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Selection and Identification of a DNA Aptamer That Mimics Saxitoxin in Antibody Binding

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ABSTRACT: In this article, high-affinity single-stranded DNA (ssDNA) aptamer-targeting $F(ab')_2$ fragments of saxitoxin (STX) antibodies were selected from a random ssDNA library by the SELEX strategy. After 16 rounds of repeated selection, the enriched ssDNA library was sequenced, and all of the sequences were carefully identified by indirect enzyme-linked assay and indirect competitive enzyme-linked assay (icELISA). The candidate aptamers in the above identification were selected for further characterization by icELISA and the equilibrium filtration method. We successfully obtained an aptamer that mimics STX in antibody binding, and a substitute for STX in aptamer form has been developed. Further work is in progress aimed at using this aptamer substitute to replace the STX standard in an antibody-based, nontoxic detection method for field determination of STX in seafood products.

KEYWORDS: *saxitoxin, aptamer, $F(ab')_2$ fragments, SELEX, paralytic shellfish poisoning*

INTRODUCTION

Paralytic shellfish poisoning (PSP) is one of the most serious naturally occurring seafood poisonings, and it is caused by the ingestion of shellfish containing PSP toxins. PSP toxins are found primarily in several marine dinoflagellates, such as *Alexandrium tamarense*, *Alexandrium fundyense*, and *Alexandrium catenella*, and can accumulate in filter-feeding shellfish using the dinoflagellates as a food source.[1−4] PSP toxins consist of at least 18 different toxin components, including saxitoxin (STX), neosaxitoxin (neoSTX), and gonyautoxin (GTX). Among the PSP toxins, STX is the most potent neurotoxin present globally in aquatic environments.[5] STX blocks sodium channels in mammalian nerve cells, preventing conductance of signals along the neuron, and the net result is paralysis and potential death from respiratory failure.[6] The LD_{50} of STX in mice is approximately 8 μg/kg, which means that a single dose of 0.2−1 mg would prove fatal for the average human.

To protect public health and ensure safety from toxic hazards, STX can be detected by mouse bioassay,[7] sodium channel blocking assay,[8] high-performance liquid chromatography (HPLC),[9,10] and immunological methods.[11,12] The development of STX detection methods has gradually increased the demand for STX standards, but because the STX standard separation and purification process is complex and chemical synthesis is not currently possible, an STX standard is expensive and difficult to obtain. Furthermore, because STX is a highly toxic substance, the use of STX standards in the detection process increases safety risks of experimental operators. Therefore, an alternative STX standard is essential to solve the above problems.

Aptamers are DNA or RNA oligonucleotides that bind to a wide range of target molecules with high affinity and specificity, ranging from small molecules[13,14] to large proteins[15,16] and even cells.[17] Aptamer selection by an in vitro process called systematic evolution of ligands by exponential enrichment (SELEX) was first reported in 1990.[18,19] During the SELEX selection process, a large library of DNA or RNA oligonucleotides is screened by repeated selection and amplification to enrich the aptamers that bind the target molecule with high affinity and specificity. Aptamer molecules can form many different secondary and tertiary structures, which make the probability of finding an aptamer for the target molecule much higher, and their unique structure contributes to the high specificity for the target. Compared with some monoclonal antibodies, aptamers show a very high affinity for their targets, with typical dissociation constants (K_d) in the micromolar to the low picomolar range but sometimes even better.[20] In addition, aptamers can be manipulated to increase their stability and improve their binding to targets. Because of this, aptamers possess many characteristics that make them good tools for experimental investigations, therapeutics,[21,22] and drug development. Recently, aptamers have been developed against STX,[23] abrin toxin,[24] and ochratoxin A.[25]

In this study, we applied the SELEX strategy to select aptamer-targeting $F(ab')_2$ fragments of STX IgG antibody. Recent advances in SELEX methodology and numerous reports of the successful generation of aptamers against proteins

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prompted us to use the microwell plate screening technique and lambda exonuclease method to generate an ssDNA aptamer by SELEX. Our work is the first to describe the selection of DNA aptamers that mimic STX in antibody binding. The aptamer obtained in this report could be used as an alternative to the STX standard in an aptamer/antibody-based nontoxic detection method to monitor STX in seafood products.

EXPERIMENTAL PROCEDURES

Chemicals and Apparatus. The STX IgG antibody used to prepare F(ab′)$_2$ fragments and three IgG antibodies against shellfish toxins were previously generated.[26,27] The F(ab′)$_2$ preparation kit (Pierce) and lambda exonuclease were purchased from Thermo Scientific (Rockford, IL, USA). The initial ssDNA random library and primers in Table 1 were chemically synthesized and HPLC-purified by Sangon Biotech Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) and *o*-phenylenediamine (OPD) were purchased from Sigma Chemical Co., Ltd. (Zhengzhou, China). Yeast tRNA was purchased from Invitrogen (Karlsruhe, Germany). Taq DNA polymerase, DL 2000 DNA marker, and DNA mate were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). Streptavidin−peroxidase complex was purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). Other unspecified chemicals and reagents were analytical purity grade, and all solutions were made with Milli-Q quality deionized water and filtered through a 0.22 μm filter (Millipore, Nepean, ON, Canada).

Table 1. DNA Sequences Used in This Work

sequence ID	sequence (5′→3′)
library	5′-CATCTGCAGTGTGGCACCATG-N40-CGTGCTGAGCGTGAATTCGC-3′
forward primer (P_1)	5′-CATCTGCAGTGTGGCACCATG-3′
reverse primer (P_2)	5′-GCGAATTCACGCTCAGCACG-3′
biotin-P_1	5′-Bio-CATCTGCAGTGTGGCACCATG-3′
phosphate-P_2	5′-Pho-GCGAATTCACGCTCAGCACG-3′

The pH of all buffer solutions was measured by a Denver basic pH meter, UB-7 (USA). For ssDNA library immobilization and relevant ssDNA separation, 96-well EIA/RIA flat-bottom plates (Costar, Corning, NY, USA) were used. All polymerase chain reactions (PCRs) were carried out in a GeneAmp PCR system 9600 (Applied Perkin-Elmer, USA). Polyacrylamide gel electrophoresis assays were recorded on a UVI gel autoimaging system (DNR Company, Inc. Israel). Absorbance was recorded on an Epoch Micro-Volume spectrophotometer system (Bio-Tek Instruments, Inc., USA). The equilibrium−filtration analyses of selected aptamers were performed on a Microcon YM-50 (Millipore, Bedford, MA, USA). Quantitative PCR was performed on an ABI 7500 system (Applied Biosystems, USA).

Antibody Purification and F(ab′)$_2$ Fragment Preparation. The STX IgG antibody was previously described elsewhere, and it strongly binds STX.[28] The STX IgG antibody was purified according to the method of McKinney and Parkinson.[29] In brief, albumin and other non-IgG proteins were precipitated with caprylic acid (octanoic acid), and the IgG antibody fraction was precipitated with ammonium sulfate. The purified IgG antibody was subjected to electrophoresis on a 12% reducing SDS-PAGE and stained with Coomassie brilliant blue R-250. The concentration of the IgG antibody was measured by absorbance at 280 nm using a spectrophotometer.

F(ab′)$_2$ fragments were generated by pepsin digestion of whole IgG antibody with the Pierce F(ab′)$_2$ preparation kit according to the manufacturer's protocols.[30] This digestion removed most of the Fc region while leaving some of the hinge region intact. Complete separation of F(ab′)$_2$ fragments from undigested IgG antibody was assessed using nonreducing SDS-PAGE and Coomassie brilliant blue R-250 staining.[31]

SELEX Procedure. The SELEX procedure was performed as previously described[32] with some modifications. Briefly, F(ab′)$_2$ fragments were coated on a 96-well ELISA plate with 0.05 M sodium bicarbonate buffer (pH 9.6) overnight at 4 °C. The coated wells were washed three times with 200 μL of washing buffer (binding buffer + 0.05% Tween 20). Coated wells and blank wells were subsequently blocked by 3% BSA at 37 °C for 1 h. The ssDNA library was denatured by heating at 95 °C for 10 min in binding buffer (20 mM PB, pH 7.35, 120 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) and immediately cooled on ice for 15 min. To obtain ssDNA secondary structures, the ssDNA library was equilibrated to room temperature prior to use.[33] Yeast tRNA (2−10 μg) was added to the ssDNA library to reduce background binding.[34]

To screen out ssDNA bound to BSA, the ssDNA library was added to BSA-blocked blank wells at 37 °C for 1 h. The unbound ssDNA was incubated with F(ab′)$_2$ fragment-coated wells at 37 °C (incubation times are shown in Table 2). The wells were then washed five times with 200 μL of washing buffer. Eluting buffer (20 mM Tris-HCl, 4 M guanidinium isothiocyanate, 1 mM DTT, pH 8.3) was added and incubated at 85 °C for 10 min, and the species bound to F(ab′)$_2$ fragments were eluted and mixed with phenol-chloroform. The mixture was centrifuged at 12000*g* for 5 min at 4 °C. The supernatant was mixed with dehydrated alcohol and 3 M NaAc overnight at −20 °C and centrifuged at 12000*g* for 20 min at 4 °C. After the supernatant was removed, 75% alcohol was added to the sediment and centrifuged for 10 min. The precipitate was dissolved in 20 μL of TE buffer (pH 8.0) and stored at −20 °C.

Table 2. Selection Parameters of F(ab′)$_2$ Fragment Antibodies

SELEX round	ssDNA library (pmol)	F(ab′)$_2$ (μg)	tRNA (μg)	incubation time
1	2000	10	2	3 h
2	1000	10	2	2.5 h
3	500	5	2	2.5 h
4	400	4	2	2.5 h
5	200	2	2.5	2 h
6	200	2	2.5	2 h
7	200	2	2.5	2 h
8	200	2	5	1.5 h
9	200	1	5	1.5 h
10	100	1	5	1.5 h
11	100	1	7.5	1 h
12	100	1	7.5	1 h
13	50	0.5	7.5	1 h
14	50	0.5	7.5	50 min
15	50	0.5	10	50 min
16	25	0.25	10	50 min
17	25	0.25	10	45 min
18	25	0.25	10	45 min

PCR and Generation of ssDNA. The F(ab′)$_2$ fragment-bound ssDNA was amplified by PCR with a biotin-labeled forward primer (biotin-P_1) and a phosphate-labeled reverse primer (phosphate-P_2). The double-labeled PCR product was then converted to ssDNA by lambda exonuclease before the next round of selection. The PCRs were performed in a final volume of 50 μL that contained 2.5 ng of template DNA, two oligonucleotide primers (20 μM), 10 mM dNTP mixture, 0.5 mM Tris-HCl, 0.5 mM KCl, 0.075 mM $MgCl_2$, and 1.25 U Taq DNA polymerase. The PCRs consisted of 96 °C for 2 min; 20 cycles of 96 °C for 30 s, 64 °C for 30 s, and 72 °C for 60 s; and a final extension of 72 °C for 5 min. The ssDNA was prepared as previously described.[35,36] The PCR product was incubated with lambda exonuclease, which preferentially degraded DNA that was 5′-phosphorylated to leave biotin-labeled ssDNA. Gel purification of

biotin-labeled ssDNA was performed after amplification following rounds 3, 5, 7, 9, and 12 to remove nonspecific products. The biotin-labeled ssDNA was purified by DNAmate and resuspended in 20 μL of water. The purified biotin-labeled ssDNA was electrophoresed on 8% polyacrylamide/7 M urea gels and silver stained. The quantity of the recovered biotin-labeled ssDNA was measured by absorbance at 260 nm using a spectrophotometer. The biotin-labeled ssDNA was used to analyze the affinity of the selected round and as the enriched library for the next selection round.

ssDNA–Indirect Enzyme Linked Assay. The increase in affinity of the library from every selected round was assessed by indirect enzyme linked assay (iELISA). The $F(ab')_2$ fragments (0.5 μg) were coated on a 96-well ELISA plate with 0.05 M sodium bicarbonate buffer (pH 9.6) overnight at 4 °C. The wells were washed three times with 200 μL of washing buffer. The biotin-labeled ssDNA was diluted to 300 nM in 100 μL of binding buffer, heated to 95 °C for 10 min, and then cooled on ice for 15 min. Biotin-labeled ssDNA (100 μL) was incubated with $F(ab')_2$ fragments in microtiter plates at 37 °C for 2 h on a plate vortex with gentle shaking. The wells were washed four times for 1 min with 200 μL of washing buffer with shaking. The streptavidin–peroxidase complex was diluted 1:1000 in binding buffer and incubated with the bound ssDNA in wells for 1 h at 37 °C. The wells were washed again five times as described above, and then 100 μL of OPD was added and incubated at 37 °C for 15 min in the dark. The binding response was stopped with the addition of 50 μL of 2 M H_2SO_4 and measured by absorbance at 490 nm using a spectrophotometer.

Cloning and Sequencing of ssDNA Aptamers. After 16 rounds of aptamer selection, the selected aptamers were amplified with unmodified primers (P_1 and P_2) and purified using the QIA quick PCR purification kit. The purified PCR products were cloned into a pMD 18-T vector by the TA cloning method according to the manufacturer's protocol and transformed into chemically competent *Escherichia coli* DH5a cells. White colonies were confirmed by PCR. Thirty recombinant clones were picked for analysis and sequenced by Sangon BiotechCo., Ltd. (Shanghai, China).

The multiple sequence alignment of the selected ssDNA was performed with DNAMAN software. Predictions of ssDNA secondary structures were performed by a free energy minimization algorithm available in the mfold program (http://www.bioinfo.rpi.edu/applications/mfold).[37]

Identification of Selected Aptamers. To determine the binding characteristics of all sequenced aptamer clones, we performed iELISA with $F(ab')_2$ fragments as described above. Briefly, the biotin-labeled aptamer clones were incubated with $F(ab')_2$ fragments and the same volume of the initial ssDNA library (test and control groups, respectively), and the absorbance at 490 nm of each group was recorded. The difference between these two values was defined as the ability of the selected aptamer to bind $F(ab')_2$ fragments. This calculation was made by comparing the OD_{490} value of the test group (P) to the OD_{490} value of the control group (N). If the ratio of P/N was more than 2.1, the result was scored as positive.

To identify potential internal image candidate aptamers, the positive aptamers (P/N > 2.1) in the above identification were selected for further characterization by indirect competitive enzyme linked assay (icELISA).[38] Briefly, the wells of the microtiter plate were coated with the $F(ab')_2$ fragments as described for the iELISA procedure. Then, 50 μL of the STX standard solution (1.0, 2.5, and 5.0 ng in binding buffer) or binding buffer alone was added to each well, and 50 μL of positive aptamer (300 nM in binding buffer) was subsequently added to the same well. After immediate mixing, the plate was incubated for 1 h at 37 °C and washed with washing buffer. The streptavidin–peroxidase complex was diluted and incubated as described above. The absorbance values were measured at 490 nm using a spectrophotometer and converted into their corresponding binding ratio (A/A_0) as follows:

$$(A/A_0) = \frac{A}{A_0} \times 100\% \quad (1)$$

in which A is the absorbance value of the competitive assay and A_0 is the absorbance value of the noncompetitive assay.

Identification of Aptamer That Mimics STX Antibody Binding. In order to obtain the aptamer that mimics STX in antibody binding, the competitive activity of four candidate aptamers was analyzed by icELISA as described above. The STX standard solution (0, 0.5, 1.0, 2.5, 5, 10, or 20 ng in 50 μL of binding buffer) or binding buffer alone was added to each well, and 50 μL of candidate aptamer (300 nM in binding buffer) was subsequently added to the same well. After immediate mixing, the plate was incubated for 1 h at 37 °C. The absorption at wavelength 490 nm was determined on a spectrophotometer.

The aptamer/$F(ab')_2$ complexes were analyzed by the equilibrium filtration method.[39,40] Briefly, $F(ab')_2$ fragments (250 nM) were added to selected ssDNA aptamers (300 nM final concentration) in 400 μL of selection buffer. Each binding reaction was incubated for 45 min at 37 °C. The reactions were then filtered through Microcon YM-50 molecular cutoff filters (50 000) and centrifuged for 20 min at 12000*g* to separate the unbound aptamers. The unbound ssDNA was quantified by real-time PCR detection system.[35] Amplification reactions were carried out with EvaGreen PCR master mix, 1 μL of unbound ssDNA, and 0.5 μM each primer (P_1 and P_2). The following PCR conditions were used: 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 60 °C for 10 s, and 72 °C for 60 s. Each reaction was run in triplicate. The amount of unbound DNA in the binding reaction mixtures was estimated from the standard curve generated by plotting the threshold cycle (CT) versus the starting aptamer quantity (aptamer concentrations ranging from 10^{-1} to 10^{-8} μM). To compensate for unspecific sorption of selected aptamers, an equivalent concentration of selected aptamer solutions without $F(ab')_2$ fragments was passed through the same procedure, and the difference between the normal sample and the $F(ab')_2$ blank sample was estimated as the amount of aptamer/$F(ab')_2$ complexes. Before the binding measurements, the ssDNA aptamers were denatured in binding buffer at 95 °C for 10 min and cooled on ice for 15 min.

Aptamer Specificity and Affinity Assays. The aptamer that mimics STX in antibody binding was tested for binding to the $F(ab')_2$ fragments and to four IgG antibodies against different shellfish toxins [STX, okadaic acid (OA), brevetoxin (BTX), and tetrodotoxin (TTX)] using the ssDNA-iELISA method described above. In concentrations of 0.0325, 0.075, 0.15, 0.3, 0.6, 0.8, 1.6, and 3.2 μM, the aptamer was incubated with each antibody (0.5 μg), and the absorbance was measured at 490 nm using a spectrophotometer. The apparent dissociation constant (K_d) of the best binding aptamer was further analyzed by the above-mentioned equilibrium filtration method. In this assay, the concentration of ssDNA was keep at 300 nM, but the concentration of $F(ab')_2$ fragments was set at 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 nM. After equilibrium filtration analysis, the concentration of the aptamer/$F(ab')_2$ complex versus the concentration of added $F(ab')_2$ fragments was plotted. The dissociation constant (K_d) was calculated via linear regression of a Scatchard plot as shown in eq 2, where B is the concentration of aptamer/$F(ab')_2$ complex, F is the concentration of $F(ab')_2$ fragments, and B_{max} is the intercept of the straight line in the B-axis.[41]

$$\frac{B}{F} = -\frac{1}{K_d}B + \frac{1}{K_d}B_{max} \quad (2)$$

RESULTS AND DISCUSSION

Purification of IgG Antibody and Preparation of $F(ab')_2$ Fragments. The STX IgG antibody was purified as described in the Experimental Procedures. The reducing polyacrylamide gel electrophoresis is shown in Figure 1a. As expected, there are two pure bands at 55 kD (heavy chain) and 25 kD (light chain). To isolate DNA aptamers that specifically interact with the variable domain of the STX IgG antibody, we used $F(ab')_2$ fragments as the selection targets; $F(ab')_2$ fragments lack most of the Fc region but retain two antigen-

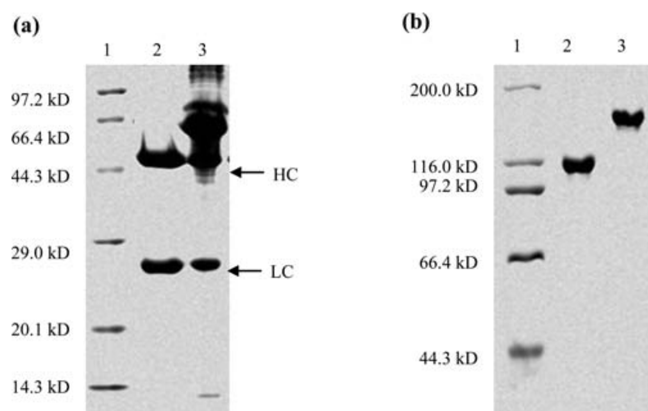

Figure 1. Characterization of IgG antibody and F(ab′)$_2$ fragments using gel electrophoresis. (a) Reducing SDS-PAGE of IgG antibody and ascites. Lane 1: protein marker; lane 2: purified IgG antibody; lane 3: ascites. The positions of HC and LC are indicated at right; protein molecular weight markers are indicated at left. (b) Nonreducing SDS-PAGE of F(ab′)$_2$ fragments and IgG antibody. Lane 1: protein marker; lane 2: F(ab′)$_2$ fragments; lane 3: IgG antibody. Protein molecular weight markers are indicated at left.

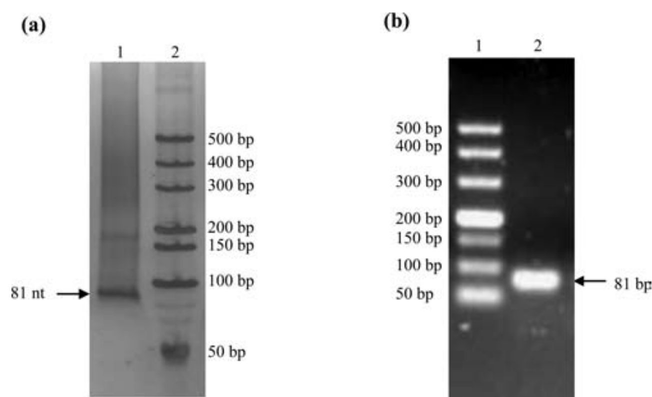

Figure 2. Electrophoresis of PCR products and ssDNA. (a) ssDNA was generated with lambda exonuclease and run on 8% polyacrylamide/7 M urea gels. Lane 1: ssDNA; lane 2: DL500 DNA molecular weight markers. The location of ssDNA is indicated by arrows; DL500 DNA molecular weight markers are indicated at right. (b) Agarose gel electrophoresis of PCR products. Lane 1: DL500 DNA molecular weight markers; lane 2: PCR products. The location of dsDNA is indicated by arrows; DL500 DNA molecular weight markers are indicated at left.

binding sites and a partially intact hinge region. The use of F(ab′)$_2$ fragments also avoids unspecific aptamer binding to the Fc region, which increases the specificity of the aptamer to the STX antibody. Digestion of STX IgG antibody with pepsin and nonreducing SDS-PAGE detected one band corresponding to the F(ab′)$_2$ fragment molecular weight (110 kD) (Figure 1b). This result indicated that pure F(ab′)$_2$ fragments were successfully obtained.

Enrichment of ssDNA Aptamers Targeting F(ab′)$_2$ Fragments. In vitro selection was initiated with a random ssDNA library (2.0×10^{15} molecules) that consisted of 81 nt ssDNA containing randomized 40-nucleotide inserts. After the first round of SELEX, the ssDNA pool was amplified by PCR, and the ssDNA was prepared by lambda exonuclease. Gel electrophoresis of the ssDNA is shown in Figure 2a. In each round of selection, the ssDNA library was incubated with the immobilized F(ab′)$_2$ fragments, and the bound ssDNA was eluted. The quantity of the ssDNA library, F(ab′)$_2$ fragments, and tRNA and the incubation times for the reactions are shown in Table 2. Since extremely strict conditions were applied after six cycles, high-affinity DNA aptamers seemed to be enriched with increasing SELEX rounds (Figure 3).

With each additional SELEX round, the absorbance gradually increased from 0.128 in the first round to 0.787 in round 11 and 1.298 in round 16. However, the value did not increase in the next two rounds of selection, indicating that the ssDNA, which specifically bound to F(ab′)$_2$ fragments, had been maximally enriched after 16 rounds of selection. Hence, the eluted ssDNA library from the 16th selection was amplified by PCR and purified by DNAmate. The purified PCR products are shown in Figure 2b. The purified PCR products were cloned, and their inserts were PCR-confirmed and sequenced. The sequences of 30 clones obtained from the 16th round are shown in Figure 4. Five ssDNA sequences were identical; therefore, 25 ssDNA aptamer sequences were selected for the following experiments.

Identification of Selected Aptamers. The binding affinity of 25 selected aptamer sequences was measured by iELISA. As shown in Figure 5, the absorbance increased in most of the aptamer identification groups compared with the control group. The absorbance of the F2, F3, F12, F19, F30, F32, and F39 aptamers was higher than the others, and significance analysis showed that the increase in absorbance in these aptamers was extremely significant ($p < 0.01$). To selected potential internal image candidate aptamers, 25 positive aptamers (P/N > 2.1) were checked for their ability to bind to the binding site of F(ab′)$_2$ fragments by icELISA.

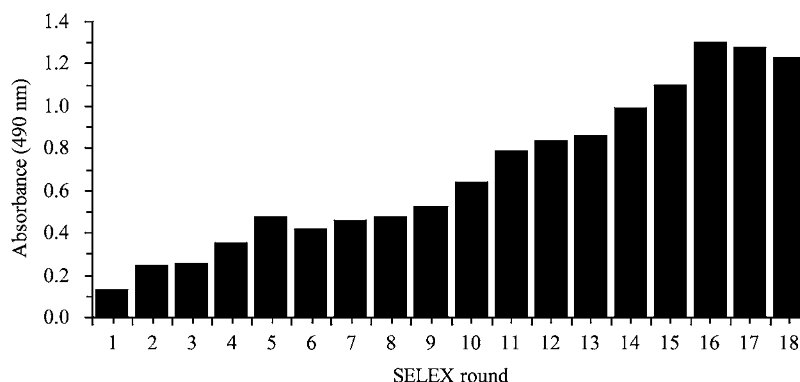

Figure 3. Binding of the ssDNA library to F(ab′)$_2$ fragments during 18 SELEX rounds.

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              1         10        20        30        40        50
Name          |---------|---------|---------|---------|---------|

f12     ..TGACGTG.GGCGGGAGGGGGGACGGTGTTTCGGG.CCG.....TGTT..   40
f13     ..GGATATG.CTGGTATTTTTAG.TCTCGTTGTGGTTCCT.....GGTG..   40
f14     ...CGAGACATT.TGGGAGATCGATGTTAGCCCTGGGTTC.....TTGA..   40
f15     ...AGGTGTGTTCTGGGGGGGGTTTGTTTGTGCTGGGTTG......TGG..   40
f16     ..GGAATAGGGTTAGGCCGTAGGTTTGAGCTT..TGTGTT.....GGTG..   42
f17     ..GTGGTGTATTGTGGGTAGACGTGGTTAGGTGTGCAGTG.....TGGT..   40
f18     ..AAGGGAGATGTGTGCCGGGCTGTTTCCCGT..TGTTGG.....TGGC..   40
f19     ..GCGGGAAAGGGTCCGGTTGTGATGTTGTTTCTGTTTTG.......GT..   38
f2      ..TCACCGGTTCGCGGTATGTGGTCGCAGGATGGTGTTC.........A..   35
f20     ..TGGCTGTGTGCTTGGCGAGGTGCGTG..TGATTGTGC............   38
f21     ..GTACTCTGGTGAAGTTATTTGCTGGACGCAGCTTAGT.........A..   40
f22     ..TGGGCTGTAGTTTCTTATTGGCTCTCTTCTTGTGTTTT.......GC..   46
f23     ..GTCATCTGCAGTGTGGCACCAT.GTTGGGTGTGGGTTGTTGTCCTGA..   39
f24     ..TGGGGTTTTGGTTGTGTTTGTATGTGGTTAGTTTTGCT........A..   40
f3      ...GAATACGACATTGTTGGCTGTTCTTGGTTATGCGGTT......TTT..   40
f30     ..GGCTGTAGTATGTCCGATGGGAAGCTATCGATGATAGT.......GT..   40
f31     ..TCGATAG.TGTGTTTCGGCTTCTTTTCCTTG.TGTCTC.....TGGG..   40
f32     ..TCCCGGAAACATCTCCCCCAGTACAAGAGCATATGTTG.......TG..   40
f33     ..TCGATAG.TGTGTTTCGGCTTCTTTTCCTTG.TGTCTC.....TGGG..   40
f34     ..GCCGGCAGGTAAGTGGTTCAGGTGGGACTAGTTTGTTT.......GG..   40
f35     ..GGCTGTAGTATGTCCGATGGGAAGCTATCGATGATAGT.......GT..   40
f36     ..TTACCTCATTTCTAATACCATATGTGCAATGTCGGAGT.......GT..   40
f37     ..AAGGGAGATGTGTGCCGGGCTGTTTCCCGT..TGTTGG.....TGGC..   40
f38     ...TCCTCCGTTAGTTATGAGTGTGTTGGGTTCTTTGTTT......TGG..   40
f39     ..GCACTTT..GAGGGTTGAACACCAGGCGGAGGTGTGAT.....GCGG..   40
f4      ..TGACGTG.GGCGGGAGGGGGGACGGTGTTTCGGG.CCG.....TGTT..   35
f7      ..TGGCTGTGTGCTTGGCGAGGTGCGTG..TGATTGTGC............   40
f1      ..TACGCGTTAGACGGTTCATGAATCTGCATGTTCTTCGT.......TT..   40
f8      ..AGCAATG.GTAGTGATATGGT.TGCCGTATCAACTCCG.....TGTT..   40
f9      .....CATCTGCAGTGTGGCACCAT.GCAGCGTGT...........TTT..   32

Consensus          t   tg  g   g tgt   tt ttgt         g
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Figure 4. Multiple sequence alignments of selected ssDNA. The sequences shown are the 40-nucleotide region that was completely randomized at the beginning of the SELEX experiment. Lowercase letters in the consensus line indicate more than 50% similarity with the consensus, and the aptamer sequence nucleotides are highlighted in viridescence. Numbers on the right-hand side indicate the length of each aptamer sequence. The number of nucleotides in the randomized region may have been changed by PCR mutations.

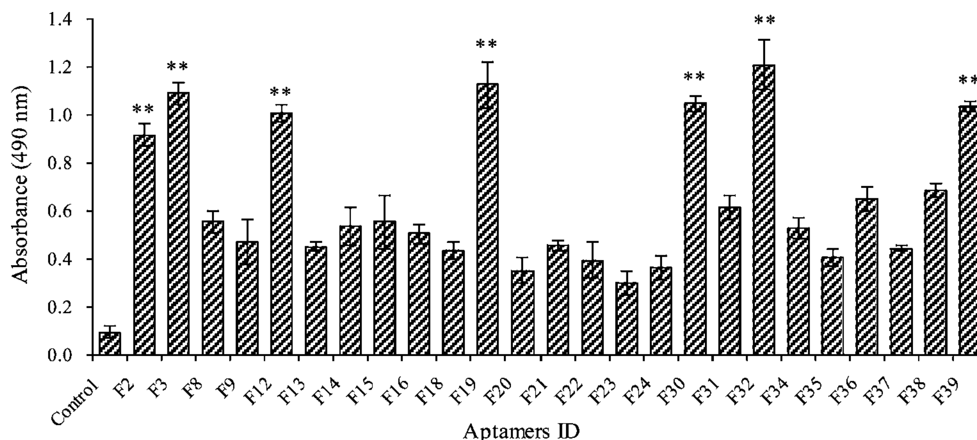

Figure 5. Binding affinities of selected aptamers and $F(ab')_2$ fragments. The initial ssDNA library was used as the negative control. Asterisks indicate that the value is significantly different from the value for other aptamers in the corresponding assay, with $p < 0.01$ (**). p values were calculated using a two-tailed, paired t test with 95% confidence intervals. The data shown are the means of three replicates, and error bars represent the standard errors of the means.

Figure 6 shows that the binding ratio of F2, F3, F19, and F32 was significantly lower than the others and decreased with increasing concentrations of STX. These results showed that the binding of an aptamer (F2, F3, F19, or F32) and $F(ab')_2$ fragments was inhibited by STX. The results suggested that four aptamers recognized an idiotypic determinant of the STX antibody. These aptamers mimicked the structure of STX. They appeared to attach near the binding site of STX antibody, and the binding was subject to STX interference.

Identification of Aptamers That Mimic STX in Antibody Binding. The potential internal image candidate aptamers in the above identification were selected for further characterization by icELISA and the equilibrium filtration method. The results are shown in Figure 7a. With increasing STX concentration, the absorbance of the four aptamers

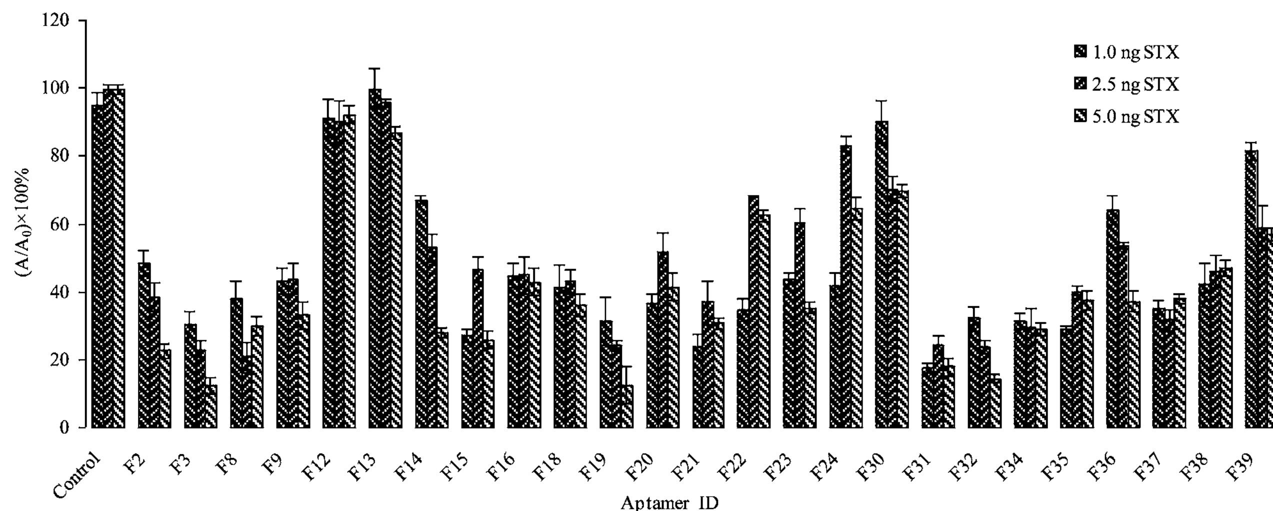

Figure 6. Determination of the specificity of aptamers by indirect competitive enzyme linked assay. The data shown are the means of three replicates, and error bars represent the standard errors of the means.

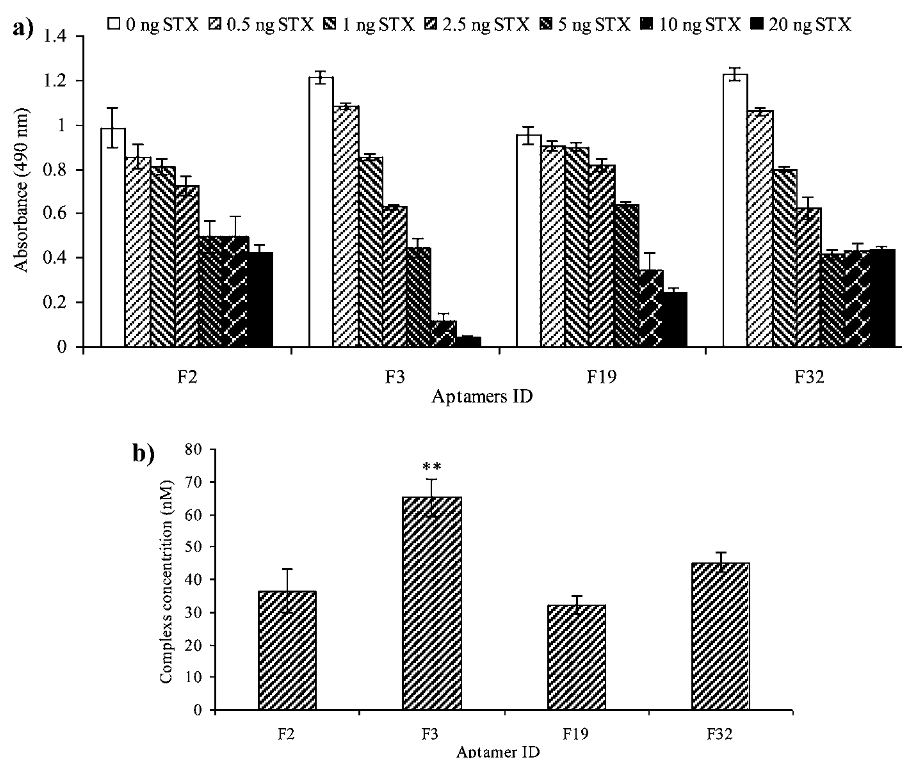

Figure 7. Identification of the aptamers that mimic STX in antibody binding. (a) The competitive activity of four candidate aptamers was analyzed by icELISA. (b) The aptamer/F(ab′)$_2$ complexes were analyzed by the equilibrium filtration method. Asterisks indicate that the value is significantly different from the value for other aptamers in the corresponding assay, with $p < 0.01$ (**). p values were calculated using a two-tailed, paired t test with 95% confidence intervals. The data shown are the means of three replicates, and error bars represent the standard errors of the means.

gradually decreased. However, the value of aptamers F2 and F32 did not decrease when 5, 10, and 20 ng of STX standard was added, indicating that aptamers F2 and F32, which may bind near the binding site of the antibody, had reached maximum binding that was no longer subject to STX interference. Therefore, we concluded that aptamers F3 and F19 recognized an idiotypic determinant of the STX antibody. Compared with aptamer F19, the competitive activity of aptamer F3 was lower. As shown in Figure 7b, aptamer F3 showed the strongest ability to capture F(ab′)$_2$ fragments. When 300 nM of this ssDNA was incubated with 250 nM F(ab′)$_2$ fragments, 65.1 nM aptamer/F(ab′)$_2$ complexes were formed. Hence, aptamer F3 was selected for further study.

Determination of Specificity and Affinity. The specificity of the F3 sequence was analyzed by comparing its ability to bind F(ab′)$_2$ fragments and four IgG antibodies against shellfish toxins (STX, OA, BTX, and TTX). The results (Figure 8a) showed that F3 selectively bound STX F(ab′)$_2$ fragments and STX IgG, while the absorbance did not significantly change when aptamer F3 was incubated with the other three shellfish toxin antibodies. The apparent dissociation constant (K_d) of the F3 sequence was further analyzed. The aptamer F3 was

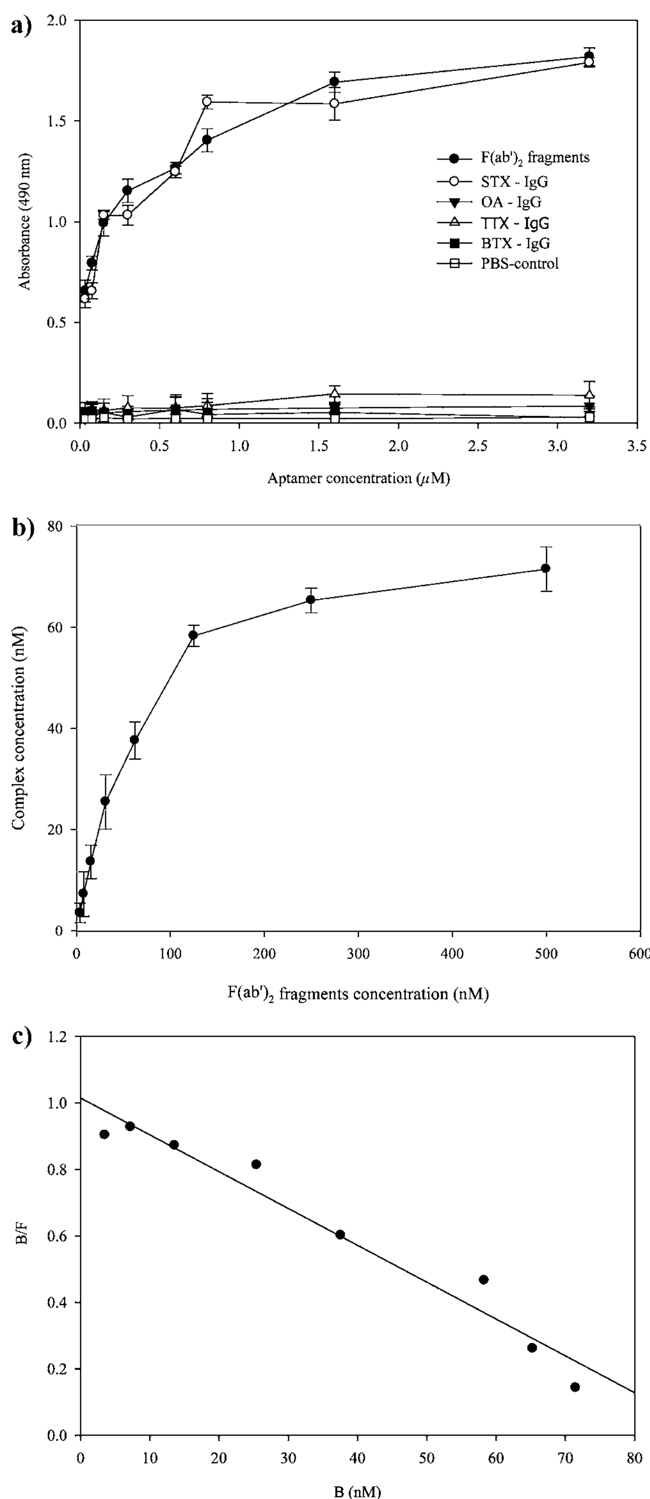

Figure 8. Determination of the specificity and affinity of F3 using indirect enzyme linked assay and equilibrium filtration method, respectively. (a) The specificity of F3 was analyzed by comparing its ability to bind with different shellfish toxin IgG antibodies. The binding buffer was used as the negative control. The data shown are the means of three replicates, and error bars represent the standard errors of the means. (b) The affinity of F3 was detected by the binding assay. The data shown are the means of three replicates, and error bars represent the standard errors of the means. (c) The dissociation constant (K_d) of F3 was analyzed using Sigma Plot 10. B and B/F are the independent variable and the dependent variable, respectively.

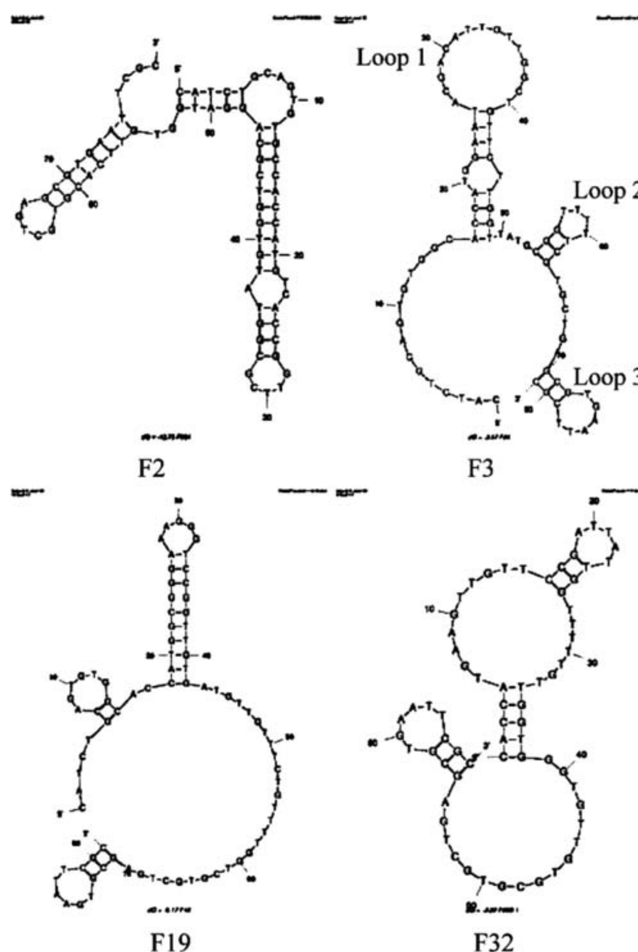

Figure 9. Predicted secondary structures of four candidate aptamers.

incubated with $F(ab')_2$ fragments of different concentration, and the concentration of unbound free aptamers was detected by a real-time PCR detection system. The aptamer/$F(ab')_2$ fragment complex concentration versus the concentration of $F(ab')_2$ fragments is plotted in Figure 8b; the concentration of the complex increased with the concentration of $F(ab')_2$ fragments. After the results were converted into B/F versus B format, the apparent dissociation constant (K_d) of F3 was estimated at 96.37 nM (Figure 8c).

Prediction of the Aptamer Structure. The secondary structure of most selected aptamers is characterized by stems, loops, bulges, hairpins, triplexes, or quadruplexes. The binding of aptamers to their targets typically requires the following: structural compatibility; stacking of aromatic rings; electrostatic, van der Waals, and hydrogen bond interactions; or a combination of these effects.[42−44] To predict the aptamer/$F(ab')_2$ complex-forming process, the most likely minimum energy secondary structure of four selected aptamers (F2, F3, F19, and F32) was analyzed with the mfold program (Figure 9). The secondary structure analysis clearly exhibited three loops, but loop 3 was formed by the constant region. Aptamer F3, which is G-rich, is likely to form a G-quartet structure. The G-rich sequence has a very important role in the binding mechanism of aptamers.[45] Hence, the G-rich loop 1 was predicted to be the target-binding region of F3.

The efficient preparation of ssDNA is one of the most crucial aspects of SELEX.[36] An asymmetric PCR method[46] or magnetic bead-based approach[47] can be used to prepare

ssDNA. We used the lambda exonuclease method and obtained a high-purity product. Aptamers form complex structures that have high degrees of specificity for their target molecules, and they are sensitive to small structural changes in their target. According to the network hypothesis,[48] anti-idiotypic antibodies express an idiotypic determinant that mimics the antigen structure. This indicates that the aptamers of antibodies and anti-idiotypic antibodies all can mimic the structure of antigens. However, the aptamer and the anti-idiotypic antibody were generated by an in vitro and a biological selection process, respectively, which have major distinctions. In this study, we selected the ssDNA aptamer but not the anti-idiotypic antibody because the aptamer is more stable and consistent, and its selection conditions can be optimized according to the nature of the target. Compared with some published works,[49] our enriched ssDNA sequences were very diverse (25/30 were unique sequences), which showed that the ssDNA library could be further enriched. However, the ssDNA yield did not increase with continued screening after the 16th round of selection, most likely because nonspecific elution prevented the efficient separation of target-binding and nonbinding ssDNA.[40] Therefore, selecting optimal screening conditions for the target was very important for the aptamer selection strategy.

Analysis of the 25 ssDNA sequences revealed that some aptamers (F16, F20, F4, F19, F22, F2, F23, and F9) contained approximately 40 bp of random nucleotides. This result may have occurred via template mutations during PCR, which is a common problem for amplification of multiple templates. It was previously reported that the obtained aptamer sequence was shorter than the design of the random sequence.[50] Taken together with our experimental results, we conclude that a small number of mutations during PCR are most likely harmless for aptamer selection. Because aptamer selection is a process of in vitro adaptation, the appropriate mutation is part of the evolutionary process.

In summary, a highly specific ssDNA aptamer (F3) that mimicked STX in antibody binding was selected from a random ssDNA library by SELEX. The F3 aptamer exhibited a dissociation constant (K_d) in the nanomolar range. The binding affinity and specificity were successfully confirmed by iELISA, icELISA, and the equilibrium filtration method. Further investigation of the construction and function of this aptamer is needed in order to assess its potential for detecting STX.

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Notes

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