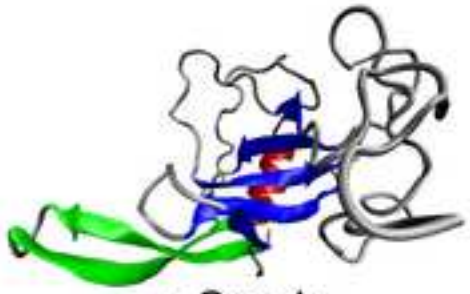
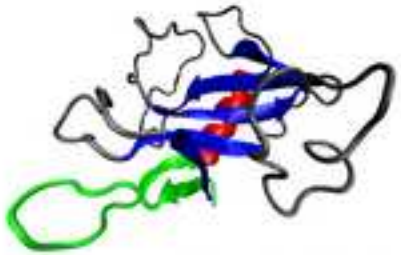
*Graphical Abstract (for review)

Fungal ribotoxins

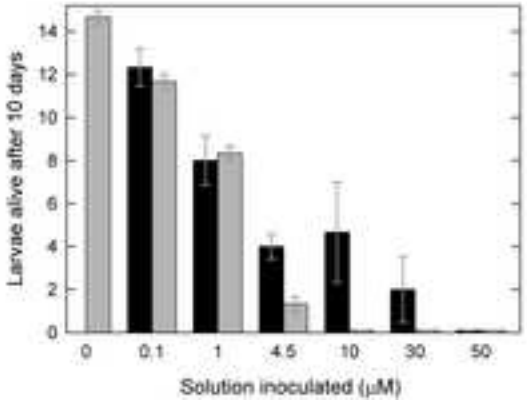


α-Sarcin

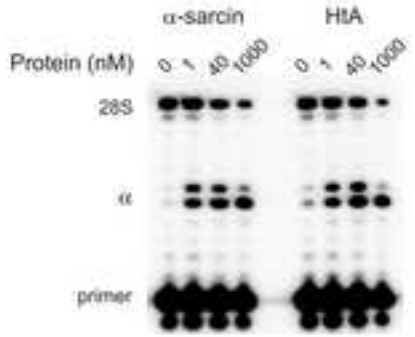


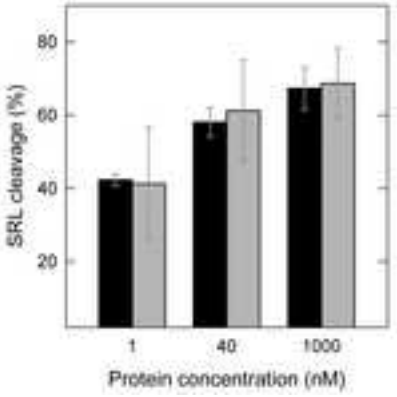
Hirsutellin A (HtA)

Galleria Mellonella **killing assay**



Ribosome inactivation





Fungal extracellular ribotoxins as insecticidal agents

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Abstract

Fungal ribotoxins were discovered almost 50 years ago as extracellular ribonucleases (RNases) with antitumoral properties. However, the biological function of these toxic proteins has remained elusive. The discovery of the ribotoxin HtA, produced by the invertebrates pathogen *H. thompsonii*, revived the old proposal that insecticidal activity would be their long searched function. Unfortunately, HtA is rather singular among all ribotoxins known in terms of sequence and structure similarities. Thus, it was intriguing to answer the question of whether HtA is just an exception or, on the contrary, the paradigmatic example of the ribotoxins function. The work presented uses HtA and α-sarcin, the most representative member of the ribotoxins family, to show their strong toxic action against insect larvae and cells.

Abbreviations: HtA, hirsutellin A; RNases, ribonucleases; SRL, sarcin/ricin loop; TLC, thin layer chromatography.

Keywords: fungal ribotoxin; ribonuclease; ribosome inactivation; insecticide.

1. Introduction

Microorganisms populate almost any ecological niche and establish complex and sometimes essential relationships with higher organisms. Consequently, a high diversity of interactions can be found, most of them based on biological mutualisms or antagonisms (Berenbaum and Eisner, 2008). In this

regard, fungi constitute a rich source of nitrogen and phosphorous for arthropods and therefore share a long evolutionary history with them. Thus, not only fungi are under constant attack by fungivorous animals such as collembolan, mites and insects (Boddy and Jones, 2008; Ruess and Lussenhop, 2005) but also display frequent mutualistic relationships (Scott et al., 2008), as can be exemplified by fungus farming ants (Currie et al., 1999). Underlying these relationships there is a complex interaction network involving preying, defense, and feeding. Elucidation of these interactions can drive to the discovery and understanding of natural products of unforeseen function (Berenbaum and Eisner, 2008).

Within this idea, it is noticeable that the fungal genus *Hirsutella* contains over 50 fungal species which are known to be entomopathogens. Under *in vivo* conditions, conidia contact the host, attach to the cuticle, germinate, and penetrate through it (Liu et al., 1995). Along the 1990s, crude filtrates of *H. thompsonii,* a particular species of this genus, were found to be toxic to a wide variety of arthropods including moth, fly, and mosquito larvae, aphids and mites (Liu et al., 1995; 1996; Omoto and McCoy, 1998; Vey et al., 1993). A toxic protein, Hirsutellin A (HtA), was then isolated from these cultures and proved to show broad pathogenic activity against insects (Krasnoff and Gupta, 1994; Mazet and Vey, 1995). HtA was lethal to *Galleria mellonella* larvae upon injection (Mazet and Vey, 1995) and caused detectable cytopathic effects on *Spodoptera frugiperda* cells (Sf9), inhibiting cell growth (Liu et al., 1995), for example. The ribosomal RNA (rRNA) extracted from these cells contained a small fragment of about 500-600 nt (Liu et al., 1996) resembling the α-fragment produced by fungal ribotoxins upon inactivation of eukaryotic ribosomes (Chan et al., 1983; Endo and Wool, 1982; Endo et al., 1983; Schindler and Davies, 1977). Accordingly, HtA was demonstrated to be a ribotoxin (Herrero-Galán et al., 2008) and subjected to detailed structural and functional characterization (Herrero-Galán et al., 2012a, 2012b; Viegas et al., 2009).

Fungal ribotoxins are extracellular and highly specific ribonucleases which behave as potent inhibitors of protein biosynthesis by being able to inactivate ribosomes from almost any organism (Gasset et al., 1994; Kao et al., 2001; Lacadena et al., 2007; Martínez-Ruiz et al., 2001). They cleave the larger rRNA component at a single phosphodiester bond located within the universally conserved sarcin/ricin loop (SRL) (Chan et al., 1983; Endo and Wool, 1982; Endo et al., 1983; Schindler and Davies, 1977), leading to complete inactivation of the ribosome and cell death by apoptosis (Olmo et al., 2001). Unfortunately, apart from generalized assertions such as being involved in defense or predation, the biological function of these unique ribonucleases still remains undetermined.

α-Sarcin, restrictocin, and Aspf1 are the most exhaustively characterized ribotoxins (Arruda et al., 1992; García-Ortega et al., 2005; Gasset et al., 1994;

Kao et al., 2001; Martínez-Ruiz et al., 2001; Wool, 1997), but many others have been identified and partially characterized within different fungal species (Huang et al.,1997; Lin et al., 1995; Martínez-Ruiz et al., 1999a, 1999b; Parente et al., 1996; Varga and Samson, 2008; Wirth et al., 1997). These studies have shown a high degree of conservation among ribotoxins, as most of those so far characterized display amino acid sequence similarities above 85% (Lacadena et al., 2007). Surprisingly, HtA is a much smaller protein (130 amino acids against 149/150 of the other known ribotoxins) which displays only 25% sequence identity with previously known family members but still contains the same elements of periodic secondary structure and identical arrangement of the active site residues (Boucias et al., 1998; Herrero-Galán et al., 2008; Martínez-Ruiz et al., 1999a). Interestingly, other closely related fungal extracellular RNases, such as RNases T1 and U2, which are only about 100 amino acids long, still show high sequence identity with ribotoxins and contain identical elements of ordered secondary structure. However, they are non-toxic and show low substrate specificity upon cleaving RNA (Martínez-Ruiz et al., 1999a; 2001). Could we then extrapolate HtA insecticidal properties to the whole ribotoxins family? In that case, it would contribute to answer the long time question about the unknown function of this family of toxins (Brandhorst et al., 1996, 2000; Herrero-Galán et al., 2008; Viegas et al., 2009).

In fact, the assignment of an insecticidal function to fungal ribotoxins has been suggested before (Brandhorst et al., 1996, 2000). These authors studied the behavior of the beetle *Carpophilus freemani* against two different *Aspergilli*: The ribotoxin producer *A. restrictus,* and *A. nidulans,* which genome does not contain a gene for this type of proteins (Brandhorst et al., 1996). These results showed a significant decrease in feeding on the first of these two fungi when conidia were starting to develop, while no change in the insect behavior was observed against maturing *A. nidulans*. The period of not consumption was coincident with a dramatic build up of restrictocin upon the surfaces of conidia and phialides (Brandhorst and Kenealy, 1992) suggesting that it could deter insects from feeding until conidia were fully formed. Once the spores were mature, the ribotoxin levels would decrease allowing insects to carry spores to new locations for spreading the fungus.

We now show compelling additional evidence of how two of the most different ribotoxins known, α-sarcin and HtA, display similar strong insecticidal properties which are highly dependent on their specific ribonucleolytic activity. Altogether these results are discussed in terms of this insecticidal activity being the biological function of fungal ribotoxins.

2. Materials and methods

2.1. Protein production and purification

Fungal natural wild-type α-sarcin and HtA were produced and isolated as reported before (Herrero-Galán et al., 2008; Martínez-Ruiz et al., 2001). *E. coli* BL21 (DE3) cells, previously cotransformed with a thioredoxin-producing plasmid (pT-Trx) and the corresponding plasmid (pINPGαSH137Q), were used to produce the catalytically inactive α-sarcin H137Q mutant, also as previously described (García-Ortega et al., 2000; Lacadena et al., 1994, 1995, 1999). This mutant retains the structural features of the wild-type protein, as well as its ability to interact with membranes, but lacks the characteristic ribonucleolytic activity of ribotoxins (Lacadena et al., 1995, 1999). SDS-PAGE of proteins, Western blots, protein hydrolysis, amino acid analysis, and spectroscopic characterization were performed according to standardized procedures described before (García-Ortega et al., 2000, 2002; Lacadena et al., 1994; Martínez-Ruiz et al., 2001). According to all these criteria, the three proteins used in this study were purified to homogeneity and retained their structural and functional properties.

2.2. Galleria mellonella killing assay

G. mellonella caterpillars in the final instar larval stage were obtained from the company Animal Center (Pobla de Vallbona, Valencia, Spain), stored at 4ºC in the dark and used within 7 days from the day of shipment. Caterpillars with body weights ranging between 0.2 and 0.4 g and absent of any grey markings on the cuticle were employed in all assays. Fifteen randomly chosen caterpillars were used per group in every assay. Results shown are the average of at least three independent assays.

Assays were performed as described (Fuchs et al., 2010; Mylonakis et al., 2005; Mylonakis, 2008). A 10-µl Hamilton syringe was used to inject 8-µl aliquots of the inoculum into the hemocoel of each caterpillar via the last left proleg. The inoculum contained different protein concentrations, ranging between 0 and 50 µM, dissolved in autoclaved 0.9% (w/v) NaCl. After injection, caterpillars were incubated in plastic Petri dishes at 30ºC in the dark. The number of dead caterpillars or pupae formed was scored daily. Caterpillars were considered dead when they displayed no movement in response to touch. Dead larvae were removed from the Petri dish housing the remaining viable larvae. Controls included a group that did not receive any injection in order to monitor the overall quality of the larvae during the course of the experiment and a 0.9% (w/v) NaCl injection group to ensure that death was not due to trauma.

2.3. Insect cells culture and toxicity assays

The insect cell lines *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (Tni High Five) were cultured as described (Tello et al., 2010) in Insect-XPRESS™ Protein-free Insect Cell medium (BioWhittaker) at 27 ºC as indicated by the manufacturer. Toxicity assays were made seeding 80% confluent cells,

supplemented with 10 mg/L gentamycin, into 24-well plastic plates and then adding 500 µl of the same medium containing different protein concentrations. Protein solutions were prepared in Insect X-press medium and sterilized by ultrafiltration. Plates were then incubated for 60 hours at 27ºC. Dead cells were counted by the dye exclusion method using a Neubauer chamber and a Nikon Eclipse TE 2000-U microscope, after convenient Trypan blue staining of the corresponding cells suspensions.

2.4. Protein biosynthesis inhibition assay using S. frugiperda cells

Sf9 cells were seeded into 24-well plates at a cell density of 10^5 cells/well and were maintained under standard culture conditions up to 80% confluency (2 days). Then, monolayer cultures were incubated in 0.5 mL of fresh medium with serial dilutions of ribotoxin from 10 µM to 1 nM final concentrations. Following 18 hr of incubation at 27ºC the medium was replaced by culture medium supplemented with 0.5 µCi/well of [^{3}H]leucine. After 5 hours of incubation the medium was removed and cell protein content was precipitated with 5 % trichloroacetic acid (TCA) and washed three times with ethanol. The precipitate was dried, dissolved in 200 µl of 0.1 N NaOH, 0.1% SDS and radioactivity was measured in a Beckman LS 3801 liquid scintillation counter. Results are expressed as percentage of incorporated radioactivity relative to samples without protein added.

2.5. *Isolation of ribosomes from S. frugiperda cells*

Ribosome preparation was obtained from 100% confluency monolayer of Sf9 cells grown in Insect-XPRESS™ Protein-free Insect Cell medium (BioWhittaker). Cells from eight F300 flasks were recovered in 20 mL of cold buffer A (50 mM Tris-HCl pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM EDTA, 5 mM β-mercaptoethanol) supplemented with 0.2 mM PMSF and a proteases inhibitor cocktail. Cell lysis was performed with a French press at 18.000 psi. Ribosome purification followed a standard protocol described before (Powers and Noller, 1991), including a continuous sucrose gradient fractionation. Ribosomes were stored in buffer A, lacking EDTA, at -80ºC.

2.6. Ribonucleolytic activity assays

The specific and unique ribonucleolytic action of ribotoxins on ribosomes results in the cleavage of a single phosphodiester bond located at the SRL with the subsequent release of a characteristic rRNA fragment (the α-fragment) which can be visualized by different means. Thus, one first set of experiments was aimed at detecting the presence of this α-fragment in the rRNA extracted from insect cells previously treated with different amounts of α–sarcin or HtA. With this purpose, cells treated as described in the previous protein

biosynthesis inhibition section were recovered in 200 μL of 100 mM HEPES-HCl pH 7.5, pelleted and resuspended again in 200 μL QIAzol lysis reagent (Qiagen). RNA was then extracted from the aqueous phase, following addition of 40 μL of chloroform, and precipitated with isopropanol at RT. Following a 70% ethanol washing step the RNA was finally resuspended in 10 μL of H_2O. This RNA preparation was analyzed using two different approaches (Garcia-Ortega et al., 2010). First, a denaturing 2% (w/v) agarose electrophoresis in MOPS buffer followed by ethidium bromide staining, was used to analyze the rRNA integrity and detect the presence of the α-fragment. Second, the extent and position of cleavage was also determined by poison primer extension as described before. In this case, reverse transcription was performed using the primer 5'-ACCAAATGTCTGAACCTGCGG-3', which complements the sequence downstream of the SRL in 28S rRNA of *S. frugiperda*. This reaction gave different products for an intact and a cleaved template. The uncleaved rRNA was transcribed up to the first uridine in the sequence due to the ddATP in the extension mixture. The extension of the cleaved template stopped at the cleavage site. The products of reverse transcription were then separated in a 10% denaturing polyacrylamide gel and quantitated with a PhosphorImager (Molecular Dynamics). Percentage of sarcin/ricin cleavage was obtained as α/(α+28S) x 100, where α and 28S correspond to the amounts of α-fragment and intact RNA found in each case, respectively.

In a second set of experiments, identical analyses were made but now ribotoxins were assayed against isolated Sf9 ribosomes as substrates. Within this purpose, reactions were performed in 25 μL of 30 mM Tris-HCl pH 7.5, 150 mM NH_4Cl, 5 mM $MgCl_2$, 2 mM EDTA and 4 mM β-mercaptoethanol. Ribosome concentration was 0.2 μM. Incubation times varied from 30 seconds to 90 min and ribotoxin concentrations from 10 to 200 nM. In order to stop the reaction, 125 μL of 0.36 M NaAc, 1.1% (w/v) SDS were added and then RNA was extracted with phenol/chloroform and precipitated with ethanol. The RNA obtained was analyzed by denaturing agarose electrophoresis and poison primer extension as described above.

2.7. Leakage of aqueous contents from lipid vesicles

The breakdown of the permeability barrier of lipid bilayers can be analyzed using an assay employing the fluorescence probe 8-aminonaphtalene1,3,6-trisulfonic acid (ANTS) and its colisional quencher N,N-p-xilene-bispyridinium bromide (DPX) (Ellens et al, 1985, 1986). When they are encapsulated into lipid vesicles, the release of the intravesicular content to the external medium results in dilution of probe and quencher, with the concomitant increase in ANTS fluorescence. Plasma membrane of Sf9 cells was isolated and separated from the endoplasmic reticulum and Golgi apparatus fractions using a discontinuous sucrose gradient (Hu and Kaplan, 2000). Total lipids from

the plasma membrane were then isolated using a described procedure (Folch et al., 1957): The plasma membrane fraction was centrifuged for 1h at 20000 rpm in a TLA120.1 rotor, resuspended in 3 ml of chloroform/methanol (2:1 v:v) and shaken gently at room temperature for 1 h. After washing with 0.2 volume of 0.9% NaCl solution, the mixture was centrifuged at low speed to separate the two phases. The lower chloroform phase was recovered, evaporated under a stream of nitrogen and stored at -20 ºC. Analysis of the phospholipids content in this cell membrane preparation was performed using thin-layer chromatography (TLC) plates (silica gel 60, 20x20 cm, Merck) as described (Gavilanes et al., 1981). TLC fractionation of the lipid plasma membrane extracts revealed high amounts of phosphatidylcholine and phosphatidylethanolamine (data not shown) in good accordance with the high abundance of these phospholipids in total lipidic extracts of Sf9 cells (Yeh et al., 1997). This pool of insect lipids was used to prepare vesicles as described before (De los Ríos et al., 1998; Herrero-Galán et al., 2008; Martínez-Ruiz et al., 2001) by hydrating dry lipid films with 15 mM Tris-HCl, pH 7.0, containing 0.1M NaCl and 1 mM EDTA, for 60 min at 37ºC and in the presence of 12.5 mM ANTS and 45 mM DPX. The lipid suspension was then subjected to five cycles of extrusion through two stacked 0.1 µm (pore diameter) polycarbonate membranes (Mancheño et al., 1994; Martínez-Ruiz et al., 2001). Unencapsulated material was separated from the vesicles by gel filtration on a Sephadex G-75 column in the same buffer. Phospholipid concentration was determined as described (Barlett, 1959). Leakage of vesicle aqueous contents was then measured by adding small volumes of freshly prepared solutions of proteins in the corresponding buffer and recording the emission above 530 nm (using a 3-68 Corning cutoff filter) upon excitation at 386 nm. Polarizers were used to eliminate potential contribution of sample turbidity to the signal registered. The extent of leakage was defined as *Relative leakage*= $(F_P-F_0)/(F_T-F_0)$, where F_P and F_0 are the fluorescence intensity values after and before the addition of protein, respectively, and F_T is the value after addition of 10%Triton X-100 (total vesicle lysis) (Martínez-Ruiz et al., 2001).

2.8. Statistical analysis

Statistical analyses were carried out by using the statistics utility of the Sigma Plot v11.2 Program (Systat Software, Erkrath, Germany). The normality of the data was checked by using the Shapiro-Wilk test and equal variance test. Percentage values were Arcsine-Square Root transformed before statistical analysis. ANOVA analyses were used for comparison of multiple groups of factors. Statistical significance was considered to be achieved at the $p<0.05$ level.

3. Results

3.1. Galleria mellonella killing assay

Injection of either fungal natural wild-type α-sarcin or HtA resulted in larvae death. Toxicity was dependent on ribotoxin concentration, HtA being more effective in terms of less amount of protein needed to produce the same mortality levels (Fig. 1). Upon toxin injection larvae begun to lose mobility. A brownish coloration slowly appeared, becoming dark brown or even black, upon death. These color changes were most probably due to an overactivation of the phenoloxidase cascade, as it has been described as one of the key defense mechanisms against pathogens in insects (Soderhall and Cerenius, 1998). Differences in larvae survival were especially evident after ten days of incubation at 30ºC (Fig. 1). On the other hand, injection of the catalytically inactive α-sarcin H137Q mutant had almost a negligible effect on survival for identical doses and incubation times (Fig. 1), suggesting that the highly specific RNase activity of ribotoxins is important for their insecticidal lethal action. The toxic effect of ribotoxins against *G. mellonella* larvae was also evident in terms of pupation delay (data not shown).

3.2. Toxic effect on insect cell lines in culture

Both ribotoxins, α-sarcin and HtA, displayed toxicity when assayed against two different insect cell lines (Fig. 2), being slightly more effective against *Spodoptera frugiperda* cells (Sf9) than against *Trichoplusia ni* (Tni High Five) cells, in terms of the estimated IC_{50} values.

Given the higher sensibility of *S. frugiperda* cells to the action of both ribotoxins, this cell line was chosen to further study the effect of the two proteins at the molecular level. Both α-sarcin and HtA showed a dramatic effect on the inhibition of *in vivo* protein biosynthesis with IC_{50} values in the nanomolar range (Fig. 3). These values were in the same order of magnitude as those obtained from cell viability assays, considering the different nature of both experiments. On the other hand, they were around two orders of magnitude smaller than those obtained for human rhabdomyosarcoma cells commonly used in the standard assay for evaluating the ribotoxins antitumoral activity (Herrero-Galán et al., 2008; Olmo et al., 2001; Turnay et al., 1993). The production of α-fragment in these cultures was analyzed by poison primer extension once total RNA was isolated (Fig. 4). The result showed that 28S rRNA was cleaved by both ribotoxins with an identical pattern as that described for *E. coli* ribosomes (Garcia-Ortega et al., 2010). Thus, the extent of cleavage of the sarcin/ricin loop was consistent with the inhibition of protein synthesis, supporting the notion that the specific ribonucleolytic action of α-sarcin and HtA is the cause for their toxicity against this insect cell line.

3.3. Ribotoxin effect against purified insect ribosomes

Insect ribosomes were isolated from Sf9 cells by a two step purification protocol based on a salt wash and a continuous sucrose gradient, as described elsewhere for prokaryotic and eukaryotic organisms (Spedding, 1990). Ribosomal RNA analysis showed the unique pattern for these ribosomes of an apparent single band in agarose gels (Fig. 5A). This is a consequence of a natural hidden break in the 28S rRNA without affecting the ribosome integrity (Winnebeck et al., 2010).

α-Sarcin and HtA treatment of these ribosomes produced an additional RNA band, the known α-fragment (Fig. 5A), as a result of their specific activity against these substrates. Different protein concentrations and reaction times were analyzed (Fig. 5B). Quantification of the extent of specific cleavage by primer extension showed an important difference between the activities of both ribotoxins. HtA was dramatically more efficient than α-sarcin (Fig. 5C), with an apparent affinity constant in the nanomolar range in these conditions.

3.4. Leakage of aqueous contents from lipid vesicles

Regardless of their ribonucleolytic activity, HtA and α-sarcin also show significant differences when interacting with biological membrane model systems (Herrero-Galán et al., 2008). HtA membrane permeabilizing activity is higher than that of α-sarcin. In this case, we studied the ability of both proteins to promote leakage of aqueous contents from vesicles made of lipids extracted from the plasmatic membrane of Sf9 cells. The results obtained confirmed that both toxins retained the membrane interaction ability in this insect model system but also that HtA was more effective as a leakage inducing agent (Fig. 6).

4. Discussion

The first fungal ribotoxin was discovered almost 50 years ago during a screening program of the Michigan Department of Health searching for antibiotics and antitumor agents. The culture filtrates of a mold isolated from a sample of farm soil were found to contain a substance inhibitory to both sarcoma and carcinoma induced in mice (Olson et al., 1965). The mold was identified as *Aspergillus giganteus* MDH18894, and the molecule responsible for these effects proved to be a protein, named α-sarcin after its anti-sarcoma activity (Olson and Goerner, 1965). Not much later two more ribotoxins were discovered but further studies revealed an unspecific cytotoxicity of these proteins, which limited their potential clinical uses (Roga et al., 1971) and caused the abandonment of their study. A few years later it was demonstrated that they inhibited protein biosynthesis by specifically cleaving a unique phosphodiester bond of the large rRNA fragment (Endo and Wool, 1982; Schindler and Davies, 1977) located at an evolutionarily conserved site with important roles in ribosome function. This observation prompted the

development of their molecular characterization (Lacadena et al., 2007). Much later, more systematic analysis of fungal genomes revealed that ribotoxins were a more widespread group of proteins within the filamentous fungi than had been previously believed (Lin et al., 1995; Martínez-Ruiz et al., 1999b; Varga and Samson, 2008). All this exhaustive work dealing with the characterization of their mechanism of action at the molecular level has not only helped to understand the basis of their antitumoral action (Lacadena et al., 2007) but also has established the principles to transform some of them into engineered chimeras with potential therapeutic uses (Carreras-Sangrà et al., 2008, 2012).

It seems clear, however, that fungi are not secreting these lethal toxins with the purpose of combating mammalian cancer. In fact, the real biological function of these toxic proteins has remained elusive. The discovery of the insecticidal protein HtA and the demonstration that it was another member of the ribotoxins family (Herrero-Galán et al., 2008) opened the door to speculate about insecticidal activity being their so long searched function. A function that had been already suggested by some other authors (Brandhorst et al., 1996, 2001) but that was far from being proven in full. Furthermore, HtA is the most singular of all ribotoxins known, given its small size and its much lower degree of sequence identity with the other members of the family. Thus, it was intriguing to answer the question of whether HtA was just an exception or, on the contrary, a paradigmatic example of their function. The present study was therefore performed with the aim of discerning this dilemma, comparing HtA and α-sarcin, the most representative member of the ribotoxins family.

The results obtained show how both ribotoxins are highly toxic against *G. mellonella* larvae (Fig. 1) and insect cells in culture (Figs 2 and 3). Indeed, their insect killing activity is linked to their specific ribonucleolytic action on insect ribosomes as proven by the production of the α-fragment within treated cells (Fig. 4). Within this same idea, the ribonucleolytically inactive H137Q α-sarcin variant is also much less toxic when assayed against larvae (Fig. 1). This toxicity is two orders of magnitude higher than that one described for their antitumoral activity, supporting the biological function of this family of proteins as insecticidal agents. The explanation lies precisely at the heart of the unique action of ribotoxins. Thus, in addition to their ribonucleolytic activity, ribotoxins are extracellular RNases that cross lipid membranes to reach their target in the absence of any known protein receptor (Oñaderra et al., 1993; Gasset et al., 1994; Martínez-Ruiz et al., 2001). Consequently, although any ribosome could be potentially inactivated by these proteins, due to the universal conservativeness of the SRL, ribotoxins have been described as especially active on transformed or virus-infected cells (Fernández-Puentes and Carrasco, 1980; Olmo et al., 2001; Olson et al., 1965). This observation has been explained in terms of an altered permeability of these cells combined with the ability of ribotoxins to interact with acid phospholipid-containing membranes

(Gasset et al., 1989, 1990, 1994; Herrero-Galán et al., 2008, 2012b; Martínez-Ruiz et al., 2001; Olmo et al., 2001). Insect cells have a different plasma membrane composition from mammalian cells due to their higher content of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol and a significant lower cholesterol/phospholipid ratio (Marheineke et al., 1998). Therefore insect plasma membranes are thinner and probably more fluid than those in mammalian cells being better candidates as targets for ribotoxins.

Unexpectedly both proteins showed large differences when assayed against isolated insect ribosomes (Fig. 5). In the conditions assayed, HtA is significantly more active than α-sarcin, although both proteins still recognize specifically the SRL as proven by the release of the α-fragment (Fig. 5A). The apparent discrepancy of both proteins displaying very similar toxicity against larvae and cells but different affinities against ribosomes is consistent with passage across the cell membrane being the rate limiting step (Olmo et al., 2001; Turnay et al., 1993;). Thus, considering that cellular internalization takes hours to complete, the kinetic and affinity differences observed against purified ribosomes (Fig. 5) would be negligible in establishing a different cytotoxic activity when employed against intact cells. Although HtA still shows higher membrane permeabilizing activity than α-sarcin when tested against model vesicles made of the insect plasmatic lipid membranes (Fig. 6), this difference is not large enough to expect significant cytotoxic activities between both ribotoxins.

The different behavior of HtA and α-sarcin when interacting with ribosomes is however very interesting at the molecular level. This action has been now quantified not only with insect ribosomes but also with ribosomes from other origins like yeast or bacteria (unpublished results). It has been so far presumed that the extra loops in the structure of ribotoxins were responsible for their specific ribosomal recognition when compared with their structurally related non-toxic T1-like ribonucleases. Considering that HtA essentially differs from the rest of the family in having different and shorter non ordered loops, the detailed analysis of the mechanisms of ribosome interaction of both HtA and α-sarcin is now a challenging question. This means that not only their specific ribonucleolytic activity and their lipid interaction are important factors for their specificity of action but also aspects like the efficiency of their natural biosynthesis and extracellular export, their stability in a particular environment or the accessibility to their target. Supporting these ideas is the fact that *Aspergillus*, the main producer of ribotoxins, and *Hirsutella* are very different fungal geni which also thrive in very different environments.

In summary, the so long studied antitumoral action of fungal ribotoxins seems to be just a side effect of their insecticidal function. Depending on the environment and the producing fungus this toxicity could be involved in protecting from predation of different arthropods or in parasitism. So far we

have proved their biological toxicity against insects. Further studies in closer to nature niches will precise their natural function. Within this idea, the study of ribotoxins would constitute a nice example of how understanding the molecular mechanism of toxic proteins can help to yield beneficial therapeutic uses of the proteins involved in that characterization.

Acknowledgments

We want to thank Dr. Antonio Di Pietro from the Universidad of Córdoba (Spain) for his advice in dealing with the *G. mellonella* larvae. Javier Merino provided the graphical abstract picture. This work was supported by projects BFQ2009-10185, from the Spanish Ministerio de Ciencia e Innovación, and ESFUNPROT-UCM, from Universidad Complutense. Miriam Olombrada is recipient of a FPU predoctoral fellowship from the Spanish Ministerio de Educación.

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Figure legends

Fig. 1.- *G. mellonella* larvae survival after 10 days of inoculation with 8.0 µl of either natural fungal wild-type α-sarcin (black bars), HtA (grey bars) or the catalytically inactive α-sarcin H137Q mutant (dark grey bars). The 0 bar corresponds to control larvae inoculated only with 0.9% NaCl. Fifteen larvae were injected for each protein concentration employed. Results are the average ± SD of three different sets of experiments. ANOVA analysis revealed that there was a statistically significant difference between the treatment with α-sarcin or HtA at the concentrations assayed respect to the control (NaCl treatment, $p < 0.001$), but not in the case of the α-sarcin H137Q mutant ($p = 0.598$).

Fig. 2.- Toxic effect of the addition of increasing ribotoxin concentrations to the culture medium of two different insect cell lines. Results are expressed as viability ratios (proportion of cells remaining alive for an initial population of 3 x 10^5 cells) ± SD after 60 hours of incubation in the presence of α-sarcin (black bars) or HtA (grey bars). ANOVA analysis of Arcsine-Square Root transformed values was performed. No significant differences were observed for the toxicity of both ribotoxins on the two insect cell lines, except for Tni High Five cells at 10 and 100 nM toxin concentration ($p = 0.002$ and $p < 0.001$, respectively), and Sf9 cells at 10 nM ($p = 0.025$).

Fig. 3.- Protein biosynthesis inhibition ± SD in Sf9 insect cells cultured in the presence of different α-sarcin (black dots) or HtA (grey dots) concentrations. Results are the average of three different sets of experiments. ANOVA analysis of Arcsine-Square Root transformed values revealed that the effect of both proteins was significantly different ($p = 0.003$) in the overall concentration range.

Fig. 4.- rRNA analysis of Sf9 insect cells treated with different concentrations of ribotoxin. The poison primer extension assay showed two additional bands corresponding to the α-fragment (α) when cells were incubated with the ribotoxin. Quantification was expressed as the percentage of specific cleavage at the sarcin/ricin loop obtained. Black bars and grey bars correspond to α-sarcin and HtA, respectively. Results are the average ± SD of three different sets of experiments. ANOVA analysis of Arcsine-Square Root transformed values showed no significant differences between both toxins.

Fig. 5.- α-Sarcin (black dots) or HtA (grey dots) activity on isolated Sf9 ribosomes. (**A**) Integrity of ribosomal RNA after ribotoxin treatment analyzed by denaturing agarose gel electrophoresis. Positions corresponding to the different rRNA molecules, α-fragment included, are indicated. (**B**) Two time courses of SRL cleavage quantified by primer extension analysis are shown: α-Sarcin 0.2 µM and HtA 10 nM. (**C**) Dependence of the reaction rate obtained from different time courses as shown in B with ribotoxin concentration.

Fig. 6.- Leakage of aqueous content of vesicles prepared with lipids extracted from cultured Sf9 cells upon addition of different α-sarcin (black bars) or HtA (grey bars) concentrations. Relative leakage, considering that produced by detergent as unit, is expressed as a function of protein/lipid molar ratios. Results are the average of two experiments. ANOVA analysis of Arcsine-Square Root transformed leakage values showed significant differences between both toxins ($p < 0.001$).

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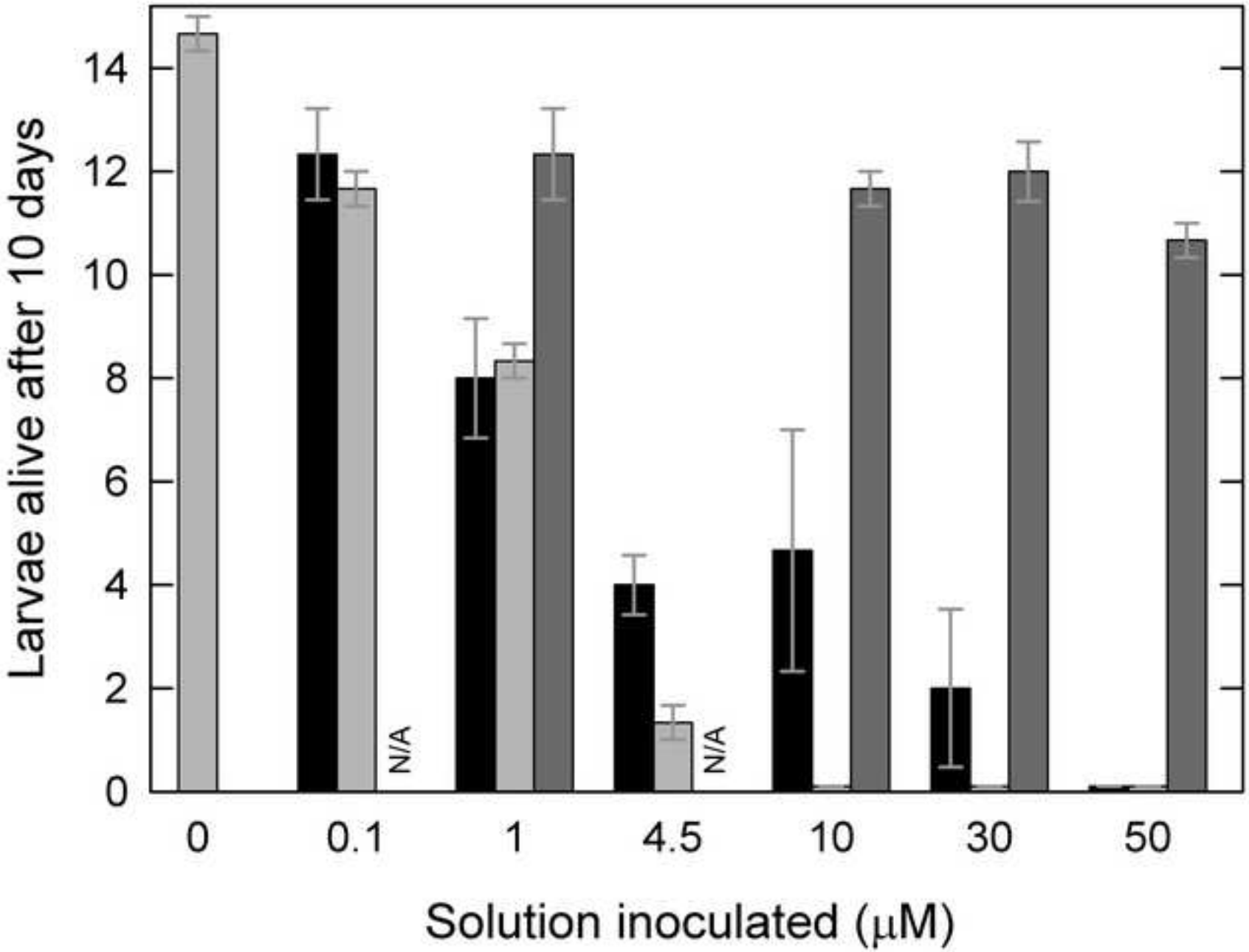

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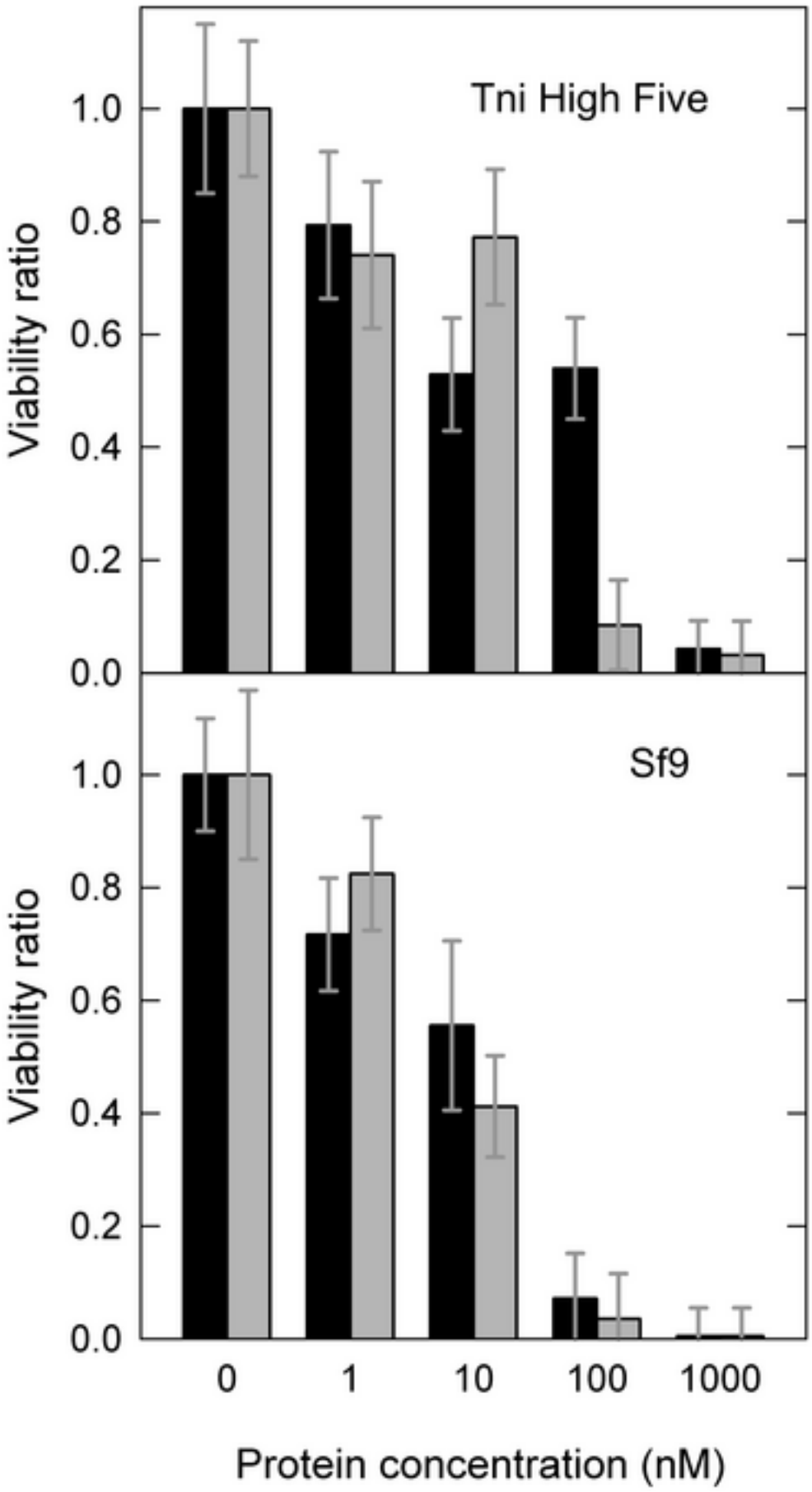

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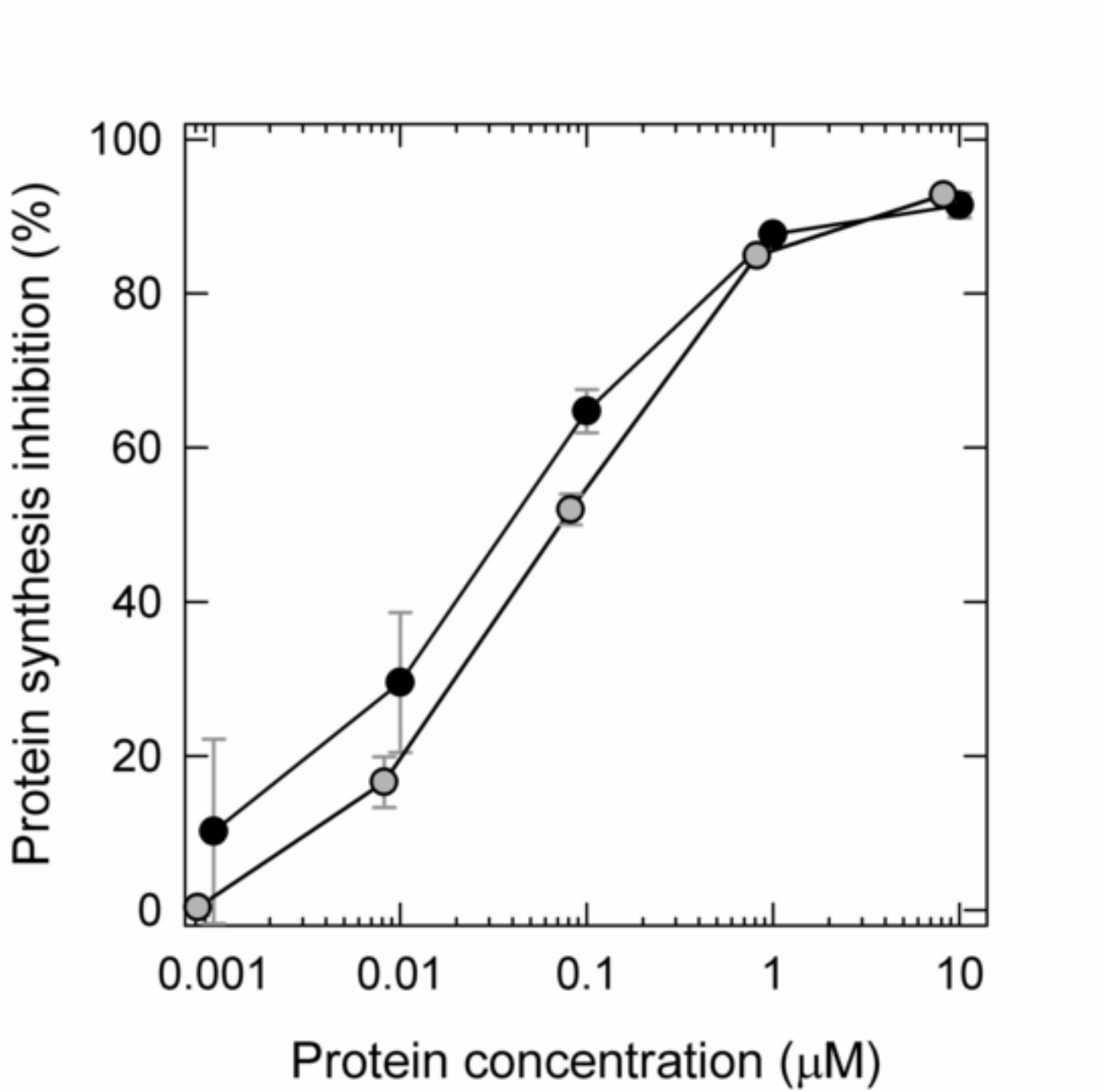

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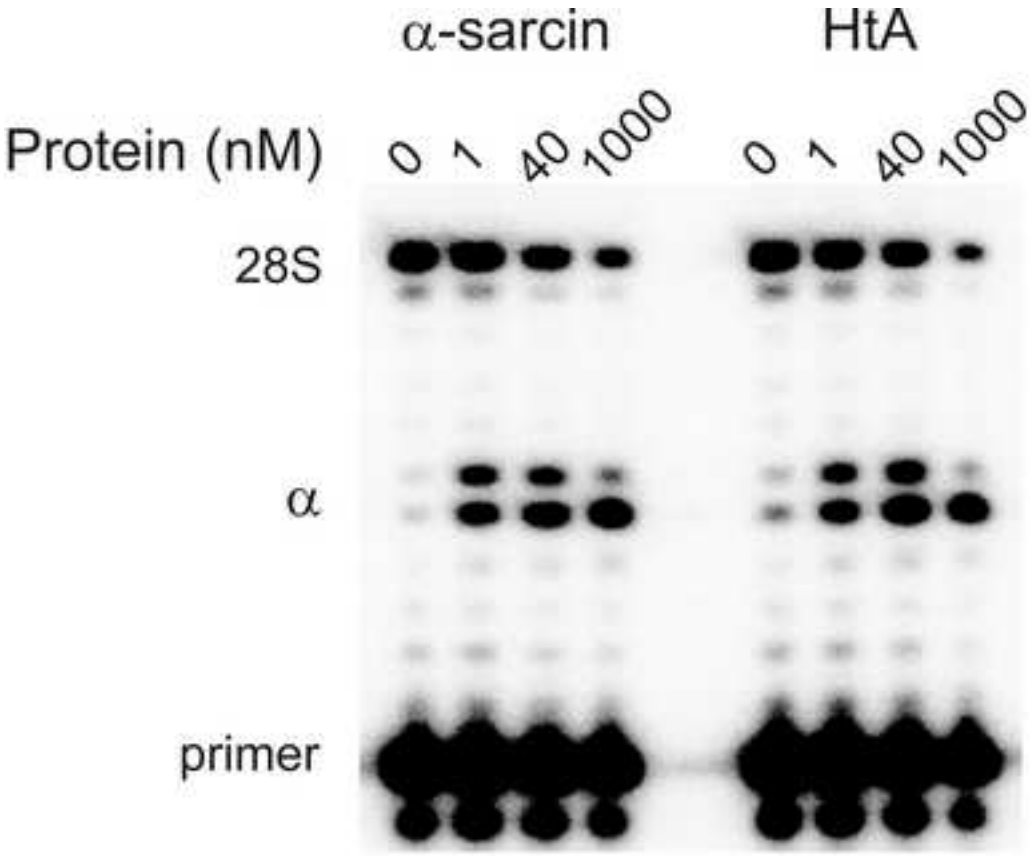

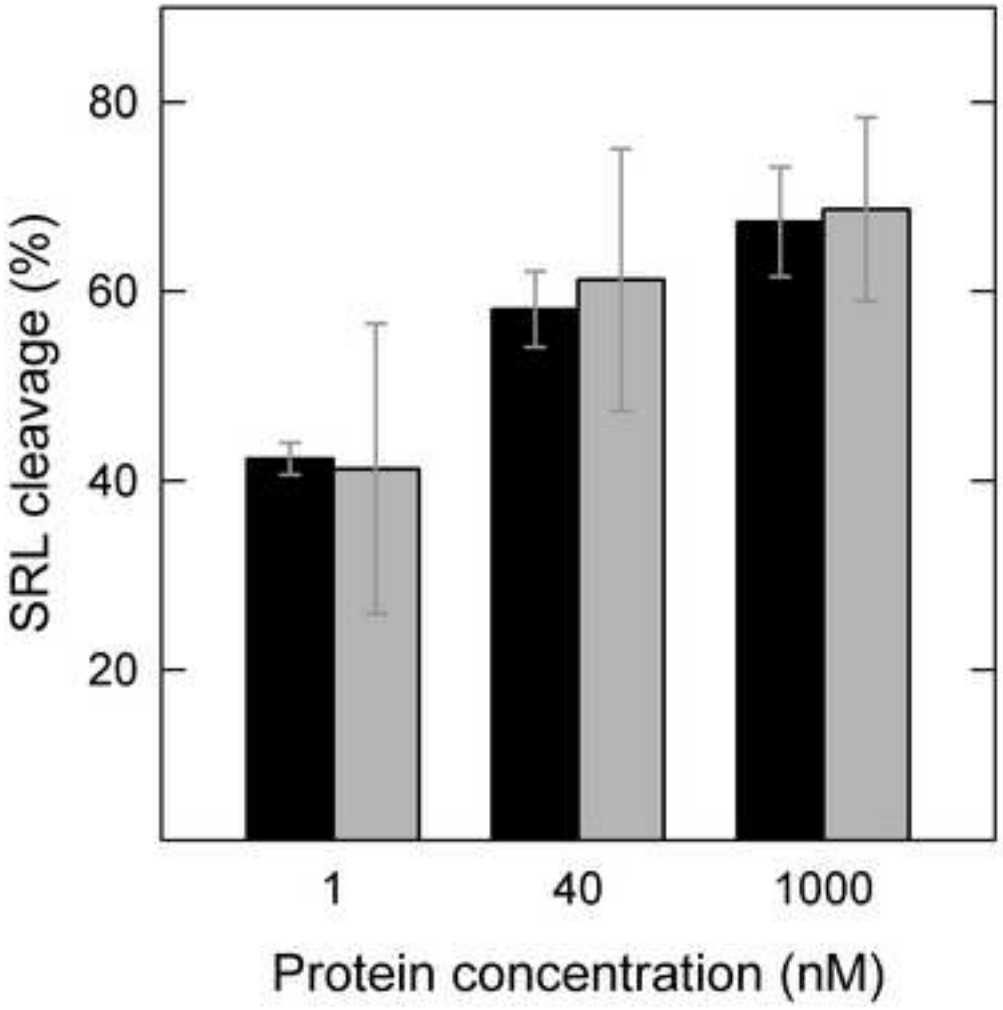

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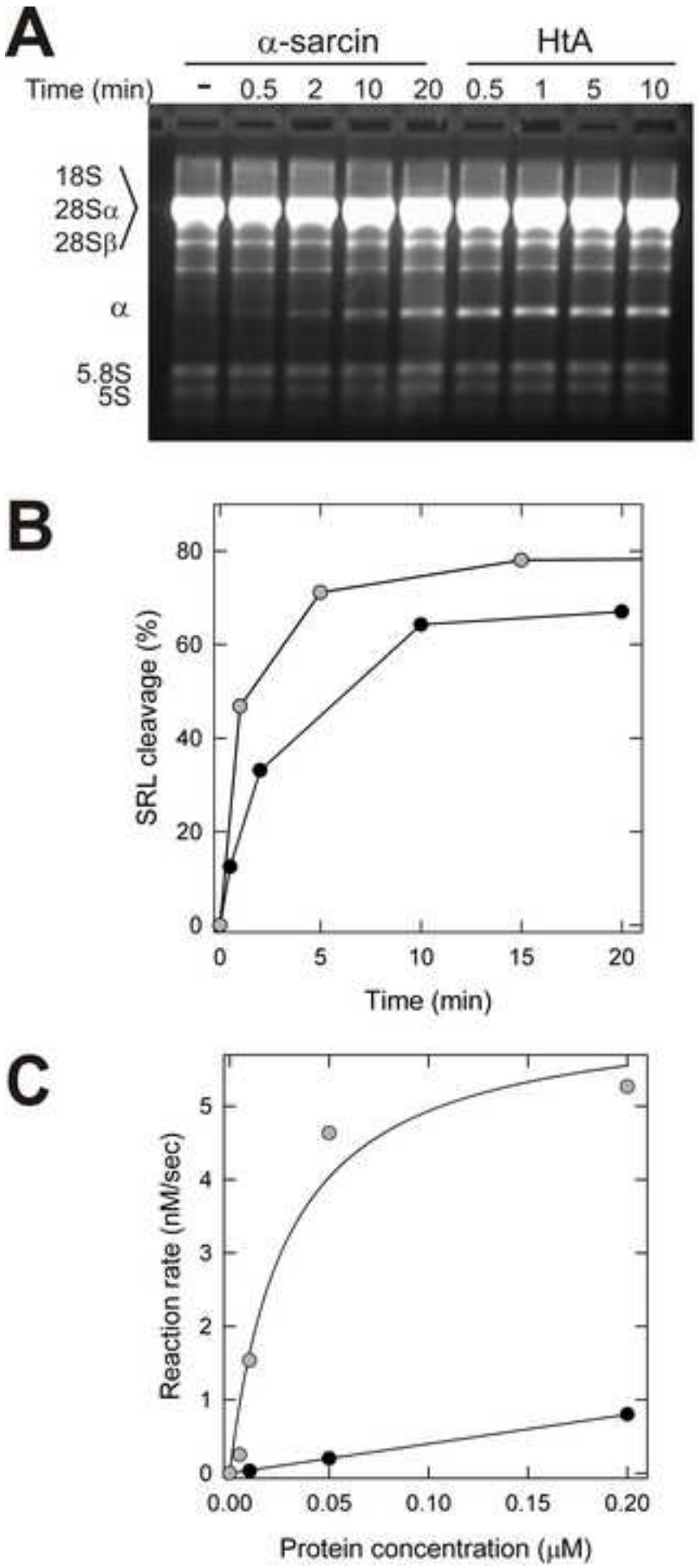

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