Biochemical and Biophysical Research Communications 386 (2009) 544–548

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A DNA aptamer recognizes the Asp f 1 allergen of *Aspergillus fumigatus*

Swee Yang Low [a], Jane E. Hill [b], Jordan Peccia [a,*]

[a] Department of Chemical Engineering, Environmental Engineering Program, Yale University, New Haven, CT 06520, USA
[b] College of Engineering and Mathematical Sciences, University of Vermont, Burlington, VT 05405, USA

ARTICLE INFO

Article history:
Received 15 June 2009
Available online 21 June 2009

Keywords:
Aptamer
Asp f 1 allergen
SELEX
Aspergillus fumigatus
Biosensor
Allergenicity
Bioaerosol exposure
Asthma

ABSTRACT

Allergies are caused by the binding of IgE antibodies onto specific sites on allergens. However, in the assessment of exposure to airborne allergens, current techniques such as whole spore counts fail to account for the presence of these allergenic epitopes that trigger allergic reactions. The objective of the research is to develop a DNA aptamer for the Asp f 1 allergen of the pathogenic fungus *Aspergillus fumigatus*, using an IgE-binding epitope of the allergen as the target for aptamer selection. Through *in vitro* SELEX, an aptamer has been produced that binds with nanomolar affinity to the Asp f 1 IgE-epitope. The aptamer is also able to recognize the native Asp f 1 allergen, and does not bind to allergenic proteins from non-target mold species such as *Alternaria alternata*. Production of this aptamer provides proof-of-principle that allergen measurement methods can be developed to indicate the potent fraction, or allergenicity, of allergens.

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Introduction

Allergenic disease is a major cause of illness and disability. In the US, up to 40% of the members of the general public have developed IgE antibodies against environmental antigens, and 20% demonstrate upper respiratory symptoms typical of rhinitis [1]. Additionally, both the presence of IgE and exposure to airborne allergens are strong risk factors for asthma. Allergies trigger attacks in 60–90% of asthmatic children and 50% of asthmatic adults. Annual costs of treating asthma in the US exceed $10 billion [2,3].

In the assessment of human exposure to allergens, however, current measurement techniques neglect the allergenicity or potency of allergens. Allergic diseases stemming from type I hypersensitivity reactions are mediated by IgE antibodies in the serum of atopic individuals. The binding of IgE to specific epitopes on allergenic proteins results in the activation of a pharmacological cascade of reactions that ultimately causes the allergic reaction in the sensitized individual. Mass or number measurements of environmental allergen content based on spore counts from culture-based methods [4] and PCR [5], or surrogate approaches that measure fungal-specific polysaccharide masses [6], are unable to account for this IgE-binding. Commercial immunodetection methods [7] are based on anti-allergen capture antibodies that often target the allergen or allergen extract, but might not target human immunogenic epitopes of allergens.

Measuring these allergenic epitopes (and therefore accounting for the human IgE-binding event) rather than measuring the whole cell (e.g. through the use of culturable spore counts) provides a more accurate determination of allergenic potential of a specific environment. Spores may not be culturable, but the presence of these epitopes on spores can still initiate IgE-binding and therefore cause allergies. Further, allergenic epitopes could be damaged, and therefore two different samples having similar allergen levels as quantified by, for instance, quantitative PCR can, in fact, have very different allergenicities. Finally, expression levels of allergenic proteins could be influenced by environmental conditions, such as temperature, thereby affecting the number of epitopes that are available for IgE-binding. Currently, allergenicity can be determined through the use of atopic serum from the blood of sensitized patients containing specific IgE [8,9]. However, the high costs and lack of serum availability have restricted the use of allergenicity determination in environmental studies.

In this study, we developed a DNA aptamer that binds with high affinity and selectivity to the major allergen Asp f 1 of the allergenic mold *Aspergillus fumigatus*. Aptamers are functional binding biomolecules, and the *in vitro* selection method for aptamers [10] enables the direct targeting of specific IgE-binding sites of allergens. The set of aptamers specific for all IgE-binding sites across all known allergens of *A. fumigatus* would allow for the full characterization of allergenicity. The *in vitro* development process and their low production costs mean that it is more feasible to

* Corresponding author. Address: Department of Chemical Engineering, Environmental Engineering Program, Yale University, Mason Laboratory, 9 Hillhouse Avenue, P.O. Box 208286, New Haven, CT 06520-8286, USA. Fax: +1 203 432 4387.
E-mail address: jordan.peccia@yale.edu (J. Peccia).

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doi:10.1016/j.bbrc.2009.06.089

develop aptamers, rather than antibodies, for targeting multiple allergenic epitopes. The development of the Asp f 1 aptamer, using an IgE-epitope of the allergen as the target for selection, brings measurements closer to allergenicity. The results presented in this study provide the first proof-of-principle that aptamer measurement methods can be developed to indicate allergen mass as well as allergenicity. Allergenicity measurements are a more direct indicator of human allergen exposure, and should better elucidate the link between environmental allergen characterization and eventual health outcomes.

Materials and methods

Immobilization of target for aptamer selection. The target for aptamer selection is a 10 amino acid long peptide having the sequence N-Q-G-D-L-R-L-C-S-H located at the C-terminal end of the Asp f 1 major allergen of *A. fumigatus*. This peptide region of the allergen has been shown to demonstrate strong IgE-binding reactivity to pooled serum from patients with allergic bronchopulmonary aspergillosis (ABPA), a complication of allergic asthma caused by *A. fumigatus* [11]. Anhydrous biotinylated Asp f 1 decapeptide (with aminohexanoic acid as a linker) was synthesized (Sigma-Genosys, St. Louis, MO) and solubilized in sterile water to a final concentration of 1 mg/ml. The peptide was subsequently immobilized onto columns (Hydros, Inc., Bourne, MA) – each containing 20 μg of streptavidin covalently coupled to a porous plastic polymer matrix – by incubating 50 μl of 1 mg/ml peptide solution in the column at room temperature for 1 h. Unbound biotinylated peptide was removed through two washing steps using a wash solution composed of 0.15 M NaCl, 0.001 M $MgCl_2$, and 0.01% SDS.

In vitro selection of aptamers. A starting aptamer library consisting of 95-mer oligonucleotides with central 60-base long randomized sequences was synthesized (Sigma-Genosys, St. Louis, MO). The sequence of each aptamer is 5′-TACTAACGGTACAAGCTA-N_{60}-AACGTTGACCTAGAAGC, where N represents a randomized nucleotide of either A, G, C or T. Primer 1 (5′-TACTAACGGTACAAGCTA) and primer 2 (5′-GCTTCTAGGTCAACGTT) were used for PCR amplification of the DNA library. Prior to *in vitro* selection of Asp f 1 decapeptide-binding aptamers, the library was subjected to PCR amplification for 20 cycles in a 50 μl mixture containing 0.4 pmol DNA template, 15 pmol each of primer 1 and 2, 0.2 mM each dNTPs, 1.5 mM $MgCl_2$, and 2.5 U Taq DNA polymerase. To generate single-stranded aptamers, the resulting amplicons were then subjected to eight cycles of asymmetric PCR containing 15 pmol of primer 1. As a control, the amplified aptamer pool was then incubated (∼1 h) with an unmodified streptavidin column to remove DNA molecules that bind non-specifically to streptavidin. To start the selection process, the flow-through fraction from this control incubation was subjected to PCR followed by asymmetric PCR under the same conditions as mentioned above, and subsequently transferred to a peptide-immobilized affinity column and incubated for 5 min. Following the incubation period, the column was washed four times with 50 μl of wash solution to remove unbound DNA molecules. Bound aptamers were eluted by incubating 50 μl of a solution composed of 7 M urea, 0.01 M EDTA and 0.001% SDS for 1 h, followed by flushing and collecting into a 1.5 ml microcentrifuge tube. The eluted aptamers were then amplified by PCR for 20 cycles, followed by eight cycles of asymmetric PCR. This new aptamer pool was then applied to a fresh peptide-immobilized affinity column for the next round of selection. A total of 13 selection cycles were completed for the *in vitro* selection process.

Cloning and sequencing of aptamers. After *in vitro* selection, aptamers were cloned using a TOPO TA cloning kit with the pCR® 4–TOPO® vector (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Colonies were subsequently picked and grown overnight in a culture of 5 ml LB medium under vigorous shaking, and plasmid DNA from these colonies was harvested with a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). Cloned inserts from the plasmid DNAs were sequenced at the DNA Analysis Facility at Yale University using an Applied Biosystems 3730 DNA analyzer (Foster City, CA). Sequence analysis and alignments were performed using the ClustalX program. The *mfold* tool [12] was used to estimate the secondary structures of sequenced aptamers.

Comparative binding assays. Ten clones were randomly selected from the pool of sequenced aptamers in the final round of selection to test for their individual binding characteristics to immobilized Asp f 1 decapeptide. In addition, a control aptamer with a random central 60-base sequence was also tested. For each binding assay, approximately 3800 pmol of aptamer ssDNA was incubated with a peptide-immobilized column for 1 h, followed by 4 × flushing steps with wash solution to remove unbound aptamers. Bound aptamers were subsequently eluted by urea solution following a 1 h incubation, and quantitated using absorbance at 260 nm with a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

Specificity testing of individual aptamers by affinity elution assays. Based on comparative binding results, promising aptamer clones were tested for their ability to bind to free Asp f 1 decapeptide (∼1.18 kDa; 21st Century Biochemicals, Marlboro, MA), Asp f 1 whole protein (∼16.8 kDa) and *A. fumigatus* allergen extract containing Asp f 1. Non-targets for testing specificity included aminohexanoic acid linker (∼131 Da; Sigma–Aldrich, Product No. A2504), bovine serum albumin (∼66.4 kDa; New England Biolabs, Catalog No. B9001S), Alt a 1 allergen (∼16 kDa) of *Alternaria alternata*, and allergen extracts of the common indoor and outdoor mold species *A. alternata*, *Cladosporium cladosporoides* and *Penicillium* spp. Purified Asp f 1 and Alt a 1 allergens were obtained from Indoor Biotechnologies (Charlottesville, VA), while allergenic extracts (1:10 wt/vol) of *A. fumigatus*, *A. alternata*, *C. cladosporoides* and *Penicillium* spp. were obtained from Hollister-Stier Laboratories (Spokane, WA). Peptide, linker and proteins were tested at 1 μg/ml while allergen extracts were tested at 1 mg/ml.

Specificity testing was performed though the use of affinity elution assays. These assays were carried out by first incubating an average of ∼3000 pmol of fluorescein-labeled aptamer with a peptide-immobilized column for 1 h. Following a series of 4 washing steps to remove unbound aptamers, the peptide column with bound aptamers was incubated for 1 h with 50 μl of an affinity wash solution containing the respective target or non-target competitor, and subsequently flushed into a 1.5 ml microcentrifuge tube. Remaining aptamers not eluted by the affinity elution wash were removed by incubation for 1 h with 50 μl of urea wash, flushed, and collected into a 1.5 ml microcentrifuge tube. The amounts of aptamers in both the affinity elution wash and urea wash were quantified by relative fluorescence measurement using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

ELISA for measuring Asp f 1 content in A. fumigatus allergen extract. Asp f 1 content in *A. fumigatus* allergenic extract was measured using ELISA kits from Indoor Biotechnologies (Code: EL-AF1), using anti-Asp f 1 mAb 4A6 for antigen capture and polyclonal rabbit anti-Asp f 1 for detection. Experimental procedures followed manufacturer's recommended protocol. Calibrated Asp f 1 standard (0.04–40 ng/ml) was used to form a standard curve.

Dissociation constant (K_D) determination. Radiolabeled aptamers were prepared by incubating 500 pmol of a single aptamer in a 50 μl reaction containing 1 × T4 polynucleotide kinase reaction buffer, 100 μCi [γ-^{32}P]ATP (3000 Ci/mmol) (Perkin-Elmer, Waltham, MA) and 50 U of T4 polynucleotide kinase (New England Biolabs, Ipswich, MA), at 37 °C for 30 min. Unincorporated nucleotides were removed using G-25 sephadex columns (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions.

Saturation binding assays for K_D determination were carried out in accordance with the previously discussed procedure for the binding and elution stages of the selection process (~1 h incubation for each stage), with the exception that increasing concentrations of radiolabeled aptamer (between 0.2 and ~400 nM) were incubated with peptide-immobilized affinity columns. The amount of aptamer clone that was bound to the column for each input aptamer concentration was quantified by liquid scintillation counting in a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin-Elmer, Waltham, MA). Based on these data, a saturation curve was plotted and the dissociation constant (K_D) computed by non-linear regression analysis (PRISM 5, GraphPad Software, Inc., La Jolla, CA).

Results

In vitro selection and sequence analysis of Asp f 1 decapeptide-binding aptamers

Thirteen rounds of *in vitro* selection were used to isolate aptamers that show favorable binding properties to the Asp f 1 IgE-binding decapeptide. Thirty-three clones derived from aptamers remaining in the final round were sequenced. Sequence alignment using the ClustalX software program revealed no single consensus sequence that is conserved among the entire population of sequenced clones (Supplementary Table S1). Examination of the sequences reveals a strong tendency towards C-richness, with most clones containing close to or above 50% cytosine residues. Secondary structural prediction using *mfold* did not reveal any two aptamers that share an exact similar structural folding.

Binding studies

Ten clones randomly selected from the 33 sequenced aptamers, together with a control random aptamer, were tested for their individual abilities to bind immobilized Asp f 1 decapeptide. The results of the binding assays are shown in Fig. 1.

The amounts of bound aptamers among most of the selected final round clones did not differ significantly from each other, ranging from ~40 pmol to just over 50 pmol. The lone exception is clone 13.52, where ~27 pmol were eluted. As expected, the final round Asp f 1 aptamers display significantly higher binding ability compared to the control aptamer ($p < 0.05$, t test). The three aptamers that showed the highest amount of bound ssDNA, 13.56 (53 pmol bound), 13.63 (52.7 pmol bound) and 13.78 (51.3 pmol bound) were selected for further characterizations of binding specificity. The *mfold*-predicted structures of the three chosen aptamers consist of various different smaller stem-loops branching off from a larger, central loop (Supplementary Fig. S1).

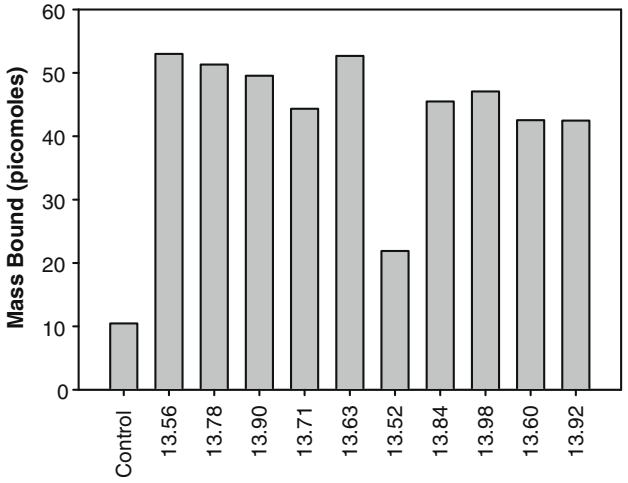

Fig. 1. Comparison of Asp f 1 decapeptide binding abilities among selected clones derived from final round sequenced aptamers and a control random aptamer. Triplicate runs of selected aptamers yielded standard error values ranging from approximately 1 to 8 pmol.

Determination of aptamer specificity by affinity elution tests

Aptamers 13.56, 13.63 and 13.78 were tested for their abilities to bind free-solution Asp f 1 decapeptide and Asp f 1 whole protein. The whole protein was tested in purified form as well as in *A. fumigatus* allergenic extract. The Asp f 1 content in 1:10 wt/vol *A. fumigatus* allergenic extracts used for specificity testing, as quantified by Asp f 1 ELISA, is ~23 μg/ml. This concentration is in good agreement with a previous study [13] which found Asp f 1 content of 17.5–61 μg/ml in *A. fumigatus* extracts obtained from the same company (Hollister-Stier Laboratories, then Bayer). *A. fumigatus* allergenic extract was tested at 1 mg/ml (corresponding to ~0.23 μg/ml Asp f 1), while Asp f 1 purified protein was tested at 1 μg/ml concentration in order to achieve approximately the same scale of Asp f 1 content. Proteins BSA and Alt a 1, along with free peptide and linker, were also tested at 1 μg/ml, with non-target allergenic extracts tested at 1 mg/ml. The results of the specificity testing are shown in Fig. 2.

All three selected aptamers were found to be eluted in significantly higher amounts by the Asp f 1 whole allergen protein and *A. fumigatus* extract (60–70% elution), versus non-targets aminohexanoic acid linker (~4% elution), BSA (~4.7% elution), Alt a 1 allergen (~6.3% elution), and mold extracts from *A. alternata* (~29.6% elution) and *Penicillium* spp. (~29.4% elution) ($p < 0.05$, t test). Only aptamer 13.56, however, displayed significantly higher elution amounts ($p < 0.05$) for Asp f 1-related targets versus *C. cladosporoides* extracts (~28.9% elution). Interestingly, for all three aptamers, affinity washes comprising the Asp f 1 decapeptide in free solution were not able to elute a substantial amount of bound aptamers (~5.4% elution).

Dissociation constant (K_D) of aptamer

The aptamer displaying the best specificity for Asp f 1 versus non-target competitors, 13.56, was selected for characterization of binding affinity. Fig. 3 shows the saturation curve obtained from the K_D experiment. Using non-linear regression analysis, the aptamer was found to have a K_D value of 93.62 ± 21.84 nM (standard error).

Discussion

In this report, we describe the development of an aptamer that could bind with high affinity and specificity to the *A. fumigatus* major allergen Asp f 1. This represents, as far as we know, the first study chronicling aptamer development for common allergens. The use of allergen-specific aptamers in sensors or detection assays should lead to increased functionality and decreased expense in exposure monitoring and epidemiological investigations for environmental respiratory diseases. In addition, by being able to potentially block IgE-binding, such aptamers may also provide a basis for therapeutics.

Aptamer selection

The peptide corresponding to the amino acid sequence of a previously-identified immunodominant epitope of Asp f 1 [11] was immobilized and used as the target for aptamer selection. On the

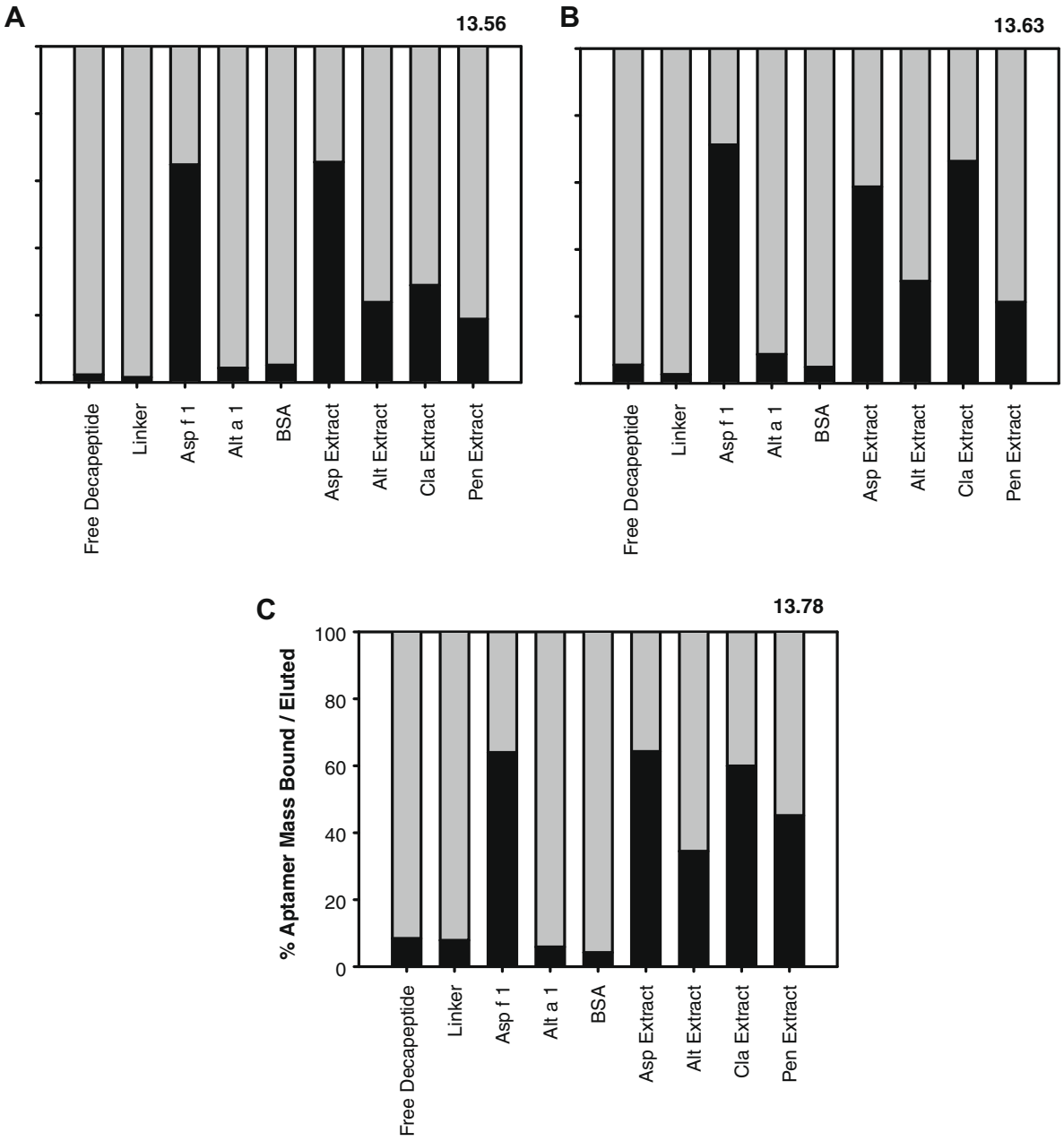

Fig. 2. (a–c) Specificity of selected aptamer clones, 13.56, 13.63 and 13.78, for Asp f 1 decapeptide in free solution, Asp f 1 whole allergen protein, and *A. fumigatus* allergenic extract containing Asp f 1, as well as non-target competitors. Affinity elution washes comprising the respective targets or non-targets followed by urea washes were carried out in turn to remove fluorescein-labeled aptamers bound to an immobilized decapeptide affinity column. Relative amounts of aptamers removed by the affinity (■) and urea (□) washes are shown as percentages of the total amount of column-bound aptamers in a stacked column bar graph. Triplicate runs of experiments yielded standard errors ranging from 0.1% to 6.5%.

whole allergen protein, this antibody epitope is located at the C-terminal end. The high segmental flexibility of this end-terminal peptide and its hydrophilicity were postulated to be among the factors for its antigenic properties. By immobilizing the synthesized peptide at the amino terminus and leaving the carboxy terminus free, the column-based peptide should simulate the conformation of the IgE-epitope on the native protein. In this way, aptamers that recognized the Asp f 1 whole allergen could be isolated.

Specificity of selected aptamers

Three aptamers, 13.56, 13.63 and 13.78, were selected for further characterizations of binding specificities. The similar affinities of the aptamers for *A. fumigatus* allergenic extracts containing comparable amounts of Asp f 1 to the purified protein tested (~60–70% elution for both targets across all three aptamers) suggest that the aptamers were able to identify their Asp f 1 target contained in the complex *A. fumigatus* extract.

To verify that the aptamers were selected for the immobilized peptide and not the aminohexanoic acid linker that formed the bridge between the immobilized peptide and the column during SELEX, we tested the specificity of the aptamers for aminohexanoic acid. The low amounts of aptamers eluted by the linker affinity washes (~4%) demonstrate that the aptamers were specific for the column-based peptide, and were not binding to the aminohexanoic acid. However, the Asp f 1 decapeptide in free solution was not found to be able to appreciably elute the bound aptamers

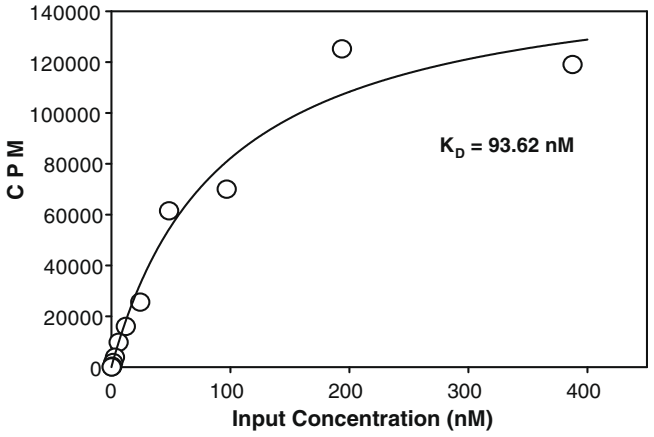

Fig. 3. Saturation curve of aptamer 13.56. A concentration series of the radiolabeled aptamer, ranging from ~0.2 to 400 nM, was incubated with Asp f 1 decapeptide-immobilized affinity columns. (K_D) of the aptamer was computed by non-linear regression analysis.

during the affinity assays (~5.4% elution), suggesting a poor affinity between the aptamers and the unbound peptide. We postulate that this low affinity is due to the aptamers binding to a particular conformation of the peptide, which is only present as the N-terminal end is immobilized, either on a column through a biotin–streptavidin bond, or when the peptide is attached as the native C-terminal end of the much bigger Asp f 1 molecule. There is precedence for such binding behavior. It has previously been noted [14] that an aptamer for a similarly-sized (11-residue) peptide, substance P, was unable to recognize the reverse orientation of the same amino acid sequence. This was attributed to the target possessing a unique structure, with unique relative positions of amino acid side chains, and it was this distinctive conformation of the peptide that was recognized by the aptamer ligand.

All three aptamers exhibited high specificity for their Asp f 1-related targets versus non-target proteins BSA, Alt a 1 allergen and extracts of *A. alternata* and *Penicillium* spp. ($p < 0.05$). Only aptamer 13.56, however, displayed high specificity against non-target proteins in *C. cladosporoides* allergenic extracts ($p < 0.05$). For the other two aptamers, the elution amounts did not differ significantly from that of Asp f 1 or *A. fumigatus* extract ($p > 0.4$), suggesting a potential for cross-reactivity. This cross-recognition of aptamers 13.63 and 13.78 for *C. cladosporoides* extract suggests the formation of aptamer–peptide complexes in the *C. cladosporoides* extract which are equally favorable to that of the aptamer–Asp f 1 decapeptide complex [15].

Binding affinity to target

The aptamer displaying the best specificity for Asp f 1 in comparison to non-target proteins and fungal allergen extracts, 13.56, was found to display good affinity for its target, with a nanomolar dissociation constant (~90 nM) to the IgE-binding epitope. The presence of positively charged residues on the peptide target could have facilitated the successful production of a high affinity aptamer [16]. This affinity is in good agreement with prior work on aptamers targeting various peptides and proteins, such as arginine-rich motifs and thrombin, which have found K_D values ranging from 5 to 300 nM [14,15,17].

In conclusion, an aptamer has been developed that is selective for and binds with high affinity to the Asp f 1 allergen of *A. fumigatus*. The results from this study proved the feasibility of producing aptamers for whole allergens using immunogenic protein subsegments as targets, and provide the platform for the potential future development of aptamers that target other IgE-binding regions in all known allergens of *A. fumigatus*, thus mimicking IgE-binding and enabling the full characterization of allergenicity.

Acknowledgment

This work was supported by NIH Grant RES015312A.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.089.

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Supplementary Table S1. Central nucleotide sequences of round #13 aptamers (5' – 3' direction). The main 60-base section of the aptamer is shown and the primer-binding sites are not displayed. Of the 33 sequenced clones, all display a tendency towards C-richness, and no sequence homology was observed.

Aptamer	Central region of aptamer
13.49	caccctccgtcccccactagactctaccctacacaaccttaatcccccgcacctcgtccc
13.51	cagacgatccgcacctctcataaaaaagtctgtagattgtttttatctcccccggccc
13.52	cacacgcctcttcccccgcctcacagtacgtttctgctccctatttcctgctcccgcccc
13.53	gccacacacctaccacactaacacatattcaccatccacctctctatttccatcctgtcc
13.54	ctgacacttcacacaccttcccaataccccaccaccactgaactccctgcatccacatcc
13.55	cccacttccacaccaccccacctactcacaatgttcactaccttctttcattccccgtcc
13.56	cacacccgccccgctccatccgcccatcgtcacctacccacctcgtgatccccttccccc
13.58	cacaaccacgctacaacccaaccaacacaaccccgctactcatgcacgaacctacctccc
13.59	tacacagaccacccccgcctccaccaatcgttagcaccatccatacttcattcctaccc
13.60	ccaaacctctccgctcggctccccctatcacatatacctttttatttcccctccccggcc
13.62	gcataccatcaacctccccgccgttcttgtattcataacccgccccacgtacgtctcccc
13.63	gcccaccagaacaccccgtcccatcgacctccctcctcatatccctgcccctctgcctcc
13.64	cacattccaccacacccatacgcacctcacccatcacccagcccctataccttcctgcc
13.65	cgccacacctcacaacccccccccggccggcactcaactctactcctcactctcacaccccc
13.67	cgtcatcctatacaccgctactctatccgtacagagtgttcccgtttcgctctatcccc
13.71	tatcaacctaccttcccttagacatcaacgcatccttcccccctcgttgacaccctcctcc
13.72	cacctccaacactaactttaccttcctacgttctccttaccactcgtcctctccgcccccc
13.73	caaacccccccccaccgccccaacctacctcaaccctatcctctcgtccaccaccccgcc
13.75	ccctcctgtagaacacctgaggcccgtcaatttacgttttacggccgtctgaacccc
13.77	cacccataataacctcatccacctctcatgcatatctcgccaatcagccctctgtgccc
13.78	caaaccccaacgcacgtacacctgtctcccgcactagttcccccctcatgactgccctccc
13.79	ccacaacctgcttccctttccctcacatcctttcctctttataccattgcccctctcccc
13.80	tcaatctatcatcccgctaacctcaatcttccgtactttctcttccttactctttcctcc
13.83	caaactcaccacccatcactctttacccgcactcccgctccgtccctcccccgcctctcc
13.84	gcctacccccgctaccacaatcccgtcccactcccgccgactctaaccacgactccgccc
13.85	cacgcaccggaccactacccctcccccaagcccccaacgagaccgtcccccctcccctcgac
13.89	aaatccaaccacttccatccgagtccttacattccggaccagttcccggatgtctagtcc
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13.92	accccgcagcaaccacagcctaccactccgttacccccttacactctccatctctgcaccc
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13.97	cacacatgaccctgccgcatcaccttcccccttacaactactgctattcgcatccgcccc
13.98	ttagcccaccctcccgctcttcctcttagtctttccttatgttccgtgtcgttcccgtcc
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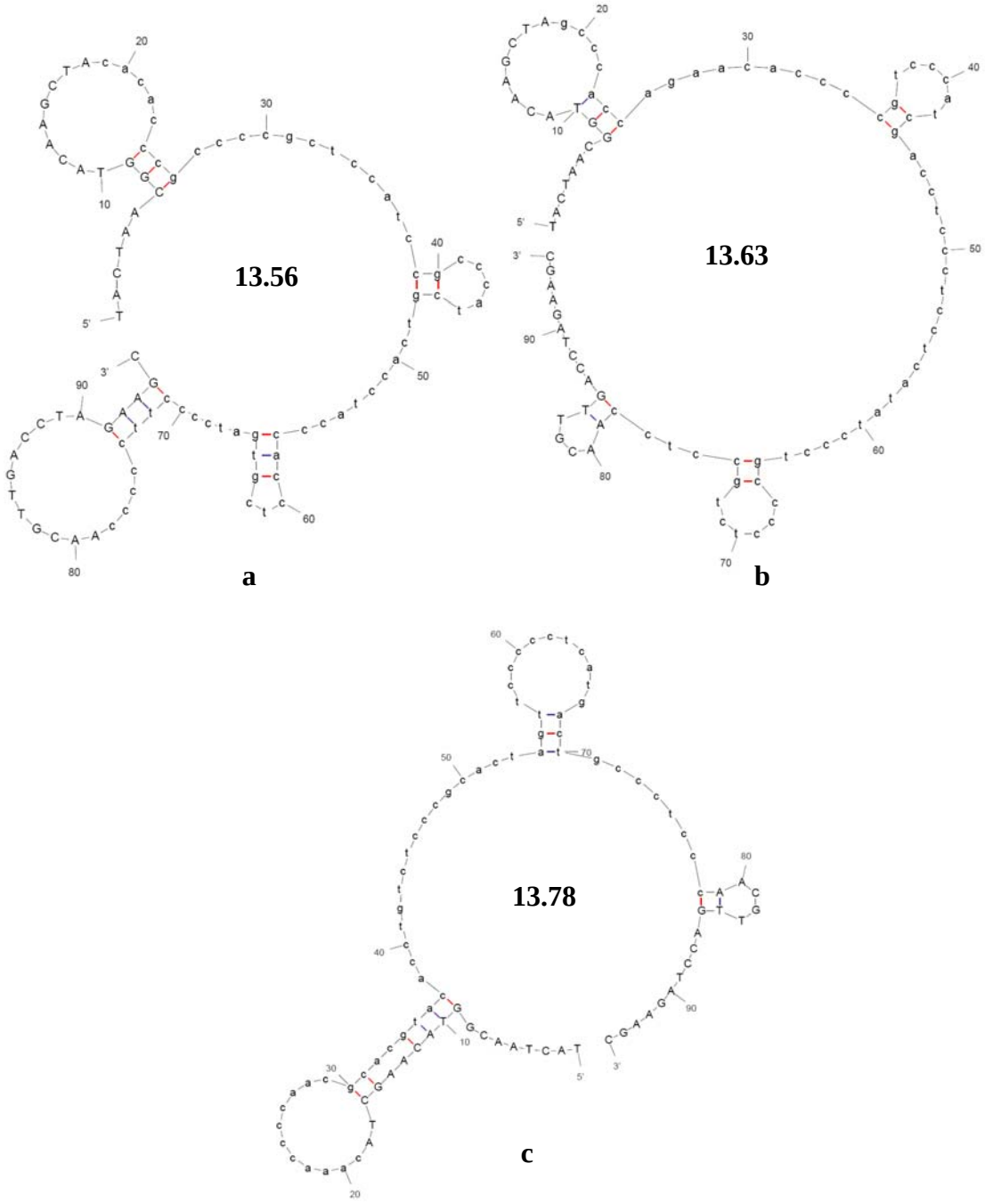

Supplementary Figure S1a, b, c. Secondary structural predictions of round #13 aptamers, 13.56, 13.63 and 13.78. The primer regions are in upper-case letters.