious sample preparation [14]. Therefore, to overcome the challenges in the early detection of insecticides, a simple, quick, sensitive, specific, and rapid sensor assay is required [16,17]. Aptamer sensors provide an attractive alternative in biowaste management via effective and efficient detection. Aptamers are single-stranded DNA or RNA (ssDNA or ssRNA) molecules that can selectively bind to specific targets [18]. Recently, aptameric sensors for detecting small molecules have drawn the intense attention of researchers and technologists as the method of choice because they meet several criteria that fulfill the demands for cheaper, quick, simple, easy, and sensitive detection [13,19,20]. For instance, Coonahan *et al*. [21] estimated the cost of aptamer-based assays to be as low as 0.25 USD/sample, while Trinh *et al*. [15] compared the cost of HPLC to be 80 to 100 times higher than that of aptasensors. Moreover, aptamers can be further developed as label-free and machine-independent sensing platforms [15,21,22].

Aptamers present several advantages over traditional methods of detection. It can form a versatile three-dimensional structure that helps form a precise binding pocket to recognize its cognate target [21]. Interactive binding via van der Waals forces, hydrogen bonding, electrostatic interactions, stacking of flat moieties, pi-pi interactions, pi-anionic interactions, steric hindrances, and shape complementarity contribute to the specificity and high affinity. Aptamers are isolated using a stringent selection protocol of the SELEX process [23]. The principle of the SELEX method is based on the binding to targets of ssDNA or RNA from a random pool (library of 10^{15} DNA molecules) [22] through several cycles of affinity binding, separation, PCR amplification, and sequencing. After the SELEX process was discovered in 1990, several aptamers have been reported to detect small molecules such as antibiotics, toxins, food contaminants, environmental pollutants, and carcinogens [8,24–26].

Here, we report the development of novel ssDNA aptameric sensors to detect toxic food and the environmentally contaminating insecticide diazinon. We successfully identified three ssDNA candidate aptamers that bind to diazinon efficiently and effectively. The isolated aptamers displayed robustness in detecting diazinon even at a very low concentration of up to 148 nm. Moreover, DIAZ-03 could be differentiated from other related organophosphorus insecticides (fenthion, fenitrothion, and malathion). Next, we demonstrated its applicability in detecting diazinon from real samples of vegetables and fruit. We believe these ap-

tamers can be custom designed as recognition elements in biosensing coupled with nanoparticles for bioremediation to remove contaminants [13]. The novel sensors present a promising future in bioprocessing, agriculture, food production, agri-waste treatment, and environmental protection. Overall, aptameric sensors could be pivotal in helping environmentalists and technologists meet several sustainable development goals (SDGs), including clean food, water, and a safe environment.

2. Materials and Methods

2.1 Chemical Compounds and Oligonucleotide Preparations

The chemicals diazinon, fenthion, fenitrothion, and malathion (HPLC purification grade) were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). All chemical insecticides were dissolved in acetone and then diluted in 1x SELEX buffer (20 mM HEPES, 1 M NaCl, 10 mM MgCl$_2$, 5 mM KCl, pH 7.5) to formulate the desired working concentration and were stored at 4 °C.

The size of ssDNAs in the library used was 72 bp, which included the following: N-30 region generated by randomly mixing the A-C-T-G bases and primer region (forward and reverse): (1) Library: 5′-GGA GGC TCT CGG GAC GAC GAC-(N30)-GTC GTC CCG ATG CTG CAA TCG TAA-3′; (2) Biotinylated column immobilizing capture strand: 5′-GTC GTC CCG AGA GCC ATA - BioTEG-3′; (3) Forward primer: 5′-GGA GGC TCT CGG GAC GAC GAC-3′; (4) Reverse primer: 5′-TTA CGA TTG CAG CAT CGG GAC G-3′; and (5) Biotinylated reverse primer: 5′-Biotin-TTA CGA TTG CAG CAT CGG GAC G-3′. All oligonucleotides were dissolved in nuclease-free water and stored at –20 °C.

2.2 The SELEX Process for the Selection of ssDNA that Binds to Target

A 500 pmol ssDNA library was mixed with 2500 pmol capture strand (1:5 ratio) in 250 μL of 1x SELEX buffer. The mixture was incubated in a hot water bath (95 °C) for denaturation. After 5 minutes, the mixture was removed and then allowed to cool at room temperature for 10 minutes. The oligonucleotide mixture was transferred to a micro biospin chromatography column (Cat. No. 732-6204, Bio–Rad, Hercules, CA, USA) containing 200 μL of streptavidin agarose resin (1–3 mg biotinylated BSA/mL resin, Thermo Scientific, Waltham, MA, USA). The oligonucleotide mixture was slowly passed through the column three times, and the eluent was collected. Then, the buffer was washed fifty times with 250 μL of 1x SELEX buffer each time. The eluent from the first wash (W1) and the final wash (W50) was collected and used to monitor the efficiency of washing. We then added three aliquots of 100 μM diazinon to the column, and each 250 μL aliquot was collected as the eluent. Small-scale PCR was run with 1 cycle of 95 °C for 2 min, N cycles of 92 °C for 15 s, 59 °C

for 30 s, and 72 °C for 45 s and 1 cycle of 72 °C for 2 min. Then, the PCR products were separated by 3% agarose gel electrophoresis (100 V, 20 min) to evaluate the efficiency and specificity of the selection procedure.

Three aliquots of eluent after diazinon addition were concentrated by a 3 KDa filter (EMD Millipore Amicon Ultra0.5 Centrifugal Filter Units, Millipore, cat. No. 732-6204) and were used as the templates for large-scale PCR. The reverse primer was replaced by a biotinylated reverse primer in this step. Four PCR tubes containing 30 μL samples were run similarly to a small-scale PCR program with 8, 10, 12, and 14 cycles. All the PCR products were loaded in the gel, and the optimal number of PCR cycles to amplify the template was estimated. Then, PCR was run with the rest of the master mix. Using a 10 KDa MWCO filter, the concentrated PCR products were passed through a mini gravity column containing 0.2 mL of streptavidin agarose resin. The eluent was collected after incubating with 400 μL of 0.2 M NaOH, and 0.2 M HCl was used to adjust pH 7.5. After adding 750 μL 2x SELEX buffer, the eluent was concentrated by a 3 KDa MWCO filter. This concentrated product was used as a new library for the next round of selection.

2.3 Sequencing and Confirmation of Selected ssDNA that Bind to Target

Purified DNA from the last round of library selection was used to clone the candidate aptamers. As mentioned earlier, PCR amplification of selected DNA was carried out, and cloning was performed using the T-Blunt™ PCR Cloning kit (SolGent, Daejeon, Korea) following the manufacturer's instructions. A total of 96 individual colonies were randomly picked for plasmid extraction. Plasmid DNAs were extracted by using the HiGene™ Plasmid Mini Kit (Ver 2.0; BIOFACT, Daejeon, Korea) following the manufacturer's instructions. All plasmids were sent for sequencing service by COSMO Tech Company (Seoul, Korea). The candidate aptamer sequences were analyzed and classified into groups using the Clustal Omega program [27] and aligned manually to remove spaces. Next, SELEX was performed with a few representative clones whose copy numbers were predominant in sequence data to confirm diazinon binding.

2.4 Design and Analysis of Sensors

Sequences were analyzed to make a 2D structure using the Mfold program [28]. The aptamer sequences that form a stem–loop structure with eight base-pair stems were selected for the aptamer assay with fluorescein (FAM) conjugated to the 5′-end of the sensor. A 13-bp quencher sequence modified with dabcyl dye at the 3′-end (5′-GTCGTCCCGAGAG -Dab-3′) was used to quench the fluorescence signal.

The quenching efficiency was determined by optimization of the ratio of the sensor to the quencher. The 2x concentration of FAM-sensor 100 nM was mixed with a 13-bp quencher by twofold serial dilution of range from 0 nM to 500 nM with an equal volume (60 μL). The sensor and quencher mixture was heated to 96 °C for 5 min and gradually cooled to room temperature for 30 min. One hundred microliters of each mixture was transferred into a 96-well black polystyrene assay plate (Cat. No. 3340, Corning, NY, USA). The fluorescence was measured by a 485 nm excitation wavelength and emission collection with a 515 nm to 535 nm cutoff on a SpectraMax GeminiTM XPS/EM Microplate Reader (San Jose, California 95134, United States). F/F0 was used to generate the binding curve, where F is the FL intensity in the presence of the quencher and F0 is the FL intensity without the quencher. Each binding curve was plotted through nonlinear least-squares progression using GraphPad Prism 5.0 software (GraphPad Software, 2365 Northside Dr., Suite 560, San Diego, CA 92108). All measurements were performed in nine replicates. The quenching efficiency was defined as q% = (1 – F/F0) %.

The K_d values of the sensor against the target were calculated. The final concentrations of both the sensor and quencher were set at a ratio that was determined by a previous quencher testing experiment, which has more than 80% quenching efficiency and is used at a sensor: quencher ratio of 1:5. To make a standard curve, the concentration of diazinon was made from 0 mM to 1 mM by twofold serial dilution. Briefly, 30 μL of each 4x sensor and a quencher were mixed and heated at 96 °C for 5 min and cooled gradually to room temperature for 30 min. Then, 60 μL of various concentrations of 2x diazinon (0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, and 50 μM) was added and incubated at room temperature for 40 min. A total of 100 μL of each solution was transferred onto a 96-well microwell plate, and the FL intensity was measured. All measurements were performed in nine replicates.

The LOD for each sensor was calculated based on the formula CL = K*Sd/S [29], where Sd is the standard deviation of blank sample; K refers to a signal-to-noise ratio (S/N) (which was used in this study with 3.3); S is the slope of detecting linear between the fluorescence intensity enhancement versus low concentrations [29] of diazinon. The specificity of the sensors toward diazinon was estimated via cross-reactivity testing. Three organophosphate pesticides, fenthion, fenitrothion, and malathion, were used to compare the cross-reactivity of the selected aptamers.

2.5 Diazinon Detection Assay in Vegetable and Fruit Tissue Extracts

The optimal conditions for detecting diazinon from vegetable and fruit tissue extracts were evaluated using different pH values, temperatures, and incubation times. The effect of pH was studied using all samples (sensor/quencher and diazinon) by preparing in 1x SELEX buffer with differ-

ent pH values (5.0, 6.0, 7.0, 7.5, and 8.0) incubating at room temperature for 40 min before measurement. The effect of incubation time on all samples was studied by preparing samples in 1x SELEX buffer at pH 7.5 and measuring the signal at different time points from 0 min to 60 min. Finally, the effect of temperature was estimated by performing the reaction in 1x SELEX buffer (pH 7.5 and 40 min) at various temperatures, such as 20 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, and 60 °C.

To prepare plant tissue extracts, tomato, Chinese cabbage, and apples were selected as examples to develop a biosensor assay. Chinese cabbage (cultivar: Wa Wa Wa Sai), tomato (cultivar: Chal), and apple (cultivar: Fuji) were purchased from TopMart (Jinju, Gyeongsangnam-do, Korea). Fifty grams of sample was first cut into small pieces and homogenized with 50 mL of 20 mM Tris-HCl buffer (pH 7.5) by using a food blender and then centrifuged for 10 minutes at 4 °C and 14000 rpm. The supernatant was collected and filtered using a Miracloth filter (Cat. No. 475855-1R, Sigma–Aldrich, St. Louis, MO, USA) and then used as an original plant extract. To obtain the proper dilution series of plant extract samples, we prepared five different plant extract solutions by 10 times serial dilution (0, 10X, 100X, 1000C, and 10000X), and each sample was spiked with 10 μM of diazinon. Additionally, we used different concentrations of diazinon to obtain optimal spiked concentrations and found that a concentration of 10 μM, which was within the linear range of detection, was most suitable.

3. Results and Discussion

3.1 Identification of Single-Stranded DNA that Binds to Diazinon

The structure-switching SELEX process was employed [23] to identify ssDNA aptamers specific to diazinon. This method was based on the principle of structure switching and elution upon target binding [15,18]. The random ssDNA library (of $>10^{15}$ DNA molecules) was hybridized with a short capture strand with a biotinylated complementary sequence (Fig. 1A, Ref. [28]). The candidate aptamer oligonucleotides were immobilized on agarose beads containing streptavidin through a biotin-functionalized capture strand. First, diazinon was introduced as a specific target at a concentration of 100 μM from the 1st round to the 10th round of the SELEX process. Later, its concentration was reduced to 10 μM from the 11th round to the 15th round. Next, three rounds of negative selection were performed using fenthion, fenitrothion, and malathion (nonspecific target molecules). To generate the enriched library, the ssDNA oligonucleotides were eluted with diazinon, collected, and amplified via polymerase chain reaction (PCR). With an increase in the round of the SELEX process, the proportion of diazinon binding sequences in the regenerated ssDNA pool was increased. Subsequently, lower affinity bound or free nucleotide sequences were washed off. After washing 50 times with 1x SELEX buffer, unbound sequences in the ssDNA pool were removed from the column (Fig. 2C).

The selected PCR products were cloned, and numerous individual colonies were obtained that carry plasmids with a particular PCR fragment representing each candidate aptameric ssDNA. A total of 96 colonies were randomly picked for sequencing. The sequences were subjected to bioinformatics analysis; sequence alignment and similarity were measured. Each sequence contained an 18 bp forward primer and 22 bp reverse primer at the 5′ end and 3′-end, respectively, while the middle part consisted of a random variable region of 30 nucleotides (Fig. 1B). Of the 96 colonies sequenced, 64 yielded high sequence similarity and high abundance, with 18 unique sequences (Table 1). The remaining 32 clones showed high sequence divergence and low abundance; some clones failed to yield good sequence data (**Supplementary Table 1**). The sequence alignment and frequency helped select and classify aptamers into three classes: DIAZ-01, DIAZ-02, and DIAZ-03 (Table 1). Next, to confirm the binding affinity of the aptamers for diazinon, DIAZ-01, DIAZ-02, and DIAZ-03 were selected as representatives of each class (Fig. 2A). The results indicated that all three aptamers displayed a high binding affinity to diazinon.
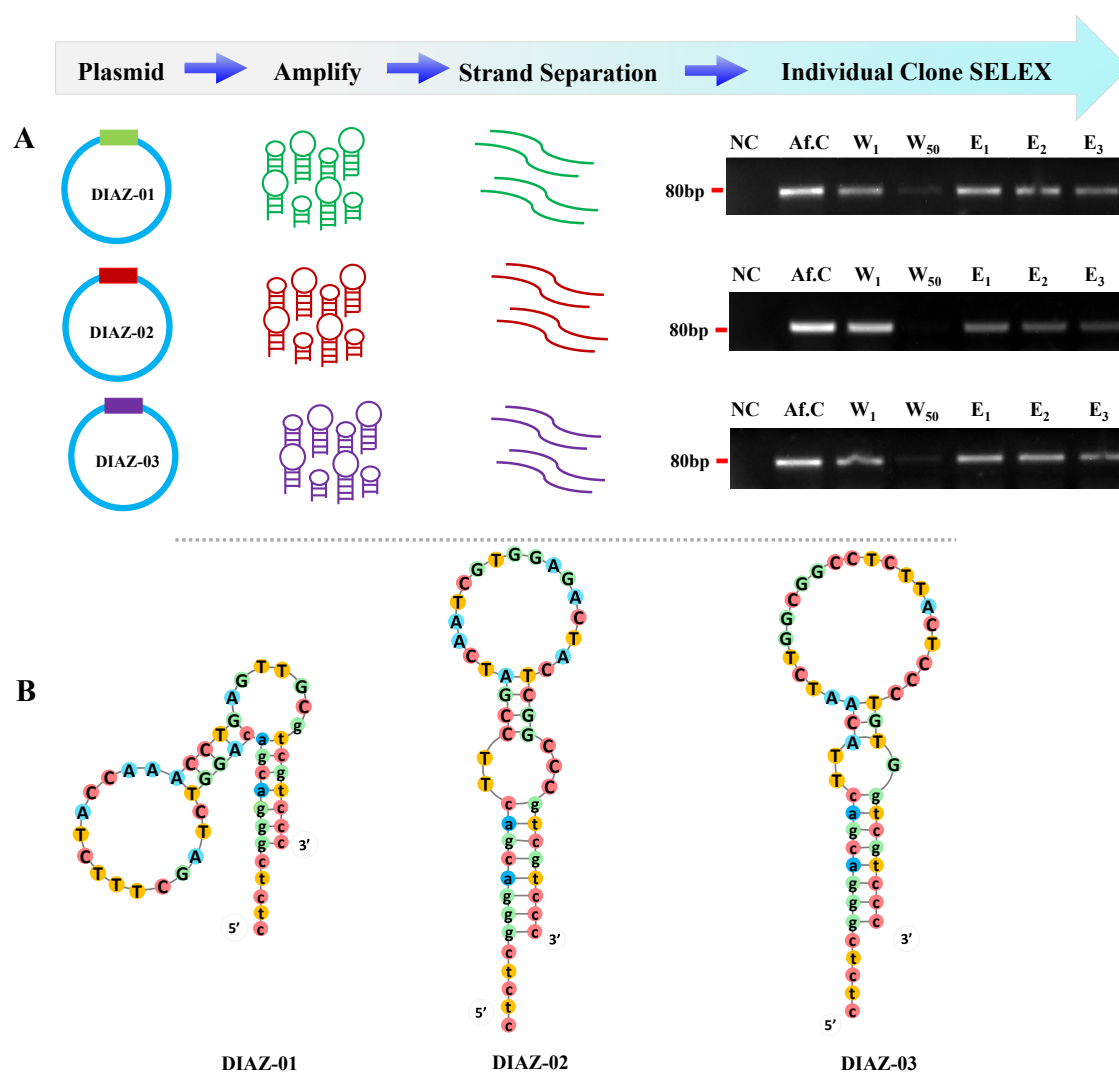
3.2 Design of a Fluorescence-Based Strategy to Detect Diazinon Using ssDNA Aptameric Sensors

The secondary structure of the newly discovered candidate aptameric sequences was predicted to examine the suitability and stability of the novel biosensor design (Fig. 2B). The sequences were analyzed by Mfold software [28]. The free energy of the sequences was compared, and all aptamers were found to have a short stem of 11 to 13 bp that could be used to form base pairing with a 13-bp dabcyl quencher.

The unique properties of ssDNA aptamers provide unparalleled opportunities to develop novel biosensors that can specifically bind to target molecules and produce a detectable signal. To obtain the fluorescence signal, the candidate ssDNA aptamer was modified with fluorescein amidite (FAM) at the 5′-end. Fluorescein is one of the most commonly used fluorophores for labeling biomolecules (DNA, RNA, and proteins) [15–17,30]. It possesses a relatively high quantum yield, strong emission peak, specific excitation wavelength, and acceptable water solubility. Moreover, the fluorophore's small size has minimal impact on ssDNA properties. However, dabcyl has been widely used as a universal quencher for many fluorophores, as it has strong potential to absorb emitted photons [30]. Hence, 6-FAM was successfully quenched, and it lacked any residual fluorescence. Additionally, 6-FAM or dabcyl molecules are relatively easy to introduce at any position in ssDNA or dsDNA sequences, including at the 5′- or 3′-end [30]. A 13-mer quencher carrying dabcyl dye was used to quench

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Fig. 1. Design of oligonucleotide probes and monitoring of the SELEX process. (A) An overview of a typical SELEX cycle to identify and isolate specific ssDNA aptameric sequences. Random ssDNA libraries flanked by fixed primer annealing sites for PCR amplification are incubated with the target molecule, and aptamer-target complexes are separated from unbound ssDNA. PCR amplification with the selected ssDNA followed by cloning, sequencing, and strand separation for the next SELEX round. The SELEX cycle was repeated several times to enrich the high-affinity binding ssDNA. (B) The random ssDNA library was hybridized with a short capture strand via biotinylated complementary sequences, wherein 'N' means any nucleotide generated by random mixing of A-T-G-C bases. (C) Electrophoresis of eluent obtained after the 10th SELEX round (10 μM diazinon) and the 16th SELEX round (1 μM fenthion, 1 μM fenitrothion, 1 μM malathion and 10 μM diazinon); where NC, Negative control; Af. C, the mixture obtained after addition to the column; W1, the first wash; W50, the 50th wash; Ex, the eluent after incubation with target diazinon; N-f, negative selection of fenthion; N-fn, negative selection of fenitrothion; N-m, negative selection of malathion.

Fig. 2. Confirmation and prediction of the secondary structures of candidate aptamers. (A) Stepwise illustration of aptamer selection—individual clones (plasmids), sequence amplification, strand separation, and confirmation of size and band intensity via electrophoresis, where NC, Negative control; Af. C, the mixture obtained after adding a column; W1, the first wash; W50, the 50th wash; Ex, the eluent after incubation with the target diazinon. (B) The secondary structure of three candidate aptamers was predicted using the Mfold program [28].

(block) the fluorescence signal. When the sensors were allowed to hybridize with a quencher, the dabcyl could successfully come in proximity to the FAM to form an F-Q complex, resulting in quenching of the FAM signal. Subsequently, the diazinon sensors undergo structural switching due to target-induced conformational change. This change in conformation leads to a unique 3D folding that causes dissociation of the quencher strand [15,30], the quenching effect is eliminated, and a measurable fluorescence signal is regenerated (Fig. 3A). Achieving higher quenching efficiency (>80%) is essential for evaluating successful and effective aptameric sensors for detecting the target. To determine the optimal concentrations of the DIAZ-F sensors and quencher for the detection of the target molecule diazinon, we performed quencher testing in the absence of

diazinon. We observed that the ssDNA aptameric sensors showed varied quencher efficiency; DIAZ-01-F, DIAZ-02-F, and DIAZ-03-F reached 89%, 86%, and 92% quenching when the concentration of quencher was applied at 125 nM, 250 nM, and 50 nM, respectively (Fig. 3B).

Next, the binding affinity of aptameric sensors with diazinon was determined by measuring the gain of fluorescence intensity (FL) by applying various concentrations of the target molecule. DIAZ-01-F, DIAZ-02-F, and DIAZ-03-F were incubated with quencher-strand in the optimal ratio that was determined by prior quenching analysis. A total of 8 different concentrations of diazinon (0 μM to 50 μM) were used, and a change in the FL intensity was observed. The FL intensity was found to increase due to diazinon concentration (Fig. 3C). The binding curve was further

Table 1. The list of ssDNA sequences obtained after the SELEX process for the specific target insecticide diazinon.

Aptamer ID	Sequence	Counts
DIAZ-01	5′-AGGTCTAGCTTTCTACCAAACCTGAGTTGC-3′	21
D1.1	5′-AGGTCTATCTTTCTACCAAACCTGAGTTGC-3′	2
D1.2	5′-GGGTCTAGCTTTCTACCAAACCTGAGTTGC-3′	2
D1.3	5′-AGGTCTACTTTGCTCTCCTTCTGTGAGTTGC-3′	1
D1.4	5′-AGGTCTACTTTGCATTGCTACAGTGAGTTGC-3′	1
D1.5	5′-AGGTCTACTTTGTTCTGCTTCAGTGAGTTGC-3′	1
DIAZ-02	5′-TTCCGATCAATCGTGGAGACTACTCGGCCC-3′	13
D2.1	5′-CGCCGAACAATCGTGGATTTGACTCGGCCC-3′	2
D2.2	5′-GATGCACCGTGGCGGCTCTCACGCCCTGCG-3′	1
D2.3	5′-TGCTTTCCTCCCGGGTGGGGTCCGACGGCCC-3′	1
D2.4	5′-GACCCAATTCAGTGGGTCAATTTCCGGCCC-3′	1
D2.5	5′-GACCCAATTCGTGGTGTCAATTTCCGGCCC-3′	1
D2.6	5′-CAAGCATACAATAGTGGGTTAAGGCGGCCC-3′	1
D2.7	5′-CAGAGCAATTCATGTGGTGAGTCCCGGCCC-3′	1
DIAZ-03	5′-TTACAATCTGGCGGCCTCTTACTCCCTGTG-3′	10
D3.1	5′-GTAAATCTGTCGGCTCCATAACTCCCTGTG-3′	1
D3.2	5′-TGCAATCTGGCGGCTCTCACTCACTACCGT-3′	2
D3.3	5′-TGCATACTGTCGGCTCACGTGTCTCTGTGG-3′	2

analyzed to measure the K_d value, and the binding analysis revealed that DIAZ-02-F had the lowest K_d value of 4.571 ± 0.714 μM among all aptamers tested. The K_d values were estimated following the method described by Hu and Easley [31]. Furthermore, by exploring the linearity of detection of the target molecule using each sensor, we calculated the limit of detection (LOD) value [29]. Linear detection was observed based on the change in FL intensity in the presence of a wide range of diazinon concentrations. The LOD values for each aptameric sensor were determined as follows: DIAZ-01-F, DIAZ-02-F, and DIAZ-03-F, 226 nM, 148 nM, and 341 nM, respectively. The analysis showed that the DIAZ-02-F aptamer had the highest affinity for diazinon among all aptamers used. As per US EPA guidelines, the maximum residue level for diazinon is 0.7 mg/Kg [32], which corresponds to 2.3076 μM for Brassica sp; whereas our assay could detect the pesticide with LOD of 44.89 μg/Kg (148 nM). Hence, the biosensor has high potential to recognize pesticide contamination of foods.

3.3 Analysis of the Cross-Reactivity of Fluorescent Aptamer Sensors Against Nonspecific Targets

To determine the specificity of the novel ssDNA aptameric sensors developed in this study, we tested the cross-reactivity of the DIAZ-F biosensors against three other organophosphate insecticides, fenthion, fenitrothion, and malathion (Fig. 4). The selected insecticides possess structural and physicochemical properties similar to those of diazinon's specific target. Detailed analysis of specificity showed that both DIAZ-01-F and DIAZ-02-F had a rel-

atively high level of cross-reactivity to fenthion and fenitrothion. The organophosphorus insecticides shared an aromatic ring structure (Fig. 4B–E). Notably, all three aptamers successfully discriminated between malathion and diazinon's specific target. Moreover, malathion did not bind to any aptasensors. Among all aptamers, DIAZ-03F was found to be the most specific, as it did not bind to fenitrothion or malathion and showed weaker binding to fenthion insecticide. Altogether, the ssDNA aptamers exhibited high affinity and specific binding to the target molecule diazinon. However, DIAZ-1-F and DIAZ-2-F showed weaker binding to the nonspecific targets fenthion and fenitrothion due to the presence of core ring structures and phosphorothioate bonds. Overall, DIAZ-03-F performed best in the specific recognition of the target molecule.

3.4 Optimization of the Sensor to Detect Diazinon in Real Vegetable and Fruit Samples

Vegetables and fruits form a significant ingredient of food and nutrition and are essential for a balanced human diet. This study assessed the applicability of newly designed aptameric sensors to detect insecticide contamination in food. The effect of pH, temperature, and incubation time was investigated as food samples present complexities. The DIAZ-02-F sensor and quencher mixture (1:5 ratios, i.e., 50 nM:250 nM) was incubated with 10 μM diazinon under different conditions and a control experiment sensor-quencher mixture without diazinon. We found that the DIAZ-02-F sensor worked well at pH values of 7.0, 7.5, and 8.0 (Fig. 5A). The FL of the sensor was similar to pH 7.5 and 8.0. Since the SELEX process was performed at pH
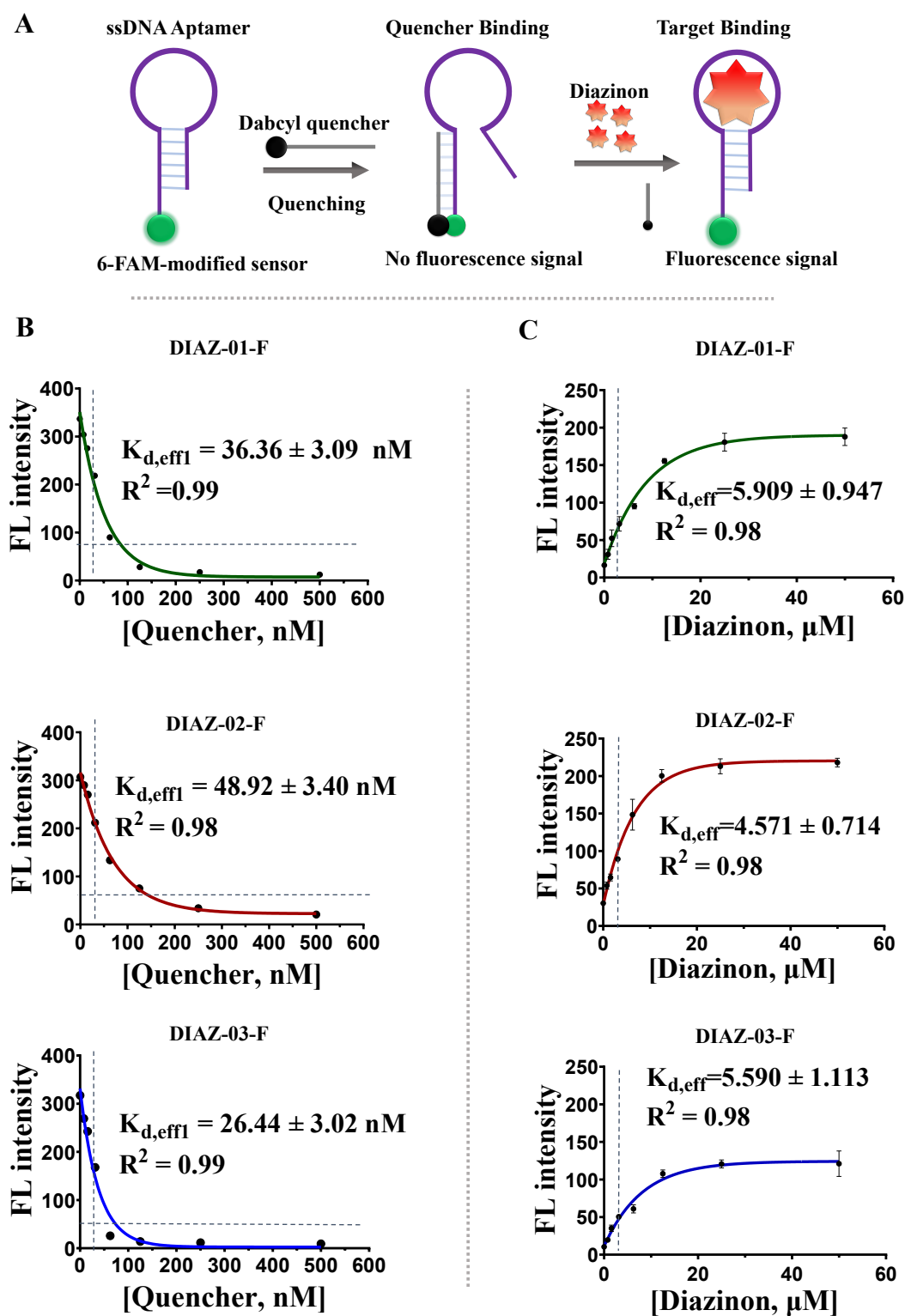
Fig. 3. Detection strategy and characterization of fluorescent aptamer sensors against diazinon. (A) Graphic illustration of detection using a strand-displacement-based fluorescence assay and quenching with a dabcyl-modified quencher. Sensors are shown in blue line with 6-FAM (green circle) at the 5′-end, quencher-strand shown in gray with dabcyl (black circle), and diazinon molecule (red star). (B) Evaluation of the quenching efficiency of selected ssDNA aptamers. (C) Evaluation of the binding potential of 6-FAM-modified sensors to diazinon.
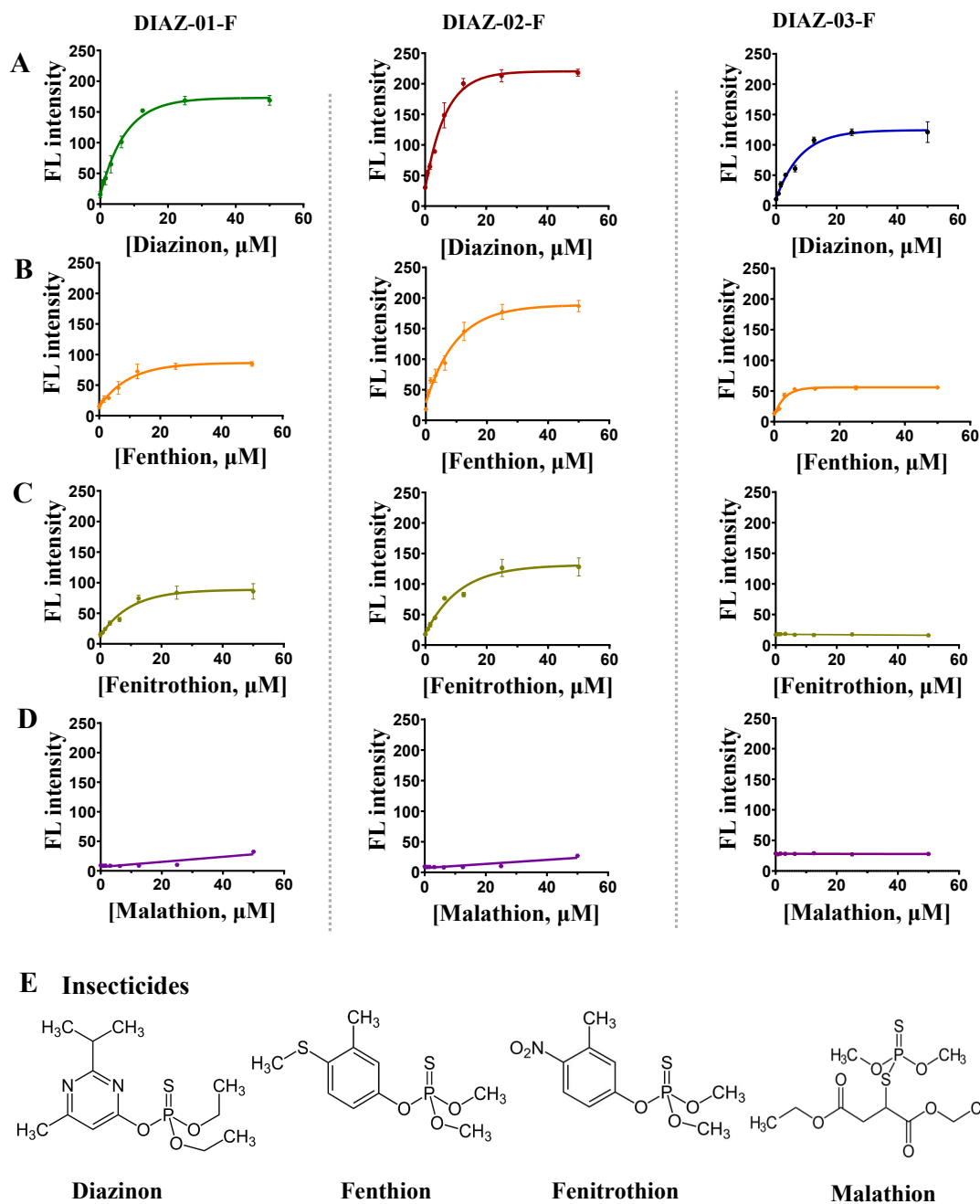
Fig. 4. Measurement of the cross-reactivity of aptamer sensors. Cross-reactivity analysis using three other insecticides: (A) diazinon; (B) fenthion; (C) fenitrothion; (D) malathion; and (E) chemical structure of insecticides.

7.5, the food testing assay was conducted at pH 7.5. However, we noticed that under acidic conditions (at pH 5.0 or 6.0), the DIAZ-02-F sensor and quencher exhibited weaker quenching efficiency, and the aptamer did not bind to diazinon effectively. Hence, the observed FL was similar to that in the control experiments (Fig. 5A). Next, we probed the effect of incubation time on assay performance, for which we used 0 to 60 min; the sensor performed best at 40 min. Subsequently, we examined the effect of temperature on the FL and found that the highest signal was achieved at 30 °C.

Altogether, the optimization experiments provided the best protocol to conduct the experiments using the following conditions: 1x SELEX buffer (for sensor: quencher mixture or food extract), pH 7.5, incubation at 30 °C for 40 min. We eventually explored the potential application of the DIAZ-02-F sensor in real sample testing. Plant tissue extracts of Chinese cabbage, tomato, and apple were analyzed for diazinon contamination (Fig. 5B). Undiluted plant tissue extracts interfered with the FL signal. Hence, we diluted the extract to improve the assay's performance. Therefore,
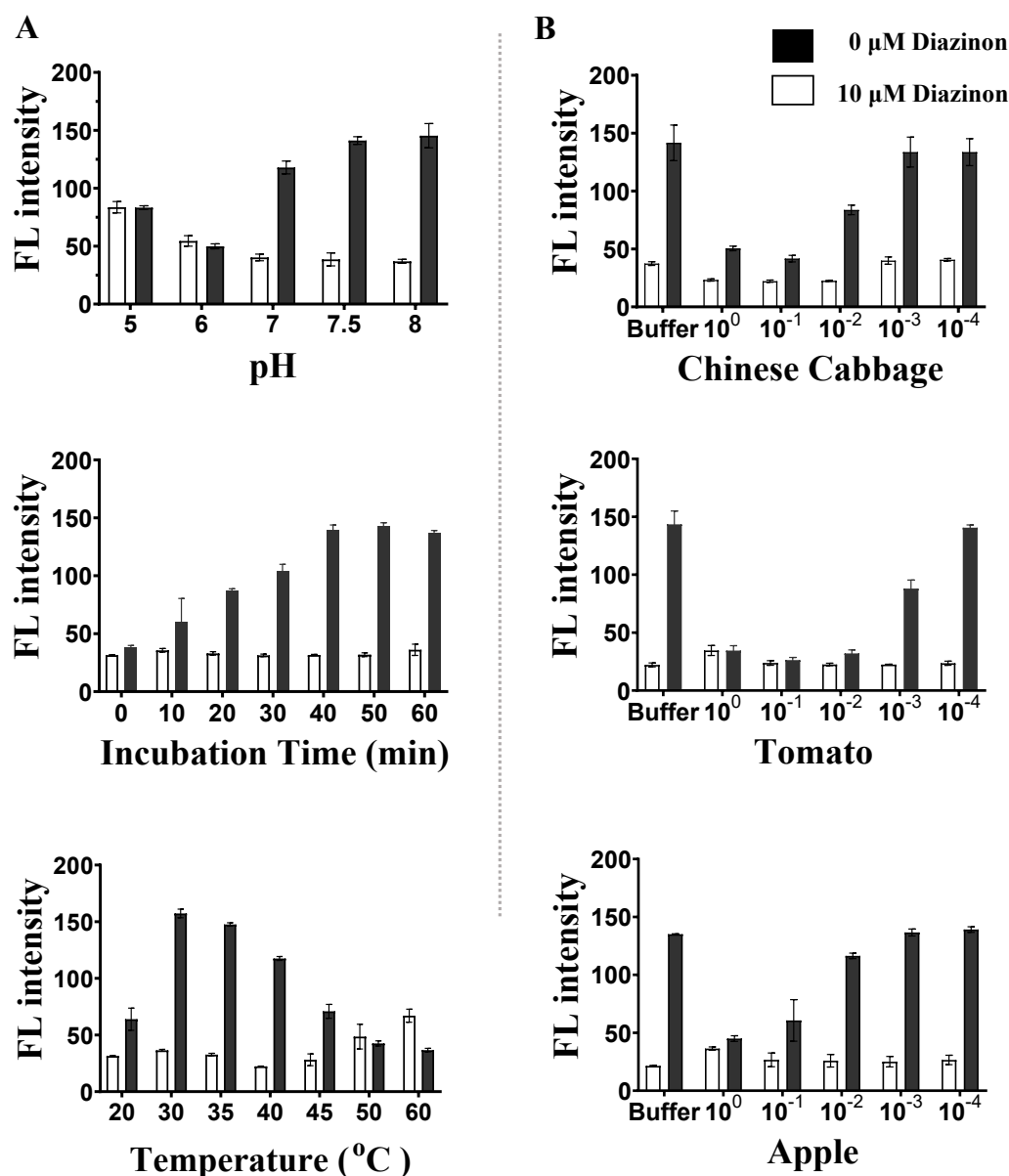
Fig. 5. Analysis of diazinon concentration using the aptameric sensor in vegetable and fruit tissue extracts. (A) Determination of optimal conditions for diazinon detection using the DIAZ-02-F sensor. (B) Analysis of diazinon concentration using the DIAZ-02-F sensor in tissue extracts of vegetables/fruits—tomato, Chinese cabbage, and apple.

we diluted the tissue extracts and found that 1000X dilution produced a higher signal than undiluted extracts, while for tomato extract, we needed to dilute 10000X. In the case of apple extract, the assay worked well even with 100X dilution. Notably, the sensor DIAZ-02-F performed well and could detect diazinon from pure Chinese cabbage extract. Overall, these results provide strong potential for sensing toxic insecticide contamination in food samples such as vegetable and fruit extracts. The sensor could be developed as a point-of-care tool to monitor the entry of toxic agri-waste contaminants into the human food chain and protect the environment, fulfilling several SDGs and ensuring a clean and safe planet for future generations.

4. Conclusions

In conclusion, we report the discovery of three novel ssDNA aptameric sensors that bind to diazinon with a low K_d value. We show the applicability of DIAZ-02-F in detecting diazinon from food samples such as vegetables (Chinese cabbage and tomato) and apple fruit. Moreover, these sensors showed specific detection of diazinon in samples directly obtained from vegetable and fruit samples. In the future, these aptameric sensors could be further developed as recognition elements in biosensing platforms to be used as a point-of-care tool and help to protect the environment and biowaste management effectively.

Author Contributions

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/FBL/27/3/10.31083/j.fbl2703092.

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