INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–0109/2019/22–2–369–374
DOI: 10.17957/IJAB/15.1073
http://www.fspublishers.org

Full Length Article

Expression of *Ganoderma lucidum* Fungal Immunomodulatory Protein (*FIP-glu*) in *Saccharomyces cerevisiae* and its Immunoreactivity

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Abstract

A fungal immunomodulatory protein isolated from *Ganoderma lucidum* is a bioactive substance with small molecular weight. In this study, the full-length cDNA fragment of the fungal immunomodulatory protein from *G. lucidum*, designated as *FIP-glu*, was amplified and subcloned into an expression vector pYES2/NT b. A recombinant vector pYES2: *FIP-glu* was constructed and transformed into *Saccharomyces cerevisiae* INVSc1. The recombinant positive clones grown on SC-U selective medium were identified by PCR technique. Meanwhile, statistical optimization of media composition (carbon and nitrogen sources) and pH were employed for increasing the production of recombinant *FIP-glu* (r*FIP-glu*) by transformants. Cobalt metal affinity resin was used for purification of r*FIP-glu*. After detected and analyzed by SDS-PAGE and Western Blot, the recombinant protein was used to evaluate the immunoreactivity on murine splenocytes. The results indicated that r*FIP-glu* was successfully expressed in *S. cerevisiae* INVSc1. After optimizing media composition and pH, the transformant could produce enough r*FIP-glu* for meeting the need for further experiments. Within a certain range of concentrations, r*FIP-glu* could significantly increase the mRNA levels of *IFN-γ* and *IL-2* in murine splenocytes. The strategy of functional expression of *FIP-glu* gene in *S. cerevisiae* may lay the foundation for the further exploration of the biological activity and security application of *FIP-glu*.

Keywords: Expression system; Fungal immunomodulatory protein; Immunoreactivity; *Ganoderma lucidum*; Yeast

Introduction

Fungal immunomodulatory proteins (FIPs), extracted from some edible or medicinal mushrooms such as *Ganoderma lucidum*, *Flammulina velutipes* and *Volvaria volvacea*, are a group of small molecule proteins with activities of immune regulation and anti-tumor (Li *et al.*, 2011). They have a potential to be developed as new immunomodulatory protein drugs or clinical immunomodulatory adjuvant therapeutic agents (Li *et al.*, 2019). Since 1989, this protein was firstly isolated from fruiting body of *G. lucidum* and named as ling zhi-8 (LZ-8) (Kino *et al.*, 1989). A gene encoding the protein was successfully cloned and was used for expression *in vitro* to study its activity and function (Li *et al.*, 2010; Lin *et al.*, 2011). It has been reported that *FIP-glu* (LZ-8) can promote spleen cell proliferation (Xue *et al.*, 2008; Lin *et al.*, 2009), activate macrophages and T lymphocytes (Yeh *et al.*, 2010), and kill tumor cells (Liang *et al.*, 2012; Cong *et al.*, 2014). For example, recombinant *FIP-glu* (r*FIP-glu*) produced from prokaryote could significantly increase transcription levels of *IL-2* and *IFN-γ* in mouse (*Mus musculus*) splenocytes (Li *et al.*, 2010). rFIP-glu-treated mouse peritoneal macrophages increased the release of *IFN-γ* and *IL-2* by murine $CD4^+$ and $CD8^+$ T cells (Yeh *et al.*, 2010). rFIP-glu can also promote T cell development and maturation and increase dramatic CD154 and CD44 expression on $CD3^+$ T cells and *IL-2* and IFN-γ secretion on $CD4^+$ and $CD8^+$ T cells. Deeper researches exhibit that *FIP-glu* has wide application.

Heterologous expression is one of the most effective methods for the production of FIPs (Zhou *et al.*, 2012). There are plenty of studies on heterologous expression of r*FIP-glu*. These studies involve a variety of host cells, such as *Escherichia coli* (Li *et al.*, 2010; Cong *et al.*, 2014), yeast (Yeh *et al.*, 2010), *Nicotiana tabacum* (Bai *et al.*, 2006) and insect cells (Jinn *et al.*, 2006). *Saccharomyces cerevisiae* is one of the ideal expression hosts for producing recombinant proteins due to its easy-culturing, fast-growing and genetic manipulatable ability in large-scale fermentation (Zhang *et al.*, 2015). In addition, *S. cerevisiae* which is "generally recognized as safe" has been approved by some food and drug administrations for the production of recombinant therapeutic drugs. Most of the recombinant protein drugs currently in the market are produced by *S. cerevisiae*

To cite this paper: Zheng, Y.Z., Q.Z. Li, K.D. Shao, P.W. Mao, Y. Liu and X.W. Zhou, 2019. Expression of *Ganoderma lucidum* fungal immunomodulatory protein (*FIP-glu*) in *Saccharomyces cerevisiae* and its immunoreactivity. *Intl. J. Agric. Biol.*, 22: 369–374

(Huang *et al.*, 2010), including insulin, hepatitis B surface antigen, glucagon and platelet-derived growth factor (Demain and Vaishnav, 2009). In the present study, the *FIP-glu* gene was subcloned into plasmid pYES2 to construct yeast expression vector and transformed into *S. cerevisiae* INVS1 cells for expression of r*FIP-glu*. Subsequently the r*FIP-glu* was purified with cobalt metal affinity resin. The biological activity of the purified r*FIP-glu* was determined by a test using mouse spleen cells as responding cells. This study lays a foundation for further elucidating a possible role of the r*FIP-glu* in the immune response. It is also benefit for the research of industrial production of this medicinal protein.

Materials and Methods

Strains and Reagents

Escherichia coli DH5α and *FIP-glu* from *G. lucidum* subcloned in pMD18-T (TaKaRa, Beijing, China) were already preserved in our lab. *S. cerevisiae* INVS1 and vector pYES2/NT b (pYES2) were provided by Prof. Yidong Zhang from School of Agriculture and Biology, Shanghai Jiao Tong University. KM mice were purchased from JSJ-Lab Co., Ltd (Shanghai, China). Yeast Nitrogen Base (YNB) was bought from Sangon (Shanghai, China). DO Supplement-Ura was obtained from Clontech (TaKaRa, Beijing, China). RPMI 1640 was from Hyclone (Logan, UT). Anti-6×His tag mouse monoclonal antibody and HRP-conjugated goat anti-mouse IgG were from Sangon (Shanghai, China). All chemicals were of analytical grade.

Construction of Expression Vector

The gene encoding *FIP-glu* has already been cloned into pMD18-T vector (TaKaRa, Beijing, China). To facilitate the cloning of *FIP-glu* ligased with the vector pYES2, a restriction enzyme site of *Hin*d III underlined below was added into a forward primer: 5'-CCCAAGCTTATGTCCGACACTGCCTTGATC-3' and an *Eco*R I site underlined below was added into a reverse primer with a 6×His-tag marked by a box: 5'-CGGAATTCCTAATGATGATGATGATGATGGGAT CCGTTCCACTGGGCGATGATG-3'. After PCR amplification, the products were analyzed by gel electrophoresis and digested by *Hin*d III and *Eco*R I. The digested products were subcloned directly into pYES2 vector predigested with the same restriction enzymes. After being transferred into *E. coli* strain DH5α, the resulting recombinant plasmid, designated as pYES2::*FIP-glu*, was sequenced.

Transformation of rFIP-glu into *S. cerevisiae*

The expression vector pYES2::*FIP-glu* was transformed into *S. cerevisiae* INVS1 according to Invitrogen™ pYES2 User Manual. Briefly, 1 μg of plasmid and 100 μg of denatured salmon sperm DNA were transformed into *S. cerevisiae* INVS1 competent cells by lithium acetate method. Then, *S. cerevisiae* INVS1 transformants were spread on SC-U selective plates (6.7 g of YNB and 0.77 g of DO Supplement-Ura (DO-U) for 1,000 mL) containing 2% glucose and incubated at 28℃ until colonies appeared and further confirmed by PCR with primers T7-Forward: 5'-TAATACGACTCACTATAGGG-3' and CYC1T-Reverse: 5'-GTGACATAACTAATTACATGATG-3' (Zhou *et al.*, 2007).

Expression and Purification of rFIP-glu

A single colony was picked and inoculated in SC-U medium containing 2% glucose at 28ºC in a shaking incubator. The cells were harvested by centrifuging and resuspended in SC-U medium containing 2% raffinose with an OD_{600} of 0.4. After incubated for 24 h, the cells were collected and broken by glass beads. Crude proteins were dissolved in lysis buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 10 m*M* imidazole, pH 7.4), and the rFIP-glu was purified with TALON® metal affinity resin (TaKaRa, Beijing, China). The rFIP-glu was ultrafiltrated through 3.5 kD membrane (Millipore, M.A., U.S.A.) to exclude salts with PBS. rFIP-glu was identified by SDS-PAGE and Western Blot.

Medium Optimization Using One-Factor-at-a-Time

The growth of the *S. cerevisiae* INVS1 transformant was optimized based on varying the concentrations of carbon source and nitrogen source and initial pH of culture condition. Five initial pH (5.0, 5.5, 6.0, 6.5 and 7.0), five concentrations of galactose (16, 18, 20, 22 and 24 g/L) and five concentrations of nitrogen with a ratio of YNB to DO-U is 67: 8 (6.0, 6.75, 7.5, 8.25 and 9.0 g/L) were used.

Orthogonal Experimental Design

Based on the results of a preliminary study on the growth of *S. cerevisiae* INVS1 transformant, an orthogonal experiment was performed to identify the significant variables affecting the growth of the transformant. A set of L_9 (3^3) experiments was conducted (Table 1).

Quantitative RT-PCR (qRT-PCR) Analysis

Spleen cells were obtained from KM mice (4 ~ 8 weeks) killed by cervical dislocation method with sterile technique and suspended in RPMI 1640 medium at 10^7 cells in 24-well plate. Cells were treated with PBS as control and various concentrations of r*FIP-glu* (1, 2 and 4 μg/mL) and were incubated at 37℃ in a 5% CO_2 incubator for 4 h. The total RNA was isolated by RNAprep pure Cell/Bacteria Kit

(Tiangen, Beijing, China), and cDNA was synthesized using FastQuant RT Super Mix (Tiangen, Beijing, China). IFN-γ (forward primer: 5'-CGGCACAGTCATTGAAAGCCTA-3' and reverse primer: 5'-GTTGCTGATGGCCTGATTGTC-3') (Ren *et al.*, 2013) and IL-2 (forward primer: 5'-TGAGCAGGATGGAGAATTACAGG-3' and reverse primer: 5'-GTCCAAGTTCATCTTCTAGGCAC) were detected. β-actin (forward primer: 5'-ATCGTGCGGGACATCAAGG-3' and reverse primer: 5'-TCGTTGCCGATGGTGATGAC-3') was used as internal control. qRT-PCR was performed using 2×T5 Fast qPCR Mix (SYBRGreenI) (Tsingke, Beijing, China) and Roche LightCycler® 96 Application. For PCR, samples were heated to 94℃ for 5 min, denatured at 94℃ for 30 s, annealed at 60℃ for 30 s, extended at 72℃ for 30 s and cycled 40 times. All reactions were performed in triplicate. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

All experiments were performed in triplicate. All values are presented as the mean ± SD. Data analysis was performed using SAS 8 statistical software (S.A.S. Institute, Cary, N.C.). Results were considered statistically significant when $P < 0.05$.

Results

Construction of Expression Vector

About 350 bp DNA fragment containing *FIP-glu* was cloned by PCR (Fig. 1A). After digested with *Hin*d III and *Eco*R I, the fragment was subcloned into pYES2 predigested with the same restriction enzymes (Fig. 1B) and a recombinant vector pYES2::*FIP-glu* was constructed (Fig. 2). The molecular weight of the fusion protein with a 6× His tag at C-terminal was about 13.5 kDa.

Transformation of *S. cerevisiae* with pYES2::*FIP-glu*

To identify the positive transformants, their genomic DNA was extracted and used as a template for PCR. As shown in Fig. 3, the PCR product was amplified, which was consistent with the expected size (573 bp). This indicated that the expression vector pYES2::*FIP-glu* had been successfully transferred into *S. cerevisiae*.

Expression, Purification and Identification of r*FIP-glu*

According to the result of SDS-PAGE analysis, the components of total protein of transformants were complicated (Fig. 4A). After purification, rFIP-glu protein in the total cellular protein exhibited two specific bands with a molecular mass of approximately 13.5 kD and 15 kD (Fig. 4A). The results of Western Blot analysis showed that the

Table 1: Factors and levels of orthogonal experiments

Levels	Factors		
	A	B	C
	pH	Concentration of galactose (g/L)	concentrations of nitrogen (g/L)
1	5.0	16	7.5
2	5.5	18	8.25
3	6.0	20	9.0

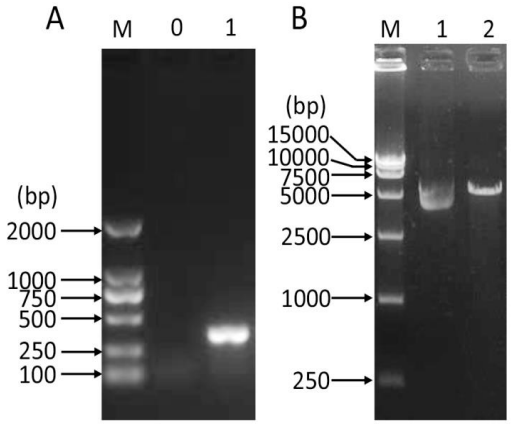

Fig. 1: Electrophoresis of PCR product of *FIP-glu* (**A**) and plasmid pYES2 (**B**). **A**, lane M: DL-2000 DNA marker; lane 0: negative control of PCR; lane 1: PCR product of *FIP-glu*. **B**, lane M: DL-15000 DNA marker; lane 1: plasmid pYES2 before digestion; lane 2: plasmid pYES2 after digestion

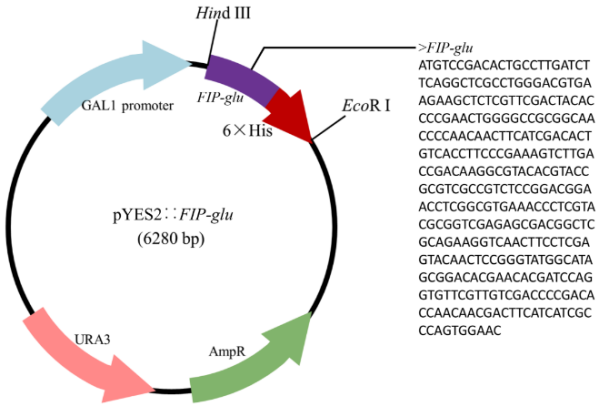

Fig. 2: Physical map of recombinant expression plasmid pYES2::*FIP-glu*

rFIP-glu was successfully produced in *S. cerevisiae* after galactose induction (Fig. 4B).

Optimization of the Media Component and Culture Condition

When *S. cerevisiae* transformants cultured with different pH, their OD_{600} were in the order of 5.5 > 6.0 > 5.0 > 6.5 > 7.0 and the OD_{600} values at pH 5.5 and 6.0 were significantly

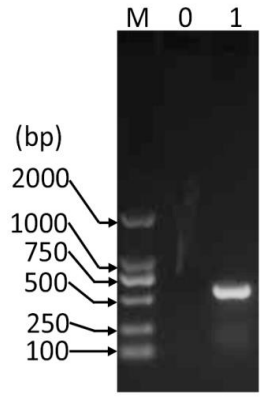

Fig. 3: PCR identification of *S. cerevisiae* transformant with pYES2::*FIP-glu*. Lane M: DL-2000 DNA marker; lane 0: negative control of PCR; lane 1: product of PCR identification

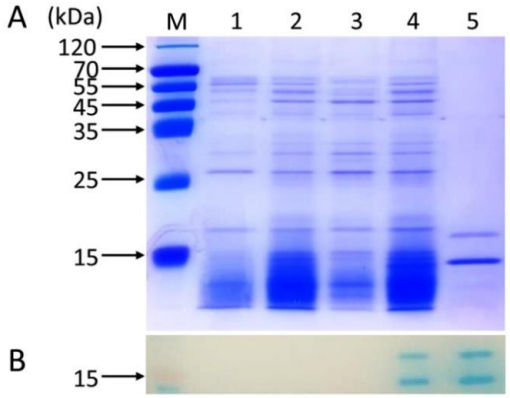

Fig. 4: Analysis of SDS-PAGE (**A**) and Western blot (**B**) of r*FIP-glu*. Lane M: protein molecular mass markers; lane 1: intracellular proteins of the transformant with pYES2 before inducing; lane 2: intracellular proteins of the transformant with pYES2 after inducing; lane 3: intracellular proteins of the transformant with pYES2::*FIP-glu* before inducing; lane 4: intracellular proteins of the transformant with pYES2::*FIP-glu* after inducing; lane 5: purified *FIP-glu*

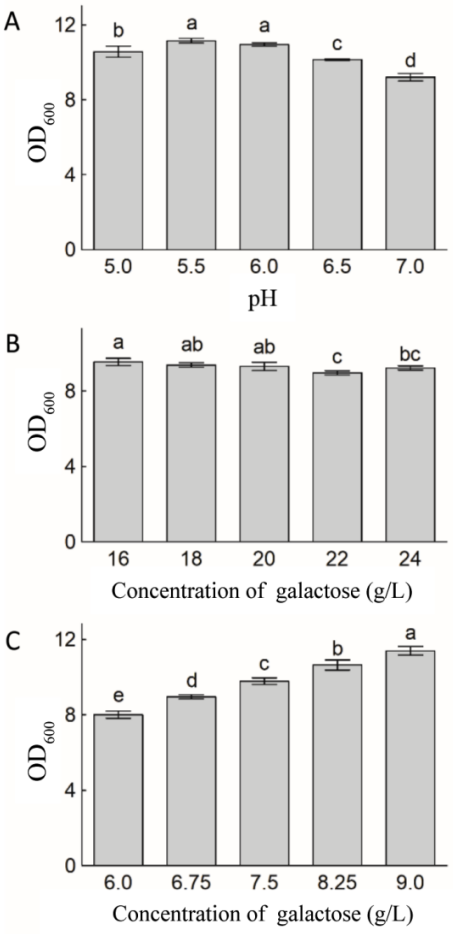

Fig. 5: OD_{600} of *S. cerevisiae* transformant in One-Factor-at-a-Time experiments. Letters were marked as the result of multiple comparisons. Different letters indicated significant differences ($P < 0.05$), while the same letters indicated no significant differences

higher than others ($P < 0.05$) (Fig. 5A). When the transformants were induced by different concentration of galactose, their OD_{600} were in the order of 16 > 18 > 20 > 24 > 22 (g/L) (Fig. 5B). There was no significant difference among the three highest OD_{600} values. As the same analytical method, OD_{600} of transformants with different concentrations of nitrogen in this order 9.0 > 8.25 > 7.5 > 6.75 > 6.0 (g/L), and there were significant differences between every two groups ($P < 0.05$) (Fig. 5C).

As shown in Table 2 and 3, the order of influence of three factors on OD_{600} was: C > A > B. The mixing proportion of optimal media component and culture condition was A2B3C3. This result was confirmed by another test and the OD_{600} value was the highest (11.42 ± 0.38) as expected. Therefore, 20 g/L of galactose, 9.0 g/L of nitrogen and initial pH 5.5 were the optimized media composition and culture condition for *S. cerevisiae* transformants containing *FIP-glu* to growth.

Effects of rFIP-glu on Mouse Spleen Cells

As shown in Fig. 6, compared with the control group, 1 μg/mL of r*FIP-glu* could significantly increase *IFN-γ* mRNA level after treated by FIP-glu for 4 h in mouse spleen cells ($P < 0.01$, Student's t-test) (Fig. 6A). However, the mRNA level of IFN-γ was gradually decreased with the concentration increased. r*FIP-glu* increased the mRNA level of IL-2 in a dose-dependent manner during the concentration of 1, 2 and 4 μg/mL ($p < 0.01$, Student's t-test) (Fig. 6B).

Discussion

In the present study, r*FIP-glu* was produced in *S. cerevisiae* cells, which provides a basis for development of production and application of r*FIP-glu*. For the convenience of

Table 2: Results of orthogonal experiments

Runs	A	B	C	OD600
1	1	1	1	9.41 ± 0.12
2	1	2	2	9.96 ± 0.33
3	1	3	3	10.97 ± 0.10
4	2	1	2	10.17 ± 0.14
5	2	2	3	11.05 ± 0.22
6	2	3	1	9.63 ± 0.09
7	3	1	3	10.19 ± 0.34
8	3	2	1	8.79 ± 0.25
9	3	3	2	9.39 ± 0.26
k_1	10.11	9.92	9.28	
k_2	10.28	9.93	9.84	
k_3	9.46	10.00	10.74	
R	0.82	0.08	1.46	

k_i is the average of all the factors at the same level, and *R* is the extreme difference of every factor

Table 3: Results of variance analysis

Factors	DF	SSD	F-value	*P*-value	Significant
A	2	1.143	304.48	0.0033	**
B	2	0.009	2.53	0.2836	
C	2	3.253	866.17	0.0012	**
Error	2	0.004			

**, $P < 0.01$

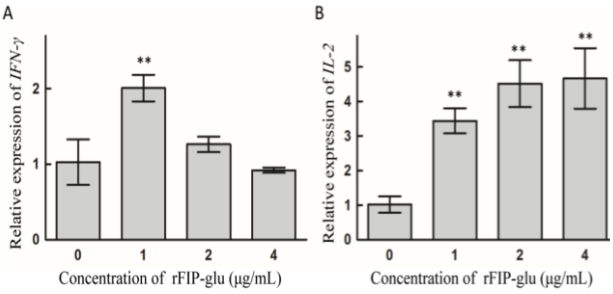

Fig. 6: Effects of rFIP-glu on RNA levels of *IFN-γ* (**A**) and *IL-2* (**B**) in murine splenocytes. ∗∗, $P < 0.01$ versus control

purification of recombinant proteins, a 6× His-tag at C-terminus of r*FIP-glu* was introduced. This strategy could remain complete N-terminal domain of r*FIP-glu*, which is needed in forming a dimer of *FIP-glu* (Lin *et al.*, 1997).

Glycosylation often occurs when foreign proteins are expressed in yeast expression system (Lin *et al.*, 2016). For instance, Bastiaannet *et al*. (2013) gained glycosylated FIP in *Pichia pastoris*. In this experiment, we inferred that the target protein had a molecular weight of 13.5 kD approximately based on the amino acid sequence of r*FIP-glu*. In addition to the 13.5 kD band, actually, there was another band with a molecular weight slightly more than 15 kD (Fig. 4), which was speculated to be the glycosylated r*FIP-glu*. This phenomenon that glycosylated proteins were obtained in *S. cerevisiae* INVSc1 had been reported in previous studies (Chen *et al.*, 2005). Interestingly, Yeh *et al.* (2010) found that r*FIP-glu* was not glycosylated with pYEX in DBY747 strain. This might be attributed to the differences of expression vector and host.

Generally, media components and culture conditions are optimized to produce more proteins for subsequent researches (Daly and Hearn, 2005; Xu *et al.*, 2016). Exogenous genes were regulated by promoter *Cal* in pYES2/NT b, and their expression was induced by galactose. In this experiment, the media component (the concentrations of carbon and nitrogen sources) and culture condition (initial pH) were optimized. For convenience, the OD_{600} value of yeast medium was used as the index to predict the content of the r*FIP-glu*. The resulting optimal medium components for growth of *S. cerevisiae* transformant were 20 g/L of galactose and 9.0 g/L of nitrogen, and the optimal medium condition was pH 5.5 when the fermentation began. The concentration of galactose was the same as that published before (Zhang *et al.*, 2012). According to the results, it was speculated that the amount of yeast would be increased with the concentration of nitrogen source.

Recombinant proteins expressed in *S. cerevisiae* will be diverse, excessively glycosylated and multimerized, because these products can be affected by many factors such as copy number and stability of expression vector, transcription level and post-translational modification. This will result in functional differences between recombinant and natural proteins. In order to evaluate biological activity of r*FIP-glu* produced from *S. cerevisiae*, transcription levels of cytokines in mouse spleen cells were measured. Similar to the findings in previous researches, r*FIP-glu* could stimulate lymphocytes to secrete *IL-2* and *IFN-γ* (Li *et al.*, 2010; Yeh *et al.*, 2010). Our studies showed that r*FIP-glu* could significantly increase the RNA levels of *IFN-γ* and *IL-2* in mouse spleen cells.

Conclusion

In the present study, a yeast expression vector pYES2::*FIP-glu* was constructed and transformed into *S. cerevisiae* INVSC1, the r*FIP-glu* had been successfully expressed and could significantly increase the RNA levels of *IFN-γ* and *IL-2* in mouse spleen cells. So we speculate that the recombinant protein has immunomodulatory activity. The optimized media composition and culture condition for the *S. cerevisiae* transformants were: pre-fermentation temperature 30°C, seed solution pH 5.5, galactose 20 g/L, nitrogen 9.0 g/L and fermentation time 24 h. It will lay the foundation for further studies on the biological activity, mechanisms and synthesis of r*FIP-glu*, and promote the development of new anti-tumor immunomodulatory reagents with application safety.

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[Received 18 Jan 2019; Accepted 09 Mar 2019; Published (online) 21 Jun 2019]