Short Communication

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Characterization of a new toxin from the entomopathogenic fungus *Metarhizium anisopliae*: the ribotoxin anisoplin

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Abstract: *Metarhizium anisopliae* is an entomopathogenic fungus relevant in biotechnology with applications like malaria vector control. Studies of its virulence factors are therefore of great interest. Fungal ribotoxins are toxic ribonucleases with extraordinary efficiency against ribosomes and suggested as potential insecticides. Here we describe this ribotoxin characteristic activity in *M. anisopliae* cultures. Anisoplin has been obtained as a recombinant protein and further characterized. It is structurally similar to hirsutellin A, the ribotoxin from the entomopathogen *Hirsutella thompsonii*. Moreover, anisoplin shows the ribonucleolytic activity typical of ribotoxins and cytotoxicity against insect cells. How *Metarhizium* uses this toxin and possible applications are of interest.

Keywords: hirsutellin; insecticide; ribonuclease; ribotoxin; sarcin.

Entomopathogenic fungi are of interest in the search for alternative biopesticides needed for crop protection and other pest biocontrol. *Metarhizium anisopliae* and *Beauveria baussiana* are the most studied for these purposes (Schrank and Vainstein, 2010). Several formulations of these fungi are already in the market, designed to target a multitude of acari and insects (Faria and Wraight, 2007). Some interesting examples are the studies with *Metarhizium* to control the mite *Varroa destructor* in honey bees (Kanga et al., 2002), and established commercial formulations like Bioblast® against termites (*Metarhizium*) or Mycotrol® against aphids and other organisms (*Beauveria*) (Kanzok and Jacobs-Lorena, 2006).

Metarhizium was first employed by Elie Metchikoff in the late 1800s for biological control of wheat-grain beetles. Since then, biopesticides based on this fungus have greatly evolved. Recently, *M. anisopliae* has also become an interesting and promising alternative for the control of adult malaria vectors like the *Anopheles gambiae* mosquito as the appearance of resistance to insecticides hampers the efforts to control the disease (Kanzok and Jacobs-Lorena, 2006; Mnyone et al., 2009; Howard et al., 2010; Fang et al., 2011; Abdul-Ghani et al., 2012).

Due to its wide biotechnological applications, *M. anisopliae* has been extensively characterized in terms of its biology and pathogenesis, including the recent sequencing of its genome which constitutes an important resource to identify new virulence factors (Gao et al., 2011; Pattemore et al., 2014). Regarding factors involved in its pathogenicity, the most studied ones are destruxins, a family of ciclodepsipeptides also present in other insect pathogenic fungi (Schrank and Vainstein, 2010; Liu and Tzeng, 2012; Wang et al., 2012). However, identification and characterization of new toxins will help, not only to better understand the biology of this fungus but also to provide new tools for the improvement of biopesticides. This should be an essential line of research because the Achilles' heel in the use of insect–pathogenic fungi for biocontrol is their speed of killing (Scholte et al., 2004). Detractors argue that the effect should be as immediate as possible, because in the pre-lethal (incubation) period these pests can still cause damage to crops or transmit disease. Therefore, one recent strategy with success in

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this regard consists in the genetic manipulation of fungal species to increase their virulence via expression of insecticidal protein/peptide toxins (Ortiz-Urquiza et al., 2015).

A well-known family of toxic proteins secreted by fungi are ribotoxins, specific ribonucleases (RNases) against the large rRNA in the ribosome with lethal consequences for the target cell (Lacadena et al., 2007). The main producer is *Aspergillus*, although the discovery of an entomopathogenic fungus, *Hirsutella thompsonii*, producing the ribotoxin hirsutellin A (HtA) suggests a wider distribution among fungi as well as their insecticide properties (Martínez-Ruiz et al., 1999; Herrero-Galán et al., 2008; Olombrada et al., 2013, 2014a). Ribotoxins specifically cross some lipid barriers and then efficiently inactivate any kind of ribosome. In *Aspergillus,* they seem to be produced during conidia maturation, most probably as a defense mechanism against predators (Brandhorst and Kenealy, 1992). They have been extensively studied (Lacadena et al., 2007; García-Ortega et al., 2010; Olombrada et al., 2014a,b) with the most recent studies focused on their antitumor properties conjugated as immunotoxins and their toxicity against insects (Carreras-Sangrá et al., 2012; Olombrada et al., 2013; Tomé-Amat et al., 2015).

Here we describe a new ribotoxin produced by *M. anisopliae* ARSEF23 as we found a 'hirsutellin A toxin' gene (accession number MAA_10099) in its sequenced genome (Gao et al., 2011) with a protein BLAST using HtA from *H. thompsonii* (accession number AAM95629) (Figure 1A). The sequence alignment with the mature HtA of the potential ribotoxin (accession number EFY94422.1), named here as anisoplin, showed 70% sequence identity. Cysteines and active site residues described for HtA (Herrero-Galán et al., 2008) were also conserved in anisoplin. In comparison with HtA and α-sarcin, the best characterized ribotoxin from *Aspergillus*, pre and pro peptides were assigned (Figure 1A) (Oka et al., 1990; Endo et al., 1993a,b; Boucias et al., 1998; Martínez-Ruiz et al., 1998). Therefore, anisoplin also seems to be an extracellular ribotoxin. Moreover, efficient compartmentalization and secretion by the producer fungus seems to be the best self-defense mechanism against endogenous ribotoxin production (Endo et al., 1993a; Martínez-Ruiz et al., 1998), so signal peptide in anisoplin might have evolved in this sense. From an evolutionary point of view, the entomopathogens *Metarhizium* and *Hirsutella* are closer in the phylogenetic tree than *Aspergillus* (Lai et al., 2014). It is not surprising then that anisoplin better resembles HtA than any other known ribotoxin.

M. anisopliae ARSEF23 was studied for ribotoxin production *in vitro* by growing it in liquid media as it was previously described for *A. giganteus* and *H. thompsonii,* producers of α-sarcin and HtA, respectively (Olson et al., 1965; Mazet and Vey 1995; Herrero-Galán et al., 2008). Culture progression was followed by pH measurements (Figure 1B) and the extracellular medium at different growth times was assayed against reticulocyte lysates to quantify the specific cleavage that ribotoxins produce in the large rRNA. Primer extension results showed the characteristic RNA α-fragment in both culture media from the first day of growth (Figure 1C), independently of the culture progression (Figure 1B). This unique RNase activity is specific of ribotoxins, so this result constitutes the first evidence of a ribotoxin production by *M. anisopliae*. However, an isolated and clear protein band of the expected size was not found by SDS-PAGE (Supplementary Figure 1). Silver-staining showed several bands of similar mobility but sample concentration and mass spectrometry of them did not succeed in anisoplin identification, concluding that the ribotoxin was produced in very low levels. *M. anisopliae* behavior was different from that of *A. giganteus* which, in similar *in vitro* conditions, produces α-sarcin mainly when glucose is depleted and pH starts rising, correlating α-sarcin production to stress conditions (Olson et al., 1965). Still, results with *M. anisopliae* are not surprising considering that it is a very ubiquitous fungus that grows on the soil and not in submerged cultures. This is, however, an interesting aspect to be explored in a near future: how and when, in its natural environment, *M. anisopliae* produces this ribotoxin.

In order to characterize this ribotoxin, it was recombinantly produced in *Escherichia coli* with the same expression system previously optimized for the production of α-sarcin (Lacadena et al., 1994; García-Ortega et al., 2000) (see Supplementary material). It produces a fusion protein directed to the periplasm and the coexpression of thiorredoxin to favor disulphide bridges formation. Protein purification followed the procedure described for the recombinant α-sarcin (Lacadena et al., 1994) yielding 0.5 mg of pure protein per liter of culture. Protein size and homogeneity was checked by SDS-PAGE (Supplementary Figure 2). Amino acid composition analysis agreed with the theoretical values and together with the absorbance spectrum allowed the calculation of the extinction coefficient: $E^{0.1\%}$ (280 nm, 1 cm)=1.62. Tryptic digestion and MALDI-TOF/TOF mass spectrometry identified the mature anisoplin with 60% sequence coverage.

The structural characterization of the recombinant anisoplin was performed by circular dichroism (CD) and intrinsic fluorescence emission spectroscopy. Far-UV wavelength range CD spectrum showed slight differences in comparison with HtA (Herrero-Galán et al., 2008) (Figure 2A). Prediction of secondary structure content

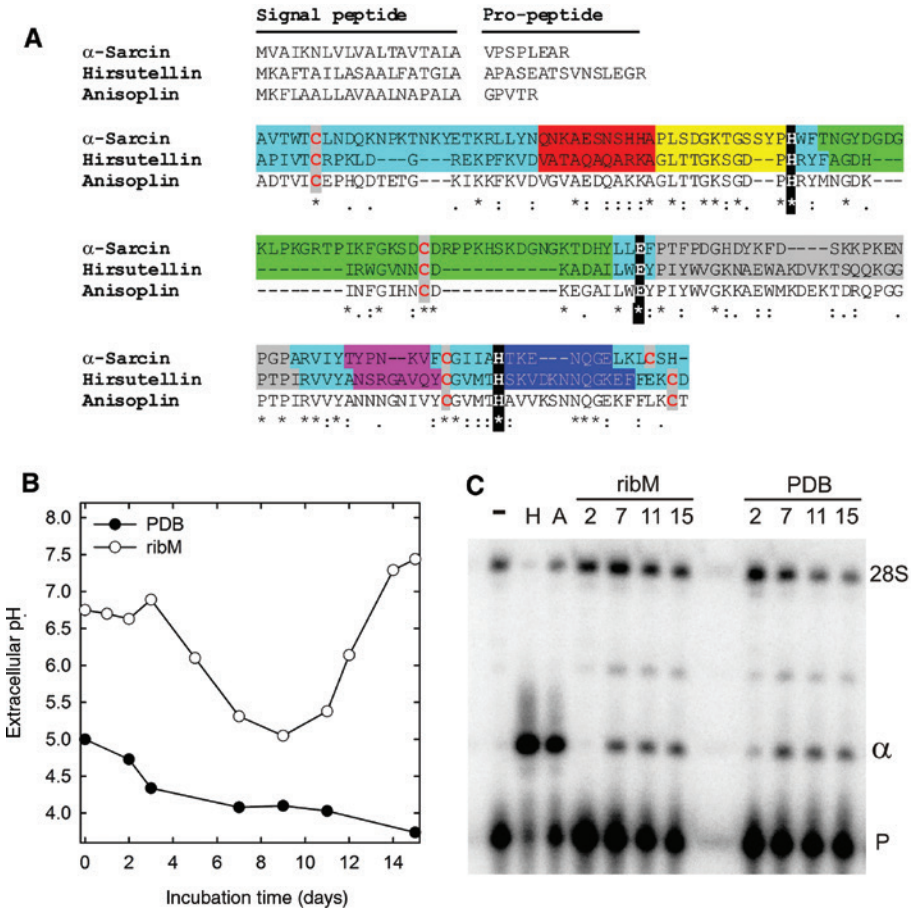
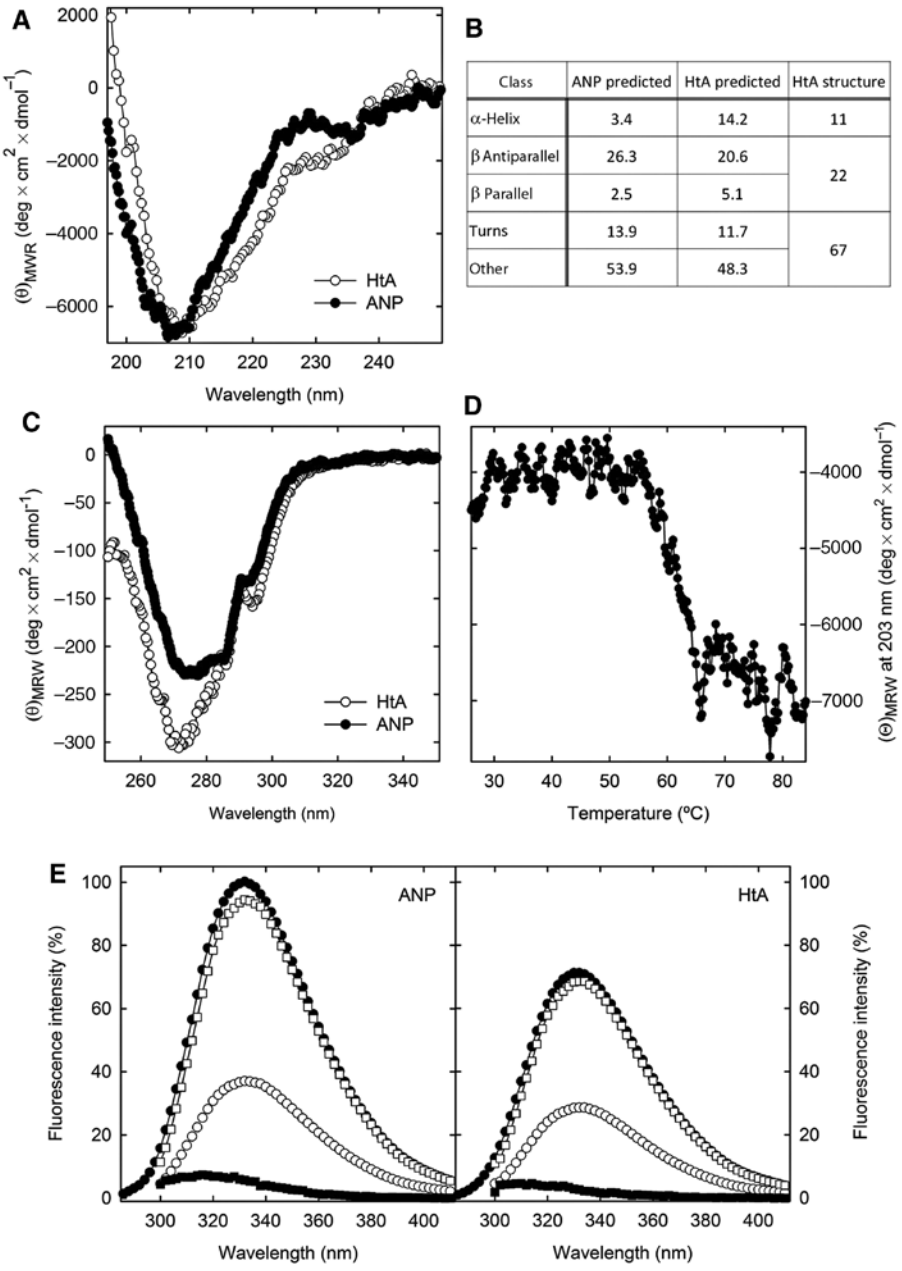


Figure 1: Analysis of the sequence of anisoplin and its production by *M. anisopliae*.
(A) Sequence alignment of anisoplin (accession number EFY94422.1), HtA and α-sarcin (Lacadena et al., 2007). *Indicates identical amino acid in the three sequences. Colors indicate the secondary structure elements from the structures of HtA and α-sarcin: N-terminal β-hairpin and β strands in blue; α-helix in red and surrounding non-regularly structured loops 1–5 in yellow, green, gray, pink and purple, respectively. Catalytic residues are marked in black and cysteines in red/gray. Pre and pro peptides are indicated by comparison with those published for HtA and α-sarcin (Boucias et al., 1998; Martínez-Ruiz et al., 1998). (B) Extracellular pH progression of *M. anisopliae* in two different liquid media: PDB and ribotoxin production medium (ribM). (C) Ribotoxin specific activity against rabbit reticulocyte lysates of extracellular media from *M. anisopliae* cultures in B (days 2, 7, 11 and 15th of growth). Poison primer extension analysis of RNA extracted from the reaction. The band corresponding to the specific cleavage is marked with α. 'P' corresponds to the free primer and '28S' to the resulted product of primer extension of intact 28S rRNA. Controls with purified fungal HtA (H), recombinant anisoplin (A) and in the absence of ribotoxin (–) are included. Detailed methods are described in the Supplementary material.

from the CD data gave similar results to those of HtA (Figure 2B). Considering these data and the high sequence identity between anisoplin and HtA, the former seems to keep the same overall protein fold of ribotoxins, with a core of antiparalel β-sheet, a short α-helix and long loops with non-regular secondary structure surrounding it. A closer look to both sequences highlights only remarkable differences in the NH_2-terminal β-hairpin (Figure 1A). It is possible that the β content of the Nt-hairpin accounts for the difference in far-UV CD spectra. Near-UV wavelength range CD spectrum of anisoplin was similar to that of HtA (Figure 2C) (Herrero-Galán et al., 2008), corresponding to that of a folded protein where the microenvironment of aromatic residues seems to be conserved. Thermal denaturation profile was obtained by recording the ellipticity at 203 nm against temperature increment (Figure 2D). It corresponded to a folded-unfolded transition with a T_m of 61°C, similar to HtA, pointing out that the characterized recombinant anisoplin is a folded and fairly stable protein. Finally, and in addition to the near-UV CD spectra, the fluorescence emission spectra for excitation at both 275 and 295 nm were obtained in comparison to HtA (Figure 2E and F) in order to analyze the structural microenvironment of Trp and Tyr residues. As it occurs with HtA, the total emission spectrum mainly corresponds to Trp contribution with almost negligible Tyr contribution. However, the Trp emission is significantly increased in anisoplin despite harboring three instead of four Trp,



Class	ANP predicted	HtA predicted	HtA structure
α-Helix	3.4	14.2	11
β Antiparallel	26.3	20.6	22
β Parallel	2.5	5.1	
Turns	13.9	11.7	67
Other	53.9	48.3	

Figure 2: Structural characterization of recombinant anisoplin (ANP) compared to hirsutellin A (HtA).
(A) Far-UV CD spectra. (B) Secondary structure prediction from the spectra in panel (A). Real data from HtA NMR structure (Viegas et al., 2009; PDB ID 2KAA) are also included. (C) Near-UV CD spectra. (D) Thermal denaturation profile of anisoplin by means of the temperature dependence of the ellipticity at 203 nm. (E) Fluorescence emission spectra of anisoplin and HtA for excitation at 275 nm (filled circles, total tyrosine and tryptophan contribution) and 295 nm (open circles). Open squares spectra correspond to tyrosine contribution (after normalization of 295 nm spectra) and filled square spectra to tryptophan contribution. Fluorescence units are arbitrary. Procedures are detailed in the Supplementary material.

suggesting local differences in the microenvironment of Trp between anisoplin and HtA.

All together, the structural characterization herein performed of recombinant anisoplin shows a folded protein, similar in structure to its homologous HtA. It has to be mentioned, however, that other structural data are still missing like the determination of disulphide bonds arrangement and, more crucial, its structural comparison with fungal anisoplin. Therefore, recombinant anisoplin behavior might not exactly correspond to that of the natural ribotoxin.

Recombinant anisoplin was next assayed to describe its ribotoxin activity. As mentioned before, ribotoxins are extremely specific RNases against a unique element

present in any kind of ribosome: the sarcin/ricin loop (SRL), a conserved sequence of about 35 nucleotides of the large ribosomal RNA (Schindler and Davies, 1977; Endo and Wool, 1982). The role of the SRL can be impaired by two types of toxins: N-glycosidades such as ricin, that depurinate one nucleotide (Stirpe and Battelli, 2006), and ribotoxins, that cleave the contiguous phosphodiester bond (Lacadena et al., 2007; Olombrada et al., 2014a). These modifications in the SRL, completely inactivate the mechanisms of correct positioning and GTPase activation of elongation factors that it is involved in, leading to protein biosynthesis inactivation and finally cell death (García-Ortega et al., 2010; Voorhees et al., 2010; Shi et al., 2012; Koch et al., 2015). In this regard, several studies have tried to specify the structural elements in the ribotoxin, the ribosome and the SRL responsible for this fine and efficient recognition (García-Ortega et al., 2002; García-Mayoral et al., 2005; Olombrada et al., 2014b).

Here, recombinant anisoplin was first tested against a 35-mer SRL oligonucleotide in comparison to HtA and α-sarcin (García-Ortega et al., 2002; Herrero-Galán et al., 2008) (Figure 3A, B and Supplementary Figure 3A, B). Anisoplin specifically cleaved the SRL with the typical pattern shown by ribotoxins. Moreover, this specific cleavage activity was slightly lower than that of HtA but higher than that of α-sarcin. So we can say that recombinant anisoplin recognizes and cleaves the SRL within the range of other fungal ribotoxins. We next analyzed anisoplin, HtA and α-sarcin against a rabbit reticulocyte lysate as previously described (García-Ortega et al., 2002; Herrero-Galán et al., 2008) (Figure 3C, D and Supplementary Figure 3C, D). In this assay, the specific cleavage of the 28S RNA within ribosomes by the ribotoxin releases a RNA fragment of about 400 nucleotides, the so-called α-fragment. Again, recombinant anisoplin behaved as a ribotoxin. However, when compared to HtA, it was considerably less active. Therefore ribosome recognition seems to be different in these two proteins, at least in terms of efficiency. Important differences in this regard have also been observed in our laboratory between α-sarcin and HtA (Herrero-Galán et al., 2008; Olombrada et al., 2013) (Supplementary Figure 3C and D). Here, however, it cannot be discarded that recombinant anisoplin were less active than its fungal counterpart due to subtle structural disarrangements. Even so, further characterization of ribosome recognition by these ribotoxins will shed light into the molecular mechanism of this process. To date, the only structural element of ribotoxins involved in ribosome recognition that has been described is the Nt β-hairpin (García-Ortega et al., 2002; García-Mayoral et al., 2005; Álvarez-García et al., 2009). Others like loop 2 have been suggested to participate in the process (García-Mayoral et al., 2005). Interestingly, a close examination of α-sarcin, HtA and anisoplin sequences reveals major differences in these two elements (Figure 1A). Both elements are shorter in anisoplin and HtA than in α-sarcin. However, anisoplin β-hairpin is longer than that of HtA, and with a theoretical pI of 5.5 instead of 9.2. This different Nt β-hairpin might be responsible for the differences observed in activity against ribosomes.

Finally, ribotoxins are cytotoxic against a variety of cells thanks to their ability to cross some lipid membranes previous to their inactivating function over the ribosomes. The specificity here, since they do not interact with any membrane receptor, is the lipid composition of the membrane of the target cell. Recently, it has been shown that ribotoxins display a much higher toxicity against insect than mammalian transformed cells and their insecticidal properties have been described (Olombrada et al., 2013). A similar experiment of protein biosynthesis inhibition with Sf9 insect cells has been performed here (Figure 3E). Anisoplin was able to inhibit protein biosynthesis with an IC_{50} of 120 nM, just slightly higher than HtA and α-sarcin. Moreover, in order to correlate this effect with ribosome inhibition, RNA from Sf9 cells treated with anisoplin was extracted and analyzed by primer extension (Figure 3F). Results showed the specific fragment due to ribotoxin cleavage in percentages similar to those obtained with HtA and α-sarcin (Olombrada et al., 2013). Therefore, anisoplin, as a typical ribotoxin, causes cytotoxicity in cultured insect cells by entering into them and specifically cleaving their ribosomes.

In this report we describe anisoplin, a ribotoxin from the entomopathogenic fungus *Metarhizium anisopliae*. However, it is still not known how and when this toxin is produced by the fungus in nature. The toxic character of anisoplin suggests some relevant function during insect infection but further research needs to be done. Other ribotoxins like restrictocin have a deterrent effect on insects feeding as a protection mechanism during conidia maturation of *Aspergillus* (Brandhorst and Kenealy, 1992; Brandhorst et al., 1996). Considering the importance of *Metarhizium* in a broad spectrum of biotechnological applications, including malaria vector control, the characterization of its molecules involved in pathogenesis is a useful tool (Schrank and Vainstein, 2010; de Bekker et al., 2013; Staats et al., 2014). Even efforts have been recently made in order to predict, characterize and compare the secretome of diverse entomopathogenic fungi (de Bekker et al., 2013; Staats et al., 2014). However, there was not previous evidence of ribotoxins in *Metarhizium* apart from the results obtained from various sequenced genomes. In

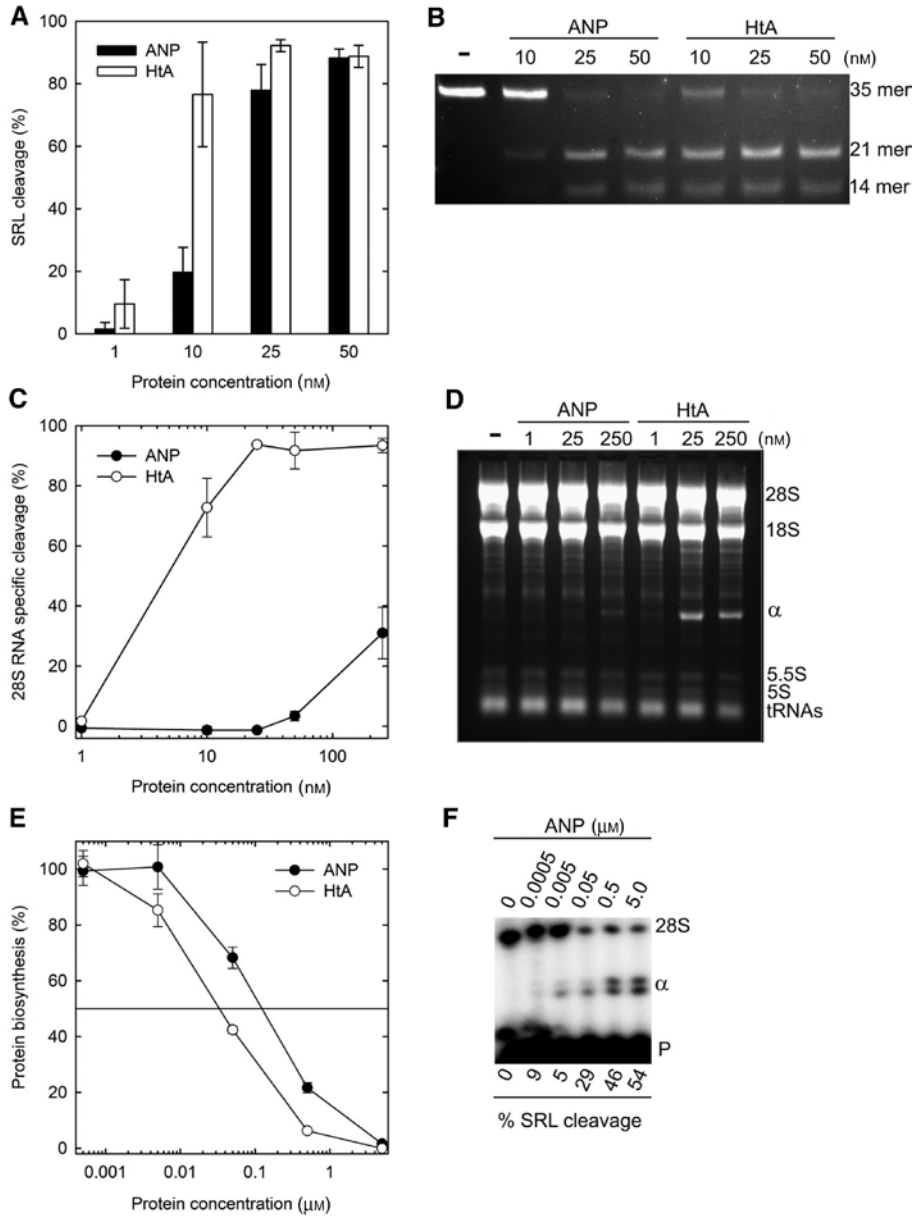


Figure 3: Ribonucleolytic and cytotoxic activities of recombinant anisoplin (ANP) compared to hirsutellin A (HtA).
(A) Quantitation of specific activity against a 35-mer sarcin/ricin loop oligonucleotide. (B) Analysis of the SRL cleavage by denaturing electrophoresis separation of products and ethidium bromide staining. Sizes of substrate and products resulted from the specific ribotoxin cleavage are indicated. (C) Quantitation of specific ribonucleolytic activity of ribotoxins against a rabbit reticulocyte lysate (Promega). (D) Analysis of the specific cleavage of 28S rRNA in reticulocytes lysates by denaturing electrophoresis and ethidium bromide staining. Bands corresponding to the different ribosomal RNAs and the α-fragment (α) released by the ribotoxin are indicated. (E) Protein biosynthesis inhibition by ribotoxins on Sf9 insect cells. Protein biosynthesis was quantitated by the incorporation of ^{3}H-Leu to newly synthesized proteins. (F) Poison primer extension analysis of RNA extracted from Sf9 cells treated with anisoplin as in panel (E). Bands corresponding to the ribotoxin specific cleavage (α), free primer (P) and not cleaved 28S are indicated. All results are expressed as the average of at least three independent experiments ±SD. Procedures are detailed in the online Supplementary material.

this regard, the characterization herein performed shows anisoplin as a protein cytotoxic to insect cells with interesting features when compared to other fungal ribotoxins in terms of its structure, as well as its ribonucleolytic activity. Moreover, fungal ribotoxins have been recently described as insecticidal agents (Olombrada et al., 2013). With all this, anisoplin seems to be an interesting biological weapon of *Metarhizium*. Its potential as a biotechnological tool in agricultural pest and disease-vector control encourages further studies regarding its molecular

features as a ribotoxin as well as its insecticide properties not to forget its role in nature.

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