Ribotoxins ribonucleolytic activity is not essential for in vitro protein biosynthesis inhibition

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Highlights:

1. Ribotoxins inactivate ribosomes by cleaving a conserved single phosphodiester bond.
2. Ribosome inactivation leads to protein biosynthesis inhibition and cell death.
3. Catalytically inactive α-sarcin mutant H137Q inhibits *in vitro* protein synthesis.
4. Wild-type and H137Q α-sarcin bind to and cosediment with ribosomes.
5. Ribotoxins' binding to ribosomes seems to suffice for impairing protein synthesis.

The ribonucleolytic activity of the ribotoxin α-sarcin is not essential for *in vitro* protein biosynthesis inhibition

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ABSTRACT

Fungal ribotoxins are toxic secreted ribonucleases that cleave a conserved single phosphodiester bond located at the sarcin/ricin loop of the larger rRNA. This cleavage inactivates ribosomes leading to protein biosynthesis inhibition and cell death. It has been proposed that interactions other than those found at the active site of ribotoxins are needed to explain their exquisite specific activity. The study presented shows the ability of a catalytically inactive α-sarcin mutant (H137Q) to bind eukaryotic ribosomes and interfere with *in vitro* protein biosynthesis. The results obtained are compatible with previous observations that α-sarcin can promote cell death by a mechanism that is independent of rRNA cleavage, expanding the potential set of activities performed by this family of toxins.

Keywords: ribotoxin; restrictocin; ribosome; protein-synthesis.

Abbreviations: DEPC, Diethyl pyrocarbonate; H137Q, a mutant version of α-sarcin where His-137 has been substituted by Gln; PMSF, phenylmethylsulfonyl fluoride; polyPhe, polyphenylalanine; RNase, ribonuclease; SRL, sarcin/ricin loop; YPD, 1.0 % yeast-extract-2.0 % peptone-2.0 % dextrose medium.

1. Introduction

Ribotoxins are a family of cytotoxic secreted fungal RNases, best represented by α-sarcin [1]. These proteins cleave a single phosphodiester bond of the larger molecule of rRNA. This bond is unique because it is located at a conserved site, known as the sarcin/ricin loop (SRL), with important roles in ribosome function. Thanks to the pioneering work of Ramakrishnan and colleagues the role of this SRL in GTPase activation of elongation factors is now known in good detail [2]. The atomic-resolution of a ternary complex (EF-Tu●GTP●tRNA) structure revealed that codon recognition leads to a series of conformational changes, needed for GTP hydrolysis, that position a conserved EF-Tu histidine by the *E. coli* SRL residue A2662. [2]. According to these authors, this GTPase activation by the SRL would be a universal mechanism for triggering elongation factors GTP hydrolysis. Consequently, cleavage of SRL impairs elongation factors function leading to protein biosynthesis inhibition and cell death by apoptosis [1,3-7]. However, it has been also recently shown how the SRL is more critical for EF-G than ternary complex binding to the ribosome, implicating additional different requirements in this region of the ribosome during protein biosynthesis elongation [7]. Finally, it is also remarkable that ribotoxins are especially active on transformed or virus-infected cells [6,8,9], what has been related to their ability to interact with acid phospholipid-containing membranes [1,3,5,6,10-12].

The catalytic mechanism of α-sarcin ribonucleolytic action has been studied in detail [13-19] showing that it is a cyclizing RNase [14]. Cleavage of its target phosphodiester bond occurs through a reaction which involves a general base/general acid pair (Glu-96 and His-137, respectively) and an electrostatic catalyst (His-50) [13,15]. Mutagenesis studies have also revealed the involvement in catalysis of some other residues conserved in ribotoxins [16-19], but only His137 and Glu96 seem to be essential for performing the acid-base type reaction [13,15,20-25].

However, the interactions established between the active site of ribotoxins and their rRNA target, the SRL, do not seem to be enough to explain their exquisite specificity. Additional interactions with other ribosomal elements have been proposed to be required [26-31]. Results obtained with a deletion mutant suggested that, in addition to the SRL rRNA sequence, α-sarcin would

recognize at least two more ribosomal regions involving eukaryotic proteins L9 and L23 [26,27,30].

Substitution of His-137 by a Gln residue renders a properly folded ribotoxin variant (α-sarcin H137Q) devoid of ribonucleolytic and cytotoxic activity but still retaining its ability to interact with phospholipid membranes [6,13,15]. This mutant shows the wild-type protein fold [15,32,33], including the spatial general arrangement of the loops and the amino-terminal β-hairpin protrusion, the structural elements potentially involved in binding to ribosomal proteins L9 and L23 [26,27]. Thus, although catalytically inactive, the H137Q α-sarcin mutant could specifically recognize the eukaryotic ribosomes. Furthermore, it has been recently published how intracellular *in vivo* induction of this mutant results in cell death by a mechanism that it is independent of rRNA cleavage [34]. According to these authors' results, wild-type and H137Q α-sarcin colocalize with ribosomal marker RP56 in the nucleus and cytoplasm [34]. In the present work we report how *in vitro* this α-sarcin variant can bind to ~~the~~ ribosomes interfering with protein biosynthesis.

2. Materials and Methods

2.1 Proteins production and purification

E. coli BL21 (DE3) cells previously cotransformed with a thioredoxin-producing plasmid (pT-Trx) and the corresponding α-sarcin H137Q plasmid (pINPGαSH137Q) were used to produce the mutant, as previously described [13,15,35,36]. Fungal natural wild-type α-sarcin was obtained as reported before [5]. SDS-PAGE of proteins, Western blots, protein hydrolysis, and amino acid analysis were performed according to standard procedures [5,35]. Identical wild-type or H137Q mutant protein batches were employed for all the experiments described in this work.

2.2 Ribonucleolytic activity assays

The ribonucleolytic activity of α-sarcin on rabbit ribosomes was followed by detecting the release of a specific 400-nt α-fragment from a cell-free rabbit reticulocyte lysate (Promega) as described [4]. With this purpose, the lysate was previously two-fold diluted with 40 mM Tris-HCl, pH 7.5, containing 40 mM

KCl and 10 mM EDTA. Then, 50 µl aliquots of this dilution (containing 5-6 pmol of ribosomes approximately) were incubated for 15 min at room temperature with different concentrations of the natural wild-type and mutant versions of α-sarcin. The reaction was stopped by addition of 250 µl of 50 mM Tris-HCl, pH 7.4, containing 0.5% (w/v) SDS followed by strong vortexing. RNA phenol/chloroform extraction was then performed and the RNA was precipitated from the aqueous phase by addition of isopropanol. The resulting pellet was washed with 70% (v/v) ethanol, dried and finally resuspended in 10 µl of 0.5% (w/v) SDS. Production of the 400-nt α-fragment was visualized by ethidium bromide staining after electrophoresis of these samples, previously heated at 90ºC for 5 min, on denaturing 2.4% agarose gels. The absence of contaminating non-specific RNase-like activities in the protein preparations employed was ruled out in all protein batches employed by means of a zymogram assay against poly(A) [4,13,15,35].

2.3 Ribosome binding and purification

Aliquots of the same diluted reticulocyte lysate mentioned above were also incubated in identical conditions, with or without the proteins, and used to isolate and purify tight coupled 80S ribosomes by means of a sucrose density gradient performed essentially as described [7,37,38]. Accordingly, the cellular lysate was first centrifuged at 45000 rpm and 4ºC for 2h in a Beckman SW65 rotor containing 4 ml of 10 mM HCl-Tris, pH 7.4, 80 mM KCl, 12.5 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 0.2 mM PMSF (phenylmethylsulfonyl fluoride), supplemented with a commercial protease inhibitors cocktail (Roche) (Buffer A). The pellet obtained was resuspended in 75 µl of ice-cold 20 mM HCl-Tris, pH 7.4, 0.5 M ammonium acetate, 0.1 M $MgCl_2$, 5 mM β-mercaptoethanol (Buffer B), supplemented with 0.2 mM PMSF and the inhibitors cocktail (Roche), and centrifuged again on a discontinuous sucrose gradient (20/40%) in buffer B at 45000 rpm and 4ºC for 16h in a Beckman SW65 rotor. The resulting ribosome pellets were resuspended in 20 µl of standard loading buffer and analyzed by SDS-PAGE and Western Immunoblot analysis using a rabbit antiserum risen against wild-type α-sarcin. The ribosome concentration in the pellets was estimated spectrophotometrically before loading onto the gel, while the amount of ribotoxin sedimented was calculated from the volumograms of the specific

Western blot bands (based on integration of the pixel intensities composing the spot) obtained with the photo documentation system UVI-Tec (Cambridge, UK) and the software facility UVIsoft UVI band Windows Application V97.04 [26]. These data were used to estimate the ribosome-ribotoxin dissociation constant (K_d). Protein sedimentation was not observed when either the wild-type α-sarcin or the H137Q mutant were subjected to identical treatment in the absence of ribosomes.

2.4 In vitro protein translation

In vitro protein translation was analyzed essentially as described [39] using the Flexi Rabbit Reticulocyte Lysate System (Promega). Each reaction tube contained 7 µl of the mentioned lysate, 0.2 µl of the supplied amino acid mixture (without methionine), 0.4 µl of ^{35}S-methionine (Promega, specific activity >1000 Ci/mmol, concentration 14.3 mCi/ml), 0.3 µl of 2.5 M KCl, 0.2 µl of 40 U/µl RNasin (Promega, as inhibitor of potential non-specific RNases) and 0.4 µl of sterile DEPC-water. The reaction was started by adding 0.5 µl of the provided luciferase mRNA, previously diluted 2.5-fold with sterile DEPC-water, to this 8.5 µl mixture. Incubations were carried out, in the absence or in the presence of different α-sarcin concentrations, for 90 min at 30ºC and then stopped by boiling the samples after the addition of the corresponding amount of a standard SDS-PAGE loading buffer. Aliquots of 10 µl were finally loaded onto 0.1% (w/v) SDS – 10% polyacrylamide gels and subjected to electrophoretic fractionation. The amount and position of the labelled luciferase produced was analyzed by autoradiography.

2.5 Inhibition of in vitro polyPhe synthesis

S. cerevisiae S30 fraction was used as the source of ribosomes and additional translation factors for this assay. For this preparation, W303 *S. cerevisiae* cells were grown in 200 mL of YPD medium up to an OD = 0.3. After harvesting, cells were resuspended in 400 µl of 10 mM Tris-HCl pH 7.4, 80 mM KCl, 12.5 mM $MgCl_2$ and 5 mM β-mercaptoethanol supplemented with a protease inhibitors cocktail and 0.2 mM PMSF, and disrupted by vigorous shaking with glass beads. The S30 supernatant fraction was obtained by centrifugation of this extract at 20,000 rpm for 30 min at 4ºC in a Beckman TLA

120.1 rotor. Concentration of the supernatant was performed in NANOSEP 3K MWCO (Pall Corporation) down to an approximate volume of 100 µL (100 absorbance units at 260 nm). Aliquots were frozen in liquid nitrogen and kept at -80ºC.

PolyPhe synthesis reaction was performed in a total volume of 25 µL containing 2 µL of S30, 0.1 mg/mL polyuridylic acid, 50 µg/mL $tRNA^{Phe}$ (from brewer's yeast, Sigma), 20 µM Phe, 0.5 µM [^{3}H]-Phe (25 µCi/pmol), 0.5 mM GTP, 1 mM ATP, 4 mM phosphocreatine and 25 µg/mL creatine phosphokinase in a buffer solution of 30 mM HEPES-HCl pH 7.4, 5 mM $MgCl_2$, 50 mM KCl and 2 mM β-mercaptoethanol. Immediately after mixing all these components, 2.5 µl of the desired ribotoxin concentration were added. Samples were incubated for 30 min. at 30ºC. The reaction was stopped by addition of 1 ml of 5% trichloroacetic acid and heated at 90ºC for 15 min. After spinning down the precipitate and washing it with ice-cold ethanol, it was dissolved in 100 µl of 0.1 M NaOH, 0.1% SDS and ^{3}H counted in a Beckman LS3801 scintillation counter. Data were presented as percentage of polyPhe synthesis, being 100% the value corresponding to the sample with no ribotoxin added.

3. Results

The standard assay used to monitor the specific enzymatic activity of α-sarcin relies in the use of a cell-free rabbit reticulocyte lysate where the ribosomes constitute the substrate [1,4,5]. The enzymatic action of ribotoxins releases a 400 nt rRNA fragment (α-fragment) resulting from the specific cleavage of a single phosphodiester bond located at the SRL. Thus, while the wild-type protein released this α-fragment (Figure 1), the rRNA remained intact after treatment with the H137Q mutant, even when protein concentrations assayed were as high as 3.3 µM (Figure 1, lane 4). Furthermore, zymogram assays against poly(A) are also routinely used not only to detect the presence of other potential contaminating RNase or phosphodiesterase-like activities but also to evaluate the non-specific ribonucleolytic activity of the ribotoxins studied [1,4,5]. In this regard, no traces of homopolymer degradation were either observed in these zymograms, as it had been reported before [13,15], even though amounts as high as 5 µg (about 10 µM) of the H137Q α-sarcin mutant were tested (data not shown). Finally, whereas wild-type α-sarcin is cytotoxic

when added to the extracellular culture medium of rhabdomyosarcoma cells, the H137Q mutant remained completely ineffective (data not shown), also in accordance with the results previously published for this particular mutant [6]. Thus, it was concluded that the mutant preparation used was catalytically inactive and innocuous when added to the extracellular medium of transformed cell lines.

However, both wild-type α-sarcin and the H137Q mutant inhibited *in vitro* protein biosynthesis when analyzed using a translation system based on a cell-free rabbit reticulocyte lysate and luciferase mRNA. This inhibition was effective in the 0.05-0.60 μM protein concentration range (Figure 2). The wild-type ribotoxin showed 100% inhibition along the whole protein concentration range studied, as revealed by the absence of a luciferase protein band in the electrophoretic assay system (see Materials and Methods section). For the H137Q mutant, although very scarcely (Figure 2), a minor residual luciferase production was observed at the lowest concentration employed (0.05 μM). Accordingly, a very similar result was obtained when the ribosomal function was studied *in vitro* using yeast ribosomes and poly(U) as template. As it can be observed in Figure 3, 50% inhibition of poly-Phe synthesis could be achieved by concentrations of the α-sarcin H137Q mutant of only 30 nM, although this value was still ten times higher than that one for the wild-type protein (Figure 3). Given the nature and concentrations of the H137Q mutant employed, this protein synthesis inhibition should not be attributed to a ribonucleolytic action on the SRL. The existence of a very low residual ribonucleolytic activity, undetectable by the usual activity assays used for ribotoxins, cannot be completely ruled out, but it would not explain the results obtained in these conditions. Therefore, a new set of experiments were performed to try to explain why this inhibition of protein biosynthesis appeared to be independent of ribonucleolytic activity.

Within this idea, rabbit reticulocyte lysates were incubated in the presence of a great excess of either wild-type or H137Q α-sarcin (protein/ribosome molar ratio of about 20), and the ribosomes were then sedimented by ultracentrifugation. Western immunoblot analysis of the obtained pellets showed that both proteins cosedimented in the ribosomal fraction (Figure 4), revealing that H137Q α-sarcin was able to bind to the ribosomes in

spite of being catalytically inactive. Quantification of sedimented protein and ribosomes revealed the presence of near stoichiometric amounts of ribosomes and ribotoxins within the pellet (protein/ribosome molar ratios of 0.4-0.6). Based on these experiments, the K_d values for both wild-type and H137Q α-sarcin with ribosomes was estimated to be micromolar, at least one order of magnitude higher than the K_M value (30 nM) described for the reaction of ribotoxins on rat ribosomes [28], maybe because this catalytic reaction takes place at a much lower ionic strength than the sedimentation experiments herein described.

Wild-type α-sarcin and its H137Q mutant display identical conformations, preserving the protein structural elements needed for specific recognition of the SRL and showing only very minor local changes at the active site microenvironment [27,32,33]. Thus, it was feasible to presume that the H137Q α-sarcin mutant could bind to the same ribosomal location as the wild-type protein, blocking the protein biosynthesis machinery. To test this hypothesis, competition of the mutant with the wild-type α-sarcin was studied using the rabbit reticulocyte lysate system. As shown in Figure 1 (lanes 5 to 9), the specific ribonucleolytic action of the wild-type protein (production of the α-fragment) was observed even in the presence of a large excess of the mutant. Given the incubation time used during these assays, this result could be explained as both protein forms binding to an identical location, the SRL surroundings, with the catalytically irreversible cleavage performed by the wild-type protein prevailing above the equilibrium-based reversible binding of the mutant lacking of ribonuclease activity. The possibility of H137Q and wild-type α-sarcin binding to different ribosomal locations is also plausible and the results presented do not allow discarding it. Indeed, it is known that, when first encountering the ribosomes, ribotoxins bind to different ribosomal regions to explore its electrostatic ribosomal surface and then, very quickly, locate to the SRL [28,30,31]. However, this interpretation would require a very different mechanism of protein biosynthesis inhibition for the mutant, without involving the direct participation of the SRL.

4. Discussion

Different living organisms use structurally and functionally distinct ribosomes. However, given the importance of protein biosynthesis, several functional regions are highly conserved because they are essential to preserve the protein biosynthesis machinery. In this regard, the SRL is one of the most universally conserved ribosomal domains in good agreement with its essential role in ribosomal good function [2,7,40,41]. The results presented above suggest that blocking the ribosome with a catalytically inactive α-sarcin variant can be enough to impair translation without cleaving any phosphodiester bond. The fact that the ribosomes act as polysomes and one inactive and stalled would inactivate all behind, besides that they are a more dynamic structure while translating, can help to sustain this actual effectiveness of the inactive ribotoxin. This observation seems to contradict previous results regarding the absence of toxicity of this particular mutant when assayed against human rhabdomyosarcoma cells [6]. However, when added to the extracellular medium, only a minor amount of protein enters the cells. If the protein assayed is wild-type α-sarcin, this small protein concentration is enough to catalytically inactivate ribosomes and arrest protein biosynthesis (Figure 5). In fact, it was stated long time ago that only one molecule of α-sarcin is needed to kill a cell [42]. On the other hand, if the H137Q mutant is the protein assayed, the amount of protein that enters the cell would not be be enough to be non-catalytically cytotoxic. These results would agree with the previous observation that intracellular overexpression of α-sarcin can promote cell death through a mechanism that is independent of rRNA cleavage [34]. These authors did not appreciate significant protein biosynthesis inhibition *in vivo* by the H137Q mutant, an observation that could be explained by low cytoplasm concentrations since they showed that it mainly localized to the nucleus. Still their results drew them to conclude that binding to the ribosome might be more important for cytotoxicity than cleavage of the SRL [34].

The results presented now also show how wild-type and H137Q α-sarcin remain attached to the ribosomes with enough affinity as to withstand cosedimentation. This suggests that ribosomal architecture is preserved after inactivation of the SRL, in good accordance with previous results in the same direction [7]. Taking into account that the ribonucleolytic inactivation of the SRL

by ribotoxins disturbs the elongation cycle during protein biosynthesis by impairing elongation factors function [7,40], the most probable explanation (although certainly not the only possible) for the inhibitory effect produced by the inactive H137Q α-sarcin mutant on protein biosynthesis would be interfering with their binding by competing for the same ribosomal location. Ribosomes would be locked on an elongation-incompetent conformation either affecting ternary complex attachment or elongation factor mediated translocation.

In summary, these results provide *in vitro* support for the hypothesis that ribotoxins can be cytotoxic even in the absence of their specific ribonucleolytic activity against ribosomes [34]. Binding to the ribosome seems to be enough to interfere with protein biosynthesis. This observation, altogether with the fact that the H137Q colocalizes with ribosomes at the eukaryotic cell nucleus [34], expands the potential set of abilities performed by this family of toxins. In fact, it is striking to recount how in such a small polypeptide (about 150 amino acid residues) there are three different and mostly independent abilities: (i) interaction with membranes, promoting even fusion of these structures; (ii) ribonuclease activity, degrading from dinucleosides to rRNA; and (iii) binding to ribosomes with enough affinity as to inhibit protein biosynthesis. Maybe that is why these proteins constitute one of the most potent groups of known cytotoxins.

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References

[1] J. Lacadena, E. Álvarez-García, N. Carreras-Sangrà, E. Herrero-Galán, J. Alegre-Cebollada, L. García-Ortega, M. Oñaderra, J.G. Gavilanes, A.

Martínez del Pozo, Fungal ribotoxins: molecular dissection of a family of natural killers. FEMS Microbiol. Rev. 31 (2007) 212-237.

[2] R.M. Voorhees, T.M. Schmeing, A.C. Kelley, V. Ramakrishnan, The mechanism for activation of GTP hydrolysis on the ribosome. Science 330 (2010) 835-838.

[3] M. Gasset, J.M. Mancheño, J. Lacadena, J. Turnay, N. Olmo, M.A. Lizarbe, A. Martínez del Pozo, M. Oñaderra, J.G. Gavilanes, α-Sarcin, a ribosome-inactivating protein that translocates across the membrane of phospholipid vesicles. Curr. Topics Pept. Protein Res. 1 (1994) 99–104.

[4] R. Kao, A. Martínez-Ruiz, A. Martínez del Pozo, R. Crameri, J. Davies, Mitogillin and related fungal ribotoxins. Methods Enzymol. 341 (2001) 324–335.

[5] A. Martínez-Ruiz, L. García-Ortega, R. Kao, J. Lacadena, M. Oñaderra, J.M. Mancheño, J. Davies, A. Martínez del Pozo, J.G. Gavilanes, RNase U2 and α-sarcin: A study of relationships. Methods Enzymol. 341 (2001) 335–351.

[6] N. Olmo, J. Turnay, G. González de Buitrago, I. López de Silanes, J.G. Gavilanes, M.A. Lizarbe, Cytotoxic mechanism of the ribotoxin α-sarcin. Induction of cell death via apoptosis. Eur. J. Biochem. 268 (2001) 2113–2123.

[7] L. García-Ortega, E. Álvarez-García, J.G. Gavilanes, A. Martínez-del-Pozo, S. Joseph, Cleavage of the sarcin-ricin loop of 23S rRNA differentially affects EF-G and EF-Tu binding. Nucleic Acids Res. 38 (2010) 4108-4119.

[8] B.H. Olson, J.C. Jennings, V. Roga, A.J. Junek, D.M. Schuurmans, α-Sarcin, a new antitumor agent. II. Fermentation and antitumor spectrum. Appl. Microbiol. 13 (1965) 322–326.

[9] C. Fernández-Puentes, L. Carrasco, Viral infection permeabilizes mammalian cells to protein toxins. Cell 20 (1980) 769–775.

[10] M. Gasset, A. Martínez del Pozo, M. Oñaderra, J.G. Gavilanes, Study of the interaction between the antitumor protein α-sarcin and phospholipid vesicles. Biochem. J. 258 (1989) 569–575.

[11] M. Gasset, M. Oñaderra, P.G. Thomas, J.G. Gavilanes, Fusion of phospholipid vesicles produced by the anti-tumour protein α-sarcin. Biochem. J. 265 (1990) 815–822.

[12] M. Oñaderra, J.M. Mancheño, M. Gasset, J. Lacadena, G. Schiavo, A. Martínez del Pozo, J.G. Gavilanes, Translocation of α-sarcin across the lipid bilayer of asolectin vesicles. Biochem. J. 295 (1993) 221–225.

[13] J. Lacadena, J.M. Mancheño, A. Martínez-Ruiz, A. Martínez del Pozo, M. Gasset, M. Oñaderra, J.G. Gavilanes, Substitution of histidine-137 by glutamine abolishes the catalytic activity of the ribosome-inactivating protein α-sarcin. Biochem. J. 309 (1995) 581–586.

[14] J. Lacadena, A. Martínez del Pozo, V. Lacadena, A. Martínez-Ruiz, J.M. Mancheño, M. Oñaderra, J.G. Gavilanes, The cytotoxin α-sarcin behaves as a cyclizing ribonuclease. FEBS Lett. 424 (1998) 46–48.

[15] J. Lacadena, A. Martínez del Pozo, A. Martínez-Ruiz, J.M. Pérez-Cañadillas, M. Bruix, J.M. Mancheño, M. Oñaderra, J.G. Gavilanes, Role of histidine-50, glutamic acid-96, and histidine-137 in the ribonucleolytic mechanism of the ribotoxin α-sarcin. Proteins 37 (1999) 474–484.

[16] M. Masip, J. Lacadena, J.M. Mancheño, M. Oñaderra, A. Martínez-Ruiz, A. Martínez del Pozo, J.G. Gavilanes, Arginine 121 is a crucial residue for the specific cytotoxic activity of the ribotoxin α-sarcin. Eur. J. Biochem. 268 (2000) 6190–6196.

[17] M. Masip, L. García-Ortega, N. Olmo, M.F. García-Mayoral, J.M. Pérez-Cañadillas, M. Bruix, M. Oñaderra, A. Martínez del Pozo, J.G. Gavilanes, Leucine 145 of the ribotoxin α-sarcin plays a key role for determining the specificity of the ribosome-inactivating activity of the protein. Protein Sci. 12 (2003) 161–169.

[18] A. Siemer, M. Masip, N. Carreras, L. García-Ortega, M. Oñaderra, M. Bruix, A. Martínez del Pozo, J.G. Gavilanes, Conserved asparagine residue 54 of α-sarcin plays a role in protein stability and enzyme activity. Biol. Chem. 385 (2004) 1165–1170.

[19] E. Álvarez-García, L. García-Ortega, Y. Verdún, M. Bruix, A. Martínez del Pozo, J.G. Gavilanes, Tyr-48, a conserved residue in ribotoxins, is involved in the RNA-degrading activity of α-sarcin. Biol. Chem. 387 (2006) 535–541.

[20] R. Yang, W.R. Kenealy, Effects of amino-terminal extensions and specific mutations on the activity of restrictocin. J. Biol. Chem. 267 (1992) 16801–16805.

[21] T. Brandhorst, R. Yang, W.R. Kenealy, Heterologous expression of the cytotoxin restrictocin in *Aspergillus nidulans* and *Aspergillus niger*. Protein Expr. Purif. 5 (1994) 486–497.

[22] R. Kao, J. Davies, Fungal ribotoxins: a family of naturally engineered targeted toxins? Biochem. Cell. Biol. 73 (1995) 1151–1159.

[23] R. Kao, J. Davies, Molecular dissection of mitogillin reveals that the fungal ribotoxins are a family of natural genetically engineered ribonucleases. J. Biol. Chem. 274 (1999) 12576–12582.

[24] I.D. Sylvester, L.M. Roberts, J.M. Lord, Characterization of prokaryotic recombinant *Aspergillus* ribotoxin α-sarcin. Biochim. Biophys. Acta 1358 (1997) 53–60.

[25] R. Kao, J.E. Shea, J. Davies, D.W. Holden, Probing the active site of mitogillin, a fungal ribotoxin. Mol. Microbiol. 29 (1998) 1019–1027.

[26] L. García-Ortega, M. Masip, J.M. Mancheño, M. Oñaderra, M.A. Lizarbe, M.F. García-Mayoral, M. Bruix, A. Martínez del Pozo, J.G. Gavilanes, Deletion of the NH_2-terminal β-hairpin of the ribotoxin α-sarcin produces a non-toxic but active ribonuclease. J. Biol. Chem. 277 (2002) 18632-18639.

[27] M.F. García-Mayoral, L. García-Ortega, E. Álvarez-García, M. Bruix, J.G. Gavilanes, A. Martínez del Pozo, Modelling the highly specific ribotoxin recognition of ribosomes. FEBS Lett. 579 (2005) 6859–6864.

[28] A.V. Korennykh, J.A. Piccirilli, C.C. Correll, The electrostatic character of the ribosomal surface enables extraordinarily rapid target location by ribotoxins. Nat. Struct. Mol. Biol. 13 (2006) 436-443.

[29] E. Álvarez-García, A. Martínez-del-Pozo, J.G. Gavilanes. Role of the basic character of α-sarcin's NH_2-terminal beta-hairpin in ribosome recognition and phospholipid interaction. Arch. Biochem. Biophys. 481 (2009) 37-44.

[30] S. Qin, H.X. Zhou, Dissection of the high rate constant for the binding of a ribotoxin to the ribosome. Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 6974-6979.

[31] M.J. Plantinga, A.V. Korennykh, J.A. Piccirilli, C.C. Correll, The ribotoxin restrictocin recognizes its RNA substrate by selective engagement of active site residues. Biochemistry 50 (2011) 3004-13.

[32] M.F. García-Mayoral, D. Pantoja-Uceda, J. Santoro, A. Martínez del Pozo, J.G. Gavilanes, M. Rico, M. Bruix, Refined NMR structure of α-sarcin by ^{15}N-^{1}H residual dipolar couplings. Eur. Biophys. J. 34 (2005) 1057–1065.

[33] M.F. García-Mayoral, A. Martínez del Pozo, R. Campos-Olivas, J.G. Gavilanes, J. Santoro, M. Rico, D.V. Laurents, M. Bruix, pH-dependent conformational stability of the ribotoxin α-sarcin and four active site charge substitution variants. Biochemistry 45 (2006) 13705–13718.

[34] S.C. Alford, J.D. Pearson, A. Carette, R.J. Ingham, P.L. Howard, α-Sarcin catalytic activity is not required for cytotoxicity. BMC Biochem. 10, (2009) 9.

[35] J. Lacadena, A. Martínez del Pozo, J.L. Barbero, J.M. Mancheño, M. Gasset, M. Oñaderra, C. López-Otín, S. Ortega, J. García, J.G. Gavilanes, Overproduction and purification of biologically active native fungal α-sarcin in *Escherichia coli*. Gene 142 (1994) 147–151.

[36] L. García-Ortega, J. Lacadena, V. Lacadena, M. Masip, C. de Antonio, A. Martínez-Ruiz, A. Martínez del Pozo, The solubility of the ribotoxin α-sarcin, produced as a recombinant protein in *Escherichia coli*, is increased in the presence of thioredoxin. Lett. Appl. Microbiol. 30 (2000) 298–302.

[37] F. Sánchez-Madrid, R. Reyes, P. Conde, J.P.G. Ballesta, Acidic ribosomal proteins from eukaryotic cells. Effect on ribosomal functions. Eur. J. Biochem. 98 (1979) 409-416.

[38] J. Zurdo, P. Parada, A. van den Berg, G. Nusspaumer, A. Jiménez-Díaz, M. Remacha, J.P.G. Ballesta, Assembly of *Saccharomyces cerevisiae* ribosomal stalk: Binding of P1 proteins is required for the interaction of P2 proteins. Biochemistry 39 (2000) 8929-8934.

[39] G.S. Beckler, D. Thompson, T. Van Oosbree, *In vitro* translation using rabbit reticulocyte lysate. Methods Mol. Biol. 37 (1995) 215-232.

[40] T.P. Hausner, J. Atmadja, K.H. Nierhaus, Evidence that the G2661 region of 23S rRNA is located at the ribosomal binding sites of both elongation factors. Biochimie 69 (1987) 911-923.

[41] N. Ban, P. Nissen, J. Hansen, P.B. Moore, T.A. Steitz, The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. Science 289 (2000) 905-920.

[42] B. Lamy, J. Davies, D. Schindler, The *Aspergillus* ribonucleolytic toxins (ribotoxins) in *Genetically engineered toxins*, (1992) Frankel, A.E., ed. Marcel Dekker, pp. 237-258.

Fig. 1. Ribonucleolytic activity assay of wild-type α-sarcin and its H137Q mutant is shown by the release of the 400-nucleotide α-fragment (which position is indicated by an arrow) from the 28 S rRNA of rabbit ribosomes. This α-fragment corresponds to the 3'-end of 28S rRNA because α-sarcin cleaves the SRL phosphodiester bond at the 3'-side of G2661 (*E. coli* numbering; G4325 in rat). Cell-free reticulocyte lysates were incubated in the absence (lane 1) or in the presence of 0.12 μM of wild-type α-sarcin (lane 2) or two different amounts (0.12 and 3.30 μM) of the H137Q mutant (lanes 3 and 4). Lanes 5 to 9 correspond to competition assays performed ~~in identical conditions but~~ in the presence of 0.12 μM of wild-type α-sarcin and increasing amounts of the H137Q mutant (0.12, 0.24, 0.60, 1.18, and 3.30 μM). The reactions were made in 40 mM Tris-HCl, pH 7.5, 40 mM KCl, 10 mM EDTA and incubated for 15 min at room temperature. The reaction was stopped by addition of 50 mM Tris-HCl, pH 7.4, 0.5% (w/v) SDS, followed by strong vortexing.

Fig. 2. Luciferase mRNA was translated in the presence of 0.60, 0.20, and 0.05 μM wild-type or H137Q α-sarcin as described in Materials and Methods section. The first two lanes correspond to controls performed without the addition of ribotoxins and in the absence (C-) or the presence of luciferase mRNA (C+). Translational products were analyzed by 15% SDS–PAGE and autoradiography.

Fig. 3. *In vitro* polyPhe biosynthesis by yeast ribosomes in the absence and in the presence of different concentrations of wild-type (●) and H137Q (○) α-sarcin. This reaction was performed in a total volume of 25 μL, containing 2 μL of S30, 0.1 mg/mL polyuridylic acid, 50 μg/mL $tRNA^{Phe}$, 20 μM Phe, 0.5 μM [^{3}H]-Phe (25 μCi/pmol), 0.5 mM GTP, 1 mM ATP, 4 mM phosphocreatine, 25 μg/mL creatine phosphokinase, and 2.5 μl of the desired ribotoxin concentration, in 30 mM HEPES-HCl, pH 7.4, containing 5 mM $MgCl_2$, 50 mM KCl and 2 mM β-mercaptoethanol for 30 min. at 30ºC. The reaction was stopped by addition of 1 ml of 5% trichloroacetic acid and heating at 90ºC for 15 min.

Fig. 4. SDS-PAGE (lanes 1 to 4) and Western immunoblot (lanes 5 to 8) analysis of proteins cosedimented with 80S ribosomes by ultracentrifugation.

Rabbit reticulocyte lysates were previously incubated in the absence (lanes 2 and 6) or in the presence of 2.2 μM wild-type α-sarcin (lanes 3 and 7) or its H137Q mutant (lanes 4 and 8). The arrows indicate the positions corresponding to dimeric and monomeric versions of α-sarcin. Lanes 1 and 5 correspond to 5 μl of Biorad Precision Plus Protein Standards (molecular masses represented are: 250, 150, 100, 75, 50, 37, 25, 20, and 15 KDa). Presence of α-sarcin dimer fraction has been described before in many different instances and shown to simply be an artifactual result of the electrophoretic separation [8].

Fig. 5. Schematic explanation of the different actions exerted by wild-type catalytically active α-sarcin and its ribonucleolitically inactive H137Q variant. (A) Extracellular addition of the protein to the tumoral cells culture results in the internalization of a small amount of catalytically active protein but enough to irreversibly inactivate the number of ribosomes needed to render protein biosynthesis inefficient and trigger apoptosis. (B) If an identical experiment is performed with the H137Q mutant, its intracellular concentration does not reach values high enough as to produce protein biosynthesis impairment, given the reversible and transient nature of its action. (C) Wild-type α-sarcin is extremely effective against ribosomes when assayed in a cell-free system. (D) In identical conditions, concentration of the H137Q mutant can be high enough as to compete for the SRL in a reversible manner, presumably with elongation factors, and impair most of the ribosomes present. This impairment would result in an inhibition of the protein biosynthesis machinery.

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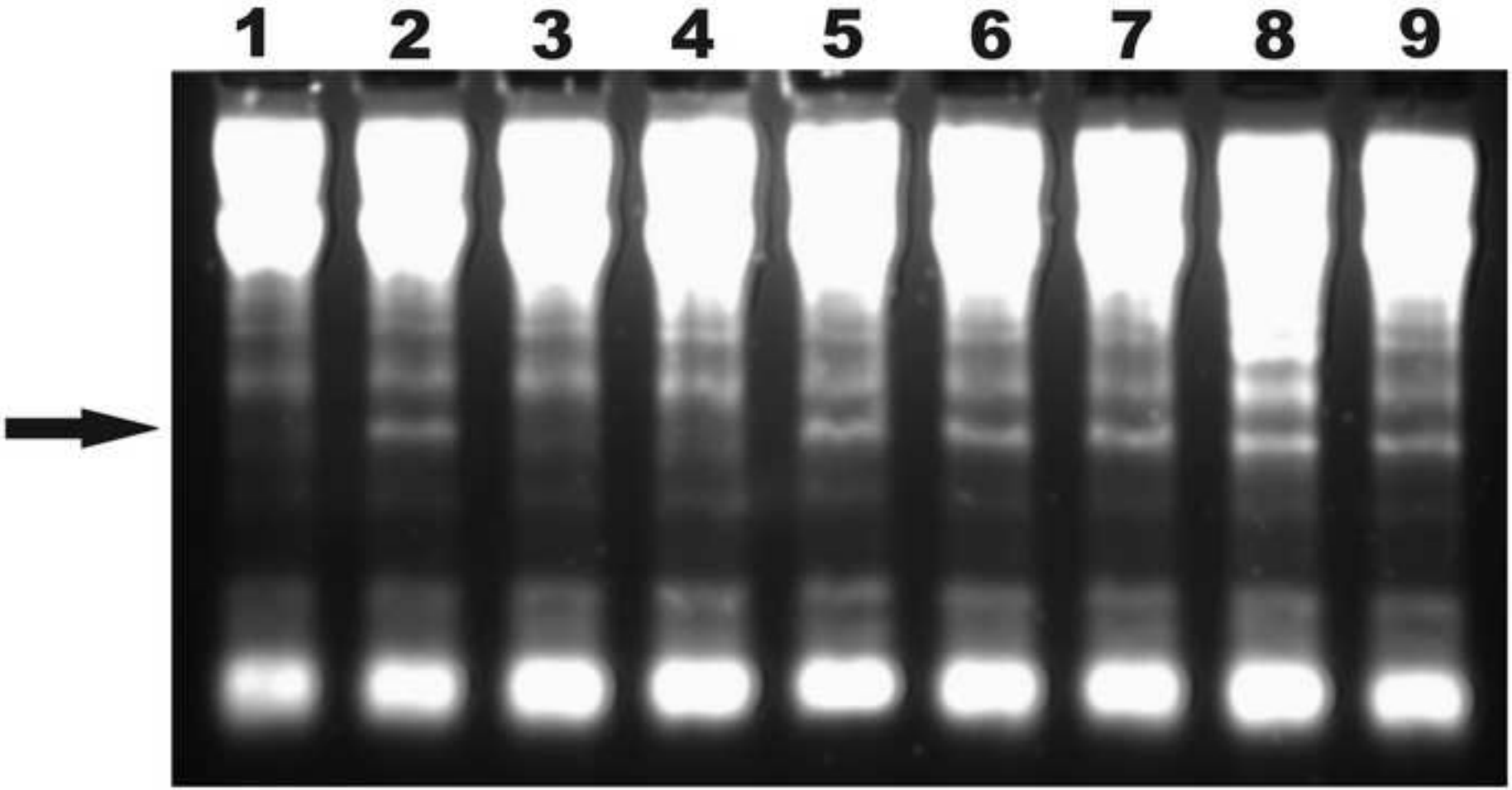

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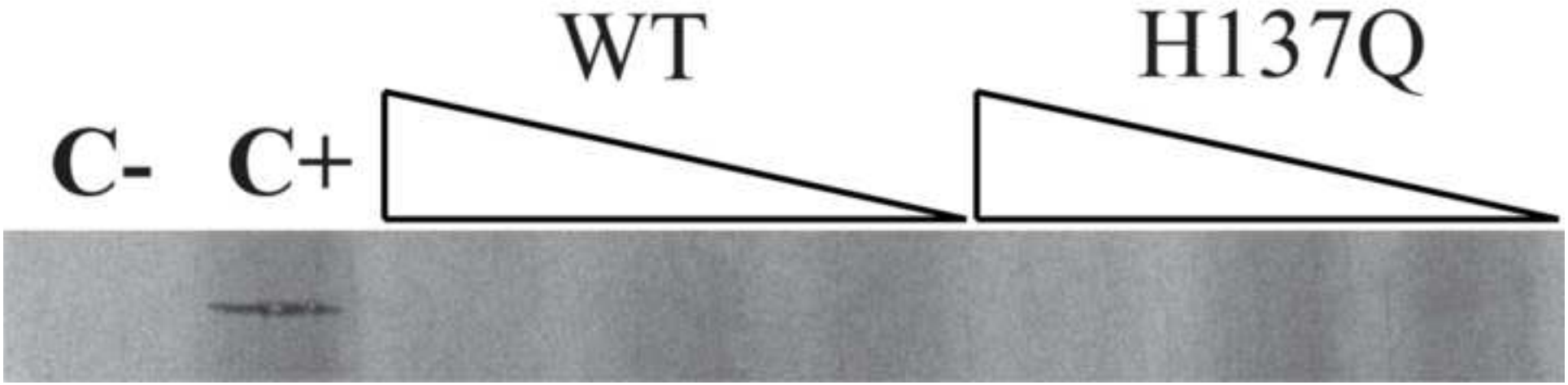

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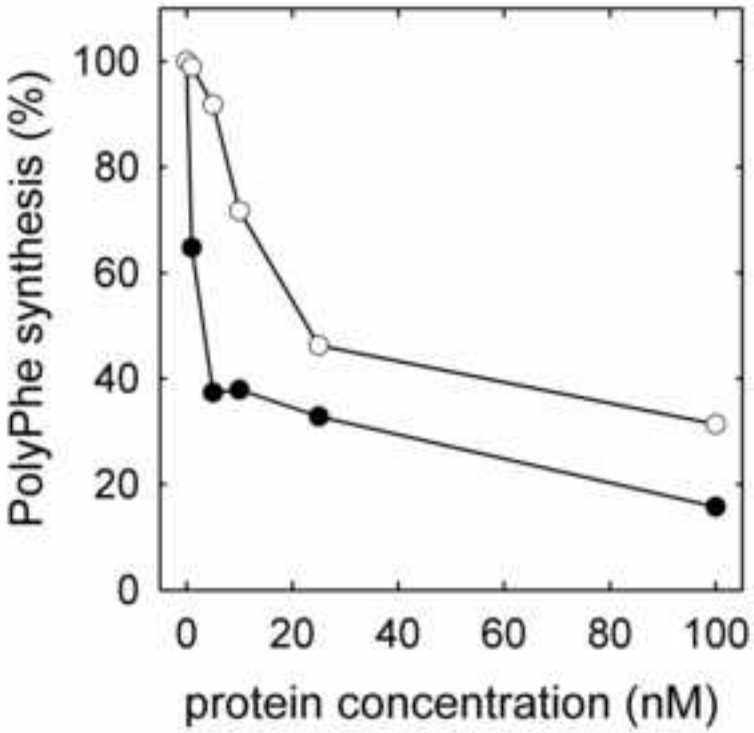

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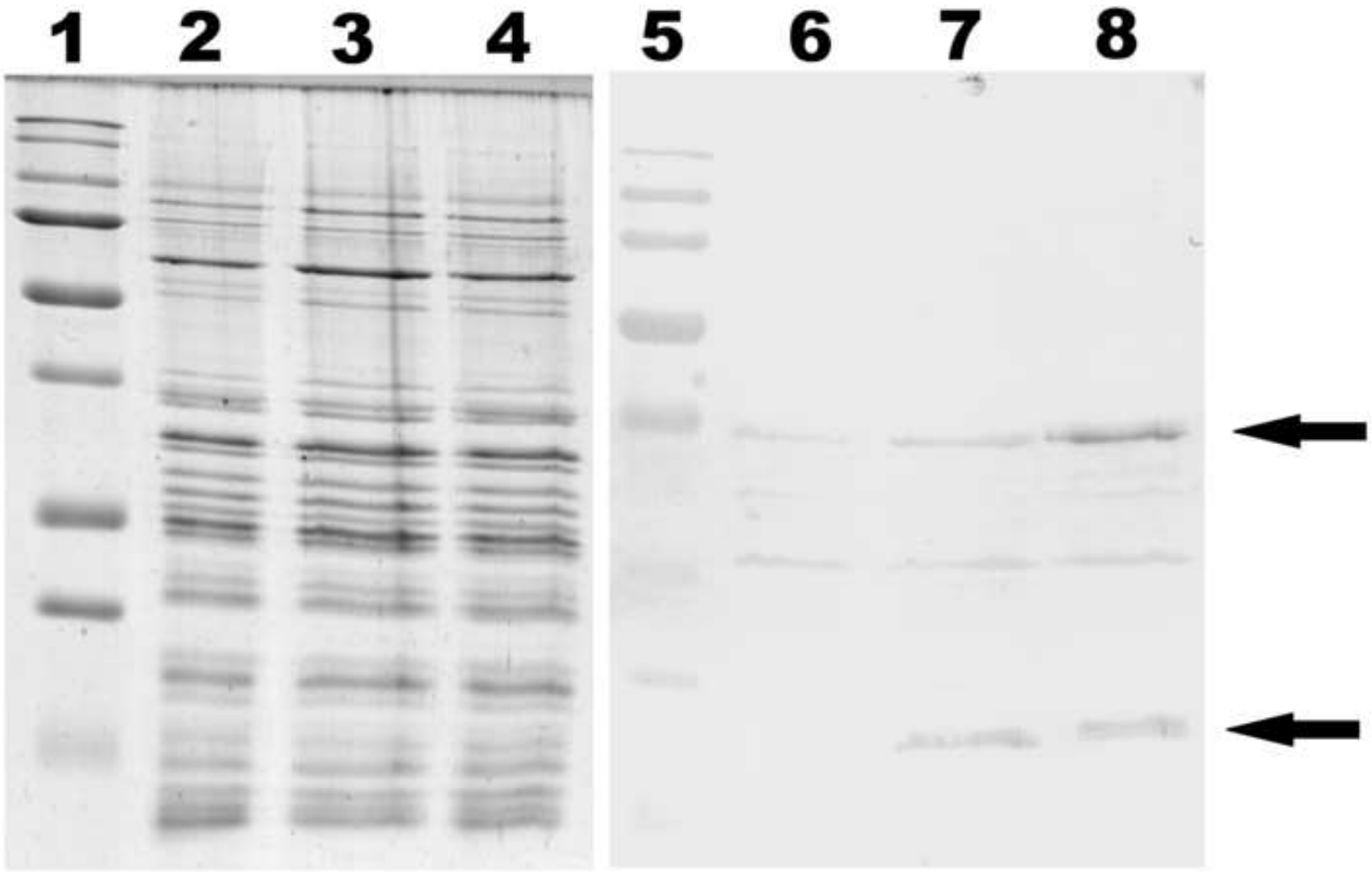

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