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Gene cloning and recombinant expression of a novel fungal immunomodulatory protein from *Trametes versicolor*

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ABSTRACT

Homology-based cloning was used to obtain a new gene (named *FIP-tvc*) from the genomic DNA of the Chinese traditional medicinal mushroom *Trametes versicolor*. *FIP-tvc* is a member of the fungal immunomodulatory protein family, is composed of 336 bp encoding 111 amino acids, and is highly homologous with other fungal immunomodulatory proteins. In addition, an expression system for *FIP-tvc* was constructed. The results indicated that recombinant protein FIP-tvc could be expressed in *Escherichia coli* and that about 20% of the expressed protein was in soluble form. Recombinant FIP-tvc was capable of agglutinating mouse and rat red blood cells. Furthermore, recombinant protein FIP-tvc could selectively enhance the expression of interleukin (IL)-1α, IL-2, IL-5, IL-6, tumor necrosis factor-alpha (TNF-α), and lympotoxin (LT) in mouse spleen cells.

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Introduction

Trametes versicolor has received increasing attention from the researchers in food and pharmaceuticals, and is one of the few medicinal mushrooms to have had its clinical effects extensively validated [1]. The polysaccharides in this medicinal mushroom are considered to be its main pharmacological component, and commercial products based on these polysaccharides, such as Kerstin (PSK) and polysaccharopeptide (PSP), have been developed as immunomodulators in adjuvant therapy for cancer patients [2,3]. Medicinal mushrooms, however, also contain bioactive proteins but few researches focus on them [4–6]. Fungal immunomodulatory proteins (FIPs) are well-documented bioactive components, and the following FIPs have been identified from medicinal or edible mushrooms: LZ-8, FIP-fve, FIP-vvo, FIP-gts, FIP-gja, FIP-gsi, and FIP-gmi from *Ganoderma lucidum*, *Flammulina velutipes*, *Volvariella volvacea*, *G. tsugae*, *G. japonicum*, *G. sinense*, and *G. microsporum*, respectively [7–12]. These FIPs are composed of 110–114 amino acids and have a small molecular weight of approximately 13 kDa. Their immunomodulatory bioactivities result from similarity in their primary and secondary structures to those of immunoglobulin heavy chains. FIP bioactivities have been qualitatively determined to inhibit cancer cell growth, to hemagglutinate blood red cells, and to selectively enhance mRNA expression of cytokines including TNF-α, IL-1, IL-6, and IL-12 in spleen cells and in human peripheral blood lymphocytes. Therefore, FIPs are good candidates for developing new therapeutic agents or new types of functional food supplements for the treatment or prevention of cancer, autoimmune diseases, etc. The commercial development of FIPs, however, has been limited by the difficulty in directly obtaining them from mushrooms for requiring long and costly procedures and low yields. The genetic engineering technique could provide an efficient method for the mass production of these bioactive proteins.

In previous experiments, we found that crude protein extracts from fruiting bodies or mycelia of *T. versicolor*, *Grifola frondosa*, *Poria cocos*, etc. could hemagglutinate blood red cells of rat and have a proliferative effect on human peripheral blood lymphocytes [13]. The immune activities of crude protein extract from *T. versicolor* were two times higher than those of other medicinal mushrooms. We speculated that a protein may be responsible for this bioactivity, and an immunomodulatory protein from *T. versicolor* has been purified in our laboratory [14]. In the current study, a fungal immunomodulatory protein gene, named *FIP-tvc*, was cloned from *T. versicolor* and expressed in *Escherichia coli*. The activity of the expressed protein was demonstrated by its ability to hemagglutinate red blood cells and to induce the gene expression of cytokines.

Materials and methods

Cultures and materials

T. versicolor isolate N50435 was supplied by Professor Xiaoqing Zhang (Institute of Microbiology, Chinese Academy of Sciences) and maintained in our laboratory. Vector pMD18-T and rTaq DNA

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polymerase were purchased from TaKaRa Biotechnology. The expression plasmid pET21a(+) was obtained from Invitrogen. Ni–NTA resin was purchased from Novagen. Female BALB/c mice and male SD/CD rats were purchased from Beijing Vital River Company. *E. coli* DH5α and BL21(DE3) cells used for plasmid manipulation were grown and transformed according to standard procedures.

Gene cloning and construction of expression vector

Total genomic DNA was extracted from *T. versicolor* N50435 mycelia using the CTAB method. The DNA sequence encoding FIP-tvc was amplified by PCR using genomic DNA as a template. A pair of specific primers 5'-AAAAACATATGTCCGACACTGCCTTGATC-3'(the N*de* I site is underlined) and 5'-AAAACTCGAGGTTCCACTGGGCGATGATG-3' (the X*ho* I site is underlined) were designed based on the nucleotide sequence of LZ-8 (GenBank AY449805). At the C-terminus, the stop codon was eliminated and replaced with an X*ho* I restriction site so that the expressed FIP-tvc would bind to the 6 × His-tag. PCR amplification was carried out using rTaq DNA polymerase (TaKaRa). The PCR products were purified with the Cycle-Pure Kit (Omega) and then ligated into the pMD18-T vector (TaKaRa) and finally transferred into *E. coli* DH5α. Plasmid DNA of the positive clone was digested by N*de* I and X*ho* I at 37 °C overnight. The digested products encoding FIP-tvc were subcloned into the pET21a(+) vector, which was predigested with the same restriction enzymes. After transfer into *E. coli* BL21(DE3) for prokaryotic expression, the resulting recombinant plasmid, designated as pET21a-FIP-tvc, was confirmed by sequencing.

The sequence of protein FIP-tvc was deduced by gene sequence using universal codons. Molecular weight (MW) and isoelectric point (*p*I) were calculated with the ProtParam Tool (http://us.expasy.org/tools/protparam.html). Protein sequence analysis was performed using DNAMAN software for multiple sequence alignments with proteins that are available from GenBank using the BLAST program (NCBI). The phylogenetic tree was constructed using the neighbor-joining method of MEGA 4.0 software (http://www.megasoftware.net/mega4/mega.html). The statistical confidence in the phylogenetic relationships was assessed with bootstrap tests that were replicated 1000 times.

Expression and purification of the FIP-tvc in E. coli

One positive colony of *E. coli* BL21(DE3) that carried the recombinant plasmid pET21a-FIP-tvc and that was grown overnight at 37 °C was diluted 100-fold in LB broth containing 100 μg/mL ampicillin and was cultured at 37 °C until the optical density at 600 nm reached 0.4 – 0.6. Isopropyl-β-D-thiogalactoside (IPTG) was added to the culture at a final concentration of 0.5 mM, and the culture was incubated overnight at 16 °C for protein expression. A 1 mL sample of the culture was taken at different times and run on a 15% SDS–PAGE gel to determine the time of maximum protein expression. The bacterial cells were collected by centrifugation at 7000*g* for 5 min and washed twice with binding buffer (20 mM Tris/HCl (pH 7.9), 500 mM NaCl, 5 mM imidazole, and 10% glycerol). The resuspended solution was lysed by ultrasonication for 15 min with a 10 s pulse and 15 s interval. The C-terminal 6 × His-tagged recombinant protein (rFIP-tvc) was purified by Ni–NTA columns (Novagen) according to the manufacturer's protocol. The final purified proteins were examined by 15% SDS–PAGE and stained with Coomassie Brilliant Blue R-250. The concentration of protein was determined by using the BCA protein assay kit (Pierce®) with bovine serum albumin (BSA) as a standard.

Western blotting analysis

Western blot was used to analyze the expression protein rFIP-tvc with 6 × His-tag. After the samples running on SDS–PAGE, gel was washed for 5 min in transfer buffer (25 mM Tris base, 200 mM glycine, 15% ethanol) and proteins were electrophoretically transferred in the same buffer onto polyvinylidene fluoride (PVDF) membrane. Blots were blocked for overnight with 2% (w/v) milk powder in TBST (100 mM Tris, pH 8.0; 150 mM NaCl, 0.1% (v/v) Tween-20). Western blot analysis was performed using 6 × His monoclonal antibody as the primary antibody and HRP-conjugated IgG as the secondary antibody. Added luminescent mixture onto PVDF membrane and exposed to X-ray film for visible blotting.

Haemagglutination assay

For determination of the activity of rFIP-tvc, fresh whole blood was obtained from male SD/CD rats and female BALB/c mice as described previously [14]. After whole blood was washed three times with a phosphate buffered solution (PBS, 136 mM NaC1, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.0 mM Na_2HPO_4, pH 7.2), blood cells were collected by centrifugation at 200*g* for 10 min and then resuspended to 2% (v/v) with PBS. A 10 μL volume containing various concentrations of rFIP-tvc (0.25 μg/mL to 64 μg/mL at final concentration) and 100 μL of 2% red blood cells were placed in the wells of a 96-well U-bottom microtitre plate (Costar). The plate was shaken for 30 s at room temperature and then incubated at 37 °C. The degree of haemagglutination was examined after 2 h.

RNA extraction and reverse transcription–polymerase chain reaction (RT–PCR)

Female BALB/c mice (6–8 weeks old) were killed by cervical dislocation, and their spleens were removed by sterile technique. The spleen cell suspension was filtered through cell strainers, and the erythrocytes were lysed with 0.83% ammonium chloride lysis solution. After three washings, the cells were collected and resuspended in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 200 mM L-glutamate, and 10 mM HEPES at pH 7.3. The cells (10^7 cells/well in 24-well microtitre plates, Costar) were treated

Table 1
Primers used for the detection of gene expression of cytokines.

Name	Sequence	References
IL-1α F	5'-CTCTAGAGCACCATGCTACAGAC-3'	[24]
IL-1α R	5'-TGGAATCCAGGGGAAACACTG-3'	
IL-2 F	5'-TTCAAGCTCCACTTCAAGCTCTACAGCGGAAG-3'	[25]
IL-2 R	5'-GACAGAAGGCTATCCATCTCCTCAGAAAGTCC-3'	
IL-3 F	5'-GAAGTGGATCCTGAGGACAGATACG-3'	[26]
IL-3 R	5'-GACCATGGGCCATGAGGAACATTC-3'	
IL-4 F	5'-ATGGGTCTCAACCCCCAGCTAGT-3'	[27]
IL-4 R	5'-GCTCTTTAGGCTTTCCAGGAAGTC-3'	
IL-5 F	5'-ATGACTGTGCCTCTGTGCCTGGAGC-3'	[28]
IL-5 R	5'-CTGTTTTTCCTGGAGTAAACTGGGG-3'	
IL-6 F	5'-TGGAGTCACAGAAGGAGTGGCTAAG-3'	[29]
IL-6 R	5'-TCTGACCACAGTGAGGAATGTCCAC-3'	
IL-2R F	5'-ACTGTGAATGCAAGAGAGGTTTCCG-3'	[30]
IL-2R R	5'-AGCAGGACCTCTCTGTAGAGCCTTG-3'	
IFN-γ F	5'-TGAACGCTACACACTGCATCTTGG-3'	[31]
IFN-γ R	5'-CGACTCCTTTTCCGCTTCCTGAG-3'	
TNF-α F	5'-ATGAGCACAGAAAGCATGATCCGC-3'	[32]
TNF-α R	5'-CCAAAGTAGACCTGCCCGGACTC-3'	
LT F	5'-TGGCTGGGAACAGGGGAAGGTTGAC-3'	[33]
LT R	5'-CGTGCTTTCTTCTAGAACCCCTTGG-3'	
β-actin F	5'- GTGGGCCGCTCTAGGCACCAA-3'	[34]
β-actin R	5'- CTCTTTGATGTCACGCACGATTTC-3'	

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