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Orthologues of *Streptococcus pyogenes* nuclease A (SpnA) and streptococcal 5'-nucleotidase A (S5nA) found in *Streptococcus iniae*

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Running title: Novel *Streptococcus iniae* virulence factors

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Abbreviations:[1]

[1] S5nA, streptococcal 5' nucleotidase A; SpnA, *Streptococcus pyogenes* nuclease A;

Summary

Streptococcus pyogenes nuclease A (SpnA) and streptococcal 5' nucleosidase A (S5nA) are two recently described virulence factors from the human pathogen *S. pyogenes*. In-vitro studies have shown that SpnA is a nuclease that cleaves ssDNA and dsDNA, including the DNA backbone of neutrophil extracellular traps. S5nA was shown to hydrolyse AMP and ADP, but not ATP, to generate the immunomodulatory molecule adenosine. S5nA also generates the macrophage-toxic deoxyadenosine from dAMP. However, detailed *in vivo* studies of the two enzymes have been hampered by difficulties with using current animal models for this exclusive human pathogen. Here we report the identification of two novel enzymes from the fish pathogen *Streptococcus iniae* that show similarities to SpnA and S5nA in amino acid sequence, protein domain structure, and biochemical properties. We propose that SpnAi and S5nAi are orthologues of the *S. pyogenes* enzymes, providing a rationale to analyse the *in vivo* function of the two enzymes using a *S. iniae*-zebrafish infection model.

Keywords:

Cell surface-anchored enzyme, enzyme synergy, *Streptococcus pyogenes* nuclease A (SpnA), streptococcal 5'-nucleotidase A (S5nA), Group A Streptococcus, *Streptococcus iniae*, LPXTG motif, zebrafish infection.

1. Introduction

Streptococcus pyogenes also known as Group A Streptococcus (GAS) is an exclusively human pathogen that can cause a wide range of diseases ranging from non-invasive pharyngitis and impetigo to more severe invasive diseases, such as toxic shock syndrome, necrotizing fasciitis and acute rheumatic fever (ARF) (*1-3*). The number of deaths due to severe GAS disease are more than half a million per year with the majority burden due to ARF and rheumatic heart disease (RHD), followed by invasive disease. A 2005 report showed that the prevalence of severe GAS disease is at least 18.1 million cases, with 1.78 million new cases each year (*4*).

GAS produces a plethora of virulence factors that facilitate colonisation, bacterial spreading and immune evasion (*5-7*). We have recently characterised two novel cell wall-anchored immune evasion factors of GAS, the *Streptococcus pyogenes* nuclease A (SpnA) and Streptococcal 5'-nucleotidase A (S5nA) (*8, 9*). Both are expressed as precursor proteins with an N-terminal signal peptide sequence for secretion and a C-terminal cell wall-anchor (CWA) domain including a conserved sortase A recognition motif (LPXTG). SpnA is a Ca^{2+}/Mg^{2+}-dependent nuclease that cleaves, double strand (ds) linear DNA, chromosomal DNA and the DNA backbone of neutrophil extracellular traps (NETs) (*8*). NETs are innate immune structures that are released from neutrophils after activation. They consist of a DNA scaffold with several bactericidal proteins, such as neutrophil elastase, myeloperoxidase and histones, that aid in bacterial clearance (*10, 11*). SpnA is able to enhance the survival of GAS in human blood and in neutrophil killing assays and was shown to facilitate virulence in a murine infection model (*8, 12*). More recently, it was shown in a *Galleria mellonella* (wax moth) infection model that nuclease activity is not solely responsible for SpnA mediated virulence and that SpnA has another, yet unknown virulence function (*13*).

S5nA is a recently discovered nucleotidase that cleaves AMP, dAMP and ADP to generate the immunomodulatory molecules adenosine and deoxyadenosine (*9*). Adenosine antagonises the effect of ATP by stimulation of adenosine receptors suppressing the pro-inflammatory response (*14-17*). As a result, adenosine decreases the phagocytic activity of macrophages by suppressing the generation of nitric oxide (*18*), superoxide (*19, 20*) and pro-inflammatory cytokines (*21*). Adenosine also inhibits neutrophil degranulation (*22*).

Despite a wealth of knowledge on the *in-vitro* function of GAS virulence factors, information on *in vivo* function is limited due to difficulties with using current animal models for this exclusively human pathogen. *Streptococcus iniae* is a major fish pathogen that shares many virulence traits with GAS, and can also cause infections in humans who handle and prepare infected fish (*23-25*). *S. iniae* is a beta-hemolytic Gram-positive coccus that was first isolated from a subcutaneous abscess of a captive freshwater dolphin (*26*). *S. iniae* infections range from skin infections to major invasive diseases in at least 27 species of saltwater and fresh water fish (*27, 28*). Phylogenetic analysis based on 16S rRNA analysis revealed a close genetic relationship with other pathogenic streptococci, including *S. pyogenes* (*29, 30*). A zebrafish infection model has been established for *S. iniae* that allows the investigation of streptococcal virulence factors in a natural host organism (*28, 31, 32*).

We have identified two genes on the *S. iniae* genome that are similar to the GAS genes encoding SpnA and S5nA. In this study, we show that the corresponding *S. iniae* proteins, termed SpnAi and S5nAi, are also functionally similar to their GAS counterparts making them true orthologues. These results provide the basis for further studies to determine the *in vivo* function of these proteins using a *S. iniae*-zebrafish infection model.

2. Materials and methods

2.1. Bioinformatic analysis

SpnAi and S5nAi were identified by a BLAST search of the *S. iniae* 9117 genome at https://blast.ncbi.nlm.nih.gov/Blast.cgi using the amino acid sequences of SpnA (AAK33693) and S5nA (NP 269071). The Signal P 4.0 server at http://www.cbs.dtu.dk/services/SignalP/ was used to predict the presence and length of the N-terminal signal peptide. For domain structure predictions, we searched the InterProScan software at EMBL-EBI. BLAST searches were performed using the tblastn program. Sequence alignments and the phylogenetic tree were generated using the ClustalW server. The Genbank accession numbers for SpnAi and S5nAi are EKB52944 and EKB52830, repectively.

2.2. Bacterial Strains and DNA manipulations

Escherichia coli BL21 cells were grown in Luria Bertani (LB, BD Biosciences) at 37°C with aeration. When appropriate, 50 µg/ml ampicillin or 30 µg/ml chloramphenicol was added to the culture. *S. iniae* strain 9117, a human clinical isolate from a patient with cellulitis (kindly provided by Dr. Sarah Highlander, JCVI, La Jolla, CA), was grown in Todd Hewitt broth medium supplemented with 0.2% yeast extract (THY, BD Biosciences). The S*pnAi* ORF without the regions encoding the N-terminal signal peptide sequence and the C-terminal cell wall-anchor domain (nucleotide positions 103-2721) was amplified from genomic DNA of *S. iniae* strain 9117. The DNA was amplified with primers SpnAi.fw (5'-GAA**GGATCC**GAAGAAATCATAGGACCC-3') and SpnAi.rev (5'-GCC**AAGCTT**TTATACTTTTCCTTTTTTTTTGAC-3') by 25 cycles of PCR using iProof™ high-fidelity DNA polymerase (Bio-Rad) at an annealing temperature of 56°C. A stop codon (TAA) was introduced with the reverse primer at the 3' end of the gene (nucleotide position 2722-2725). The *S5nAi* ORF (nucleotide position 82-1926) was

amplified under the same conditions using the primers S5nAi.fw (5'-C**GGATCC**GATCAGGTTGATGTTCAAATTC-3') and S5nAi.rev (5'-GGC**GAATTC**TTATTCTTGTTTCTTAGCCATTG-3'). The *SpnAi* and *S5nAi* PCR products were cloned separately into the BamHI/HindIII and BamHI/EcoRI cloning sites of the pPROEX-Htb expression vector (Life Technologies), respectively, followed by transformation into *E.coli* BL21. The cloned DNA sequences were analysed by the dideoxy chain termination method using the DNA sequencing facility at the School of Biological Science, University of Auckland.

2.3. Expression and Purification of Recombinant Proteins

Recombinant SpnAi and S5nAi were expressed in *E. coli* BL21. Cultures were grown at 37°C until OD_{600} of 0.6 and protein expression was induced for 4 hrs after adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma). The recombinant His_6-tagged proteins were purified using Ni^{2+}-iminodiacetic acid (IDA) sepharose (Sigma) according to the manufacturer's instruction. The eluates containing the recombinant proteins were collected and analysed on 12.5% SDS-polyacrylamide gels according to the procedure of Laemmli.

2.4. Enzyme Activity Assays for rSpnAi

Varying concentrations of rSpnAi were incubated with lambda DNA (GE Healthcare) in nuclease reaction buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and incubated for 1 hr at 37°C. For pH titration at pH 4.0–6.5, Tris-HCl was replaced with 50 mM acetate buffer. For Mg^{2+} and Ca^{2+} titrations, $MgCl_2$ or $CaCl_2$ were omitted from the reaction buffer and added separately at indicated concentrations. After incubation, all enzyme reactions were stopped by adding EDTA to a final concentration of 20 mM. Samples were then loaded onto 1% agarose gels. Lambda DNA without the addition of rSpnAi was loaded as the control for each set of experiment. DNA bands were visualised

after SYBR® safe staining (Invitrogen) using a Gel Doc™ EZ system (Bio-Rad) and quantified using Image Lab™ Software V5.2.1 (Bio-Rad). The percentage of DNA digestion was calculated as [1 - (band intensity with rSpnAi / band intensity without rSpnAi)] × 100%. All samples were analysed in triplicates for each of the three independent experiments.

2.5. Enzyme Activity Assays for rS5nAi

Enzymatic reactions with rS5nAi were carried out in 50 mM Tris-HCl pH 7, 10 mM $MgCl_2$, 1 mM substrate and 0.1 µM of rS5nAi in a total volume of 20 µl. The reaction mixture was incubated for 20 mins at 37°C. Substrates included AMP, ADP, ATP, dAMP, GMP, CMP and TMP (Sigma Aldrich). For pH titration at pH 5.0–6.5, Tris-HCl was replaced with 50 mM acetate buffer. Dependence on metal cations was analysed for Mg^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+} at three different concentration (0.1, 1 and 10 mM) at pH7 and 37°C. The enzyme kinetics were analysed by incubation of a fixed concentration (0.1 µM) of rS5nAi with increasing amounts of AMP in a total volume of 50 µl at pH7 and 37°C. The enzymatic reactions were stopped by adding EDTA to a final concentration of 50 mM. The release of inorganic phosphate (P_i) was quantified using a malachite green phosphate colorimetic assay kit (Sigma-Aldrich) according to the manufacturer's instructions. Release of P_i was measure at Abs_{650nm} and the amount of P_i was calculated based on a standard P_i curve. Michalis-Menten curve fitting using non-linear regression was performed using GraphPad Prism version 7.03 software.

To test for synergy with SpnAi, a 200 µl reaction mix containing 50 µg/mL of UltraPure™ salmon sperm DNA (Invitrogen), 10 µg/mL rSpnAi, 50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 2 mM $MgCl_2$ and 2 mM $CaCl_2$ was incubated at 37°C. After 1 hr, 0.1 µM S5nAi and 10 mM $MgCl_2$ were added and the reaction was incubated for another 1 hr. Generation of P_i was analysed as described above.

All samples were analysed in triplicate for each of the three independent experiments.

3. Results and discussion

3.1. Identification of SpnAi and S5nAi

To investigate if recently discovered GAS virulence factor genes *spnA* and *s5nA* would also be present on the *S. iniae* genome, protein sequences of SpnA and S5nA derived from GAS strain SF370 (serotype M1) were used to search the *S. iniae* strain 9117 using the tblastn option at the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Two hypothetical open reading frames (ORFs) were identified that shared amino acid sequence identities of 63% and 62% with SpnA and S5nA, respectively, and were named SpnAi and S5nAi. These sequences were then used to search the entire nucleotide collection at NCBI. ORFs with significant sequence similarities were used to generate a phylogenetic tree (figure 1A). Notably, ORFs with significant sequence similarities to the putative *S. iniae* nuclease SpnAi (62%–70%) were only found in certain streptococcal species (*S. dysgalactiae, S. suis* and *S. equi*) and not in any other genera. In contrast, and as previously reported, S5nA related nucleotidases are found in a wide range of organisms, including Gram-positive and Gram-negative bacteria, as well as mammals (*9*). The putative *S. iniae* nucleotidase S5nAi is most closely related to the UshA protein of *S. dysgalactiae* and to an uncharacterised ORF (SEQ1278) in *S. equi*, both sharing 68% sequence identity (figure 1A).

The 2823 bp nuclease gene *spnAi* from *S. iniae* strain 9117 encodes a 940 amino acid precursor protein with a predicted signal peptide sequence (position 1–34) and a C-terminal CWA domain (907-940). The *s5nAi* gene is 2031 bp in length and encodes a 676 amino acid gene product with a predicted signal peptide sequence (position 1–27) and a C-terminal CWA domain (642-676). (figure 1B).

In order to determine if SpnAi and S5nAi are functionally similar to the GAS proteins SpnA and S5nA, soluble recombinant proteins (rSpnAi and rS5nAi) that lack the N-terminal signal peptide sequence and the C-terminal CWA domain (figure 1B) were expressed with an N-terminal $(His)_6$-tag in *E. coli*. The proteins were purified by Ni^{2+}-affinity chromatography to a purity of approximately 95%, as estimated from an SDS-polyacrylamide gel (figure 1C). rSpnA and rS5nAi migrated at ~100 kDa and ~70 kDa, respectively, which is in agreement with their calculated molecular weight of 96280.61 Da and 66935.19 Da, respectively.

3.2. Biochemical analysis of recombinant SpnAi

SpnA has a very strong dependence on Mg^{2+} and Ca^{2+} and is completely inactive if one of the cations is absent (*8*). To determine if SpnAi displays similar cation-dependence, purified rSpnAi was incubated with double-stranded linear lambda DNA in the presence of Ca^{2+} and Mg^{2+}. Complete DNA digestion by rSpnAi was observed after 1 hr incubation at 37°C. However, in the absence of either Ca^{2+}/Mg^{2+} or both, the DNase activity of rSpnAi was completely undetectable (figure 2A) indicating a similar cation requirement as SpnA. The Mg^{2+} and Ca^{2+} dependence was further analysed at different concentrations of $MgCl_2$ and $CaCl_2$ (figure 2B and C). rSpnAi was active between 0.19 mM and 25 mM Mg^{2+} with maximum activity at 0.78 mM and 3.125 mM Mg^{2+}, but was completely inactive at 50 mM Mg^{2+}. This was similar to the results previously reported for rSpnA, which showed maximum activity at 1.56-3.125 mM and no activity at 50 mM (*8*). Although, the optimum Ca^{2+} concentration was similar between SpnAi (0.78-6.25 mM) and SpnA (0.78 mM), the *S. iniae* enzyme was still active at high concentrations of Ca^{2+} (~70% activity at 200 mM), whereas the GAS enzyme was completely inactive at ≥25 mM Ca^{2+}(*8*).

Next, we examined the effect of pH on rSpnAi activity using the constant optimised Mg^{2+} concentration of 3 mM. Greater than 50% activity was observed between pH 5 and 8.5, with

maximum activity seen between pH 6.5 and 8. Reduced activity (~25%) was also measurable at pH 4, while the enzyme was almost inactive at pH 9 (~8% activity) (figure 2D). The pH range was slightly shifted into the alkaline range compared to rSpnA, which showed optimal activity between pH 5.5 and 7 (*8*). This might be due to adaptation to a different host, as most fish species have a normal blood pH in the range of 7.7–8.0 (*33*, *34*), whereas human blood has a pH of 7.3-7.4.

Recombinant SpnAi activity was determined for a range of different temperatures (12-47°C) at pH 7 and 3 mM Mg^{2+} (figure 2E). Maximum rSpnAi activity was found between 32–37°C, while activity was reduced to ~80% at 27°C, ~60% at 22°C, ~40% at 17°C and 42°C and ~25% at 47°C. Only marginal activity was observed at 12°C (< 10%). This is only slightly lower than the optimal temperature range for rSpnA which was found to between 32°C and 42°C (*8*).

The enzymatic activity of rSpnAi under optimal conditions (1 mM $CaCl_2$, 3 mM $MgCl_2$, pH 7, 37°C) was determined by serial dilution of rSpnAi starting from 35 pM. As shown in figure 2F, 20 pmol of rSpnAi is the minimum amount of enzyme required to completely digest 1 µg of lambda DNA in 1 hr. This is similar to rSpnA, for which 26 pmol was required for the same activity (*8*).

All biochemical properties for rSpnAand rSpnAi are summarised in table 1.

3.3. Biochemical analysis of recombinant S5nAi

We have previously shown that S5nA from *S. pyogenes* hydrolyses AMP and ADP, but not ATP, to produce the immunomodulatory product adenosine (*9*). We have tested rS5nAi for activity against the same components and found a similar substrate preference (figure 3A). The highest activity was observed against AMP, while ATP was not hydrolysed. In contrast to rS5nA, rS5nAi showed higher activity for ADP (*9*). There were also small differences in

the activity for other nucleoside monophosphates. rS5nAi hydrolysed GMP, CMP, and TMP with almost equal efficiency, but lower than for AMP, whereas rS5nA showed a high preference towards CMP (*9*). Both enzymes hydrolysed dAMP with similar efficiency as AMP, which is different from the NudP nucleosidase produced by *Streptococcus agalactiae* (Group B Streptococcus), which is unable to hydrolyse dAMP (*35*).

Divalent cations are important as co-factors for nucleosidase activity. We analysed the activity of rS5nAi for AMP with either Mg^{2+}, Mn^{2+}, Ca^{2+} or Zn^{2+} at 37°C and pH 7 (figure 3B). The activity of rS5nAi was slightly higher in the presence of Mg^{2+} compared to Ca^{2+} in a dose-dependent manner with highest activity at 10 mM. Highest activity was also achieved with lower concentrations of Mn^{2+} (0.1 mM and 1 mM), whereas a higher concentration of 10 mM had an inhibitory effect. In contrast, rS5nAi hydrolysis of AMP was strongly decreased with Zn^{2+}, in particular at 10 mM. These results are very similar to those observed for rS5nA (*9*).

As observed for the nuclease enzyme, the optimal pH range was higher for S5nAi (pH 5-7.5) compared to S5nA (pH 5-6.6) (figure 3C), which might reflect an adaptation to the higher pH of fish blood compared to human blood (*33, 34*). The temperature requirements for rS5nA and S5nAi are very similar (*9*). Like rS5nA, rS5nAi shows maximum activity at 42°C, but is active over a wide range of temperatures (figure 3D). Even at 21° C and 52°C, the enzymatic activity was found to be over 25% suggesting strong thermostability.

Finally we tested the enzymatic activity against AMP at 37°C, pH 7 and 10 mM $MgCl_2$. A time course showed that AMP hydrolysis with 0.1 μM rS5nAi reached equilibrium after 30 min (figure 3E), which is comparable to 25 min for rS5nA (*9*). The reaction kinetics followed the Michaelis-Menten model for a single substrate with a K_m of 121.3±1.683 mM and a V_{max} of 7808±78.3 nmol of released Pi/mg enzyme/min (figure 3F). This corresponds well with the

kinetics previously reported for rS5nA (K_m of 168.3±381.683 mM and a V_{max} of 7550±326 nmol of released Pi/mg enzyme/min) (*9*). All biochemical properties for rS5nA and rS5nAi are summarised in table 2.

3.4. Synergy between SpnAi and S5nAi

Based on work conducted with *Staphylococus aureus*, it has previously been suggested that nucleases and nucleotidases might work in synergy to avoid host immune evasion. It was proposed that the nuclease would hydrolyse DNA, including NETs, to generate the nucleotidase substrate dAMP, which would then be hydrolysed to produce deoxyadenosine (dAdo) to trigger the caspase-3-mediated death of macrophages and monocytes restricting macrophage influx into abscesses (*36*, *37*). To test the possibility that such a synergy also exists between SpnAi and S5nAi, we tested both enzymes together using salmon sperm DNA as substrate. A strong production of inorganic phosphate (P_i) was observed when both enzymes were mixed together. In contrast, addition of either SpnAi or S5nAi alone (or no enzyme) did not generate detectable Pi (figure 4).

4. Conclusion

The biochemical properties of the *S. iniae* enzymes SpnAi and S5nAi are very similar to those reported for *S. pyogenes* SpnA and S5nA, respectively. Together with similar protein lengths, domain structures and amino acid similarities, it is highly likely that these proteins are true orthologues and act together as immune evasion factors.

Future work will include the generation of *spnAi* and *s5nAi* deletion mutants in *S. iniae* and their use in a zebrafish infection model (*28, 31, 32*). Investigation into bacterial dissemination

in the host, NET degradation, and migration of neutrophils and macrophages to the site of infection will provide new insights into the function of the two enzymes. This might allow us to draw conclusions on similar virulence mechanisms used by *S. pyogenes* in the human host.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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Table 1 Summary of biochemical properties of rSpnAi and rSpnA.

Biochemical properties	**rSpnAi**	**rSpnA (*8*)**
Molecular weight[#]	96280.61	94068.15
Cell wall anchor	+	+
Ca^{2+} and Mg^{2+} dependency	Yes	Yes
Optimum $MgCl_2$	0.78–3.125 mM	1.56–3.125 mM
Optimum $CaCl_2$	0.78-6.25 mM	0.78 mM
Optimum pH	pH 6.5–7.5	pH 5.5–7
Optimum temperature	32–37°C	32–42°C
Complete digest of 1µg of lambda DNA	20 pM	26 pM

Table 2 Summary of biochemical properties of rS5nAi and rS5nA.

Biochemical properties	**rS5nAi**	**rS5nA (*9*)**
Molecular weight[#]	66935.19	66922.8
Cell wall anchor	+	+
Optimum pH	pH 5–7.5	pH 5–6.5
Optimum temperature	42°C	42°C
Substrate preference	AMP>dAMP>CMP=TMP	
	AMP=dAMP=CMP>GMP	
	=GMP>ADP>>ATP	=TMP>ADP>ATP
Enzyme kinetics		
Time to reaction equilibrium[$]	30min	25min
V_{max} (nmol P_i/mg/min)	7808±78.3	7550±326
K_m	121.3±1.683 μM	168.3±38 μM
Activating cations	Mg^{2+}, Ca^{2+}, Mn^{2+}	Mg^{2+}, Ca^{2+}, Mn^{2+}
Inhibiting cations	Zn^{2+}	Zn^{2+}

[#]without signal peptide and cell wall anchor regions.

[$]AMP hydrolysis with 0.1μM rS5nAi at 37°C, pH7, 10mM Mg_2Cl

FIGURE LEGENDS

Figure 1. Bioinformatic analysis and purification of SpnAi and S5nAi. A, Rooted phylogenetic tree (UPGMA, (unweighted pair group method with arithmetic mean) of cell wall-anchored nucleases (left) and cell wall-anchored 5'-nucleotidases (right). The trees were generated with ClustalW using complete protein sequences of SpnAi (*S. iniae*), WP_003050123 (*S. dysgalactiae*), WP_024395691 (*S. suis*), WP_012679382 (*S. equi*), S5nAi (*S. iniae*), S5nA (*S. pyogenes*, AAK33792), AdsA (*Staphylococcus aureus*, ESR29110), ecto-5'-nucleotidase A (*Nt5e*) (*S. sanguinis*, AFK32764), Ssads (*S. suis*, YP001197640), NudP (*S. agalactiae,* CDN66659), UshA (*S. dysgalactiae*, ADX24386), SEQ1278 (*S. equi*, CAW94038), 5'-nucleotidase (5'NT) (*E. coli*, AJM76137), and CD73 (*Homo sapiens*, AAH65937). The number in parentheses show amino acid sequence identities with SpnAi and S5nAi. B, schematic presentation of SpnAi (top) and S5nAi (below) domain structure based on the prediction using InterProScan software at EMBL-EBI. The numbers on top represent amino acid positions. SP, signal peptide sequence; CWA, cell wall anchor domain. The arrows above indicate the regions that were generated as recombinant proteins in *E. coli*. *C*, recombinant form of SpnAi (left) and S5nAi (right) were expressed and purified from *E. coli* by immobilised metal chelate chromatography. The purity of the proteins was ≥95% as estimated from a 10% SDS-polyacrylamide gel. M, BenchMark™ molecular weight marker.

Figure 2. Biochemical analysis of rSpnAi. (A) SpnAi activity depends on the presence of Ca^{2+} and Mg^{2+}. Lambda DNA was digested in the presence or absence of each of the cations and analysed on a 1% agarose gel. C, control: reaction mixture without rSpnAi. Lambda DNA was digested with rSpnAi in the presence of varying concentrations of $MgCl_2$ (B), varying concentrations of $CaCl_2$ (C), at different pH (D) and at different temperature (E). Digested DNA was run on a 1% agarose gel and DNA bands were visualised after SYBR® safe staining and quantified using Image Lab™ Software V5.2.1. (F) The minimum enzyme required for complete cleavage of 1 µg of lambda DNA was analysed at optimum reaction conditions (1 mM $CaCl_2$, 3 mM $MgCl_2$, pH 7, 37°C). The error bars show the standard deviation of three independent experiments performed in triplicates.

Figure 3. Biochemical analysis of rS5nAi. (A) Reaction mixtures containing 0.1 µM rS5nAi were incubated with 1 mM of various substrates for 15 mins in the presence of 10 mM $MgCl_2$ at 37°C. Hydrolysis of 1 mM AMP by 0.1 µM rS5nAi over 20 mins was analysed in the presence of different divalent metal cations at varying concentrations (B), at different pH at 37°C (*C*) and at different temperature at pH7 (D). (E) The time to reaction equilibrium was determined with 1 mM AMP and 0.1 µM rS5nAi in the presence of 10 mM $MgCl_2$ at pH7 and 37°C. (F) Enzyme kinetics of rS5nAi for AMP hydrolysis were determined by velocity measurements with various substrate concentrations in the presence of 10 mM $MgCl_2$ at pH7 and 37°C using GraphPad Prism V6.03 software. The P_i released in each experiment was quantified using a malachite green phosphate colorimetric assay kit. The error bars show the standard deviation of three independent experiments performed in triplicates.

Figure 4: Synergy between rSpnAi and rS5nAi. Incubation of salmon sperm DNA with both enzymes, but not with individual enzymes, results in the generation of P_i which was quantified using a malachite green phosphate colorimetric assay kit. This suggests that SpnAi hydrolyses dsDNA to produce deoxynucleotide monophosphates including dAMP, which can then be used as a substrate by S5nAi to generate deoxyadenosine (dAdo) and P_i.

The error bars show the standard deviation of three independent experiments performed in triplicates.

Fig. 1

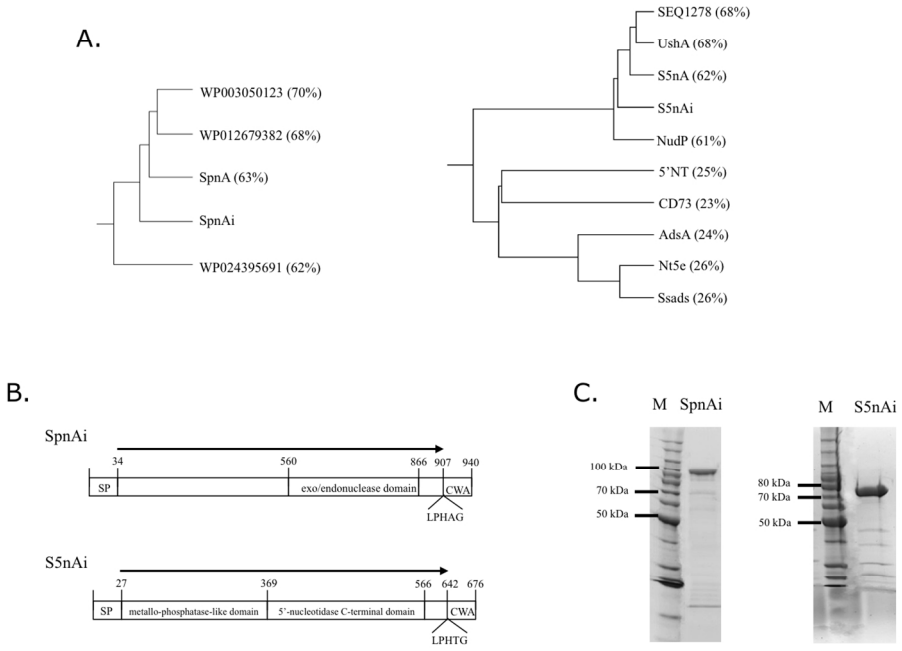

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Fig. 2

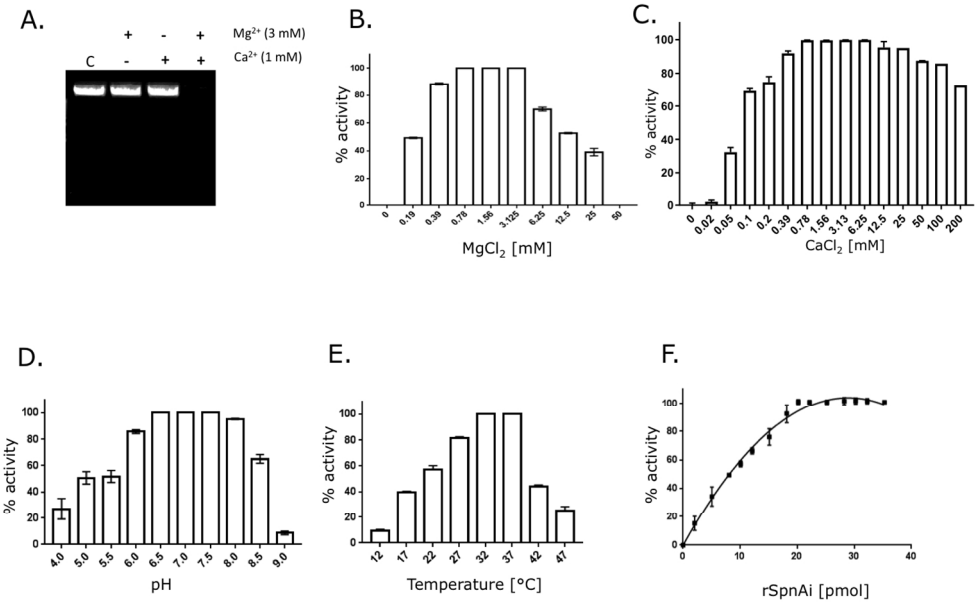

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Fig. 3

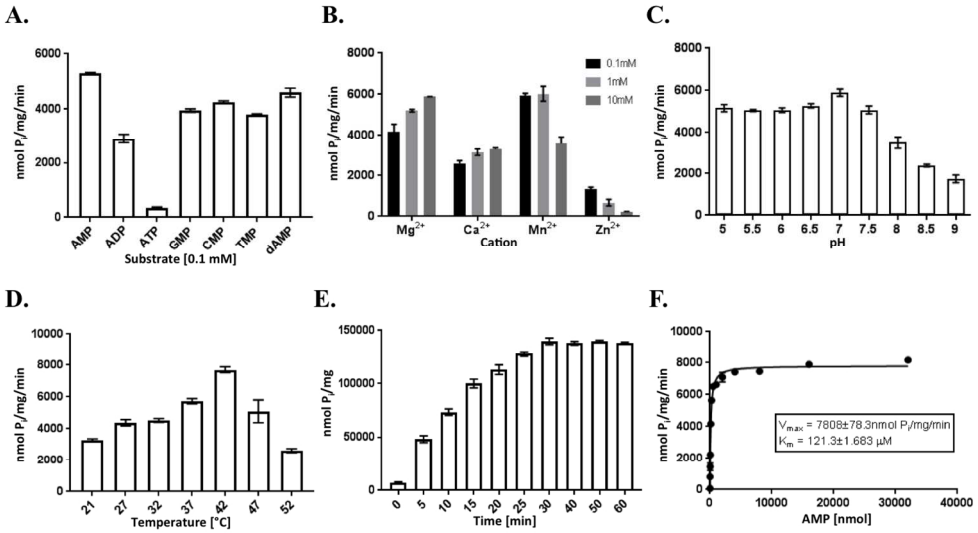

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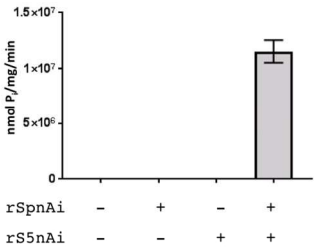
Fig. 4



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