Involvement of loop 5 lysine residues and the N-terminal β-hairpin of the ribotoxin hirsutellin A on its insecticidal activity

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Abstract: Ribotoxins are cytotoxic members of the family of fungal extracellular ribonucleases best represented by RNase T1. They share a high degree of sequence identity and a common structural fold, including the geometric arrangement of their active sites. However, ribotoxins are larger, with a well-defined N-terminal β-hairpin, and display longer and positively charged unstructured loops. These structural differences account for their cytotoxic properties. Unexpectedly, the discovery of hirsutellin A (HtA), a ribotoxin produced by the invertebrate pathogen Hirsutella thompsonii, showed how it was possible to accommodate these features into a shorter amino acid sequence. Examination of HtA N-terminal β-hairpin reveals differences in terms of length, charge, and spatial distribution. Consequently, four different HtA mutants were prepared and characterized. One of them was the result of deleting this hairpin ([Δ(8-15)] while the other three affected single Lys residues in its close spatial proximity (K115E, K118E, and K123E). The results obtained support the general conclusion that HtA active site would show a high degree of plasticity, being able to accommodate electrostatic and structural changes not suitable for the other previously known larger ribotoxins, as the variants described here only presented small differences in terms of ribonucleolytic activity and cytotoxicity against cultured insect cells.

Keywords: hirsutellin A; insecticidal; ribonucleases; ribotoxins; rRNA.

Introduction

Ribotoxins are cytotoxic members of the family of fungal extracellular ribonucleases (RNases) best represented by RNase T1 (Yoshida, 2001). They have been shown to be extremely toxic because they exert their ribonucleolytic activity just on a unique phosphodiester bond of the larger molecule of rRNA in the ribosome, leading to protein synthesis inhibition and cell death (Lacadena et al., 2007). This rRNA bond is unique because it is located at an evolutionarily conserved site, the sarcin-ricin loop (SRL), with essential roles in ribosome function (García-Ortega et al., 2010) and maturation (Lo et al., 2010), and it is also the target of the family of plant ribosome-inactivating proteins (RIPs), a group of glycosidases best represented by ricin (Nielsen and Boston, 2001).

In addition to their ribonucleolytic activity, fungal ribotoxins have the ability to cross lipid membranes in the absence of any known protein receptor, mainly due to their ability to interact with acid phospholipids (Oñaderra et al., 1993; Gasset et al., 1994; Martínez-Ruiz et al., 2001). This feature is the explanation of why these proteins display a remarkable but not highly specific antitumoral activity (Jennings et al., 1965; Olson and Goerner 1965; Turnay et al., 1993; Olmo et al., 2001; Lacadena et al., 2007).

α-Sarcin is the most extensively characterized ribotoxin (Lacadena et al., 2007), but many others have been identified and/or characterized in different fungal species (Lin et al., 1995; Parente et al., 1996; Huang et al., 1997; Wirth et al., 1997; Martínez-Ruiz et al., 1999a,b; Varga and Samson 2008). Most of them show a high degree of conservation with sequence identities above 60% and even higher than 85% in many instances. The only exception known so far is hirsutellin A (HtA), another extracellular RNase produced by an invertebrate pathogen, the fungus Hirsutella thompsonii, which has been demonstrated to be a ribotoxin even though it only displays about 25% sequence identity with the previously known members of the family (Figure 1) (Boucias et al., 1998; Martínez-Ruiz...
et al., 1999b; Herrero-Galán et al., 2008, 2013; Olombrada et al., 2014a). HtA is considerably smaller than the rest of ribotoxins known but still contains the same elements of periodic secondary structure and an identical structural arrangement of the active site residues (Figure 1) (Martínez-Ruiz et al., 1999b; Herrero-Galán et al., 2008, 2012a, b; Viegas et al., 2009). HtA is indeed a well-known insecticidal protein (Liu et al., 1995; Boucias et al., 1998). Therefore, the characterization of HtA as a fungal ribotoxin not only proved that the unique abilities of ribotoxins can be accommodated into a shorter amino acid sequence (Herrero-Galán et al., 2008), but also suggested that they might play an insecticidal role in nature (Olombrada et al., 2013, 2014a). Consequently, deciphering the distinct molecular features that enable rather different proteins like α-sarcin and HtA to show this identical and singular toxic behavior is of great interest and might be useful in the design and development of new and effective biotechnological tools for different applications like new biopesticides (Olombrada et al., 2013, 2014a), the study of ribosome-related diseases (Olombrada et al., 2014b; De la Cruz et al., 2015) or the construction of new immunotoxins (Tomé-Amat et al., 2015).

The N-terminal β-hairpin of ribotoxins has been shown to modulate their catalytic activity (García-Ortega et al., 2001, 2002). Deletion variants of α-sarcin and Aspf1 (another well-known ribotoxin), in which this hairpin had been eliminated without affecting the overall three-dimensional structure of the proteins (García-Ortega et al., 2002, 2005b; García-Mayoral et al., 2004), retained their nonspecific ribonucleolytic activity as well as their ability to specifically cleave SRL-like oligonucleotides. However, these deletion mutants were not able to inactivate rabbit ribosomes with the high specificity and efficiency displayed by the wild-type proteins and, therefore, were much less cytotoxic (García-Ortega et al., 2002, 2005b; Olombrada et al., 2014b). Comparison of α-sarcin and HtA three-dimensional structures revealed major differences precisely in the sequence, length, and conformation of their N-terminal β-hairpins (Figure 1A), suggesting that an additional protein region would be required for HtA specific recognition of the ribosome. Within this idea, close inspection of these structures suggested that this function could be assisted by the presence of a cluster of Lys residues along HtA loop 5 (Figure 1B) (Viegas et al., 2009; Herrero-Galán et al., 2012a). Therefore, and in order to investigate this matter, the work presented here deals with the production and characterization of a deletion mutant version of HtA, where amino acids 8 to 15 (PKLDGREK) were substituted by two Gly residues [Δ(8-15)], and three single mutants affecting the mentioned Lys residues, which were changed to Glu (K115E, K118E, and K123E).

Results

Protein purification and spectroscopic characterization

All four HtA mutants studied were purified to homogeneity according to their SDS-PAGE behavior. Amino acid analyses were in good agreement with the mutation produced. These analysis and the corresponding
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Table 1: Some features of the purified proteins.

<table>
<thead>
<tr>
<th>HtA variant</th>
<th>Purification yield</th>
<th>(E_{0.1%}^{280\text{ nm}, 0.1\text{ cm}})</th>
<th>(T_m (\text{°C}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.00</td>
<td>2.00</td>
<td>62.0</td>
</tr>
<tr>
<td>Δ(8-15)</td>
<td>0.50</td>
<td>2.00</td>
<td>50.0</td>
</tr>
<tr>
<td>K115E</td>
<td>0.36</td>
<td>2.01</td>
<td>57.0</td>
</tr>
<tr>
<td>K118E</td>
<td>0.60</td>
<td>1.87</td>
<td>59.0</td>
</tr>
<tr>
<td>K123E</td>
<td>0.45</td>
<td>1.90</td>
<td>58.5</td>
</tr>
</tbody>
</table>

*aMilligrams of protein obtained per liter of original culture.

*bMid-point temperature of the thermal denaturation transition measured as the ellipticity value variation at 215 nm as a function of temperature (Lacadena et al., 1995, 1999; García-Mayoral et al., 2006; Herrero-Galán et al., 2008).

UV-absorption spectra were used to calculate their \(E_{0.1\%}^{280\text{ nm}, 1\text{ cm}}\) values (Table 1). These values were very similar for all of them in accordance with the fact that there were neither Trp nor Tyr residues among the mutated amino acids. Furthermore, this invariance of the \(E_{0.1\%}^{280\text{ nm}}\) values suggested a conservation of their three-dimensional structures. In agreement with this observation, the far-UV CD spectra of the four mutant proteins (Figure 2), as well as their fluorescence emission spectra (Figure 3), showed only very minor changes. Higher differences were observed in terms of thermostability (Table 1). The Δ(8-15) protein showed a decrease of 12°C in its \(T_m\) value, and the Lys to Glu mutants displayed reductions of only 3–5 degrees respect to the wild-type HtA (Table 1). Nevertheless, the functional differences observed for these proteins should not be attributed to the lower thermostability of the mutants, since the \(T_m\) values were far above the temperature values used in the experiments herein described.

Ribonucleolytic activity against eukaryotic ribosomes

The natural optimum substrates of ribotoxins are eukaryotic ribosomes. Accordingly, the enzymatic characterization of both wild-type and mutant proteins was first performed following the release of the characteristic rRNA α-fragment resulting from their cleaving activity on the larger ribosomal subunit of ribosomes of a cell-free rabbit reticulocyte lysate. As it can be observed in Figure 4A, although all mutant proteins assayed retained this specific ribonucleolytic activity against rabbit ribosomes, it was slightly impaired when compared to the wild-type protein (Figure 4B). This effect was especially evident for the K123E and Δ(8-15) variants which showed much lower activity values at the lower enzyme concentrations employed.

![Figure 2: Far-UV circular dichroism spectra of wild type HtA (black dots) and its different mutants (white dots): K115E (A), K118E, (B), K123E (C), and Δ(8-15) (D).](image-url)

The line with black squares represents the calculated difference spectra wild-type minus mutant. Circular dichroism values are expressed as mean residue weight ellipticities (\(\theta_{MRW}\)) in units of \(\text{deg} \times \text{cm}^2 \times \text{dmol}^{-1}\).
Ribonucleolytic activity against an SRL-like oligonucleotide

Short oligoribonucleotides mimicking the SRL sequence and structure (SRL-like oligos) are frequently used to evaluate the influence of other ribosomal elements on ribotoxins activity. Thus, although ribotoxins cleave these SRL-like oligos specifically, producing only two smaller fragments which can be fractionated on a polyacrylamide gel, this activity is several orders of magnitude less efficient than that one produced on intact ribosomes (Endo et al., 1988; Wool et al., 1992; Glück and Wool, 1996; Wool, 1997; Kao et al., 2001). It is assumed that this is due to the absence of additional interactions with specific ribosomal regions such as some large subunit conserved proteins (García-Mayoral et al., 2005) as well as the contribution of electrostatic interactions with the complete ribosome (Korennykh et al., 2007). In this occasion, and in good accordance with the assays using intact ribosomes described above, all mutants studied also retained the ability to specifically cleave the SRL-like oligo employed (Figure 5A). In fact, the K115E variant displayed a cleaving pattern which was practically indistinguishable from that shown by wild-type HtA. K123E and Δ(8-15) were also the least efficient variants, showing again much lower activity values than wild-type HtA (Figure 5B) at the lower protein concentrations assayed.

Figure 3: Fluorescence emission spectra of wild-type HtA and the mutant variants studied. All spectra were recorded at identical protein concentrations. Spectra were recorded using 275 (continuous lines) and 295 (dotted lines) nm as excitation wavelengths. These spectra were normalized at wavelengths above 380 nm to obtain the tryptophan contribution (dashed lines). Finally, tyrosine contribution was calculated as described in the Materials and Methods section (dashed-dotted lines). Fluorescence emission units were arbitrary, and referred to the maximum value of wild-type HtA upon excitation at 275 nm. The table shows the relative fluorescence Tyr or Trp quantum yield for excitation at 275 nm to that of wild-type HtA considered as 1.00.

<table>
<thead>
<tr>
<th>HtA variant</th>
<th>Q_{TY}</th>
<th>Q_{TP}</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Δ(8–15)</td>
<td>1.15</td>
<td>0.89</td>
</tr>
<tr>
<td>K115E</td>
<td>0.89</td>
<td>1.02</td>
</tr>
<tr>
<td>K118E</td>
<td>1.49</td>
<td>0.98</td>
</tr>
<tr>
<td>K123E</td>
<td>1.98</td>
<td>1.01</td>
</tr>
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</table>
Toxic effect on insect cell lines in culture

It is well known the dramatic effect of HtA on the inhibition of *in vivo* Spodoptera frugiperda cells protein biosynthesis (Herrero-Galán et al., 2013; Olombrada et al., 2013; Castaño-Rodríguez et al., 2015). Therefore, this insect cellular line was chosen to evaluate the toxic effect of the mutants studied when employed against intact cells. In good accordance with the previous results shown above, K115E and K118E showed very similar insecticidal behavior as wild-type HtA, at least in terms of their ability to inhibit protein biosynthesis in *S. frugiperda* cultured cells (Figure 6). On the other hand, K123E and Δ(8-15) showed around a ten-fold increase in the value of protein concentration needed to produce a 50% protein biosynthesis inhibition (Figure 6). That is to say, these two mutants displayed lower cytotoxic activity against insect cells in comparison with the behavior shown by wild-type HtA.

Discussion

RNase T1 is not only one of the most exhaustively characterized proteins but also the best known member of the family of fungal extracellular RNases (Loverix and Steyaert 2001; Yoshida, 2001), a group that includes ribotoxins (Martínez-Ruiz et al., 2001; Lacadena et al., 2007). All members of this family, ribotoxins included, share a high degree of sequence identity (Rodríguez et al., 1982; Sacco et al., 1983; López-Otín et al., 1984; Fernández-Luna et al., 1985; Arruda et al., 1990; Wirth et al., 1997; Martínez-Ruiz et al., 1999a,b) and a common structural fold concerning the architecture and connectivity of the regular secondary structure elements (Campos-Olivas et al., 1996; Yang and Moffat, 1996; Martínez-Ruiz et al., 2001; Pérez-Cañadillas et al., 2000; Viegas et al., 2009) and the geometric arrangement of the residues involved in the active site (Pérez-Cañadillas et al., 2000; Martínez-Ruiz et al., 2001;
However, only ribotoxins show cytotoxic properties. Non-toxic fungal extracellular RNases are smaller than ribotoxins, lack a well-defined N-terminal β-hairpin, and display much shorter and negatively charged unstructured loops (Pace et al., 1991; Lacadena et al., 2007). Accordingly, it was predicted that these structural differences would account for the cytotoxic properties of ribotoxins (Martínez-del-Pozo et al., 1988).

These predictions, which were formulated at the early stages of the study of ribotoxins (Lamy et al., 1992; Mancheño et al., 1995; Martínez-del-Pozo et al., 1988), have been confirmed since then (Campos-Olivas et al., 1996; García-Ortega et al., 2001, 2002, 2005b; García-Mayoral et al., 2004, 2005). For example, the N-terminal β-hairpins of α-sarcin, mitogillin, restrictocin, and Aspf1 (four well characterized ribotoxins) modulate their catalytic activity (Kao and Davies, 1999, 2000; García-Ortega et al., 2001, 2002, 2005b). These studies included deletion variants in which the hairpin had been eliminated without affecting the overall three-dimensional structure of the proteins (García-Ortega et al., 2002, 2005b; García-Mayoral et al., 2004). Deletion mutants [α-sarcin Δ(7-22) and AspFl Δ(7-22)] which retained their non-specific ribonucleolytic activity as well as their ability to cleave SRL-like oligonucleotides, but were not able to specifically inactivate rabbit ribosomes. Therefore, they were much less cytotoxic (García-Ortega et al., 2002, 2005b). Docking studies revealed that this N-terminal β-hairpin could establish interactions with ribosomal proteins in order to direct the ribotoxin to the SRL region of the ribosome (García-Mayoral et al., 2005).

HtA was discovered and characterized as a new and singular fungal ribotoxin (Liu et al., 1995; Boucias et al., 2007).
In the two deletion mutants previously studied, α-sarcin Δ(7-22) and Aspf1 Δ(7-22), the two proteins did not recognize the SRL within the context of intact ribosomes under the assay conditions employed. However, they retained their ribonucleolytic activity against less specific substrates such as SRL-like oligonucleotides (García-Ortega et al., 2002, 2005b). The results presented now reveal that the four HtA mutants studied showed a decreased ability to generate the α-fragment when assayed against rabbit ribosomes, but all they still retained the specificity (Figure 4). In this case, however, the results obtained with the 35 mer SRL-like oligonucleotide assays were very similar (Figure 5). Overall, these results suggest that for HtA not only the N-terminal β-hairpin but also loop 5 residues are involved in ribosome recognition and that, as predicted, loop 5 would contribute to specificity since a missing N-terminal β-hairpin does not render a non-specific RNase.

It has been also proposed that the enzymatic efficiency of α-sarcin is dependent on the interactions between its catalytic His137, loop 5 residues, and the N-terminal β-hairpin (Pérez-Cañadillas et al., 2000; Masip et al., 2001; Álvarez-García et al., 2009a). After more than two decades of systematic studies, it has also become well established that the activity of ribotoxins is extremely dependent on electrostatic interactions among their active site residues (Pérez-Cañadillas et al., 1998, 2000; Masip et al., 2001; Álvarez-García et al., 2006). His 42, Glu 66 and His 113 form the catalytic triad of HtA (Figure 1), in good accordance with many other fungal ribonucleases, toxic or not (Lacadena et al., 1995, 1998, 1999, 2007;
Yoshida, 2001). However, single mutants of this catalytic triad of HtA (Herrero-Galán et al., 2012a) maintained to some extent the ability to degrade eukaryotic ribosomes, so none of these three residues is strictly essential for the ribonucleolytic activity of this protein on this substrate (Herrero-Galán et al., 2012a). On the other hand, none of the single mutants of the catalytic triad of HtA was able to cleave the synthetic SRL (Herrero-Galán et al., 2012a). These results showed the importance of a microstructural environment more than the presence of a particular individual residue in the activity of HtA. The results presented now with this new set of mutants are in perfect agreement with this hypothesis, and suggest that this microenvironment is influenced by interactions beyond the active site. Overall, HtA seems to be a more adaptable enzyme, with the ability to accommodate changes which would not be suitable for the larger ribotoxins characterized before. In this regard, it could be considered a better fitted enzyme for the purpose of inactivating ribosomes in more variable environments.

In addition to their ribonucleolytic activity, ribotoxins cross lipid membranes, showing a cytotoxic behavior against different types of cells. Of special relevance is their toxic effect against insect cells, an evidence of their potential insecticidal behavior in nature (Olombrada et al., 2013, 2014a). The results reported now would be in agreement with the observation that HtA Lys 115 and 118 residues do not play a major role in recognizing the ribosome (Figures 4 and 5) supporting their unaltered cytotoxic activity (Figure 6). On the other hand, K123E and Δ(8-15) showed a significantly impaired cytotoxic activity (Figure 6) which also correlated with the detrimental ribonucleolytic activities that they displayed against ribosomes and the SRL-like oligonucleotide (Figures 4 and 5). For these two mutants, we cannot discard however a role for the residues mutated in lipid interaction and passage across cell membranes as an additional explanation of their lower cytotoxicity. In fact, it has been reported before how α-sarcin Lys 14 and 21, with identical amino acids in equivalent positions of HtA (Figure 1), are crucial residues for the correct achievement of these interactions (Álvarez-García et al., 2009b).

In summary, the results obtained support the general conclusion that, in spite of its smaller size, HtA active site would be highly adaptable, accommodating changes which would not be suitable for the larger ribotoxins characterized before. This flexibility makes HtA active site susceptible to intramolecular interactions with the N-terminal β-hairpin and loop 5. In addition, the hydrophobic interaction between Leu 10 and Val 116 seems to be crucial for the high protein thermostability. Finally, not only the N-terminal β-hairpin but also Lys123 in loop 5 play an important role for this protein to exert its insecticidal action on cultured insect cells.

Materials and methods

DNA manipulation

All materials and reagents were of molecular biology grade. Cloning procedures, PCR-based oligonucleotide site-directed mutagenesis, and bacterial manipulations were carried out as previously described (Lacadena et al., 1994; Martínez-Ruíz et al., 2001; Álvarez-García et al., 2006; Herrero-Galán et al., 2012a,b; Castaño-Rodríguez et al., 2015). Mutagenesis constructions were performed using different sets of complementary mutagenic primers (Supplementary Table S1). Mutations were confirmed by DNA sequencing at the corresponding Complutense University facility. The plasmid used as template for mutagenesis, containing the cDNA sequence of wild-type HtA, had already been described (Herrero-Galán et al., 2008, 2012a,b, 2013).

Protein production and purification

Production and purification of HtA mutants was carried out as previously described for the wild-type recombinant protein (Herrero-Galán et al., 2008). Briefly, plasmid pTacTacHtA was used for protein production in Escherichia coli BL21 (DE3) cells, induced with 1 mM IPTG at 37°C for 4 h. Purification using a low percentage Ni²⁺ nitrilotriacetic acid agarose affinity column allowed elution of the different mutants with 10 mM MOPS buffer (pH 7.8), containing 200 mM imidazole. SDS-PAGE analysis, protein hydrolysis, and amino acid analysis were performed according to standard procedures, also as previously described (Lacadena et al., 1994; Martínez-Ruíz et al., 2001).

Spectroscopic characterization

Spectroscopic characterization was performed following well-established procedures (Lacadena et al., 1999; García-Ortega et al., 2001, 2002, 2005a; Martínez-Ruíz et al., 2001; Álvarez-García et al., 2006, 2009b). Absorbance measurements were carried out on a Beckman DU640 spectrophotometer (Beckman Coulter, Brea, CA, USA) at 260 nm/min scanning speed and room temperature. Amino acid analyses and the corresponding UV-absorbance spectra were also used to calculate their extinction coefficients (Table 1). Circular dichroism spectra were obtained in a Jasco 715 spectropolarimeter (Jasco, Easton, MD, USA), equipped with a thermostated cell holder and a Neslab-111 circulating water bath, at 0.2 nm/s scanning speed. Thermal denaturation profiles were obtained by measuring the temperature dependence of the ellipticity at 215 nm in the 25–80°C range using a rate of temperature increment of 30°C per hour. Fluorescence emission spectra were recorded on an SLM Aminco8000 spectrofluorimeter at 25°C using a slit width of 4 nm for both excitation and emission beams. The spectra were recorded for excitation at 275 and 295 nm and both were normalized by considering
that Tyr emission above 380 nm is negligible. The Tyr contribution was calculated as the difference between the two normalized spectra. Thermostated cells with a path length of 0.2 and 1.0 cm for the excitation and emission beams, respectively, were used. All these experiments were performed in 50 mM sodium phosphate, pH 7.0, containing 0.1 M NaOH.

Ribonucleolytic activity assays

All procedures were carried out using RNase-free materials and reagents. The specific ribonucleolytic activity of ribotoxins is usually detected by the release of a 400-nt fragment (α-fragment) from eukaryotic ribosomes (Schindler and Davies 1977). Therefore, HtA and its mutants were assayed against ribosomes contained in a rabbit cell-free reticulocyte lysate (Kao et al., 2001). After a 15 min incubation at room temperature of the sample with 150 nm of the different proteins in 23 mM Tris-HCl, 23 mM KCl, 6 mM EDTA pH 7.5, RNA was phenol-extracted, precipitated with isopropanol and visualized by ethidium bromide staining after electrophoresis on denaturing 2.4% agarose gels as described (Lacadena et al., 1994, 1999; Herrero-Galán et al., 2008).

Ribotoxin cleavage was quantitated by poison primer extension (PPE) as described (García-Ortega et al., 2010; Olombrada et al., 2014b). Reverse transcription where dATP is substituted by ddATP was performed using the complementary sequence downstream the SRL in the 28S rRNA of Oryctolagus cuniculus (5′-ACC AAA TGT CTG AAC CTT GGG-3′). The products of this reverse transcription were then separated in a denaturing 10% polyacrylamide gel and the amount of [32P] present in each one of the DNA bands produced was quantitated using a PhosphorImager screen (Molecular Dynamics).

Finally, cleavage of a synthetic oligonucleotide that mimics the sequence and structure of the SRL was also measured for HtA and its mutant variants. Synthesis of this SRL-like 35mer RNA was performed as previously described (Kao et al., 2001; García-Ortega et al., 2010). SRL (2 μM) was incubated with 2-100 nm protein for 15 min at 37°C in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM MgCl2. Reaction products were run on a denaturing 19% (w/v) polyacrylamide gel and visualized by ethidium bromide staining.

Insect cell culture and toxicity assays

The insect cell line S. frugiperda (SF9) was cultured at 27°C as described (Olombrada et al., 2013; Castaño-Rodríguez et al., 2015) in Insect-XPRESS™ Protein-free Insect Cell medium (BioWhittaker) as indicated by the manufacturer. Protein solutions were prepared in culture medium and sterilized by ultrafiltration. Protein biosynthesis assays were carried out by seeding SF9 cells into 24-well plates at a cell density of 10^5 cells per well, maintaining them under standard culture conditions up to 80% confluency. Then, monolayer cultures were incubated in 0.5 ml of fresh medium with serial dilutions of ribotoxin from 5.0 μM to 0.5 nm final concentrations. Following 18 h of incubation at 27°C medium was replaced by culture medium supplemented with 0.5 μCi/well of [3H]-leucine. After 5 h of incubation medium was removed and cell protein content was precipitated with 5% trichloroacetic acid 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 ribotoxin added.

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References


Martínez-Ruiz, A., Kao, R., Davies, J., and Martínez-del-Pozo, A. (1999a). Ribotoxins are a more widespread group of proteins within the filamentous fungi than previously believed. Toxicon 37, 1549–1563.


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