Appl Biochem Biotechnol (2010) 162:1403–1413
DOI 10.1007/s12010-010-8916-1

Cytokines Expression Induced by *Ganoderma sinensis* Fungal Immunomodulatory Proteins (FIP-gsi) in Mouse Spleen Cells

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Received: 20 October 2009 / Accepted: 17 January 2010 /
Published online: 20 February 2010

Abstract *Ganoderma sinensis* fungal immunomodulatory protein (FIP-gsi) was a new member of FIPs family. Based on the cloning of *FIP-gsi* gene from *G. sinensis*, this paper reported that *FIP-gsi* gene was expressed in *Escherichia coli* expression system. Then, the recombinant proteins were analyzed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Finally, the immunomodulatory activity was examined by inducing cytokine genes expression. The results showed that the recombinant FIP-gsi protein could be expressed in *E. coli* and got the yield of about 25% of the soluble form in the total soluble protein. The FIP-gsi protein was composed of 111 amino acids, and the sequence of homologous rate was 88.6% with FIP-glu (LZ-8). Furthermore, it could enhance the levels of interleukin (IL)-2, IL-3, IL-4, interferon gamma, tumor necrosis factor alpha, and IL-2 receptor (IL-2R) in mouse spleen cells.

Keywords *Ganoderma sinensis* · Fungal immunomodulatory protein · Expression · Cytokine

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Introduction

In the ancient Chinese medical encyclopedias, *Shen-Nong Ben Cao Jing* and *Ben Cao Gang Mu*, *Ganoderma* spp. (a group of medical fungus) are recorded as the king of herbs. *Ganoderma lucidum* (named as reishi in Japan, ganoderma in US, and ling-zhi in China) has been used as traditional Chinese medicine for promoting health, perpetual youth, vitality, and longevity [1]. Actually, only *G. lucidum* and *Ganoderma sinensis* are embodied by Chinese Pharmacopoeia (2005 Ed) among the more than 90 *Ganoderma* strains which are indigenous to China [2]. Unfortunately, many researches study on *G. ludicum* and *Ganoderma tsugae*, but few on *G. sinensis*. *Ganoderma* contains many bioactive substances, and the major activity components include *G. lucidum* polysaccharide, triterpene, protein, adenosine, organic acid, and alkaloid [3]. While in these compositions, lots of researches have been carried out in polysaccharide and triterpene, but few in protein [4]. The bioactive proteins responsible for the immunomodulating activities have been qualitatively described [5, 6], but the molecular mechanisms of the immunomodulating action of proteins have not been clearly elucidated.

Fungal immunomodulatory proteins (FIPs) (*ganoderma lucidum* (LZ-8) (FIP-glu), *ganoderma tsugae* (FIP-gts), *flammulina velutipes* (FIP-fve), and *volvariella volvacea* (FIP-vvo)) showed the similar structure, and the structure of FIPs was similar with variable region of immunoglobulin heavy chain in its primary and secondary structure [5, 7–10]. The natural FIPs were presented in dimerization form which showed a dumb bell-shaped structure [11]. The key amino acids, leucine (Leu-5), phenylalanine (Phe-7), and Leu-9 in the ten amino acids of N-terminal composing the first α-helix were considered to be responsible for the immunomodulatory activity [7]. Because of the similar structure, FIPs had the similar function. FIPs had the activity of hemagglutination. They could hemagglutinate rat, sheep, or human blood cells. In addition, the immunomodulatory bioactive of FIPs was confirmed by stimulating the proliferation of mouse spleen cells or human peripheral blood lymphocytes (hPBLs), selectively enhancing the levels of expression of cytokines [5, 7–9]. FIPs had the ability of induction of cytokines expression. Ling Zhi-8 (LZ-8) could stimulate interleukin (IL)-2, IL-8, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α production in the hPBLs and could modulate IL-2/IL-4 balance in the human peripheral blood mononuclear cells (hPBMCs) [12, 13]. Recombinant FIP-gts could enhance the level of IL-2 induction in murine splenocytes [14]. The FIP-fve had the ability of increasing the expression of IL-2, IFN-γ, and TNF-α in mouse spleen cells [8, 11]. While the natural FIP-vvo was able to enhance the expression of IL2, IL-4, TNF-α, IFN-γ, lymphotoxin (LT), and IL-2R in mouse spleen lymphocytes [9].

In our previous study, a gene encoding FIP-gsi was cloned from *G. sinensis* [15]. To further examine the function of the gene and the immunomodulatory activity of the protein encoded by the gene, we had focused on expressing the protein in *Escherichia coli*. In particular, the immunomodulatory activity was demonstrated by inducing the expression of cytokines. Furthermore, the results were examined by reverse transcriptase-polymerase chain reaction (RT-PCR) and showed by agarose gel electrophoresis. This study might lay a foundation for the mechanism of expression of cytokines induced by FIP-gsi.

Materials and Methods

Materials

FIP-gsi gene was preserved by our laboratory. Kunming mice were purchased from B&K Universal Group Limited (SCXK (Hu 2008-0026)).

Construction of Expression Vector

The gene encoding FIP-gsi had been cloned into pMD18-T vector (Japan) before. The *FIP-gsi* gene sequence was re-amplified from the T vector by PCR and subcloned into an expression cassette vector pQE-30 (CA, USA). The primers were designed based on the previous research [15]. To facilitate the cloning of *FIP-gsi* gene ligased with the vector pQE-30, a restriction enzyme site of *Bam*HI underlined below was added into a forward primer: 5′-CGGGATCCATGTCCGACACTGCCTTGATCTTCAGG-3′ and a *Hin*dIII site underlined below was added into a reverse primer: 5′-CCAAGCTTCTAGTTCCACTG GGCGATGATGAAGTC-3′. The PCR amplification was carried out using primers and pMD18-T vectors as template, all mentioned above under a thermocycling profile after initial denaturation at 94 °C for 5 min; the PCR reaction was performed using Taq plus deoxyribonucleic acid (DNA) polymerase (Japan). Denaturation was performed at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 40 s. A final extension at 72 °C for 10 min was added at the end of the 35 cycles. The PCR products were analyzed by gel electrophoresis and cloned into pMD18-T simple vector (Japan). After digestion of *Bam*HI and *Hin*dIII, the digested products encoding FIP-gsi were subcloned directly into pQE-30 vector predigested with the same restriction enzymes. After being transferred into *E. coli* strain M15 (pREP4) for the prokaryotic expression, the resulting recombinant plasmid, designated as pQE-FIP-gsi, was sequenced.

The Expression of the *FIP-gsi* Gene in *E. coli*

A single positive colony of transformants with plasmid pQE-FIP-gsi was grown overnight at 37 °C in Luria–Bertani (LB) medium supplemented with 100 mg/L ampicillin and 25 mg/L kanamycin. The transformed strains were then cultured in the same culture at 37 °C until the optical density (OD_{600}) reached 0.5–0.7. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM for protein expression, and the culture was incubated for another 5 h at the same condition. The cells were harvested by centrifugation at 5,000 rpm for 20 min and resuspended in 100 μL of 2× SDS-PAGE sample buffer containing 100 mM, pH 6.8 Tris-Cl, 4% (*w*/*v*) SDS, 0.2% (*w*/*v*) bromophenol blue, 20% (*w*/*v*) glycerine, and 2% (*w*/*v*) β-mercaptoethanol and boiled for 5 min at 95 °C. The expression product was analyzed by 15% SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

Induction Time Screening and Western Blotting Analysis

To find out the optimum expression time and analyzing the time course of expression, 1-mL cultures were taken at time points 0, 0.5, 1, 2, 3, 4 and 5 h after addition of IPTG (final concentration of 1 mM) for induction when the transformants were grown until OD_{600} reached 0.6. After the preparation as described before, the samples were loaded and run to SDS-PAGE on two separate gels. One of the gels was strained with Coomassie Brilliant Blue R-250, and the other gel was electroblotted onto 0.2-μm microporous polyvinylidene fluoride membrane (MA, USA) with transfer buffer containing 25 mM Tris, 192 mM glycine, methanol 20% (*v*/*v*) for western blotting. Western blot analysis was performed using 6×His monoclonal antibody (albumin-free) (Japan) as the primary antibody at a 1:20,000 dilution and anti-mouse immunuglobulin (H+L), AP conjugate (WI, USA) as the secondary antibody at a 1:10,000 dilution. The gel electroblotted onto membranes used Mini Trans-Blot® Electrophoretic Transfer Cell System (California, USA).

Purification of the Recombinant FIP-gsi

The recombinant protein FIP-gsi was purified with a column of nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (CA, USA). Cells were harvested from 50 mL cultures after inducement by centrifugation at 5,000 rpm for 20 min at 4 °C and were resuspended in 5 mL of lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0. After being frozen in liquid nitrogen and thawed in cold water, the sample was lysed by sonication six times in 10 s bursts at 200–300 W with a 10-s cooling period between each burst. The debris was removed by centrifugation of at 13,000 rpm for 20 min at 4 °C, and the supernatant containing the 6×His-tagged recombinant protein was utilized for protein purification by Ni-NTA agarose. The Ni-NTA column was washed by 10 vol of Ni-NTA agarose with wash buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0. Then, the recombinant protein was eluted in five 0.5 mL aliquots with elution buffer containing 50 mM NaH_2PO_4, 300 Mm NaCl, 250 mM imidazole, pH 8.0 and was analyzed by SDS-PAGE.

MALDI-MS of Recombinant FIP-gsi

The MALDI-MS of recombinant FIP-gsi was performed in institutes of biomedical sciences, Fudan University. The target protein bind was resolved in SDS-PAGE excised from the gel. After trypsin digestion, sulfonation, and desalination, the analysis of post-source decay MALDI-MS of the sample was applied on a 4700-Protemics Analyzer with version 3.0 software (CA, USA).

Ribonucleic Acid Extraction and Reverse Transcriptase-Polymerase Chain Reaction

Spleen cells were obtained from Kunming mice (6–8 weeks) killed by cervical dislocation method with sterile technique and suspended in RPMI 1640 medium (Shanghai, China) supplemented with 100 mg/L carbenicillin and 25 mg/L kanamycin at 10^7 cells/well (24-well microtitre plate). After being treated with various concentrations of recombinant FIP-gsi, the cultures were incubated under 5% CO_2 in air at 37 °C for 4 h. The total ribonucleic acids (RNAs) of the cells were then isolated by Column Mate RNA Isolated Mini Kit (Shanghai, China), and PCR was carried out in a volume of 25 μL mixture using one step RNA PCR kit (AMV) (Japan). All the primers used in RT-PCR amplifications to detect gene expression of cytokines (β-actin used as internal control) were designed according to the references (Table 1) and synthesized (Shanghai, China). Reverse transcription was done at 50 °C for 30 min, 94 °C for 2 min followed by 25 cycles (30 cycles for IL-3, IL-4, and LT) of 30 s at 94 °C, 30 s at 60 °C (65 °C for IL-2 primers), and 40 s at 72 °C. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

Results

Construction of Expression Vector pQE-FIP-gsi

The gene, *FIP-gsi*, was re-amplified from pMD18-T vector with a restriction enzyme site of *Bam*HI and *Hin*dIII and cloned into pQE-30 vector. The recombinant plasmid was designated as pQE-FIP-gsi. The approximate molecular mass of the recombinant protein, containing 123 amino acids, was 13.8 kDa.

Table 1 The primers used in the detection of gene expression of cytokines.

Names	Sequences	References
IL-1α F	CTCTAGAGCACCATGCTACAGAC	[16]
IL-1α R	TGGAATCCAGGGGAAACACTG	
IL-2 F	TTCAAGCTCCACTTCAAGCTCTACAGCGGAAG	[17]
IL-2 R	GACAGAAGGCTATCCATCTCCTCAGAAAGTCC	
IL-3 F	GAAGTGGATCCTGAGGACAGATACG	[18]
IL-3 R	GACCATGGGCCATGAGGAACATTC	
IL-4 F	ATGGGTCTCAACCCCCAGCTAGT	[19]
IL-4 R	GCTCTTTAGGCTTTCCAGGAAGTC	
IL-5 F	ATGACTGTGCCTCTGTGCCTGGAGC	[20]
IL-5 R	CTGTTTTTCCTGGAGTAAACTGGGG	
IL-6 F	TGGAGTCACAGAAGGAGTGGCTAAG	[21]
IL-6 R	TCTGACCACAGTGAGGAATGTCCAC	
IFN-γ F	TGAACGCTACACACTGCATCTTGG	[22]
IFN-γ R	CGACTCCTTTTCCGCTTCCTGAG	
TNF-α F	ATGAGCACAGAAAGCATGATCCGC	[23]
TNF-α R	CCAAAGTAGACCTGCCCGGACTC	
LT F	TGGCTGGGAACAGGGGAAGGTTGAC	[24]
LT R	CGTGCTTTCTTCTAGAACCCCTTGG	
IL-2R F	ACTGTGAATGCAAGAGAGGTTTCCG	[25]
IL-2R R	AGCAGGACCTCTCTGTAGAGCCTTG	
β-actin F	GTGGGCCGCTCTAGGCACCAA	[26]
β-actin R	CTCTTTGATGTCACGCACGATTTC	

Expression Recombinant FIP-gsi in *E. coli* and Purification of the Recombinant Protein

The recombinant FIP-gsi could be efficiently expressed in *E. coli* M15 cells. The prokaryotic expression vector pQE-FIP-gsi was transformed into *E. coli* M15 expression host strain, and the recombinant protein was expressed by adding 1 mM IPTG (final concentration) at 37 °C. According to the result of SDS-PAGE analysis, a major protein product in the total cellular protein expressed in *E. coli* M15 harboring recombinant FIP-gsi exhibited one specific band with a molecular mass of 14.4 kDa (lane 6 in Fig. 1), which was a similar size to the deduced molecular mass of amino acid sequence of the recombinant FIP-gsi protein.

To determine the optimal induction period, a small-scale time course experiment was performed (Fig. 2 A). The result was determined that the optimal time for the induction of the recombinant protein expression was 4 h. After Coomassie Brilliant Blue staining, SDS-PAGE analysis showed that the fusion protein had been expressed after 0.5 h, and the expression protein showed the most yield at 4 h. The expression protein was detected by western blot using mouse anti-6×His-tag (Fig. 2 B). The results showed that the recombinant FIP-gsi protein had been expressed and had the immunorecognition.

In order to determine whether the recombinant protein was soluble or not, the cells were frozen, thawed, and sonicated. After centrifugation, the supernatants and precipitates resuspended in lysis buffer were analyzed by SDS-PAGE, respectively. The results showed that the fusion protein was mainly in the soluble form (lane 3 in Fig. 3). The supernatant

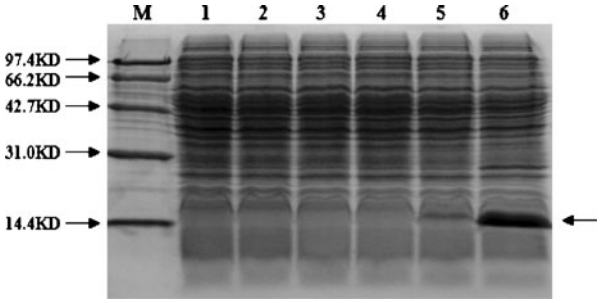

Fig. 1 SDS-PAGE of expression of recombinant FIP-gsi in *E. coli* M15 cells. *Lane M*: protein molecular mass markers. *Lane 1*, *E. coli* M15 cells without being induced by IPTG. *Lane 2*, *E. coli* M15 cells induced by IPTG. *Lane 3*, *E. coli* M15 cells containing expression vector pQE-30 without being induced by IPTG. *Lane 4*, *E. coli* M15 cells containing expression vector pQE-30 induced by IPTG. *Lane 5*, *E. coli* M15 cells containing expression vector pQE-FIP-gsi without being induced by IPTG. *Lane 6*, *E. coli* M15 cells containing expression vector pQE-FIP-gsi induced by IPTG

was purified by Ni-NTA column containing Ni-NTA agarose since the recombinant protein had a 6×His-tag. The yield accounted for about 25% of the total soluble proteins.

MALDI-MS of Recombinant FIP-gsi Protein

After purification by Ni-NTA agarose column, the recombinant FIP-gsi was identified by MALDI-MS. The results showed that the total six peptides (RLAWDVKK, MSDTALIFRL, KKLSFDYTPTWGRG, RFVDNVTPQVLADKA, RDLGVRPSYAVGSDGSQKV, and RFVDNVTFPQVLADKAYTYRV) were identified (Table 2). After being assembled, three peptides, corresponding to 68 amino acid residues of FIP-gsi, over half of the protein sequence, were verified by MALDI-MS (Fig. 4). The sequence of FIP-gsi showed that it is highly homologous with the sequences of FIP-glu, FIP-fve, and FIP-vvo after amino acid alignment (Fig. 5). This result demonstrated that the protein purification from the *E. coli* induced by IPTG was the mature FIP-gsi.

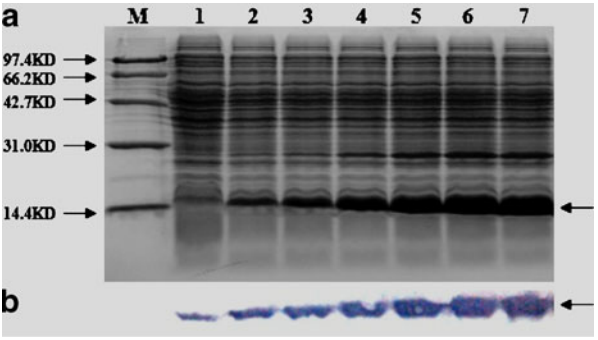

Fig. 2 SDS-PAGE analysis (*A*) and western blot (*B*) of expression of recombinant FIP-gsi at different induction times. *Lane M*: protein molecular mass markers. *Lane 1*, cells without being induced by IPTG. *Lanes 2–7*, cells induced by IPTG at 37 °C for 0.5, 1, 2, 3, 4, and 5 h, respectively

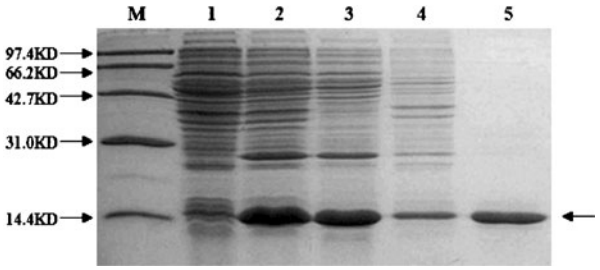

Fig. 3 SDS-PAGE analysis of recombination protein solubility and purification of the protein. *Lane M*: protein molecular mass markers. *Lane 1*, sample without being induced by IPTG. *Lane 2*, sample induced by IPTG. *Lane 3*, soluble protein fraction. *Lane 4*, insoluble protein fraction. *Lane 5*, purified protein

Induction Expression of Cytokine

Recombinant FIP-gsi protein was expressed and purification from *E. coli* M15. To determine the immunomodulatory activity, the induction of cytokine gene expression by recombinant protein FIP-gsi was examined by using murine spleen cells. The gene expression of IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IFN-γ, TNF-α, LT, and IL-2R was examined by RT-PCR. The result demonstrated that the recombinant FIP-gsi protein could induce IL-2, IL-3, IL-4, IFN-γ, TNF-α, and IL-2R gene expression, but not IL-1α, IL-5, IL-6, and LT (Fig. 6).

Discussion

Ganoderma has been used for medicinal purposes for thousands of years in China. A great deal of work has been carried out on *Ganoderma lucidum*, and many biological activities have been isolated from *G. lucidum* [27]. However, *G. sinensis*, like a number of *Ganoderma*, is another important medicinal *Ganoderma* embodied by Chinese Pharmacopoeia (2005 Ed). Few studies on *G. sinensis*, also called "Chinese *Ganoderma*", traditional cultivation using hardwood logs as raw material, is indigenous to China. Some correlation studies focus around the bioactive substances and pharmacological action, and the study of molecular biology are few [28–30]. While *G. sinensis*, the Chinese traditional *Ganoderma*, has been used to defend and treat diseases for thousands of years, the conundrum of the molecular biological mechanism is still waiting for a solution.

Prokaryotic expression is one of the usual methods to express exogenous gene and has the characteristics of easy, fastness, high yield, and fitness for industrialization. Some FIPs

Table 2 Peptide fragments identified form tryptic digest of recombinant FIP-gsi.

Parent icon (*m*/*z*)	Peptide sequence
731.4617	RLAWDVKK
922.5483	MSDTALIFRL
1470.8210	KKLSFDYTPTWGRG
1592.9153	RFVDNVTFPQVLADKA
1735.9543	RDLGVRPSYAVGSDGSQKV
2247.2695	RFVDNVTFPQVLADKAYTYRV

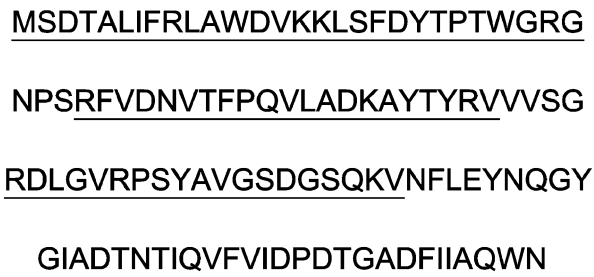

Fig. 4 Purified recombinant FIP-gsi was subjected to protein identification by MALDI-MS. Peptide fragments of recombinant FIP-gsi identified by MALDI-MS analysis are *underlined*

had already been expressed in *E. coli* using different expression vectors. *FIP-glu* (*LZ-8*) gene was ligated with expression vector pET-30a and pET-28b, and the protein encoded by *FIP-glu* (*LZ-8*) was expressed in *E. coli* BL21 with the yield of 34.46 and 70 mg/L, respectively [31, 32]. Two other proteins, FIP-gts and FIP-fve, both expressed with glutathione S-transferase as fusion protein using the expression vector pGEX-2 T, got the yield of 20 and 5 mg/L, respectively [7, 33]. In this study, FIP-gsi protein was expressed in *E. coli* M15 using pQE-30a expression vector. *FIP-gsi* gene was expressed in *E. coli* as a fusion protein of 6×His and FIP-gsi, and the yield of recombinant FIP-fve was about 25% of the soluble form in the total soluble protein. Although structures and functions of FIPs had a lot in common, the difference of genes encoding the proteins had a certain effect on the yield of these proteins expressed in *E. coli*. Furthermore, the yield of protein would be different in different cells using different expression vector. The mechanism still needs to be studied further.

MALDI-MS, which is a clinical assay in proteomics, is a rapid and high sensitivity method, with spectrum unscrambling easily and sample underivatized [34]. This technique has been widely applied in protein identification and protein structural studies [35]. Combined with other methods such as high performance liquid chromatography, MALDI-MS can conveniently provide direct measurement of absolute molecular masses [36]. In this study, recombinant FIP-gsi was routinely analyzed by MALDI-MS methods. The result determined that FIP-gsi was composed of 111 amino acids, and the sequence of homologous rate was 88.6% with FIP-glu (LZ-8) [10]. Because of the high homologous

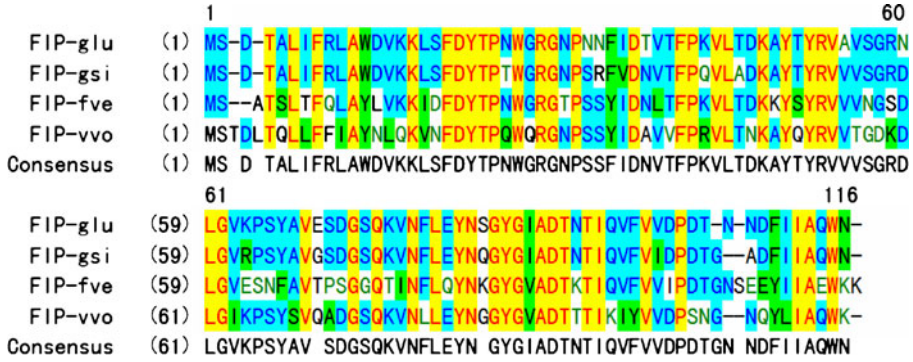

Fig. 5 Amino acid sequence alignment of fungal immunomodulatory proteins. The proteins, FIP-glu, FIP-gsi, FIP-fve, and FIP-vvo, were isolated from *G. lucidium, G. sinensis, Flammulina velutipes* and *Volvariella volvacea,* respectively

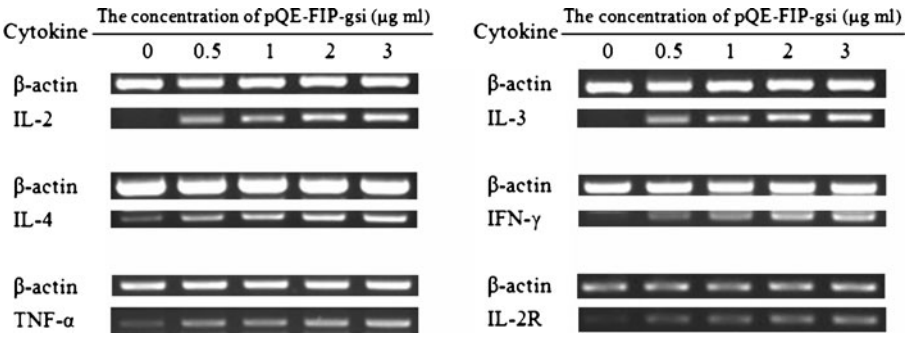

Fig. 6 Induction of cytokine gene expression by the effects of recombinant FIP-gsi protein in mouse spleen cells. The β-actin, used to confirm the equal amount of DNA, were shown

rate, FIP-gsi encoded by *FIP-gsi* gene isolated from *G. sinensis* was believed to belong to FIPs family. The result not only presented a new member in FIP family, but also proved that FIPs have a diversity characteristic.

Cytokine was a kind of protein or minor polypeptide, which had the function of transferring information among cells and is immunomodulatory. T helper (Th) cells, including Th1 and Th2 subsets, are important immune adjustment cells [37, 38]. Th1, mainly secreting IL-2, IFN-γ, LT, and TNF-β, promoting proliferation of Th and CTL, belonged to cellular immune response, while Th2, mainly secreting IL-4, IL-5, IL-6, IL-10, and IL-13, promoting proliferation and differentiation of B cells, belonged to B cell-mediated humoral immune response [39]. The researches showed that the imbalanced Th1 and Th2 subsets are associated with various diseases [40], thus keeping the balance of Th1/Th2 in keeping the health of body.

In this study, the recombinant FIP-gsi protein could induce IL-2, IL-3, IL-4, IFN-γ, TNF-α, and IL-2R gene expression. Among the cytokine-induced expressions by recombinant protein FIP-gsi, two cytokines (IL-2 and IFN-γ) secreted by Th1 and one cytokine (IL-4) secreted by Th2 were induced in mouse spleen cells. The result, recombinant FIP-gsi mainly acting on Th1 cells and less on Th2 cells, had similarity with the result of FIP-vvo which could enhance transcriptional expression of IL-2, IL-4, IFN-γ, TNF-α, LT, and IL-2R [9]. The particular IgE, produced by IL-4, was the main factor mediated anaphylaxis. However, the FIP-vvo could enhance the IL-4 gene expression. Hus et al. [9] believed that this is the reason that FIP-vvo showed little effect on the prevention of systemic anaphylaxis, and recombinant FIP-gsi could also induce the IL-4 gene expression, so maybe it was the same effect with FIP-vvo on the systemic anaphylaxis. FIP-glu (LZ-8) could prevent the systemic anaphylaxis [5], so it might not induce IL-4 gene expression. Haak-Frendscho et al. [12] showed that FIP-glu (LZ-8) could stimulate IL-2 expression in hPBLs. Furthermore, Yeh et al. [13]. found that recombinant FIP-glu (LZ-8) could improve the IL-2/IL-4 ratio in hPBMCs. So maybe, FIP-glu (LZ-8) did not induce IL-4 gene expression, or the expression of IL-2 is more higher than IL-4s. The same FIP-fve could suppress the systemic anaphylaxis, and no study showed that it could induce the expression of IL-4 [8, 11].

In the Th1 subset hyperfunction, the IFN-γ inhibited the proliferation of Th2, inhibited humoral immune response, and caused many disease like acute rejection. In the Th2 subset hyperfunction, IL-4 inhibited the proliferation of Th1, inhibited cellular immune response, and caused lots of diseases such as specific anaphylaxis [39–41]. The antagonism of IFN-γ

and IL-4 impels the balance of Th1/Th2 [42]. The balance of Th1/Th2 is therefore one of the immunomodulatory embodiments. How to maintain the balance of Th1/Th2 efficiently for the benefit of human beings and the mechanism of modulation of Th1/Th2 by FIP-gsi, are the keys to relieve and cure some diseases.

Acknowledgements This research is financially supported by the National Natural Science Foundation of China under Grant Nos. 30771500, Shanghai Science and Technology Committee under Grant Nos.05DZ05007 and Shanghai Leading Academic Discipline Project (Project Number: B209).

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