ARTÍCULO DE INVESTIGACIÓN

Finding of a novel fungal immunomodulatory protein coding sequence in *Ganoderma australe*

Hallazgo de una nueva secuencia codificadora para una proteína inmunomoduladora de origen fúngico en *Ganoderma australe*

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Abstract

Among the most common human diseases with immune system compromise are autoimmune diseases, cancer, and the acquired immunodeficiency syndrome (AIDS). Many of these diseases still have no treatment or their therapies have undesirable side effects. This has aroused a great interest in the search for new natural products with therapeutic potential and scientifically proven effects, showing minimal side effects. Formal clinical and pharmacological investigation in various medicinal fungi of the genus *Ganoderma* (Ganodermataceae) has shown immunomodulatory effects and tumor growth inhibition in mammals, attributable to the presence of immunomodulatory proteins and other secondary metabolites. To date, six fungal immunomodulatory proteins (FIPs) have been reported in *Ganoderma*. This paper seeks to advance in the discovery of immunomodulatory proteins present in *Ganoderma australe*, through mycelium transcriptome 454 Roche® pyrosequencing (RNA-seq) and bioinformatics analyses. The results suggest the presence of gene sequences related to an immunomodulatory protein which has been reported in another fungal species *Taiwanofungus camphoratus*. The candidate gene sequences found in *G. australe* exhibit high identity values in their amino acid composition and predicted protein secondary structure with the protein reported for *Tai. camphoratus*. According to present knowledge about the action mechanisms of these proteins, it is possible to suggest that this is a promising molecule for the treatment and prevention of diseases associated with certain immune deficiencies, cancer, and other diseases with compromised immune systems. Future studies are proposed in order to determine its immunomodulatory potential using *in vitro* and *in vivo* assays.

Keywords: *Ganoderma,* fungal immunomodulatory protein, immunomodulation, transcriptome, therapeutic.

Resumen

Enfermedades comunes como las autoinmunes, el cáncer y el síndrome de inmunodeficiencia adquirida aún no tienen tratamiento o sus terapias tienen efectos secundarios indeseables. Ello ha suscitado el interés en la investigación de bioproductos con potencial terapéutico, que no impliquen efectos secundarios. Investigaciones farmacológicas y clínicas en algunos hongos medicinales del género *Ganoderma* (Ganodermataceae) han comprobado efectos inmunomoduladores e inhibidores de crecimiento tumoral en mamíferos, atribuibles a la presencia de proteínas fúngicas inmunomoduladoras (FIPs) y otros metabolitos secundarios. Este trabajo busca avanzar en el descubrimiento de proteínas inmunomoduladoras presentes en *Ganoderma australe*, mediante la secuenciación del transcriptoma de micelio por tecnología de pirosecuenciación 454 Roche® (RNA-seq) y análisis bioinformáticos. Los resultados sugieren la presencia de secuencias génicas relacionadas con una proteína inmunomoduladora que se ha reportado en la especie de hongos *Taiwanofungus camphoratus.* Las secuencias génicas candidatas halladas en *G. australe* exhiben una altos valores de similitud en sus predicciones de composición aminoacídica y estructura secundaria proteica con la proteína reportada para *Tai. camphoratus*. Los mecanismos de acción de este tipo de proteínas inmunomoduladoras sugieren que se trata de una molécula con potencial promisorio para el tratamiento y prevención de enfermedades con compromiso del sistema inmunológico y el cáncer. Se proponen nuevos estudios que permitan determinar el potencial inmunomodulador de la proteína hipotética hallada mediante estudios *in vivo* e *in vitro*.

Palabras clave: *Ganoderma*, proteína fúngica inmunomoduladora, inmunomodulación, transcriptoma, terapéutica.

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Introduction

The Fungi kingdom is considered one of the great biodiversity resources, carrying out important roles in ecosystems and producing chemically diverse secondary metabolites that contribute to a wide variety of bioactive properties in many fungal species. Therefore, fungi are promising resources for the discovery of novel bioproducts with high potential for diverse biotechnological applications.

Interest in the use of therapeutic fungi has been historically restricted to popular medicine and to the manufacture of dietary supplements with health benefits. Moreover, until a few decades ago, the biological potential of these organisms stood as an unexploited resource in formal scientific investigation (Stamets, 1993). However, scientific interest in fungi, as biological resources with biotechnological and therapeutic applications, has increased considerably in the last few decades.

In particular, *Ganoderma* P. Karsten (1881) is a genus of ancient medicinal fungi, which has been used in traditional medicine for over 4,000 years (McMeekin, 2004) and is considered a "spiritual herb" or "fungi of immortality" in oriental cultures. Within the genus, the species *Ganoderma lucidum* has been the object of interest of most pharmacological investigations. It has been proven that its extracts and pure biomolecules inhibit cancer cell growth and promote an *in vivo* immune response through inducing immunoglobulin and cytokine production (Chang *et al*., 2009; Lin *et al*., 2006); in addition, its metabolites can be effective antivirals against the human immunodeficiency virus (HIV) (Gao *et al*., 2003). Recently, one of these species' bioactive therapeutic proteins has been cloned and efficiently expressed in molecular vectors (Wu *et al*., 2013). Furthermore, other *Ganoderma* species have also been shown to be effective in diverse biotechnological applications for medicinal use. *Ganoderma applanatum*, *G. tsugae*, *G. sinense*, and *G. microsporum* also promote antitumor and immunomodulatory activities (Jeong *et al*., 2008; Liao *et al*., 2008; Li *et al*., 2010a; Lin *et al*., 2010), and *G. australe* has been reported to possess antimicrobial functions as well (Albino-Smania *et al*., 2007). The most recent biotechnological applications in this genus include the development of recombinant fungal immunomodulatory proteins (Bastiaan-Net *et al*., 2013; Zhang *et al*., 2013) and nanoparticle mycosynthesis for the directed modulation of antibiotic and antimicrobial (Karwa *et al*., 2011) and antitumor activities (Li *et al*., 2010a, 2010b).

Pharmacological and formal clinical studies on *Ganoderma* have investigated its therapeutic activity with further detail and have scientifically evaluated its capacity for modulating the activation and expression of cells and messengers involved in the immune response. This immunomodulation is mediated in part by proteins of the Fungal Immunomodulatory Protein (FIP) family, among other therapeutic secondary metabolites. The biological function and action mechanisms of these proteins have been reported extensively in diverse studies (Li *et al*., 2011a; Li *et al*., 2010a; Lin, 2005), classifying them as possible effective agents for treating and preventing diseases originated by certain immunodeficiencies and other states of immune dysfunction.

In general, mature FIPs have been defined as a family of proteins with a molecular weight of around 13 kDa, with 110 to 114 amino acid residues, and exist as homodimers (Lin *et al*., 1997), except for FIP-gmi which is a tetramer (Wu *et al*., 2008). In addition, these proteins exhibit a high structural and functional similarity with the immunoglobulin superfamily (Li *et al*., 2011a). Since the first FIP was isolated and characterized from *G. lucidum* (LZ-8) (Tanaka *et al*., 1989), its biological functions have been intensively explored and its DNA and protein sequences have served as references for the identification of new FIPs in other fungi, through homology hypotheses. To date, a total of nine FIPs have been reported from diverse basidiomycetes: FIP-fve, from *Flammulina velutipes* (Ko *et al*., 1995); FIP-vvo, *Volvariella volvacea* (Hsu *et al*., 1997); FIP-tve, *Trametes versicolor* (Li *et al*., 2011b); LZ-8, *G. lucidum* (Murasugi *et al*., 1991; Kino *et al*., 1989); FIP-gts, *G. tsugae* (Lin *et al*., 1997); FIP-gsi, *G. sinense* (Zhou *et al*., 2007, 2009); FIP-gja, *G. japonicum* (GenBank accession AAX98241); FIP-gmi, *G. microsporum* (Wu *et al*., 2007); and FIP-gap, *G. applanatum* (GenBank accession AEP68179).

The use of high-throughput genomic and transcriptome sequencing technologies has generated high volumes of biological information, and the increasing development of bioinformatics tools has built the way to finding and identifying novel genes related to proteins of specific interest, as in the case of therapeutic proteins in fungi (Zhou *et al*., 2009). The main objective of this work is to identify genetic sequences related to fungal immunomodulatory proteins in *G. australe,* through transcriptome sequencing (RNA-seq) by next generation sequencing technologies (454 Roche® pyrosequencing) and bioinformatics tools. We report the finding of a candidate coding sequence for an immunomodulatory protein in *G. australe*, exhibiting high sequence identity and secondary structure similarity to a reported immunomodulatory protein in *Taiwanofungus camphoratus*. The results motivate further detailed biochemical and functional studies of this predicted hypothetical immunomodulatory protein.

Materials and Methods

Fungal culture

A mycelium sample of *Ganoderma australe* grown on potato-dextrose agar (PDA) was received as a donation from the Universidad Tecnológica de Chocó, Colom-

bia. For biomass production, the fungus was replicated on PDA and malt-extract agar (MEA). Then, these cultures were used to inoculate a liquid culture medium enriched and optimized for *Ganoderma,* according to the protocol described by Zhou *et al*. (2007), in order to obtain a clean and pure sample for RNA extraction from mycelium.

cDNA library preparation

Total RNA extraction and messenger RNA (mRNA) purification, copy DNA (cDNA) library preparation, and 454 GS FLX (Roche®) transcriptome sequencing was performed at the Centro Nacional de Secuenciación Genómica (CNSG), Sede de Investigación Universitaria (SIU), at the Universidad de Antioquia, Medellín, Colombia.

Total RNA extraction and mRNA purification from a mycelium sample of *G. australe* was done using the RNeasy Plant Mini Kit (Qiagen) and Oligotex mRNA Mini Kit (Qiagen), respectively, according to the manufacturer's instructions. Total RNA and mRNA quantification was carried out on a TBS 380 fluorometer (Turner Biosystems) and quality was determined through capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies).

Once optimal mRNA concentration and quality was determined, cDNA library preparation was done according to the Roche® manufacturer protocol. Library quality was determined through capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies) and sequencing was performed on half a plate of the 454 Sequencing System GS FLX platform (Roche®).

De novo transcriptome assembly and bioinformatics analyses

Sequenced reads were assembled using the bioinfomatics algorithms employed by the Newbler 2.6 assembler (Roche®). In order to identify mRNA transcripts related to the expression of immunomodulatory proteins in *G. australe* mycelium, a local BLAST (tblastn) search was performed against a database of GenBank-reported FIPs and other immunomodulatory proteins of other protein families, in order to broaden the search for immunomodulatory proteins in *G. australe*. Non-FIP family proteins were included if they belonged to fungal species and showed significant alignment e-values and high sequence identities to the reported FIPs.

The transcripts with the highest identity to reported immunomodulatory proteins were selected for further bioinformatics analyses using online programs and servers, such as: Translate, for translating a DNA sequence to its six open reading frames and selecting the most probable coded protein (http://web.expasy.org/translate/); MUSCLE (Edgar, 2004), for multiple alignments between the selected translated protein sequences and reported immunomodulatory proteins; Interpro, to predict protein domain architecture of the candidate translated sequences (http://www.ebi.ac.uk/interpro/); and PSI-PRED v3.0, for predicting secondary protein structure (http://bioinf.cs.ucl.ac.uk/psipred/).

Results and discussion

A total of 712,683 raw reads were obtained from the transcriptome sequencing of the mycelium sample of *G. australe*. Preprocessing and quality control filtered 679,023 reads that were used for *de novo* assembly. Table 1 summarizes the *de novo* assembly results.

Table 1. Summary of *Ganoderma australe* transcriptome sequencing and assembly.

Sequencing	
Total reads	712,683
QC-passed reads	679,023
Amount of information	277 MB
De novo **assembly**	
Total contigs	10,257
N50 (bp[b])	918
Number of large contigs[c]	4,692
Mean size of large contigs (bp)	893
Largest contig size (bp)	4,933

[a] Quality control-passed
[b] bp: base pairs
[c] Contigs of at least 500bp

The search for transcripts (contigs) potentially related to immunomodulatory proteins allowed for the identification of 24 initial candidate sequences showing significant alignments to non-FIP immunomodulatory proteins (data not shown). A further BLAST search against GenBank non-redundant nucleotide and protein databases filtered 12 of these transcripts, showing homology to non-FIP immunomodulatory proteins reported in the basidiomycetes *Trametes versicolor* (GenBank Accession AGH06133.1) and *Taiwanofungus camphoratus* (GenBank Accession AAT11911.1), which were not included in our initial protein database, since they showed no significant hit to the FIP family. Although *Trametes versicolor* has a reported FIP, homology was found with a different non-FIP immunomodulatory protein reported for this species.

Finally, only the transcripts with the highest identity were selected as definite candidates, which resulted in three candidate contigs: c2535 (e-value=3e-37), c725 (e-value=1e-41), and c13717 (e-value=6e-48), with 69%, 68%, and 66% identities, respectively, to the *Tai. camphoratus* protein. Subsequent analyses of the

candidates were performed in comparison to this particular *Tai. camphoratus* protein, due to showing higher identity values than the *Tra. versicolor* protein.

The selected contigs were translated to protein sequences (139 aa, 137 aa, and 137 aa for c2535, c725, and c13717, respectively) and a MUSCLE alignment was performed against the *Tai. camphoratus* immunomodulatory protein (figure 1). The alignment shows conserved sequence regions between the translated proteins and the reported protein. Furthermore, many mismatch positions included substitutions between amino acid residues with similar biochemical properties.

Secondary structure prediction of the hypothetical proteins showed high structural similarity between the three predicted proteins (figure 2). The protein from c13717 was further selected to be compared to the predicted secondary structure of the *Tai. camphoratus* immunomodulatory protein (figure 3), because it showed the highest prediction confidence level compared to the other two candidates. Both *G. australe* c13717 and *Tai. camphoratus* proteins consisted of two α helices and seven β sheets, with the exception of a short additional β sheet in c13717, which was considered non-significant, due to a low prediction confidence level, as represented by the height of the bars above the predicted structure (figure 3). Although secondary structure is not conclusive for determining protein function, it is nevertheless an indicator of a similar folding pattern which could give insight into similar structural motifs between the proteins, as well potential similar function.

Interestingly, the immunomodulatory protein from *Tai. camphoratus* was isolated by Sheu *et al*. (2009) from a mycelium extract and reported as a glycoprotein with a molecular weight of 27 kDa and 136 amino acid residues. Its immunomodulatory function is promoted through macrophage activation of a pro-inflammatory response (Sheu *et al*., 2009). Furthermore, it has been reported that this protein does not promote hemagglutination in humans or mice (Sheu *et al*., 2009), which makes it promising for therapeutic application.

The immunomodulatory protein from *G. australe*, inferred from contig c13717 in this study, as well as those reported from *Tai. camphoratus* and *Tra. versicolor,* show a predicted protein architecture composed of an 18 amino acid residues signal peptide and a cerato-platanin conserved domain, which is present in proteins within the cerato-platanin protein family. This family contains phytotoxic proteins of approximately 150 ami-

Figure 1. MUSCLE alignment between translated protein sequences of the candidate immunomodulatory proteins from *Ganoderma australe* against the immunomodulatory protein sequence from *Taiwanofungus camphoratus*.

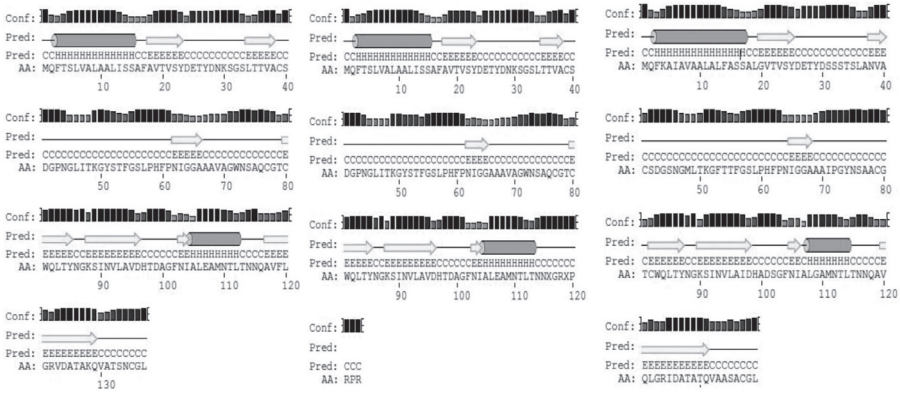

Figure 2. Predicted primary and secondary protein structures of the candidate immunomodulatory hypothetical proteins founded in *Ganoderma australe*. From left to right, blocks represent contigs: c13717, c725, and c2535, respectively. For each block: 1st row, bars indicate confidence levels and a higher confidence level of the predicted structure is indicated by a greater height of the bars. 2nd row, secondary structure predictions are indicated by two figures; cylinders represent α helices, arrows represent β sheets and straight lines represent random coils. 3rd row, strings of letters H, E and C, represent the same structures as in 2nd row, respectively. 4th row, amino acids are indicated individually by single letter code. 5th row, amino acid residues positions are numbered.

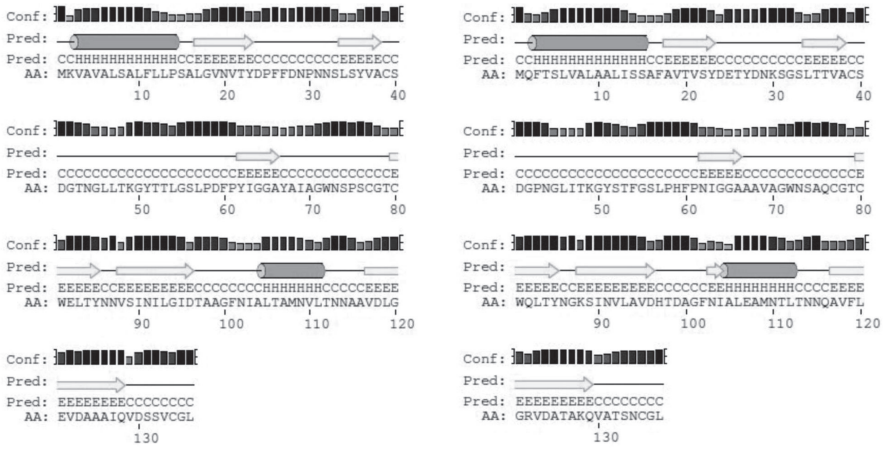

Figure 3. Comparison of primary and secondary immunomodulatory protein structures of the *Taiwanofungus camphoratus* immunomodulatory protein (left block) and the *Ganoderma australe* hypothetical immunomodulatory protein (right block), inferred from contig c13717 in this study. Key descriptions for each row same as in figure 2.

no acid residues, with four cysteine residues that form two disulfide bridges contributing to protein structure (Pazzagli *et al*., 1999). Indeed, the immunomodulatory protein predicted from *G. australe* is not, in a strict sense, an immunomodulatory protein belonging to the FIP family, like other proteins previously reported for *Ganoderma*. To this respect, strictly speaking FIPs have a molecular architecture composed of immunoglobulin-like beta-sandwich folds, characteristic of proteins within the immunomodulatory fungal family (FIP-fve), which is not the case with the *G. australe* protein reported in this study. Furthermore, the hypothesized *G. australe* protein exhibits substantial identity and structural similarity with the *Tai. camphoratus* protein, and both contain methionine, cysteine, and histidine residues that FIP-fve and LZ-8-like prototypes do not contain. This evidence supports that the *G. australe* hypothetical protein reported and modeled in this work is not properly an immunomodulatory protein belonging to the FIP family as traditionally defined, like those reported in other *Ganoderma* species and other mushrooms, but rather a novel type of immunomodulatory protein with potentially similar functionalities based on its structure.

Finally, the observed homology of the hypothetical protein from *G. australe* with the immunomodulatory protein from *Tai. camphoratus* is an interesting finding from a taxonomic perspective, since *Tai. camphoratus* has shared a taxonomic relation with the genus *Ganoderma* in the past. *G. australe* was initially described as *Ganoderma comphoratum* Zang & Su (1990), yet, due to morphological aspects of the fruiting body, it was reclassified under the genus *Taiwanofungus* (Polyporaceae) in 1994, with *Taiwanofungus camphoratus* as the only species in this genus, endemic to Taiwan (Wu *et al*., 2004).

Further analyses should include the genomic sequencing of *G. australe* and the *in vitro* testing of this sequence by means of molecular cloning of the complete gene into an appropriated vector, for example *Pichia pastoris*, or any other commercial expression vector, in order to further evaluate the therapeutic potential of the gene product through the implementation of protein recombination technology and bioassays approaches using a model organism.

Conclusions

In this study, the presence of a candidate genetic sequence coding for a hypothetical immunomodulatory protein in the mycelium of *Ganoderma australe* is reported. Moreover, transcriptome sequencing and assembly performed in this study allow an estimation of at least 8,000 to 10,000 genes in *G. australe*. In order to confirm the presence of the predicted protein and experimentally determine its potential bioactive properties, future prospects include protein isolation and purification, recombinant expression, and possible *in vitro* and *in vivo* evaluations of the predicted immunomodulatory function.

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