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A novel secreted endonuclease from *Culex quinquefasciatus* salivary glands

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Summary

Previous analysis of the salivary gland transcriptome of *Culex quinquefasciatus* showed the potential presence of an endonuclease with sequence similarities to shrimp, crab and two tsetse salivary proteins. Indeed, not only was the cloned cDNA shown to encode an active double-stranded endonuclease, but also the same activity was demonstrated to be secreted by salivary glands of *Cx. quinquefasciatus*. Preliminary studies with salivary gland extracts confirmed the presence of a highly active nuclease. This enzyme was shown to be present in the saliva of female mosquitoes by allowing starved mosquitoes to probe DNA-containing agarose gel. The recombinant *Cx. quinquefasciatus* endonuclease (CuquEndo) produced in mammalian cells showed no sequence specificity for DNA substrate except that it only cleaves double-stranded DNA. Recombinant *Cx. quinquefasciatus* endonuclease was active in the presence of Mg^{2+} ions at pH 7.0–8.0, but no endonuclease activity was detected in the presence of calcium ions. The final hydrolysis products of this enzyme, detected by ion exchange chromatography, yielded DNA fragments ranging form 8–12 base pairs. Although endonucleases have been associated with a variety of cellular functions, their role in mosquito saliva is not clear. This female-specific secreted endonuclease may assist blood meal intake by lowering the local viscosity created by the release of host DNA in the bite site and/or acting as an indirect anticoagulant factor by producing a defibrotide-like mixture of DNA haptamers.

Key words: mosquito, salivary gland, endonuclease, *Culex quinquefasciatus*, arthropod.

Introduction

Nucleases, including ribonucleases and deoxyribonucleases, are an evolutionarily old group of enzymes present in both prokaryotes and eukaryotes. In a broad sense, nucleases are enzymes capable of hydrolyzing nucleic acids. These enzymes belong to the phosphodiesterase family and are capable of cleaving phosphodiester internal bonds within double-stranded (ds) or single-stranded (ss) DNA and RNA substrates. Bacterial and eukaryotic endonucleases (EC 3.1.30.-) require a divalent ion such as magnesium for their activity under neutral or alkaline conditions. In addition to nucleotide salvage, repair, recombination, transposition, degradation and cell division, nucleases are also involved in cell defense, promoting degradation of foreign nucleic acids (Mishra, 2002).

Nonspecific nuclease family members cleave all types of nucleic acids with similar specificity for RNA and for both ss and dsDNA substrates (Friedhoff et al., 1996; Ho and Liao, 1999; Meiss et al., 1998). However, some nucleases showing little sequence specificity do recognize certain structural features of their respective DNA substrates other than ss/ds DNA. Enzymes recognizing helical parameters such as groove width and flexibility (Dnase-I) or helical distortions caused by abasic sites (exonuclease III, HAP1), and enzymes specific for such structures as flap DNA (5′ nucleases of eukaryotes and phage and eubacterial DNA polymerases) and four-way junctions (T4 endonuclease VII, RuvC) have been reported (reviewed by Suck, 1997). Shagin et al. reported (Shagin et al., 2002) a type of nonspecific nuclease from crab that displays no (or extremely low) RNase activity and a strong preference for cleaving dsDNA compared with ssDNA. Nonspecific DNases with cleavage preference for dsDNA have also been reported in the sea urchin and Kamchatka crab (Menzorova and Rasskazov, 1981; Menzorova et al., 1993).

The sialotranscriptome of *Culex quinquefasciatus* (Ribeiro et al., 2004) contains one relatively abundant cluster of sequences coding for a putative protein similar to endonucleases found in shrimps and crabs (Shagin et al., 2002; Wang et al., 2000) and also similar to two tsetse salivary proteins named Tsal1 and Tsal2 (Li et al., 2001) of unknown function. The full-length clone of the *Cx. quinquefasciatus* endonuclease has the NUC Smart motif indicative of DNA/RNA nonspecific endonucleases and phospho-diesterases. These arthropod enzymes display an activity similar to vertebrate pancreatic Dnase-I; however, they belong to a distinct protein family despite sharing similarities near the active site (Shagin et al., 2002).

Our studies provide evidence for a novel, secreted endonuclease from *Cx. quinquefasciatus* salivary glands. Although the relevance of endonuclease function in saliva is not clear, its tissue and sex specificity indicate that this enzyme plays a role in mosquito blood feeding. This type of enzymatic activity is here identified for the first time in the saliva of hematophagous animals.

Materials and methods

Mosquitoes

Mosquitoes *Culex quinquefasciatus* Say were reared in the Medical Entomology Section, National Institutes of Health, Twinbrook III, MD, USA as described previously (Ribeiro et al., 2004). Adult female *Cx. quinquefasciatus*, Vero Beach strain, were dissected at day 2–3 post-emergence to remove the salivary glands. Salivary glands (10 pairs) were dissected under a stereomicroscope in 20 mmol l^{-1} Hepes, 120 mmol l^{-1} NaCl, pH 7.4, and used immediately for total RNA extraction (Trizol; Gibco-BRL, Gaithersburg, MD, USA) and cDNA synthesis (SuperScript™ RT; Invitrogen, San Diego, CA, USA) following standard procedures.

Ex vivo endonuclease assay

To demonstrate the salivary secretion of endonuclease (CuquEndo) by *Cx. quinquefasciatus* adult females an *ex vivo* assay was designed. 5–10 adult female mosquitoes (2 days old, non-blood fed) were starved of sugar for 24 h before the test. Starved mosquitoes were allowed to probe for 15–20 min in a 1% agarose gel containing 50 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, 10 mmol l^{-1} NaH_2CO_3, and 1 mmol l^{-1} $MgCl_2$ pH 7.4 and 200 ng ml^{-1} ds circular plasmid DNA. The probing assay slide with the gel on was kept at 37°C in a slide warmer (Precision Scientific, Chicago, IL, USA). The combination of CO_2 released from the bicarbonate buffer plus the temperature stimulated the mosquitoes to probe the slide (Braverman et al., 1991). After probing, mosquitoes were removed and the agarose gel, which was kept for another 30 min at 37°C in a wet chamber, then stained with ethidium bromide and observed under UV light.

Expression of Culex endonuclease in the 293F cell system

PCR fragments coding for *Cx. quinquefasciatus* salivary (GenBank accession code AAR18449) endonuclease was amplified (Platinum Supermix; Invitrogen) from salivary gland cDNA using gene-specific primers, CxEndoFor (5′-atgatttcatttttattgagcatc-3′) and CxEndoRev (5′-ttagttctttttgttgttgatttccc-3′). PCR-amplified product was cloned into VR2001-TOPO vector (modified version of the VR1020 vector; Vical Incorporated, San Diego, CA, USA) and their sequence and orientation verified by DNA sequencing (DTCS Quick Start Kit; Beckman Coulter Inc., Fullerton, CA, USA). 3–5 mg of plasmid DNA (VR2001-CxEndo construct) was obtained using EndoFree™ plasmid MEGA prep kit (Qiagen, Valencia, CA, USA) and purified through a 0.22 μm filter.

Recombinant *Culex* endonuclease (CuquEndo) was produced by transfecting FreeStyle™ 293-F cells (Invitrogen) with 30 μg of purified VR2001 *Culex* endonuclease plasmid following the manufacturer's recommendations (Invitrogen). After 72 h, transfected cell cultures were harvested and the supernatant concentrated 60 fold in Amicon® Ultra-15 (10 kDa MW) (Millipore Corp., Bedford, MA, USA).

Assay of nuclease activity

Endonuclease reactions contained 150 mmol l^{-1} NaCl, 1 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} Tris-HCl, pH 7.5, 5 μg BSA, and 400 ng ds circular plasmid DNA (VR2001; Vical Incorporated) in a final volume of 20 μl. The reaction mixtures were incubated with different dilutions of salivary gland extracts and recombinant endonuclease. After 10 min at 37°C, samples were electrophoresed in 1.2% agarose gel (Invitrogen) and visualized under UV light.

Alternatively, DNase was detection using Hoechst dye 33258 (bisbenzimide; Sigma Chemical, St Louis, MO, USA). Briefly, the endonuclease reaction was carried out as described above: 5 μl were taken from different salivary gland extract dilutions and further diluted in 100 μl of the dye mix (20 μg ml^{-1} Hoechst dye, 2 mol l^{-1} NaCl, 1 mmol l^{-1} EDTA, 10 mmol l^{-1} Tris, pH 7.4). The fluorescence was measured using a Fluorolite 1000 fluorimeter (Dynatech Laboratories, Chantilly, VA, USA) with 365/460 nm (excitation/emission, 6.1 V) filters.

SDS–NuPAGE/Triton X-100 endonuclease assay

Recombinant CuquEndo (15 μl) in NuPage LDS sample buffer (Invitrogen) were loaded into a NuPAGE 4–12% Bis-Tris gel (Invitrogen) without heating. The sample was run in one dimension at low voltage (95 V at 4°C) under nonreducing conditions. After electrophoresis, the gel was washed once in 2.5% Triton X-100 for 1 h and then washed in 120 mmol l^{-1} NaCl, 50 mmol l^{-1} Tris, pH 7.4, twice for 30 min at room temperature. Renatured NuPAGE gel was overlaid onto a 1% agarose gel containing 0.54 ng ml^{-1} of ds plasmid DNA in 120 mmol l^{-1} NaCl, 1 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} Tris, pH 7.4. Gels were kept for 30 min at 37°C in a wet chamber. The lytic zone of activity was visualized with ethidium bromide under UV light.

Cation dependency of recombinant CuquEndo

To determine the cation dependency of the cell supernatant endonuclease activity, reaction buffers at pH 7.5 containing different concentrations of divalent cations (Mg^{2+} or Ca^{2+}) were assayed. Reaction mixtures containing 1 μl of supernatants of cells expressing recombinant CuquEndo were incubated for 10 min at 37°C in different reaction buffers (500 ng ds plasmid DNA, 50 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl pH 7.5) in a final volume of 20 μl. CuquEndo products were resolved in a 1.2% agarose gel (Invitrogen).

Determination of CuquEndo substrate specificity and degradation products

To determine substrate (ds or ss) specificity of CuquEndo,

2 μl of recombinant protein or 2 μl of 1/500th of a *Cx. quinquefasciatus* salivary gland were incubated for 60 min at 37°C with different combinations of ds circular plasmid DNA or poly nucleotides (ss and ds). Reactions were performed in 20 μl (final volume) containing 50 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, 1 mmol l^{-1} $MgCl_2$ and 400 ng of plasmid DNA or 1.5 μg of 50-mer polyC, polyT, polyAG or polyCT. Additionally, a mixture of polyAG/polyCT (1:1 molar ratio) was incubated for one cycle of 20 min at 95°C; 20 min at 85°C; 10 min at 72°C; 10 min at 60°C; and 10 min at 50°C to create a ds synthetic substrate. Approximately 2 μg of ds polyAG/CT was incubated under the same conditions as described above in the presence of recombinant CuquEndo.

The pattern of nuclease activity of CuquEndo was determined by ion exchange chromatography–high pressure liquid chromatography (IEC–HPLC). The digested samples were diluted 50 times in 25 mmol l^{-1} Tris, pH 9.0, and subjected to chromatography. Chromatographic separation of CuquEndo products was performed with a TSKgel DEAE-NPR (4.6 × 35 mm) equilibrated with 25 mmol l^{-1} Tris, pH 9.0, and the peaks resolved through 0–1 mol l^{-1} NaCl gradient over 30 min and monitored at 254 nm. All chromatography was performed using a Spectra System P400 pump in conjunction with a dual-wavelength UV-visible detector (model SM4100; Thermo Separation Products, Riviera Beach, FL, USA).

Molecular modeling

Three-dimensional structure predictions for *Cx. quinquefasciatus* endonuclease were obtained using the algorithm Phyre v 0.2 (protein homology/analogy recognition engine) (Kelley et al., 2000) (http://www.sbg.bio.ic.ac.uk/~phyre/). The active site of CuquEndo was superimposed with the crystal of *Serratia marcescens* (PDB id 1G8T; mmdbId:15096) endonuclease and the cartoon drawn by DeepView/Swiss PDB viewer v3.7 (http://www.expasy.ch/spdbv/).

Results

Initial observations

Random sequencing of 503 clones of a cDNA library from the salivary glands of adult female *Cx. quinquefasciatus* mosquitoes (Ribeiro et al., 2004) found a cluster coding for an enzyme associated with nucleotide hydrolysis. This abundant cluster of sequences (with six expressed sequence tags), annotated as a nonspecific endonuclease (CuquEndo; NCBI# AAR18449), has the NUC Smart motif indicative of DNA/RNA nonspecific endonucleases and phosphodiesterases. The cDNA of CuquEndo has an open reading frame of 1095 bp coding for a protein of 364 amino acids (aa). It has a predicted signal peptide (Nielsen et al., 1997) of 24 aa, indicative of secretion. The predicted mature CuquEndo has a molecular mass of 39 290 Da with an isoelectric point of 9.21 and does not contain any potential *N*-glycosylation sites. Alignment of the CuquEndo putative active center (Fig. 1A) with other proteins of the same family shows the presence of the conserved RGH triad found in most DNases characterized so far. Most of the other conserved residues (in red in Fig. 1) are present in the CuquEndo except for the last conserved Arg residue. A Lys exists in the mosquito sequence within one residue of the missing Arg. The importance of these residues for catalysis has been previously studied in detail (Miller et al., 1999; Shlyapnikov et al., 2000).

Using the crystal structure of *Serratia marcescens* endonuclease as a template, a model of CuquEndo was obtained using the Phyre modeling software (Kelley et al., 2000). According to Shlyapnikov et al. (Shlyapnikov et al., 2000), the structure of *S. marcescens* endonuclease – which has been extensively (biochemically and structurally) studied – reveals essential inner symmetry. The protein secondary structure can be divided into three regions: (i) a central β sheet formed by six antiparallel β strands, which is flanked by (ii) an α domain consisting of four short α helices and joining the N- and C-terminal parts of the molecule on one side, and (iii) an α/β conformational domain forming the upper layer of the globule, which consists of a ds β sheet embraced by a symmetrical system of eight helical fragments. As shown in Fig. 1B, superimposition of the CuquEndo model on that of *S. marcescens* reveals significant overlap between the two active sites. This quite similar active site geometry suggests that both enzymes might have a similar mechanism of action on DNA substrates.

To verify whether endonuclease was present in salivary glands of adult female *Cx. quinquefasciatus* mosquitoes, we used ds circular plasmid DNA as the endonuclease substrate and incubated it with salivary gland homogenates. The results, shown in Fig. 2, demonstrate the presence of a highly active salivary nuclease in these mosquitoes. Indeed, salivary gland homogenates, equivalent to 1/500th of one salivary gland, hydrolyzed approximately 50% of the plasmid DNA as measured by fluorometry using Hoechst dye (Fig. 2B). No significant DNA degradation was observed at concentrations lower than 1/4096th of one salivary gland in the agarose gel (Fig. 2A). Controls containing no salivary gland extract did not show DNA hydrolysis. No RNase activity was detected on yeast RNA (not shown).

Secretion of endonuclease activity by female Cx. quinquefasciatus

To demonstrate that adult mosquitoes secrete an endonuclease, female *Cx. quinquefasciatus* mosquitoes were starved for 24–36 h and induced to probe in an agarose gel containing plasmid DNA warmed to 37°C. The mosquito-probed gel was subsequently tested for endonuclease activity. Fig. 3 shows that female mosquitoes indeed secrete a salivary endonuclease, as predicted by the presence of a signal peptide in CuquEndo cDNA. This experiment was replicated with *Aedes aegypti* and *Anopheles gambiae* mosquitoes, producing no DNA hydrolysis (data not shown).

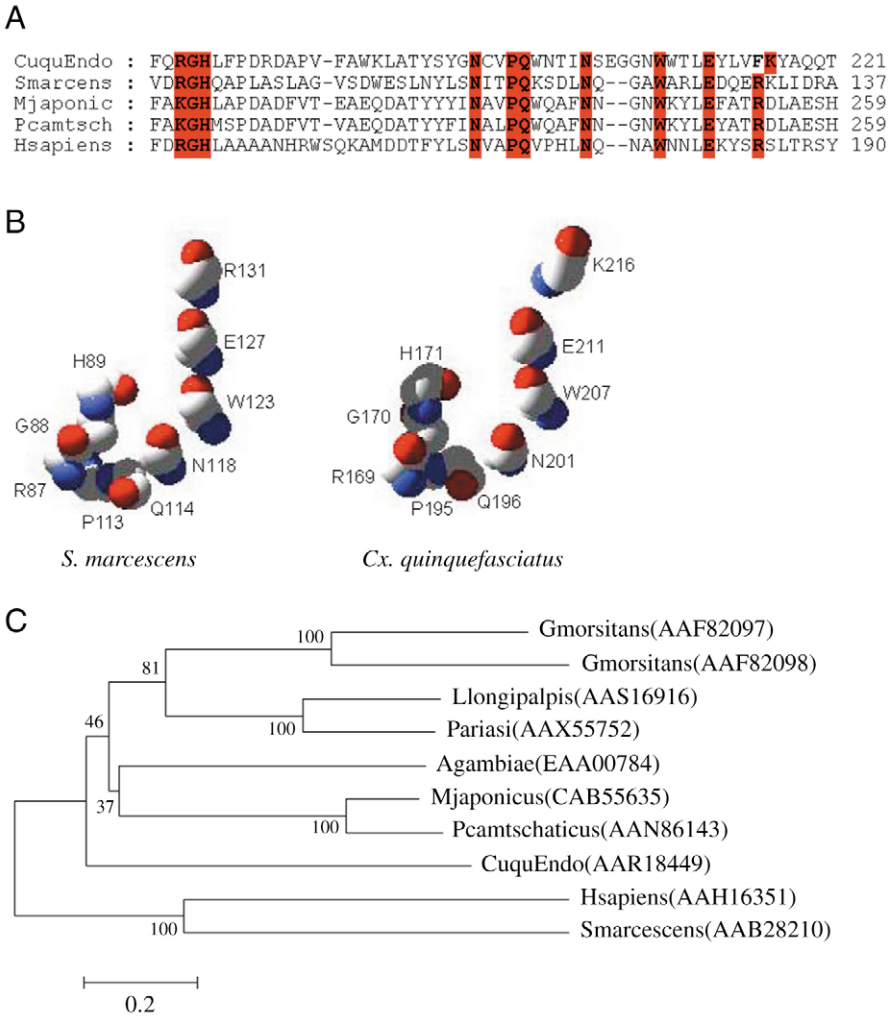


Fig. 1. (A) Amino acid (aa) sequence alignment of conserved elements among different endonuclease active sites. Conserved aa residues are highlighted in red. Most known endonucleases have the conserved R(K)GH triad. CuquEndo also contains other aa residues implicated in the nucleophilic attack of DNA substrate and stabilization of the active site. (B) Molecular modeling of CuquEndo and active site comparison with Smarcens endonuclease (PDB id 1G8T). The similarity of the active site geometry suggests that both enzymes might have a similar mechanism of action on DNA substrates. (C) Phylogenetic analysis of the endonuclease family. The unrooted neighbor-joining tree (10,000 bootstraps) was generated by MEGA 3.1 software. CuquEndo, *Culex quinquefasciatus*; Smarcens, *Serratia marcescens*; Mjaponic, *Marsupenaeus japonicus*; Pcamtsch, *Paralithodes camtschaticus*; Hsapiens, *Homo sapiens*; Gmorsitans, *Glossina morsitans*; Longipalpis, *Lutzomyia longipalpis*; Pariasi, *Phlebotomus ariasi*; Agambiae, *Anopheles gambiae*. NCBI accession numbers in parentheses.

Production of recombinant CuquEndo in a mammalian system

Recombinant CuquEndo was produced in 293F cells as described in the Materials and methods. After transfection, cells were allowed to produce recombinant CuquEndo for 48–72 h. A parallel transfection using empty VR2001 was used as a negative control. Both supernatants were assayed for endonuclease activity on ds plasmid DNA. Supernatant-containing CuquEndo (1 μl) completely hydrolyzed 500 ng of plasmid DNA at 37°C in 10 min. No degradation of DNA was observed in the negative control (data not shown). No RNase activity was detected on yeast RNA.

The SDS-PAGE agarose gel detection of endonuclease activity allowed us to analyze the 293F cell supernatant by apparent molecular mass. A unique band is seen at ~41–42 kDa (Fig. 4), consistent with the calculated molecular mass of CuquEndo. This in-gel renaturation assay allowed us to confirm the presence of recombinant CuquEndo secreted in the 293F cell supernatant.

Divalent cation dependency of CuquEndo

Recombinant CuquEndo activity was examined for divalent cation sensitivity. CuquEndo hydrolyzed 500 ng of ds plasmid DNA in the pH range 7.5–8.5 (Fig. 5A) in the presence of 1 mmol l^{-1} $MgCl_2$. We also examined the effect of Ca^{2+} on endonuclease activity of recombinant CuquEndo. Fig. 5B shows that in the presence of Mg^{2+} ions, CuquEndo hydrolyzes the DNA substrate into a smear of heterogeneous digested product after 10 min of incubation at 37°C. The endonuclease activity of CuquEndo decreases above 6.0 mmol l^{-1} and was not detected in the reaction mix depleted of $MgCl_2$. When Mg^{2+} was replaced by Ca^{2+} in the reaction mix, no endonuclease activity was detected. The data indicate that CuquEndo is divalent cation dependent, similar to *S. marcescens* endonuclease, which requires Mg^{2+} ions, but it is insensitive to Ca^{2+} ions (Friedhoff et al., 1996).

Analysis of CuquEndo digestion products and substrate specificity

To investigate the substrate preference of CuquEndo for ds or ssDNA as well as the terminal degradation products of its enzymatic activity, we developed an assay based on synthetic hetero-oligonucleotides followed by ion exchange HPLC (IE-HPLC). 50-mer ss oligonucleotides (polyC, polyT, polyAG

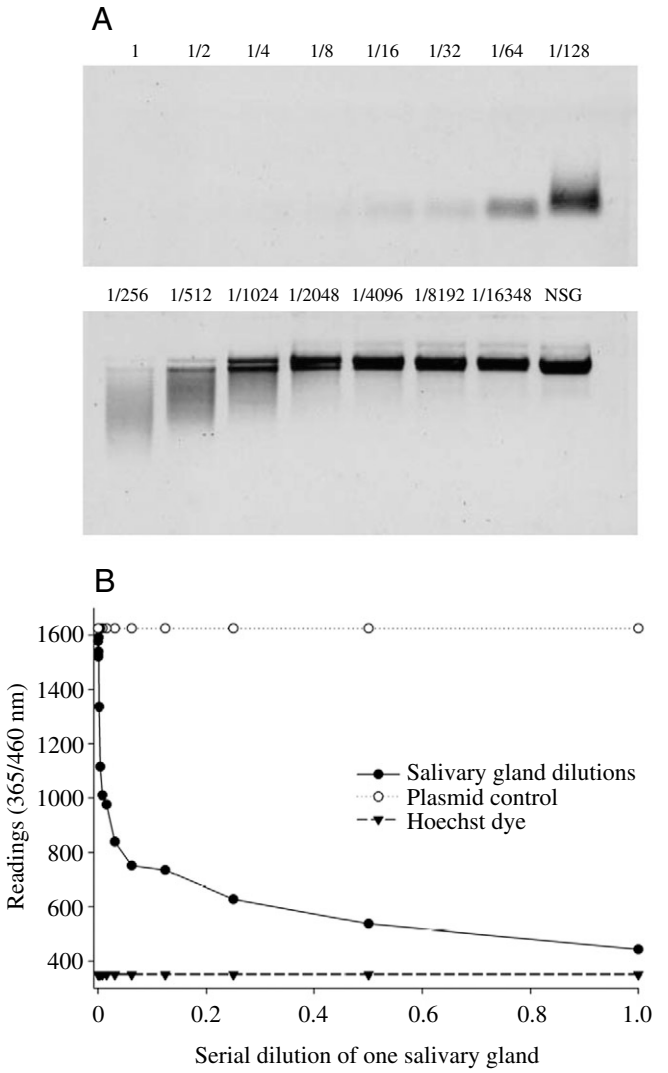


Fig. 2. Endonuclease activity in salivary gland extract of female *Culex quinquefasciatus*. Double-stranded plasmid DNA (400 ng) was incubated in a final volume of 20 μl with different salivary gland dilutions (serial dilutions of one salivary gland) and incubated for 10 min at 37°C. (A) 10 μl from each sample was electrophoresed in a 1.2% agarose gel and visualized under UV light. NSG, no salivary gland, negative control. (B) 5 μl from each reaction were diluted in 100 μl of Hoechst dye mix and the fluorescence measured using a fluorimeter with 365/460-nm (excitation/emission, 6.1 V) filters.

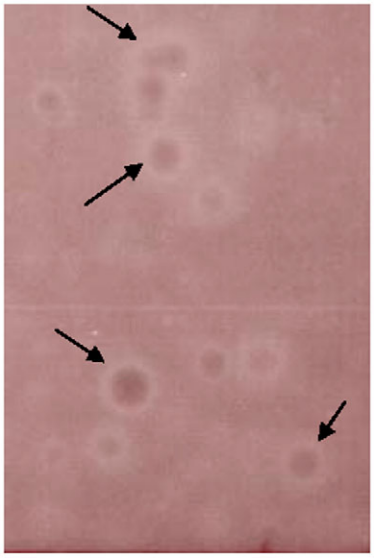
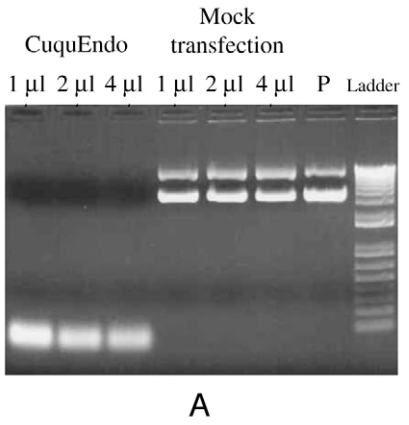


Fig. 3. Secretion of CuquEndo in *Cx. quinquefasciatus* saliva. Starved mosquitoes were allowed to probe in an agarose gel containing 10 mmol l^{-1} $NaHCO_3$, 100 mmol l^{-1} NaCl, 1 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} Tris, pH 7.5, and 200 μg ml^{-1} of double-stranded plasmid DNA. After 30 min incubation at 37°C, the gel was stained with Ethidium Bromide and visualized under UV light. Arrows indicate the hydrolysis of plasmid DNA at the biting sites within the gel.

and polyCT) or ds polyAG/CT and plasmid DNA were used for endonuclease assays in the presence of 1 μl of recombinant CuquEndo in a reaction mix supplemented with 1 mmol l^{-1} $MgCl_2$ in a final volume of 20 μl. We also assayed the action of salivary gland extracts on ds plasmid DNA. After 1 h incubation at 37°C, 5-μl aliquots from each reaction were diluted 50 times in 25 mmol l^{-1} Tris, pH 9.0, and subjected to chromatographic analysis. Separation of CuquEndo hydrolysis products analyzed by IEC-HPLC are shown in Fig. 6.

Fig. 6A shows the chromatogram used to standardize the product sizes generated by CuquEndo hydrolysis of the DNA substrates tested. The chromatograms obtained from the plasmid DNA hydrolyzed either with salivary gland extract or recombinant CuquEndo (Fig. 6B) show that salivary gland extract and CuquEndo in culture supernatants produced by 293F cells behaves in a similar way. Plasmid DNA was cleaved to fragments with retention time ranging from 17 to 22 min (5–10 nt compared with polyT standard). Similarly, Friedhoff et al. reported (Friedhoff et al., 1996) that for moderately good cleavage activity by the *Serratia* nuclease, the deoxynucleotide substrate should contain at least five phosphate residues.

Results of incubation of synthetic ss and ds deoxynucleotides in the presence of recombinant CuquEndo (Fig. 7) clearly indicate that this enzyme only cleaves dsDNA. The terminal degradation of dsDNA substrates by CuquEndo shows that the action of the enzymes gives a random distribution of sizes. The average oligonucleotide size was found to range from 5–10, in a similar way as observed when plasmid DNA was used as a substrate. Our interpretation is that fragments shorter than five are ss at the temperature (37°C) at which we carried out the enzymatic assays and thus cannot be cleaved by CuquEndo, which appears to be a ds-specific endonuclease. Other nonspecific endonucleases exhibit strong cleavage preference for dsDNA but, unlike CuquEndo, low levels of hydrolytic activity toward ssDNA have been reported (Friedhoff et al., 1996; Shagin et al., 2002; Wang et al., 2000).



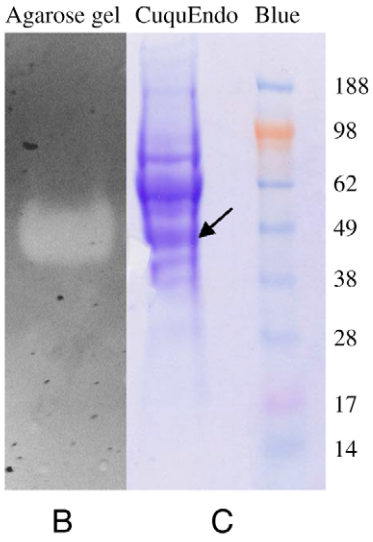


Fig. 4. Expression of recombinant CuquEndo in 293 cells and SDS–PAGE agarose gel assay. (A) *In vitro* assay showing the presence of endonuclease activity in supernatant of cells transfected with the CuquEndo-VR2001 construct. No plasmid degradation was observed in the supernatant of 293 cells transfected with an empty VR2001 plasmid (mock control) P, 500 ng of doubled stranded plasmid DN, Ladder, 1 kb Plus DNA ladder. (B) Concentrated 293 cell supernatant (crude) containing the recombinant CuqueEndo was resolved in a NuPAGE gel. After in gel renaturation (see Materials and methods) the NuPAGE gel was exposed to a 1% agarose gel containing 0.54 ng ml^{-1} of double stranded plasmid DNA. Degradation of plasmid DNA occurred at the position of the calculated molecular mass of recombinant CuquEndo, as visualized with Ethidium Bromide under UV light. (C) The Coomassie Blue-stained NuPAGE gel (arrow indicates position of CuquEndo). Blue, prestained standard proteins.

Discussion

In this work, we have characterized a potent endonuclease secreted by the salivary glands of female mosquitoes. Based on sequence analysis, this enzyme was classified as a member of the DNA/RNA nonspecific nuclease family (EC 3.1.30.-). Several endonucleases of this family have either no or low RNase activity. To the best of our knowledge, CuquEndo is the first reported secreted endonuclease found in saliva of hematophagous animals and the first example of an active recombinant insect enzyme of this class.

Vertebrate and invertebrate DNase-I have a number of properties in common: optimal pH between 6.5 and 8, molecular mass of 30–40 kDa, activation by the divalent cations and inhibition by EDTA. *S. marcescens* endonuclease is a well studied example of this family for which structural information is available (Miller et al., 1999; Shlyapnikov et al., 2000). CuquEndo shares some structural and enzymatic similarities with *S. marcescens* endonuclease. Both enzymes have strict divalent cation dependency for enzymatic activity. CuquEndo and *S. marcescens* endonuclease require Mg^{2+} for activation and are Ca^{2+} insensitive. Structurally, both endonucleases show quite similar active site geometry, suggesting that both enzymes might have similar mechanisms of action on DNA substrates.

Alignment of the endonucleases (not shown) shows the presence of ten conserved cysteine residues, of which CuquEndo does not have the tenth but has four additional Cys residues (Ribeiro et al., 2004). The shrimp enzyme, for which more detailed structural studies have been done (Shagin et al., 2002), has an extra Cys that appears to be linked to an unknown ~500 Da residue. The putative active center of the shrimp

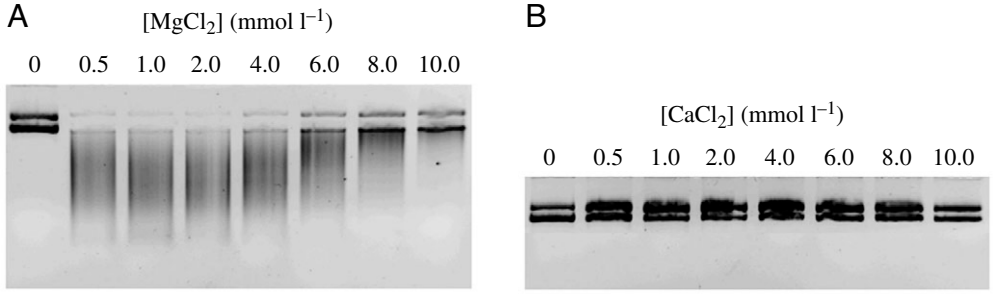


Fig. 5. Effect of divalent cations on recombinant CuquEndo activity. Reaction mixtures containing 1 µl of recombinant CuquEndo and 500 ng of plasmid DNA in a final volume of 20 µl were incubated for 10 min at 37°C in buffers with cation concentration ranging from 0 to 10 mmol l^{-1}. The digested DNA plasmids were resolved in 1.2% agarose gels and visualized under UV light. (A) $MgCl_2$. (B) $CaCl_2$.

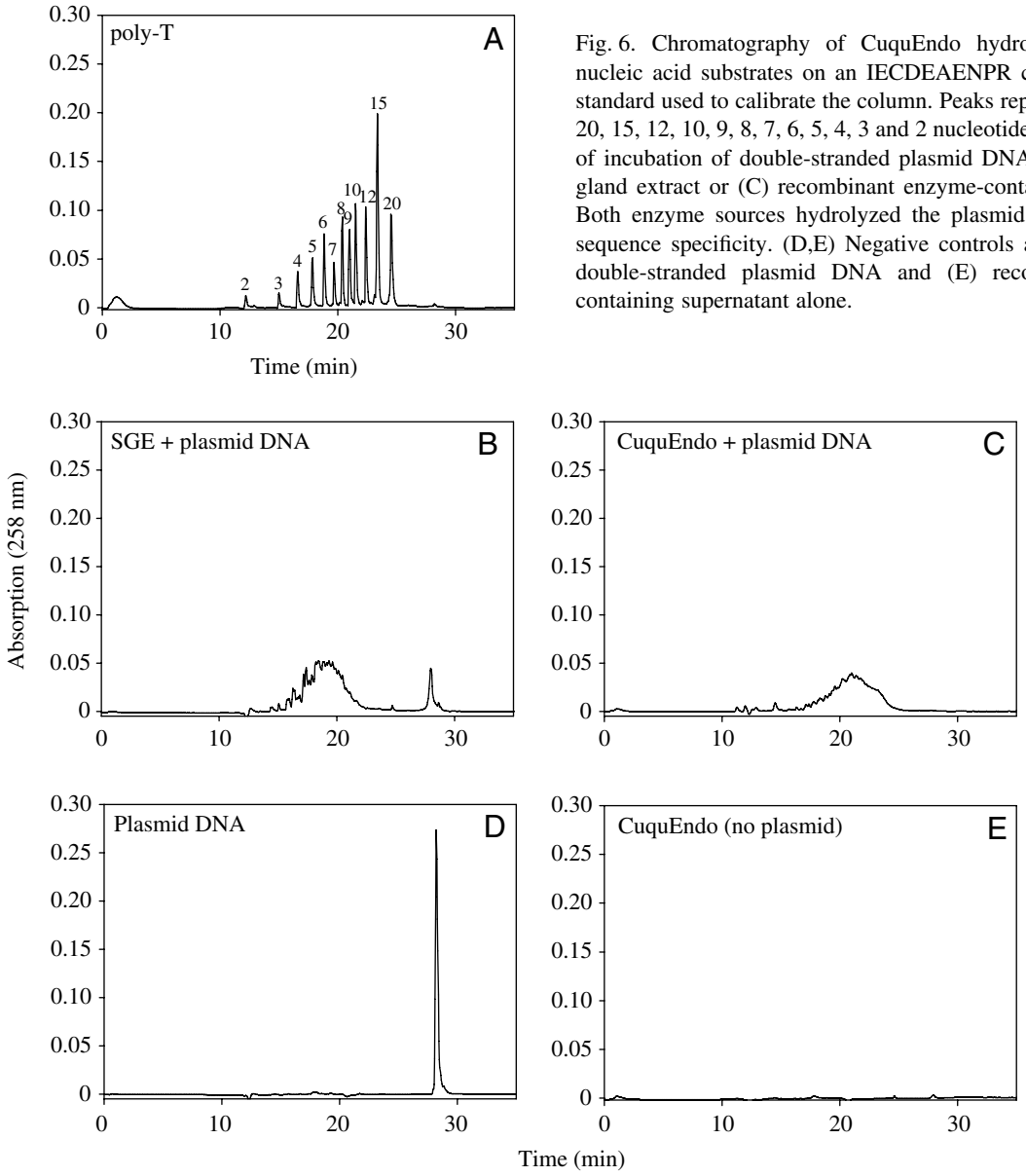


Fig. 6. Chromatography of CuquEndo hydrolysis of different nucleic acid substrates on an IECDEAENPR column. (A) PolyT standard used to calibrate the column. Peaks represent polymers of 20, 15, 12, 10, 9, 8, 7, 6, 5, 4, 3 and 2 nucleotides. (B,C) The effect of incubation of double-stranded plasmid DNA with (B) salivary gland extract or (C) recombinant enzyme-containing supernatant. Both enzyme sources hydrolyzed the plasmid substrate with no sequence specificity. (D,E) Negative controls are (D) undigested double-stranded plasmid DNA and (E) recombinant enzyme-containing supernatant alone.

enzyme has been deduced from conserved aa residues found by aligning the active sites of different DNases (Shagin et al., 2002). Most of these conserved residues are present in CuquEndo (Fig. 1). The RGH triad was found in most DNases used in the alignment and thus represents a conserved element. Except for the last conserved Arg residue (marked in Fig. 1), all the other six regions are conserved in the mosquito sequence. A Lys exists in the mosquito sequence within one residue of the missing Arg. The mosquito putative enzyme thus has eight of the nine conserved active site aa residues found in this arthropod family of DNases.

Shlyapnikov et al. reported (Shlyapnikov et al., 2000) the refinement of the *S. marcescens* endonuclease structure at 1.1 A and proposed the enzyme reaction mechanism. The active center of *S. marcescens* endonuclease has the invariable residues His89, Arg57, Asn106, Asn119 and Glu127, which are also present in equivalent positions in the CuquEndo putative active center (Fig. 1). The importance of these residues has been demonstrated by mutagenesis of every one of them, resulting in a drastic reduction in the catalytic properties of *S. marcescens* endonuclease (Friedhoff et al., 1996). His89 of *S. marcescens* (CuquEndoHis171) nuclease and the magnesium water cluster are directly involved in the phosphodiester bond cleavage of the DNA substrate. The metal ion could serve to activate the electrophile, stabilize the transition state, and protonate the leaving group (Shlyapnikov et al., 2000).

CuquEndo cleaves only dsDNA, as we have shown using synthetic oligonucleotides and plasmid DNA. The terminal degradation of dsDNA substrates by CuquEndo shows products with a random distribution of sizes ranging from 5 to 12 bases. Fragments with this size are ss at 37°C and cannot

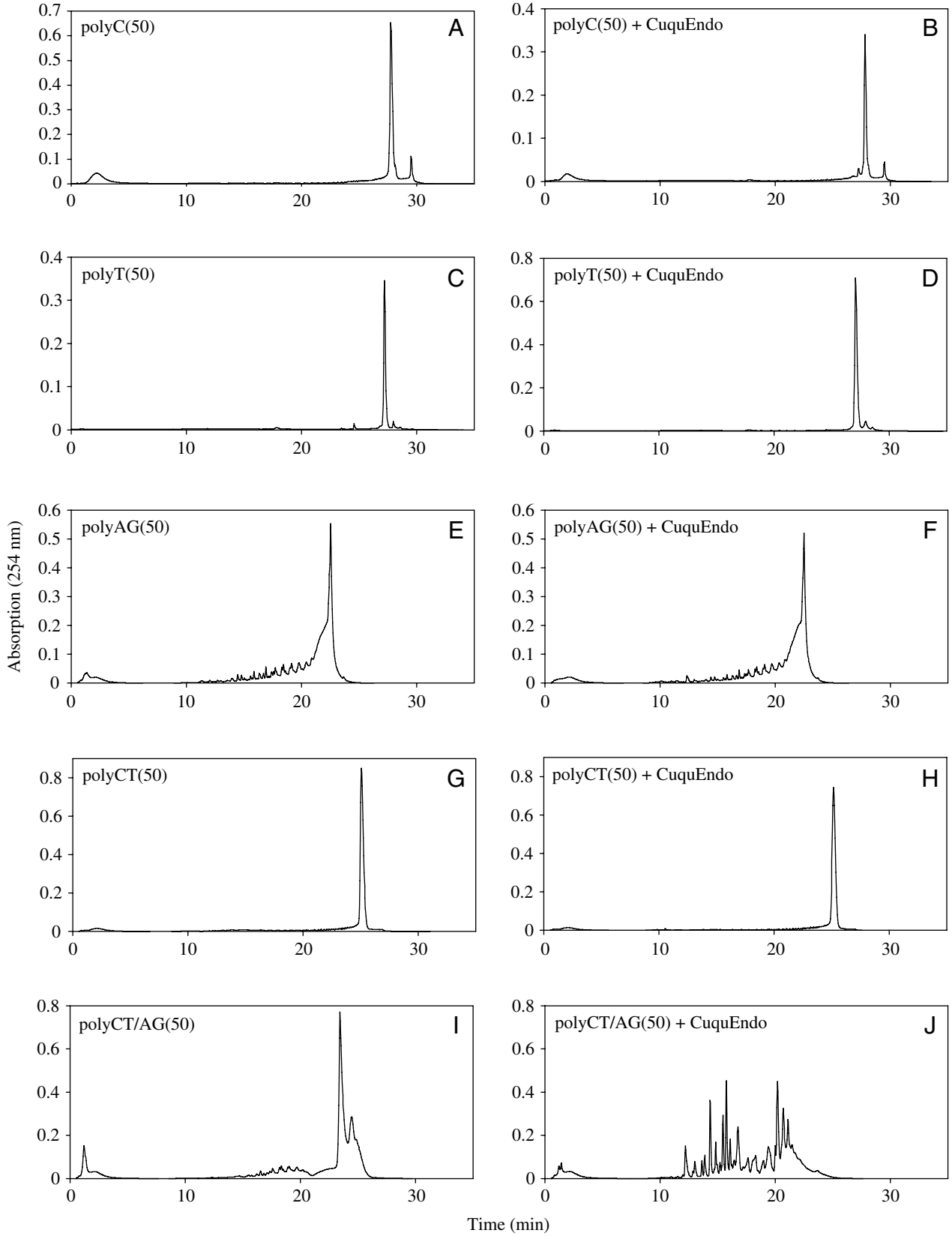


Fig. 7. Substrate specificity of recombinant CuquEndo-containing supernatant on different single-or double-stranded polynucleotides. Hydrolysis products were resolved using an IEC-DEAE-NPR column. Recombinant CuquEndo hydrolyzed only double-stranded DNA with no sequence specificity. No endonuclease activity was detected on single-stranded substrates.

be further cleaved by CuquEndo. This enzyme also does not cleave synthetic ss homo- and hetero-oligonucleotides. Endonuclease activity with demonstrated cleavage preference for dsDNA, previously reported only in the Kamchatka crab (Shagin et al., 2002) and designated a duplex-specific nuclease, has strong cleavage preference for dsDNA and little activity on ssDNA. Although this enzyme was classified as a DNA/RNA endonuclease (same as CuquEndo), no significant hydrolysis activity against RNA substrate was observed. We also did not find RNase activity in CuquEndo (not shown) – either in salivary gland extract or in recombinant CuquEndo.

It is interesting that only *Cx. quinquefasciatus*, but not *Ae. aegypti* or *An. gambiae* display significant salivary endonuclease activity. This is in agreement with the relatively abundant salivary expression of CuquEndo-coding transcripts, where 6 ESTs out of 503 randomly sequenced clones were found in a salivary gland cDNA library from adult female *Cx. quinquefasciatus* (Ribeiro et al., 2004), whereas none have been found in over 3000 ESTs from *An. gambiae* salivary glands (Arca et al., 2005), or over 3000 ESTs from a similar library from *Aedes aegypti* (Valenzuela et al., 2002). Accordingly, to the extent that *Culex* endonuclease is immunogenic, and that we can generalize the absence of salivary endonucleases in the genera *Aedes* and *Anopheles*, CuquEndo may be a good marker of *Culex* exposure in geographical areas where *Aedes* or *Anopheles* mosquitoes co-occur. This exclusive finding of salivary endonuclease in *Culex* also underlines the exclusive pathways of mosquito evolution, where, for example, serpins and tachykinins are found in *Aedes* as anti-clotting and vasodilatory molecules, whereas in *Anopheles* these functions are provided by completely different protein families (Ribeiro and Francischetti, 2003).

Mosquito salivary glands have been studied for their roles in blood feeding and in pathogen transmission to vertebrate hosts (Ribeiro, 1995); however, the biological function of some components, many of them interacting with each other, remains unclear. To acquire a blood meal, female mosquitoes need to penetrate the host's skin. In this process, mosquito mouthparts cause various degrees of damage to the host, releasing host cell contents at the bite site (including nucleic acids, among others). We propose that the primary function of CuquEndo is reducing the local viscosity at the biting site, facilitating a better intake of a blood meal and diffusion of other sialome components. CuquEndo could act as a 'spreading factor' as has been suggested for hyaluronidase in arthropod venom. Incidentally, the sialotranscriptome of *Cx. quinquefasciatus* also has a transcript coding for a hyaluronidase, which was not found in other mosquito sialotranscriptomes. CuquEndo may also produce aptamer (and defibrotide) sequences. These short ssDNA aptamers act as thrombin antagonists (Bracht and Schror, 1994) inhibiting thrombin-induced platelet aggregation and the blood-clotting cascade.

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