International Immunopharmacology 15 (2013) 167–175

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Biochemical and functional characterization of recombinant fungal immunomodulatory proteins (rFIPs)

Shanna Bastiaan-Net [a,*,1], Wasaporn Chanput [a,b,c,d,1], Amelie Hertz [a], Romy D. Zwittink [a], Jurriaan J. Mes [a], Harry J. Wichers [a,c]

[a] Food and Biobased Research, Wageningen University and Research Centre, Bornse Weilanden 9, P.O. Box 17, 6700 AA Wageningen, The Netherlands
[b] Cell Biology and Immunology Group, Wageningen University and Research Centre, De Elst1, P.O. Box 338, 6708WD, Wageningen, The Netherlands
[c] Laboratory of Food Chemistry, Wageningen University and Research Centre, Bomenweg 2, P.O. Box 8129, 6700 EV Wageningen, The Netherlands
[d] Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, 50 Phahonyothin Road, Chatuchak, Bangkok, 10900 Thailand

ARTICLE INFO

Article history:
Received 17 August 2012
Received in revised form 1 November 2012
Accepted 1 November 2012
Available online 19 November 2012

Keywords:
Fungal immunomodulatory proteins (FIPs)
Hemagglutination
Caco-2 cells
Transepithelial electrical resistance (TEER)
THP-1 macrophages
Immunomodulation

ABSTRACT

In this study, two novel FIPs have been identified and characterized. The first is FIP-*nha*, identified in the ascomycete *Nectria haematococca*, and as such, FIP-*nha* would be the first FIP to be identified outside the order of Basidiomycota. The second is LZ-9, an LZ-8 like protein identified in *Ganoderma lucidum*. Recombinant FIPs (rFIPs) were produced in *Pichia pastoris* and purified using His-affinity magnetic beads. The bioactive characteristics of FIP-*nha* and LZ-9 were compared to the well-known FIPs, LZ-8 from *G. lucidum* and FIP-*fve* from *Flammulina velutipes*, which were produced and purified using the same method. The produced rFIPs: rLZ-8, rLZ-9, rFIP-*fve* and rFIP-*nha* were investigated for their hemagglutinating activity which revealed that rLZ-8, rLZ-9 and rFIP-*nha* were able to agglutinate rabbit, mouse and sheep red blood cells while rFIP-*fve* only agglutinated rabbit red blood cells. None of the rFIPs were able to agglutinate human red blood cells unless the cells were trypsinized. In addition, all rFIPs were studied and compared to several lectins for their effect on Caco-2 intestinal cell layer integrity using transepithelial electrical resistance (TEER) measurement. rLZ-9 appeared to have the highest effect in lowering TEER, similar to one of the tested lectins. Testing of rFIPs for their activation of inflammation-related genes of THP-1 macrophages showed rFIP-*fve* to be the strongest inducer of pro-inflammatory cytokine transcription. These results indicate that each rFIP has a unique bioactive profile as well as each lectin, creating the basis for further studies to relate structure to biological activity.

1. Introduction

Fungal immunomodulatory proteins (FIPs) are a class of small proteins extensively studied for their immunomodulating activities [1–3]. They have shown to potentially exert, for instance, anti-cancer, anti-tumor, anti-allergy and anti-anaphylaxis activity and stimulation of immune cells to produce cytokines [1,4,5]. Moreover, it has been described that FIPs can be applied in pharmaceutical preparations or vaccines in order to enhance the level of immune regulation and suppress tumor and autoimmune diseases [6,7]. Several FIPs belonging to the fungal order of Basidiomycota were identified: LZ-8 from *Ganoderma lucidum* [8], FIP-*gts* from *Ganoderma tsugae* [9], FIP-*gja* from *Ganoderma japonicum* (GeneBank: AAX98241), FIP-*gsi* from *Ganoderma sinense* [10], FIP-*gmi* from *Ganoderma microsporum* [11], FIP-*gap* from *Ganoderma applanatum* (GeneBank: AEP68179), FIP-*fve* from *Flammulina velutipes* [12], FIP-*vvo* from *Volvariella volvacea* [13] and FIP-*tvc* from the mushroom *Trametes versicolor* [14]. Based on BLAST analysis, we identified a novel fungal immunomodulatory protein gene in the *Nectria haematococca* genome sequence (DOE Joint Genome Institute, http://www.jgi.doe.gov/) and designated it as FIP-*nha* (GeneBank: XP3043654). This pathogenic fungus, also commonly referred as *Fusarium solani*, is a member of the Sordariomycetes family belonging to the order of Ascomycota which makes FIP-*nha* the first FIP member identified outside the Basidiomycota order. Besides this complete new FIP, we previously identified a slightly different FIP as currently published for *G. lucidum* (in preparation). This FIP has a high similarity to LZ-8 and was therefore named LZ-9.

The resemblance in the bio-functional properties of FIPs to plant lectins has been well recognized in their ability to agglutinate red blood cells [1,14,15]. Both types of proteins are able to interact and bind to cell surface sugar moieties [16,17]. Some lectins are known for their anti-nutritional effect as they can bind to membrane glycosyl groups of the cells lining the digestive tract. As a result of that, they can damage luminal membranes of the epithelium and interfere with nutrient digestion and absorption (noticeable as causing diarrhea) as also shown by decreasing transepithelial electrical resistance (TEER) values in *in vitro* cell model studies, using Caco-2 cells [18,19]. However, it still

* Corresponding author at: Food and Biobased Research, Wageningen University and Research Centre, Bornse Weilanden 9, 6708WG Wageningen, The Netherlands. Tel.: +31 317 487 611; fax: +31 317 483 011.
E-mail address: shanna.bastiaan@wur.nl (S. Bastiaan-Net).
[1] These authors contributed equally to this work.

1567-5769/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.intimp.2012.11.003

remains unknown whether FIPs also have an effect on the intestinal cell layer integrity, which is important when developing novel functional food or feed products based on FIPs. Besides that, there is no assay available for comparing lectins and FIPs to study their specific bioactivity towards immune cells systematically, while such method could support risk-benefit analysis. Therefore, the human macrophage cell line model, THP-1, was explored as a model to analyze the bioactivity of this class of proteins.

In this study, we expressed, isolated and purified four FIPs: LZ-8 and LZ-9 from *G. lucidum*, FIP-*fve* from *F. velutipes* and FIP-*nha* from *N. haematococca* using the *Pichia pastoris* expression system. Bioactivity characteristics of the purified recombinant FIPs (rFIPs) were investigated and compared to some lectins in terms of hemagglutinating activity, kinetic of transepithelial electrical resistance (TEER) values of confluent differentiated Caco-2 monolayers and expression of inflammation-related genes in THP-1 macrophages. The characterizations of rFIPs provided in this research were aimed to support risk-benefit analysis and raise some issues which should be taken into consideration when designing human and animal intervention trials in the future.

2. Materials and methods

2.1. Samples and chemicals

Yeast extract peptone dextrose (YPD) broth, low salt Luria–Bertani (LB) broth, buffered glycerol-complex medium (BMGY), glycerol, peptone, micro agar, yeast extract and yeast nitrogen base (YNB) with ammonium sulfate without amino acids were purchased from Duchefa (The Netherlands). The methanol was obtained from Merck (Germany). All tested lectins were purchased from Sigma-Aldrich (St. Louis, MO, USA): BSI from *Bandeirae simplicifolia* (*Griffonia simplicifolia*), Concavalin A type IV from *Canavalia ensiformis* (Jack bean), SBA from *Glycine max* (soy bean), WGA from *Triticum vulgaris* (wheat germ) and LE from *Solanum esculentum* (tomato) with protein content according to manufacturer's datasheet >90%, non-determined, >80%, 100% and >40%, respectively. All PCR and RT-qPCR primers used were synthesized by Biologio (The Netherlands).

2.2. Recombinant FIPs (rFIPs) cloning and construction of expression vector

The nucleotide sequence of LZ-8 and FIP-*nha* was synthesized by BaseClear B.V. (Leiden, the Netherlands) based on the sequence identified in *G. lucidum* (GeneBank: ACD44335) and *N. haematococca* (GeneBank: XM3043608), respectively. The FIP-*fve* and LZ-9 genes were obtained by PCR based cloning from *F. velutipes* (strain M4600) and *G. lucidum* (strain M9720), respectively, in which both strains were obtained from Mycelia BVBA, Belgium. To facilitate sub-cloning of the FIP genes into Pichia expression vector pPICZα-A (Life Technologies Ltd., Paisley, UK), the following forward and reverse primers were used:

5′-GGGGAATTCTCCGACACTGCCTTGATCT-3′ (Forward LZ-8 and LZ-9),
5′-GGGGAATTCGCTACCACCAATGACTC-3′ (Forward FIP-*nha*),
5′-GGGGAATTCTCCGCCACGTCGCTCAC-3′ (Forward FIP-*fve*),
5′-GGGTCTAGA**CCTCCCCCGCCTCCCCC**GTTCCACTGGGCGATGATG-3′ (Reverse LZ-8 and LZ-9),
5′-GGGTCTAGA**CCTCCCCCGCCTCCCCCC**TTCCACTGGGCAACAAGAT-3′ (Reverse FIP-*nha*) and
5′-GGGTCTAGA**CCTCCCCCGCCTCCCCC**AGTCTTCTTCCACTCAGCG-3′ (Reverse FIP-*fve*).

An *Eco*RI and *Xba*I restriction site, as underlined above, was added to the forward and reverse primers, respectively. In addition, all reverse primers contained an additional flexible linker consisting of six glycine amino acids (6× Gly, indicated in bold) to stimulate proper structure formation and free rotation in between the rFIP and c-myc epitope/6× His tag fusion protein [20]. All FIP fragments were amplified using the AccuPrime™ *pfx* polymerase of Life Technologies Ltd. PCR amplification conditions were as follows: a 2 min denaturing step at 94 °C followed by 35 cycles of amplification, 15 s at 94 °C for denaturation, 30 s at 63 °C for annealing and 1 min at 68 °C for extension. Amplified fragments were run on a 2% agarose gel. DNA bands were purified using the agarose gel purification kit (QIAGEN, Valancia, USA) and sub-cloned into pGEM-T vector (Promega Corporation, Wisconsin, USA). The ligated plasmids were used to transform *Escherichia coli* XL1-Blue heat-shock competent cells (Stratagene, Santa Clara, USA). The transformants were checked by restriction endonuclease digestion and verified by full length sequencing (BaseClear B.V., Leiden, The Netherlands). Finally, the verified FIP genes were transferred into the pPICZα-A plasmid using the *Eco*RI/*Xba*I restriction sites to transform *E. coli* Top10 heat-shock competent cells (Life Technologies Ltd., Paisley, UK). The transitions between the FIP gene and the alpha signal and in between the c-myc epitope/polyhistidine (6× His) were checked by sequencing using the sequencing primers 5′-TCATCGGTTACTCAGATTTAGAAG-3′ and 5′-ACG GCGCTATTCAGATCCTC-3′, respectively.

2.3. Transformation of P. pastoris and selection of transformants

The pPICZα-A-FIP plasmids were introduced into *P. pastoris* strain X-33 according to the EasyComp™ *Pichia* Transformation kit (Life Technologies Ltd., Paisley, UK). Transformed cells were spread onto YPD plates with different Zeocin concentrations (100, 250, 500 and 1000 μg/ml) for direct selection of multi-copy insertion transformants. Six integrant strains, chosen from different Zeocin selection plates were checked by colony PCR using FIP forward and 3′AOX reverse primers. Of these, three integrant strains, preferably single-copy as well as multi-copy strains, were evaluated for protein expression.

2.4. Expression and purification of rFIPs from P. pastoris

A single colony, picked from a fresh plate, was grown in 25 ml of BMGY medium at 30 °C for 18–24 h to generate cell biomass. The yeast cells were collected by centrifugation at 1,500 ×*g* for 5 min and resuspended in BMMY medium (baffled flasks, 1:10 v/v medium:air) to a final OD600 of 1.0. Protein expression was induced by adding 100% methanol to a final concentration of 1.5% every 24 h for 3 days. The rFIP proteins secreted into the growth medium were purified using his-affinity electromagnetic beads (Dynabeads; Life Technology Ltd., Paisley, UK) according to the manufacturer's protocol, dialyzed twice against PBS ([−]$MgCl_2$ [−]$CaCl_2$) in the total period of 16 h and quality checked by SDS–PAGE gel and western blot. The contamination of LPS was determined in all test samples using LAL endotoxin test (L00350, GenScript, NJ).

As a negative control for protein purification, the *P. pastoris* wild type strain X33 was grown, treated with methanol and processed in exactly the same way as was done for the FIP expressing strains. The obtained control yeast protein sample, designated as X33nc, functioned as a control in all cell-based assays.

2.5. Biochemical characterization of purified FIPs

2.5.1. Protein yield

Protein concentration was determined by Bradford Assay using Coomassie Protein Assay Reagent (Thermo Fisher Scientific Inc, MA, USA). The protein yield per liter of growth medium in all purified rFIPs was calculated.

2.5.2. SDS–PAGE of purified FIPs

Purified rFIPs were visually analyzed by SDS–PAGE gel using precast NUPAGE 10% Bis-Tris gels in 1× NuPAGE® MES SDS Running Buffer, stained by SimplyBlue™ SafeStain (Life Technologies Ltd., Paisley, UK). Protein sample (7.8 μl) containing 3 μl 4× NuPAGE® LDS buffer (Life