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Minimized natural versions of fungal ribotoxins show improved active site plasticity

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ABSTRACT

Fungal ribotoxins are highly specific extracellular RNases which cleave a single phosphodiester bond at the ribosomal sarcin-ricin loop, inhibiting protein biosynthesis by interfering with elongation factors. Most ribotoxins show high degree of conservation, with similar sizes and amino acid sequence identities above 85%. Only two exceptions are known: hirsutellin A and anisoplin, produced by the entomopathogenic fungi *Hirsutella thompsonii* and *Metarhizium anisopliae*, respectively. Both proteins are similar but smaller than the other known ribotoxins (130 vs 150 amino acids), displaying only about 25% sequence identity with them. They can be considered minimized natural versions of their larger counterparts, best represented by α-sarcin. The conserved α-sarcin active site residue Tyr48 has been replaced by the geometrically equivalent Asp, present in the minimized ribotoxins, to produce and characterize the corresponding mutant. As a control, the inverse anisoplin mutant (D43Y) has been also studied. The results show how the smaller versions of ribotoxins represent an optimum compromise among conformational freedom, stability, specificity, and active-site plasticity which allow these toxic proteins to accommodate the characteristic abilities of ribotoxins into a shorter amino acid sequence and more stable structure of intermediate size between that of other nontoxic fungal RNases and previously known larger ribotoxins.

Fungal ribotoxins are a unique group of highly specific extracellular RNases [1,2] initially discovered as antitumoral agents [3]. The toxicity of these ribotoxins relies on their ability to cleave a singular phosphodiester bond strategically located at a universally conserved sequence of the large rRNA [4], known as the sarcin-ricin loop (SRL). Therefore, ribosomes are their natural substrates. Cleavage of this single bond inhibits protein biosynthesis, interfering with the function of elongation factors [5–7] and leading to cell death by apoptosis [8]. Given the universal conservation of the SRL sequence, all known ribosomes are susceptible to ribotoxins action. However, these toxic RNases show different affinity against ribosomes from different origins and, above all, they first have to cross the plasmatic membrane to be able to cleave their substrate and exert their cytotoxic activity [9–11]. This behavior explains why not all cells are equally targeted by these toxins. Intriguingly, they are especially active on transformed or virus-infected cells [3,8,12] due to an altered permeability of their membrane in combination with an enrichment in acidic phospholipids [8,11,13–15]. This feature has been lately related to the possibility of ribotoxins acting as natural insecticidal agents [16–18].

α-Sarcin, restrictocin, Aspf1, and hirsutellin A (HtA) are the most exhaustively characterized ribotoxins [10,11,19–26], but many others have been identified and partially characterized in different fungal species [27–35]. Most of them show a high degree of conservation, with similar sizes and amino acid sequence identities above 85% [1,18,33]. So far only two exceptions are known: HtA [23,24,32] and the recently discovered anisoplin [35], produced by the entomopathogenic fungi *Hirsutella thompsonii* and *Metarhizium*

Abbreviations: CD, circular dichroism; HtA, Hirsutellin A; PDB, Protein Data Bank; SEM, standard error of the mean; SRL, sarcin-ricin loop; WT, wild-type; T_m, melting temperature.

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anisopliae, respectively. Both proteins are highly similar but smaller than the other known ribotoxins (130 residues vs 150), displaying only about 25% sequence identity with them [23,24,32,35]. Therefore, they can be considered as minimized natural versions of their larger counterparts. This lower sequence identity and size do not however preclude the conservation of the elements of ordered secondary structure as well as the identity and geometric arrangement of the residues configuring the active site (Fig. 1). This structural conservation can be even extended to the other non-toxic members of the larger fungal extracellular RNases family, such as RNase T1 and RNase U2, for example [36,37]. In fact, comparison of all these RNases three-dimensional structures reveals the strict conservation of the active site residues forming their catalytic triad, His50, Glu96, and His137 in α-sarcin [38], for example, as well as the preservation of a highly hydrophobic residue at the position corresponding to α-sarcin's Leu145 [39] (Fig. 1). On the other hand, the presence of an Asp residue in HtA [40] and anisoplin [35] in a position equivalent to α-sarcin Tyr48 [41] must be highlighted as a novelty in the active site of this family of toxic RNases (Fig. 1). This is a quite intriguing observation in the context that substitution of this Tyr48 by Phe rendered a α-sarcin variant which was catalytically incompetent, unable to inactivate the ribosome [41]. Interestingly, studies with HtA mutants D40N and D40N/E66Q demonstrated an important role for Asp40 in the activity of HtA in establishing a new set of electrostatic interactions different from the one described for the already known larger ribotoxins [40].

In the work herein presented α-sarcin Tyr48 has been replaced by an Asp residue to produce and characterize the corresponding Y48D mutant. As a control, the corresponding inverse anisoplin mutant (D43Y) has been also studied. The results shown reveal the key role of these residues not only in maintaining the correct electrostatic environment and active site plasticity in each type of ribotoxins but also in the preservation of their characteristic high thermostability.

1. Materials and methods

1.1. Mutant cDNA construction

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 [11,16,41–43]. Mutagenesis constructions were performed using different sets of complementary mutagenic primers (Table S1). Mutations were confirmed by DNA sequencing at the corresponding Complutense University facility. The plasmids used as templates for mutagenesis, containing the cDNA sequence of either wild-type α-sarcin or anisoplin, have already been described [35,38,42].

1.2. Protein production and purification

Escherichia coli RB791 or BL21 (DE3) cells, the latter ones being

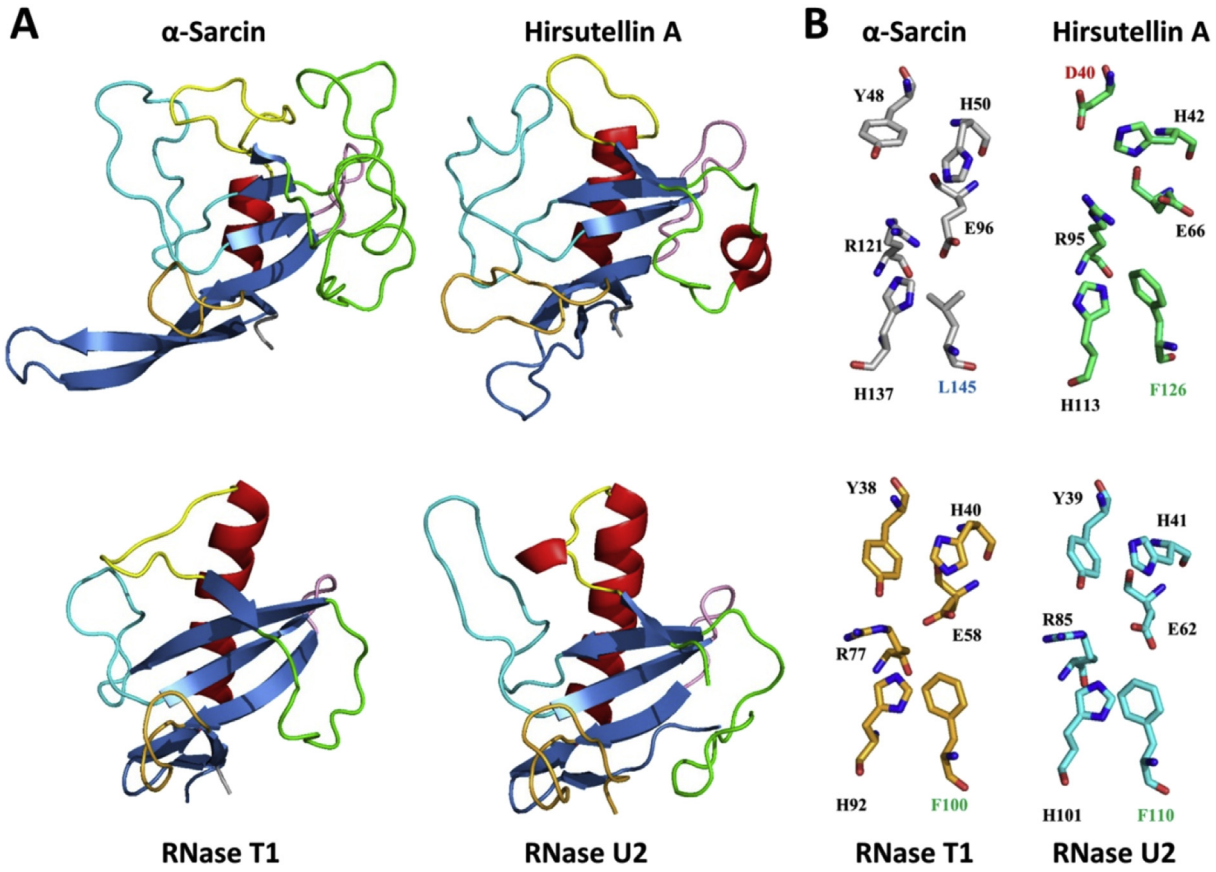

Fig. 1. Representation of the three-dimensional structure and active center geometric arrangement of representative fungal RNases. (A) Diagrams showing the three-dimensional structure of ribotoxins α-sarcin (PDB ID: 1DE3) [63] and HtA (PDB ID: 2KAA) [26], and two non-toxic fungal extracellular RNases from the same family: RNases T1 (PDB ID:9RNT) [36,66] and U2 (PDB ID:1RTU) [37,67,68]. (B) Geometric arrangement of the active site residues of these same four RNases. The catalytic triad made of two His and one Glu residues is conserved in all proteins shown while a fourth residue, the equivalent to α-sarcin Leu145, maintains its highly hydrophobic character (Phe or Leu). The position corresponding to α-sarcin Tyr48 is also conserved except for HtA and anisoplin (not shown) where the equivalent position is occupied by an Asp residue (D40, in red). Diagrams were generated using the PyMol software [69]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)