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Lactococcus lactis as a vehicle for the heterologous expression of fungal ribotoxin variants with reduced IgE-binding affinity

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Abstract

Fungal ribotoxins are a family of extracellular ribonucleases which inhibit protein biosynthesis by inactivating the ribosomes. This inactivation results in the induction of cell death by apoptosis. Ribotoxins show antitumoral properties based on their ability to cross the membrane of some transformed cells. Unfortunately, they also show an unspecific cytotoxicity which has greatly impaired their potential clinical uses. α-Sarcin, produced by *Aspergillus giganteus*, is the best-characterized ribotoxin. Asp f 1, another ribotoxin produced by *A. fumigatus,* is indeed one of its major allergens. In this work, the *Lactococcus lactis* MG1363 strain has been engineered to produce and secrete not only wild-type Asp f 1 and α-sarcin but also three different mutants with reduced cytotoxicity and/or IgE-binding affinity. The proteins were secreted in native and active form when the extracellular medium employed was buffered at pH values around 8.0. Strains producing the wild-type natural α-sarcin were proved to be innocuous when administered intragastrically to mice for a period of 14 days. Overall, the results presented are discussed in terms of its potential application as a vehicle of oral delivery of hypoallergenic variants as well as a starting point to approach the design of strategies to accomplish the safe delivery of these proteins as antitumoral agents.

Keywords: α-Sarcin; Asp f 1; Ribonuclease; Probiotic

1. Introduction

Aspergillus species are responsible for several human lung pathologies, including different allergic inhalant diseases (Kurup et al., 2002), allergic bronchopulmonary aspergillosis (ABPA) being the most severe form among them. *Aspergillus fumigatus* is usually the mold involved in most of those diseases, ABPA included, because this fungus, with small spores, optimally grows at 37 °C, a temperature that is prohibitive for most of the other environmental ubiquitous fungi. Thus, it can colonize the respiratory tract of the host leading to the onset of pathological events (Banerjee and Kurup, 2003).

Ribotoxins are a group of secreted fungal ribonucleases, best represented by α-sarcin (Lacadena et al., 2007), whose toxicity comes from their ability to reach the cytosol via endocytosis without establishing any receptor interaction (Olmo et al., 2001). They inhibit protein biosynthesis by inactivating the ribosomes (Schindler and Davies, 1977; Kao et al., 2001) which results in induction of cell death by apoptosis (Olmo et al., 2001). This ribosome inactivation is achieved by cleaving a unique phosphodiester bond at the so-called sarcin–ricin loop (SRL) of the largest rRNA (Endo and Wool, 1982; Correll et al., 1999). Ribotoxins were discovered during a screening program of the Michigan Department of Health searching for antibiotics and antitumor agents (Olson et al., 1965). Unfortunately, further studies revealed an unspecific cytotoxicity of these proteins, which limited their potential clinical uses (Roga et al., 1971).

All ribotoxins show a high degree of sequence identity with most of their differences appearing on exposed regions, such as their NH_2-terminal β-hairpin (α-sarcin residues 1–26), a domain

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; SRL, sarcin–ricin loop; GRAS, “Generally Regarded As Safe”; PCR, polymerase chain reaction; H&E, haematoxylin and eosin.

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which can be considered as two consecutive minor β-sheets connected by a hinge region. The second of them (residues 7–22) juts out as a solvent-exposed protuberance and is one of the protein regions with highest conformational flexibility (Pérez-Cañadillas et al., 2000, 2002). Deletion of this β-sheet results in a Δ(7–22) mutant that shows no significant conformational differences except for the deleted region (García-Mayoral et al., 2004) but has lost its ability to specifically recognize the ribosome and is much less cytotoxic (García-Ortega et al., 2002).

Asp f 1, the ribotoxin produced by *A. fumigatus*, is also a major and one of its best-characterized allergens (Moser et al., 1992). There is a significant prevalence of Asp f 1-specific IgE antibodies in sera from patients sensitized to *Aspergillus*. Particularly in ABPA, the detection of these antibodies seems to be a promising approach for its otherwise difficult diagnosis (Kao et al., 2001; Greenberger, 2002; Banerjee and Kurup, 2003; García-Ortega et al., 2005). Asp f 1 differs from α-sarcin in only 19 (87% sequence identity) out of 150 residues. Five of these differences are precisely located at the NH_2-terminal β-hairpin. Structural and immunogenic studies of Asp f 1, α-sarcin, and two variants where this β-hairpin had been deleted [Asp f 1 Δ(7–22) and α-sarcin Δ(7–22)] (García-Ortega et al., 2005) showed that the deleted portion is involved in at least one allergenic epitope (García-Mayoral et al., 2004; García-Ortega et al., 2005). In spite of their decreased IgE reactivity, the prevalence of the two deleted proteins among the sera of patients remained essentially unaffected while they still retained most of the IgG epitopes (García-Ortega et al., 2005). It was then concluded that these ribotoxins' variants might be suitable for use in immunomodulating therapies and diagnosis of *Aspergillus* hypersensitivity.

The digestive tract is inhabited by commensal flora, whose correct settlement has been shown to be very important for human health. In fact, some pathological states can be improved just by means of administration of certain live bacteria, the so-called probiotics (Hooper and Gordon, 2001; Schiffrin and Blum, 2001). In the last years, the setting-up of genetic engineering techniques has made possible the genetic modification of commensal bacteria in order to obtain "biodrugs", i.e., strains of bacteria capable of *in vivo* producing drugs, antimicrobial agents, or vaccines (Hooper and Gordon, 2001; Blanquet et al., 2001). One of the advantages of these "biodrugs" is the specific delivery of the therapeutic agent to their target.

Lactococcus lactis is a non-pathogenic, non-invasive, no colonizing Gram-positive bacterium, mainly used to produce fermented foods. This lactic acid bacterium holds "Generally Regarded As Safe" (GRAS) status and hence is a suitable candidate to be used as one of those "biodrugs". For example, it has been proven useful in producing IL-10 for the treatment of inflammatory bowel disease in mice (Steidler et al., 2000). In this work, the extracellular production by *L. lactis* of the above α-sarcin and Asp f 1 deletion variants is presented. Both wild-type proteins were also included in the study, as well as a properly folded, but catalytically inactive α-sarcin H137Q mutant, as a control (Lacadena et al., 1995; García-Ortega et al., 2002, 2005). The possibility of using this strategy as a potential immunomodulating therapeutic approach is discussed.

2. Materials and methods

2.1. Materials

All reagents were molecular biology grade. Restriction endonucleases and DNA modifying and synthesizing enzymes were purchased from Roche (Indianapolis, IN), New England Biolabs (Beverly, MA), or Promega (Madison, WI). Oligonucleotides were purchased from Sigma-Genosys (Cambridge, UK). PerkinElmer (Wellesley, MA) GeneAmpPCRSystem 2400 thermal cycler was used for the polymerase chain reaction (PCR)-based amplifications. DNA sequencing was performed at the facility of the Universidad Complutense (Madrid, Spain).

2.2. Media

L. lactis strain MG1363 (Table 1) was routinely grown at 30 °C in M17 (Difco) containing 0.5% (w/v) glucose (GM17) under static conditions, as described (Gil et al., 2001), adding erythromycin (5 μg/ml) when needed. In order to prepare electrocompetent cells, they were grown in GM17 medium but supplemented with 23.8 mg/ml L-threonine, and 1 mM $MgSO_4$ (GM17GT). Protein production was initially assayed in a medium (GM9) containing 0.042 M Na_2HPO_4, 0.02 M KH_2PO_4, 0.01 M NH_4Cl, 8.5 mM NaCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 g/l peptone, and 5 μg/ml erythromycin. Final production of the proteins was accomplished in 0.2 M potassium phosphate buffered GM9 (PM) adjusted at different pH values between 6.0 and 8.5.

2.3. Cloning procedures

Cloning procedures and DNA manipulations were carried out according to standard methods (Lacadena et al., 1994; Maassen, 1999; Schotte et al., 2000; Sambrook and Russell, 2001). Suitable deoxyoligonucleotides were used as primers for PCR amplification, using a series of plasmids constructed before as templates (Lacadena et al., 1994, 1995; García-Ortega et al., 2002, 2005), containing the cDNA corresponding to the five proteins studied: Asp f 1, α-sarcin, Asp f 1 Δ(7–22), and α-sarcins Δ(7–22) and H137Q. These amplified DNA fragments were flanked by NgoMIV and BamHI restriction sites that were used to clone the proteins into the corresponding cloning sites of the plasmid pT1NX, in frame with the secretion signal leader of the lactococcal *usp*45 gene placed under the control of the constitutive promoter P1 (Steidler et al., 1995, 2000). The vectors thus obtained (Table 1) were used to electroporate electrocompetent MG1363 *L. lactis* cells.

2.4. L. lactis electroporation

In order to prepare electrocompetent MG1363 *L. lactis* cells, the corresponding strain was grown overnight at 30 °C in 5 ml of GM17GT medium. Next day, this culture was diluted with fresh medium to a final volume of 25 ml and an OD_{600} of 0.1 and further incubated until reaching a value of 0.32. Cells were then harvested by centrifugation at 7500 × *g* for 15 min. The cel-