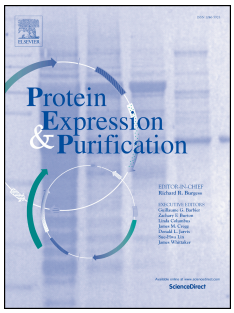
Journal Pre-proof



Optimization and bioactivity verification of porcine recombinant visfatin with high expression and low endotoxin content using pig liver as template

Hui Zhen Li, Fen Liang Xu, Abdur Rahman Ansari, Wen Jie Yang, Zhe Wei Zhang, Ling Dong, Xiao Yu Niu, Hui Song

PII: S1046-5928(20)30367-3

DOI: https://doi.org/10.1016/j.pep.2020.105776

Reference: YPREP 105776

To appear in: *Protein Expression and Purification*

Received Date: 24 May 2020

Revised Date: 8 October 2020

Accepted Date: 8 October 2020

Please cite this article as: H.Z. Li, F.L. Xu, A.R. Ansari, W.J. Yang, Z.W. Zhang, L. Dong, X.Y. Niu, H. Song, Optimization and bioactivity verification of porcine recombinant visfatin with high expression and low endotoxin content using pig liver as template, *Protein Expression and Purification*, https://doi.org/10.1016/j.pep.2020.105776.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier Inc.

Journal Pre-proof

Author Statement

None of the authors has any challenging conflicts of interests.

Journal Pre-proof

Journal Pre-proof

Optimization and bioactivity verification of porcine recombinant visfatin with high expression and low endotoxin content using pig liver as template

Hui Zhen Li[1#], Fen Liang Xu[1#], Abdur Rahman Ansari[2], Wen Jie Yang[1], Zhe Wei Zhang[1], Ling Dong[1], Xiao Yu Niu[1], Hui Song [1*]

[1]College of Animal Science and Veterinary Medicine, Huazhong Agricultural University, Wuhan, 430070, China.

[2]Section of Anatomy and Histology, Department of Basic Sciences, College of Veterinary and Animal Sciences (CVAS), Jhang; University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan.

[#] Authors contributed equally

***Corresponding Author:** songh2007@mail.hzau.edu.cn

Tel.: +86-27-87286970; Fax: +86-27-87280408;

Journal Pre-proof

Journal Pre-proof

Abstract

In order to obtain the porcine recombinant visfatin protein with high expression and low endotoxin content, the current study aims to express and verify the biological activity of the purified porcine recombinant visfatin protein. Firstly, four different expression strains were successfully constructed. Then they were simultaneously induced at 37℃ for 4 hours and 16℃ for 16 hours. The results showed that Visfatin-pET28a-Transetta was the best strain with high protein expression and purity at 16℃ induction for 16 hours. After that, endotoxin was reduced from the recombinant visfatin until the residual endotoxin was less than one endotoxin units per milliliter (EU/mL). Finally, the purified porcine recombinant visfatin protein was incubated with RAW264.7 cells. The results of cell counting kit-8 (CCK-8) showed the survival rate of the cells first increased and then decreased with the increase in visfatin concentration. When the concentration of visfatin was 700ng/mL, the survival rate of the cells was the highest. Thereafter, control (PBS), Visfatin and Visfatin+PolymyxinB (Ploy.B) groups were incubated with the RAW264.7 cells for 6 hours. Real-time quantitative polymerase chain reaction (RT-qPCR) and Enzyme Linked Immuno-Sorbent Assay (ELISA) results showed that, as compared to the control group, the expressions of interleukin (IL)-1β, tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 in Visfatin group were significantly increased ($P<0.05$). However, there was no significant difference between the Visfatin and Visfatin+Poly.B groups, indicating that porcine recombinant visfatin protein promoted the inflammatory activity of RAW264.7 cells while the residual endotoxin did not play a role, suggesting biological activity of porcine recombinant visfatin protein.

Keywords: recombinant visfatin, prokaryotic expression, endotoxin, biological activity

1. Introduction

Journal Pre-proof

Visfatin, a new adipocytokine, is also known as nicotinamide mononucleotide adenylyltransferase (NAMPT) or pre-B-cell colony-enhancing factor (PBEF) [1]. It is highly expressed in visceral adipose tissue [2], involved in DNA replication, apoptosis, repair and growth in different kind of cells [3]. Visfatin is a visceral fat cytokine that exerts similar effects to that of insulin [4]. As an adipocytokine, visfatin affects the immune function and inflammatory reaction [5]. Furthermore, visfatin plays an important role in insulin resistance [6], appetite promotion [7] and cancer [8]. These accumulating scientific reports emphasize the important role of visfatin in biological processes.

The structure of visfatin protein lacks typical signal peptide sequence [9]. Due to the existence of alternative splicing, visfatin encodes transcription products of various sizes [10]. There are multiple kinase phosphorylation sites on recombinant product sequences, which are involved in the biological effects of visfatin [11], mainly in the form of dimers [12]. The coding sequence of the NAMPT gene is highly conserved evolutionarily indicating its critical significance to different biological functions [13]. Tissue expression profiling of porcine visfatin gene revealed its expression in multiple porcine tissues. Chen cloned the porcine visfatin gene and studied its characteristics in three variants [10]. There are also other studies on the cloning and expression of visfatin gene, however the expression level and purity of the protein was not high [14].

Therefore, in the present study, total RNA of pig liver was used as a template and a large amount of highly purified and biologically active porcine recombinant visfatin protein was successfully obtained through prokaryotic expression and biological activity verification methods, which provided the basis for in-depth research of recombinant visfatin in the porcine field.

2. Materials and Methods

2.1 Construction of prokaryotic expression system

2.1.1 Gene cloning and identification

According to the sequence of coding region in porcine visfatin gene transcript 1 (DQ 020218 NM_01031793) available at GenBank, a pair of specific primers containing *Bam*H I and X*ho* I restriction sites was designed; Forward (5′-CGGGATCCATGAATGCTGCGGCAGAAGCCGAATTCAAC -3′), Reverse (5′-CCGCTCGAGTTACTAATGAGGTGCTGCTTCCAGTTCAATATTCAGCTGTGCG-3′).

We extracted the total RNA from pig liver, converted it into cDNA and then amplified the target gene using this cDNA as template with the specific primers by Polymerase Chain Reaction. We recovered the PCR products using agarose gel electrophoresis, purified it and connected it with pEASY-T1 vector. In this way, we obtained the Visfatin-pEASY-T1 recombinant plasmid. Then we transformed the recombinant plasmid into the competent cells Trans5α (*E. coli*). After selecting the positive colony, we extracted the recombinant plasmid and sent it to Shanghai biotech Co., Ltd. for sequencing.

2.1.2 Construction of recombinant expression plasmid

The Visfatin-pEASY-T1 plasmid, pET-28a and pET-30a were digested with the restriction enzyme *Bam*H I and X*ho* I respectively at the same time. The target gene fragments of visfatin obtained by the gel recovery were connected to pET-28a and pET-30a respectively. In this way, we obtained two recombinant expression plasmids, Visfatin-pET-28a and Visfatin-pET-30a. Then, the competent cells DH5α (*E.coli*) were transformed, and a single colony was selected to extract the recombinant plasmid, which was identified by double enzyme digestion and sent to Shanghai Biotechnology Co., Ltd. for sequencing.

2.2 Optimization of expression conditions of porcine recombinant visfatin protein

Journal Pre-proof

Journal Pre-proof

2.2.1 Exploration of the expression conditions of recombinant visfatin protein in a small amount

The correct recombinant expression plasmids Visfatin-pET-28a and Visfatin-pET-30a were identified by sequencing results. They were transformed into TransBL21 and Transetta respectively to construct four expression strains of Visfatin-pET28a-TransBL21,Visfatin-pET30a-TransBL21,Visfatin-pET28a-Transetta and Visfatin-pET30a-Transetta. The recombinant expression strains were inoculated in 10mL LB liquid medium to induce small amount of expression and determine the best expression condition. Four strains were simultaneously induced at 37℃ for 4 hours and 16℃ for 16 hours. After the bacteria were collected, they were broken by ultra-sonicator. The supernatant and precipitation were collected by centrifugation at 4℃. The same amount of sample buffer was added to the same volume of supernatant and precipitation samples, centrifuged and boiled. Equal volume of protein sample were loaded onto 1% SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride membranes. Transferred membranes were blocked using 5% skim milk and incubated overnight with anti-His primary antibody. After washing with TBST three times, the blots were hybridized with secondary antibodies conjugated with horseradish peroxidase for 2 h at room temperature. The antibody-specific protein was visualized by ECL detection system. In this study, the expression condition of the recombinant visfatin protein was detected by sodium dodecyl SDS-PAGE and Western Blot respectively.

2.2.2 Large expression of recombinant visfatin protein

Visfatin-pET28a-Transetta and Visfatin-pET30a-Transetta were inoculated in 300mL LB liquid medium respectively and then induced at 16℃ for 16 hours to determine the best expression strain. Subsequently, the bacteria were collected and broken down with a pressure crusher. After centrifugation at 4℃, the supernatant was passed through a 0.44 μm filter, and bound to Ni-NTA column by a protein loading pump. Then protein was purified and collected based on the protein elution peak. The imidazole and heteroproteins were removed from the protein by low-temperature ultrafiltration, and the solvent of the recombinant protein was replaced with

phosphate-buffered saline (PBS), pH=9.2. SDS-PAGE method was used to analyze the expression of the recombinant visfatin protein.

2.3 Removal of endotoxin from porcine recombinant visfatin protein

Endotoxin removal in the recombinant visfatin protein was performed according to the instructions in the kit. This study used endotoxin removal beads to remove endotoxins from the protein. Endotoxin removal beads are usually used to remove endotoxins from biological proteins. This kind of beads bind Polymyxin B to 4% agarose microspheres, and specifically removes endotoxin in the solution through the affinity of Polymyxin B. In this study, the flow rate of the recombinant visfatin protein solution through the beads were controlled at about 1 to 2 drops/min under the condition of 4 ℃, and the outflowing protein solution was collected. The removal effect of the collected porcine recombinant visfatin protein was tested by the limulus kit, and the endotoxin content was controlled within a safe range. Endotoxin-free visfatin protein was sterilized through a 0.22 μm filter. After putting it into liquid nitrogen for instant freeze crystallization, it was transferred to a -80℃ refrigerator for storage.

2.4 Verification of biological activity of porcine recombinant visfatin protein

2.4.1 Effect of recombinant visfatin protein on the viability of Raw264.7 cells

RAW264.7 cells were grown to logarithmic phase. The cells were prepared as a single cell suspension, seeded in 96-well plates, and the marginal wells were filled with PBS. We set the blank group (no cells), the control group (cells without visfatin), and the experimental group or Visfatin group (100ng/mL, 300ng/mL, 500ng/mL, 700ng/mL, 900ng/mL). Each group was set in three duplicates. The effect of the recombinant visfatin protein on the activity of RAW264.7 cells was tested by cell counting kit-8 (CCK-8) assay and calculated the cell survival rate with the following formula:

Survival rate = [(OD experimental group-OD blank group) / (OD control group-OD blank group)] x 100%

2.4.2 RAW264.7 cell related experiments to detect the activity of recombinant visfatin protein

This study examined the biological activity of the recombinant visfatin protein by culturing RAW264.7 cells and detecting changes in the expression of inflammatory factors. The phosphate-buffered saline (PBS) or control, Visfatin, and Visfatin+PolymixinB (Poly.B) groups were set up, with three replicates per group. The PBS group was exposed to the complete medium. The Visfatin group was containing 700ng/mL of recombinant visfatin only and Visfatin+Poly.B group with 700ng/mL recombinant visfatin protein and 10μg/mL Poly.B. After 6 hours of incubation, cells and cell supernatants were collected respectively to perform RT-qPCR technology and ELISA kits (Meimian Biotechnology, Yancheng, Jiangsu, China) for detection of interleukin (IL)-1β, tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 expressions. The forward and reverse primer sequences of IL-1β, TNF-α and MCP-1 used in RT-qPCR were listed in table 1. The RT-qPCR data were analyzed by a comparative threshold (CT) method. The results were expressed as the ratio of reference gene to target gene using the following formula: CT=CT (target genes)−CT (β-αctin). To determine the relative expression levels, the following formula was used: CT=CT (treated group)−CT (PBS group). Thus, mRNA levels were normalized to those of β-αctin. Relative mRNA levels are shown using arbitrary units and the value of the PBS group is defined as one.

3. Results

3.1 Construction of prokaryotic expression system

3.1.1 Visfatin gene amplification

Specific primers were used to amplify the visfatin gene by PCR. After agarose gel electrophoresis of the product, a specific band of about 1.5 kb was observed (Fig.1a), which was consistent with the expected size (1476 bp).

Journal Pre-proof

3.1.2 Nucleic acid and Amino acid sequence analysis

The forward and reverse sequencing results of the pEASY-T1-visfatin plasmid obtained from Shanghai Bio-Biotechnology Co., Ltd. were spliced to obtain the complete porcine visfatin gene-coding region (CDS) sequence. The sequence was compared with the GenBank sequence (NM_001031793) provided in **supplementary file 1**. Analysis revealed that the porcine visfatin CDS sequence had T-C and C-T interchange at positions 98^{th} and 240^{th} from the starting codon ATG. Then, the sequencing results were translated into amino acid sequences according to standard codons. With reference to the amino acid sequence of porcine visfatin available at the National Center for Biotechnology Information (NCBI), comparative analysis was performed using Basic Local Alignment Search Tool (BLAST). The analysis results showed that the T-C and C-T interchange at the 98^{th} and 240^{th} positions of the nucleic acid sequence did not cause amino acid mutations.

3.1.3 Construction of recombinant expression plasmid

The bacterial solution whose PCR was positive was selected for plasmid extraction. After double digestion with BamH I and Xho I, these were detected by 1% agarose gel electrophoresis. The result showed appearance of two fragments of about 5000bp and 1500bp, which were the vector and the target fragments, respectively (Fig.1b). Furthermore, these sequencing results confirmed the same results, which proved that pET-28a-visfatin and pET-30a-visfatin were successfully constructed.

3.2 Optimization of porcine recombinant visfatin protein

3.2.1 Optimization of small-scale expression conditions of recombinant visfatin protein

SDS-PAGE and Western Blot analysis were performed to determine the best expression condition of Visfatin-pET28a-TransBL21, Visfatin-pET30a-TransBL21, Visfatin-pET28a-

Transetta and Visfatin-pET30a-Transetta. The protein induced at 37℃ for 4 hours was mainly expressed in the precipitate (Fig.2a&b). However, the protein expressed in the supernatant and the precipitate after being induced at 16°C for 16 hours, so it showed that the Transetta strain was superior to the TransBL21 strain (Fig.3a&b).

3.2.2 Large-scale expression of recombinant visfatin protein

Two strains, Visfatin-pET28a-Transetta and Visfatin-pET30a-Transetta, were selected for large-scale expression at 16℃ for 16 hours. SDS-PAGE analysis showed that Visfatin-pET28a-Transetta (Fig.4a&b) had higher protein expression and purity than Visfatin-pET30a-Transetta (Fig.5a&b), so Visfatin-pET28a-Transetta was the best expression strain.

3.3 Endotoxin removal of porcine recombinant visfatin protein

After the endotoxin was removed from the porcine recombinant visfatin protein,a limulus kit assessed the residual amount. The standard curve for endotoxin detection was drawn according to the absorbance readings of the microplate reader. The effect of endotoxin removal was calculated from the standard curve. The residual amount of endotoxin was found to be less than one EU/mL (Fig.6a).

3.4 Verification of biological activity of porcine recombinant visfatin protein

3.4.1 Effect of recombinant visfatin protein on the viability of Raw264.7 cells

The effect of the recombinant visfatin protein was assessed on the viability of RAW264.7 cells by CCK-8 method. It was revealed that with the increase in visfatin concentration, the cell survival rate increased first and then decreased. When the concentration of visfatin reached at 700ng/mL in visfatin group, the cell survival rate was the highest with a significant difference ($P < 0.01$) as compared to the control group. However, when the concentration of visfatin exceeded

Journal Pre-proof

beyond 700ng/mL, cell viability began to decline (Fig.6b). In view of the needs of this experiment, the selected concentration of visfatin was set at 700ng/mL during the subsequent tests.

3.4.2 Effect of recombinant visfatin protein on expression of inflammatory factors

Using RT-qPCR and ELISA techniques to detect the expression levels of inflammatory factors of different groups after different treatments. The results showed that visfatin significantly induced the up-regulation of IL-1β, TNF-α and MCP-1 expression levels (Fig.7a-f), and the cytokine response induced by visfatin was not reduced by Poly.B, an inhibitor of negatively charged molecules like LPS that is normally used to exclude the effects of contaminating endotoxins. These results indicate that the recombinant visfatin protein promotes the inflammatory activity of Raw264.7 cells, and endotoxin does not work.

4. Discussion

The *Escherichia coli* (*E. coli*) expression system has the advantages of clear genetic background, simple molecular manipulation, short culture period, with widely used host for expression analysis of recombinant proteins [15]. The target gene cloning into the pET series vector, can be controlled by the strong T7 promoter [16]. After sufficient induction, it can express more than 50% of the total protein in just a few hours [17, 18]. Poly-Histidine tags or His tags are the most commonly used purification tags in many laboratories. The main features of the tags are small size, low cost and usually little or no impact on the structure or function of the target protein [19, 20]. Furthermore, when expressing mammalian proteins in bacterial cells, the choice of host cell is particularly important, because codon usage varies among organisms. The Rosetta (*E. coli*) is modified strain, specifically used for the expression of eukaryotic proteins with rare codons of *E. coli*, which increase the level of unique transfer ribonucleic acid (tRNA), improving the

expression efficiency of some eukaryotic genes [21, 22]. Based on the above facts, pig liver tissue was used as a template to clone porcine visfatin gene by using pEASY-T1 cloning vector in the current study. To construct four different expression strains of porcine visfatin protein, pET-28a and pET-30a were transformed into TransBL21 and Transetta, respectively, and performed the further screening of the optimal expression strain. Stability is an ongoing problem in the expression and purification of recombinant proteins [23]. Factors that cause poor protein stability during expression and purification may include, the primary structure of the protein, the construction of recombinant expression plasmids, the temperature and the expression medium used as well as the toxicity of the protein to the host. Temperature is an important factor affecting the growth of genetically engineered bacteria, plasmid stability and the formation of recombinant products. The alterations in temperature can be used to control the stability and solubility of the recombinant proteins [24]. Therefore, in this study, appropriate temperatures, 37°C and 16°C were selected to explore the protein expression induction temperatures. The protein showed expression in the form of inclusion bodies, only in the precipitate after induction at 37°C for 4 hours. However, the protein was expressed in both the supernatant and the precipitate after induction at 16°C for 16 hours. Moreover, Visfatin-pET28a-Transetta strain expressed protein in the supernatant with high concentration, purity and stability after induction at 16℃ for 16h proving the most optimized translation mechanism of producing target proteins at very low temperature. The expression of protein can last for a long time without down regulating the cloned gene. Furthermore, under low temperature conditions, the proliferation of bacterial cells ceases and the expression of endogenous proteins (such as proteases) reduces which can further protect the target protein from degradation and improves its purity [25, 26]. Moreover, lower temperatures can stabilize one or more folded intermediates [27]. Therefore, the

low-temperature overexpression of proteins in *E. coli* improves their solubility and stability [28, 29]. Thus, low temperature is conducive to the maintenance protein activity and enables the production of higher amounts of biologically active proteins through prokaryotic expression.

Endotoxin or lipopolysaccharide (LPS), a unique component of the outer cell wall of Gram-negative bacteria, is released after the bacteria die or decompose [30]. After entering into the body, endotoxin can cause shock, tissue damage, death and other adverse reactions, so it is very important to control endotoxin in biological products. Most biological products are usually prepared by Gram-negative bacteria with high endotoxin content [31, 32]. To ensure the safety of biological products, endotoxins must be removed as many countries have strict requirements on the endotoxin content in the biological products [33]. Herein, endotoxin was reduced from the porcine recombinant visfatin protein with the endotoxin removal kit to safe range of less than one EU/mL. Hence, it prevented the possible adverse effects of endotoxin on verification results of subsequent protein biological activity in our experiments.

RAW264.7 is a mouse mononuclear macrophage with leukemia, commonly used cell in biological experiments. In previous scientific studies, our laboratory has verified that commercial recombinant visfatin protein can induce the up-regulation of inflammation-related factors in RAW264.7 cells as well as in murine [34-36]. Therefore, the study verified the protein biological activity of porcine recombinant visfatin by detecting the effect of visfatin on the expression of inflammatory factors in Raw264.7 cells. The results showed that the expression of inflammatory factors IL-1β, TNF-α and MCP-1 were up-regulated after porcine recombinant visfatin protein incubated with RAW264.7 cells. Polymyxin B (Poly.B) is an inhibitor of LPS, usually used to exclude the effects of endotoxin [37, 38]. In the current investigation, the expression levels of inflammatory factors in both Visfatin and Visfatin+Poly.B groups were similar. These results

indicate that visfatin promotes the inflammatory activity of RAW264.7 cells, while the endotoxin does not affect the results. Hence, our findings are consistent with the results of previous studies, and prove that the porcine recombinant visfatin protein has biological activity.

5. Conclusion

In this study, we successfully constructed four different prokaryotic expression strains and prepared a large number of porcine recombinant visfatin protein with low endotoxin content and biological activity. Furthermore, the recombinant visfatin protein promoted the inflammatory activity of RAW264.7 cells while the residual endotoxin did not play any role, suggesting biological activity of porcine recombinant visfatin protein.

Supplementary Material

Supplemental information is provided in supplementary file 1.

Acknowledgments

National Natural Science Fund Project of China (No.31772687); Fundamental Research Funds for the Central Universities (No.2662015PY063) and National Natural Science Fund Project of China (No. 31101776).

Conflict of interest

The authors declare that they have no conflicts of interest.

Data Availability

The nucleotide sequence data has been deposited in GenBank under BankIt accession number MW052047.

References

[1] T. Filippatosm, C. Derdemezis, D. Kiortsis, A. Tselepis, M. Elisaf, Increased plasma

Journal Pre-proof

levels of visfatin/pre-B cell colony-enhancing factor in obese and overweight patients with metabolic syndrome. J. Endocrinol. Invest. 30 (2007) 323-326.

[2] K. Shi, Y. Chen, J. Qian, L. Xia, H. Zhu, L. Lin, L. Chen, X. Guo, Expression of visfatin influences the development of coronary artery disease. Int. J. Clin. Exp. Pathol. 10 (2017) 6861-6867.

[3] X.-T. Wu, A.R. Ansari, X.-X. Pang, H.-Z. Li, Z.-W. Zhang, Y. Luo, M. Arshad, H. Song, Visfatin plays a significant role in alleviating lipopolysaccharide-induced apoptosis and autophagy through PI3K/AKT signaling pathway during acute lung injury in mice. Arch. Immunol. Ther. Exp. 67 (2019) 249-261.

[4] A. Fukuhara, M. Matsuda, M. Nishizawa, K. Segawa, M. Tanaka, K. Kishimoto, Y. Matsuki, M. Murakami, T. Ichisaka, H. Murakami, E. Watanabe, T. Takagi, M. Akiyoshi, T. Ohtsubo, S. Kihara, S. Yamashita, M. Makishima, T. Funahashi, S. Yamanaka, R. Hiramatsu, Y. Matsuzawa, I. Shimomura, Visfatin: A Protein Secreted by Visceral Fat That Mimics the Effects of Insulin. Science 307 (2005) 426-430.

[5] Y. Luo, X.-X. Pang, A.R. Ansari, X.-T. Wu, H.-Z. Li, Z.-W. Zhang, H. Song, Visfatin Exerts Immunotherapeutic Effects in Lipopolysaccharide-Induced Acute Lung Injury in Murine Model. Inflammation 43 (2020) 109-122.

[6] Y.J. Heo, S.-E. Choi, J.Y. Jeon, S.J. Han, D.J. Kim, Y. Kang, K.W. Lee, H.J. Kim, Visfatin Induces Inflammation and Insulin Resistance via the NF-κB and STAT3 Signaling Pathways in Hepatocytes. Journal of Diabetes Research 2019 (2019).

[7] Z. Li, X. Liu, P. Zhang, R. Han, G. Sun, R. Jiang, Y. Wang, X. Liu, W. Li, X. Kang, Comparative transcriptome analysis of hypothalamus-regulated feed intake induced by exogenous visfatin in chicks. BMC genomics 19 (2018) 249.

Journal Pre-proof

[8] Y.-F. Chiang, H.-Y. Chen, K.-C. Huang, P.-H. Lin, S.-M. Hsia, Dietary Antioxidant Trans-Cinnamaldehyde Reduced Visfatin-Induced Breast Cancer Progression: In Vivo and In Vitro Study. Antioxidants 8 (2019) 625.

[9] S. Sonoli, S. Shivprasad, C. Prasad, A. Patil, P. Desai, M. Somannavar, Visfatin-a review. Eur. Rev. Med. Pharmacol. Sci. 15 (2011) 9-14.

[10] H. Chen, T. Xia, L. Zhou, X. Chen, L. Gan, W. Yao, Y. Peng, Z. Yang, Gene organization, alternate splicing and expression pattern of porcine visfatin gene. Domest. Anim. Endocrinol. 32 (2007) 235-245.

[11] Z. Sun, H. Lei, Z. Zhang, Pre-B cell colony enhancing factor (PBEF), a cytokine with multiple physiological functions. Cytokine Growth Factor Rev. 24 (2013) 433-442.

[12] A. Gillig, S.R. Majjigapu, B. Sordat, P. Vogel, Synthesis of a C-Iminoribofuranoside Analog of the Nicotinamide Phosphoribosyltransferase (NAMPT) Inhibitor FK866. Helv. Chim. Acta. 95 (2012) 34-42.

[13] L.Q. Zhang, D.P. Heruth, S.Q. Ye, Nicotinamide Phosphoribosyltransferase in Human Diseases. J. Bioanal. Biomed. 3 (2011) 13-25.

[14] X.Y. Liu, L. Ge, Cloning and expression of visfatin and screening of oligopeptides binding with visfatin. Int. J. Clin. Exp. Med. 7 (2014) 4828.

[15] N. Bhardwaj, V.K. Verma, V. Chaturvedi, P. Verma, Cloning, expression and characterization of a thermo-alkali-stable xylanase from Aspergillus oryzae LC1 in Escherichia coli BL21 (DE3). Protein Expr. Purif. 168 (2020) 105551.

[16] F.W. Studier, T7 expression systems for inducible production of proteins from cloned genes in E. coli. Curr. Protoc. Mol. Biol. 124 (2018) e63.

[17] G.L. Rosano, E.S. Morales, E.A. Ceccarelli, New tools for recombinant protein

Journal Pre-proof

Journal Pre-proof

production in Escherichia coli: A 5-year update. Prot. Sci. 28 (2019) 1412-1422.

[18] I. Novagen, pET system manual, Section, (2005) 34-41.

[19] J.J. Lichty, J.L. Malecki, H.D. Agnew, D.J. Michelson-Horowitz, S. Tan, Comparison of affinity tags for protein purification. Protein Expr. Purif. 41 (2005) 98-105.

[20] C.L. Young, Z.T. Britton, A.S. Robinson, Recombinant protein expression and purification: a comprehensive review of affinity tags and microbial applications. Biotechnol. J. 7 (2012) 620-634.

[21] G.J. Gopal, A. Kumar, Strategies for the production of recombinant protein in Escherichia coli. Protein J. 32 (2013) 419-425.

[22] G.R. Webster, A.Y.H. Teh, J.K.C. Ma, Synthetic gene design—The rationale for codon optimization and implications for molecular pharming in plants. Biotechnol. Bioeng. 114 (2017) 492-502.

[23] I. Ahmad, N. Nawaz, N.M. Darwesh, S. ur Rahman, M.Z. Mustafa, S.B. Khan, S.G. Patching, Overcoming challenges for amplified expression of recombinant proteins using Escherichia coli. Protein Expr. Purif. 144 (2018) 12-18.

[24] M.C. Deller, L. Kong, B. Rupp, Protein stability: a crystallographer's perspective. Acta Crystallog. F. 72 (2016) 72-95.

[25] G. Qing, L.-C. Ma, A. Khorchid, G. Swapna, T.K. Mal, M.M. Takayama, B. Xia, S. Phadtare, H. Ke, T. Acton, Cold-shock induced high-yield protein production in Escherichia coli. Nat. Biotechnol. 22 (2004) 877-882.

[26] D. Clift, C. So, W.A. McEwan, L.C. James, M. Schuh, Acute and rapid degradation of endogenous proteins by Trim-Away. Nat. Protoc. 13 (2018) 2149-2175.

[27] L. Sapir, D. Harries, How do Cosolutes Stabilize Macromolecules? From Bridging to

Depletion Attraction. Biophys. J. 112 (2017) 195a.

[28] S.K. Gupta, P. Shukla, Advanced technologies for improved expression of recombinant proteins in bacteria: perspectives and applications. Crit. Rev. Biotechnol. 36 (2016) 1089-1098.

[29] D. Correddu, J.d.J.M. López, P.G. Vadakkedath, A. Lai, J.I. Pernes, P.R. Watson, I.K. Leung, An improved method for the heterologous production of soluble human ribosomal proteins in Escherichia coli. Sci. Rep. 9 (2019) 1-8.

[30] I. Ginsburg, M. Korem, E. Koren, J. Varani, Pro-inflammatory agents released by pathogens, dying host cells, and neutrophils act synergistically to destroy host tissues: a working hypothesis. J. Inflamm. Res. 12 (2019) 35.

[31] S.-Y. Kwon, M. Ro, J.-H. Kim, Mediatory roles of leukotriene B 4 receptors in LPS-induced endotoxic shock. Sci. Rep. 9 (2019) 1-8.

[32] J. Reich, F.A. Weyer, H. Tamura, I. Nagaoka, H. Motschmann, Low endotoxin recovery—Masking of naturally occurring endotoxin. Int. J. Mol. Sci. 20 (2019) 838.

[33] J.-P. Pirnay, M. Merabishvili, H. Van Raemdonck, D. De Vos, G. Verbeken, Bacteriophage production in compliance with regulatory requirements, Bacteriophage therapy, Springer, 2018, pp. 233-252.

[34] X.-T. Wu, Z. Yang, A.R. Ansari, K. Xiao, X.-X. Pang, Y. Luo, H. Song, Visfatin regulates the production of lipopolysaccharide-induced inflammatory cytokines through p38 signaling in murine macrophages. Microb. Pathog. 117 (2018) 55-59.

[35] K. Xiao, W.-H. Zou, Z. Yang, Z. ur Rehman, A.R. Ansari, H.-R. Yuan, Y. Zhou, L. Cui, K.-M. Peng, H. Song, The role of visfatin on the regulation of inflammation and apoptosis in the spleen of LPS-treated rats. Cell Tissue Res. 359 (2015) 605-618.

[36] Z. Yang, K. Xiao, W. Wang, J. Tang, P.-P. Sun, K.-M. Peng, H. Song, The Effect of

Journal Pre-proof

Visfatin on Inflammatory Reaction in Uterus of LPS-Induced Rats. Int. J. Morphol. 33 (2015).

[37] S.P. Mukherjee, N. Lozano, M. Kucki, A.E. Del Rio-Castillo, L. Newman, E. Vázquez, K. Kostarelos, P. Wick, B. Fadeel, Detection of endotoxin contamination of graphene based materials using the TNF-α expression test and guidelines for endotoxin-free graphene oxide production. PloS one 11 (2016) e0166816.

[38] Q. Zhang, J. Huang, J. Yu, Z. Xu, L. Liu, Y. Song, X. Sun, A. Zhang, M. Jin, HP1330 contributes to Streptococcus suis virulence by inducing toll-like receptor 2-and ERK1/2-dependent pro-inflammatory responses and influencing in vivo S. suis loads. Front. Immunol. 8 (2017) 869.

Journal Pre-proof

Table 1: Primers used in amplifying RAW264.7 cell inflammatory factors

Journal Pre-proof

Gene name	Forward primer	Reverse primer
β-αctin	5´-CACTGCCGCATCCTCTTCCTCCC-3´	5´-CAATAGTGATGACCTGGCCGT-3´
IL-1β	5´-GGCTCATCTGGGATCCTCTC-3´	5´-TCTCCACAGCCACAATGAGT-3´
TNF-α	5´-TCTTCTCATTCCTGCTTGTGG-3´	5´-CACTTGGTGGTTTGCTACGAC-3´
MCP-1	5´-AGAAGGAATGGGTCCAGACATA-3´	5´-GTGCTTGAGGTGGTTGTGGA-3´

Journal Pre-proof

Journal Pre-proof

Figure Legends

Fig. 1: PCR amplification, amino acid sequence alignment and double digestion of expression plasmid

a. PCR amplification of visfatin

M: 2500bp DNA marker; 1, 2: PCR product of visfatin

b. The result of double enzyme digestion of expression plasmid

M: 5000bp DNA marker; 1 and 2, 3 and 4: the double digestion of Visfatin-pET-28a and Visfatin-pET-30a respectively

Fig . 2: Small expression induced at 37℃ for 4 hours

a. SDS-PAGE analysis

b. Western Blot analysis

M: Protein Marker; 1: The supernatant of Visfatin-pET28a-TransBL21 after induced by IPTG; 2: the precipitation of Visfatin-pET28a-TransBL21 after induced by IPTG; 3: the supernatant of pET28a-TransBL21 after induced by IPTG; 4: the precipitation of pET28a-TransBL21 after induced by IPTG; 5: the supernatant of Visfatin-pET28a-Transetta after induced by IPTG; 6: the precipitation of Visfatin-pET28a-Transetta after induced by IPTG; 7: the supernatant of Visfatin-pET30a-TransBL21 after induced by IPTG; 8: the precipitation of Visfatin-pET30a-TransBL21 after induced by IPTG; 9: the supernatant of pET30a-TransBL21 after induced by IPTG; 10: the precipitation of pET30a-TransBL21 after induced by IPTG; 11: the supernatant of Visfatin-pET30a-Transetta after induced by IPTG; 12: the precipitation of Visfatin-pET30a-Transetta after induced by IPTG; 13: the supernatant of pET30a-Transetta after induced by IPTG; 14: the

precipitation of pET30a-Transetta after induced by IPTG.

Fig. 3: Small expression induced at 16℃ for 16 hours

a. SDS-PAGE analysis

b. Western Blot analysis

M: Protein Marker; 1: The supernatant of Visfatin-pET28a-TransBL21 after induced by IPTG; 2: the precipitation of Visfatin-pET28a-TransBL21 after induced by IPTG; 3: the precipitation of Visfatin-pET28a-TransBL21 after induced by IPTG; 4: the supernatant of pET28a-TransBL21 after induced by IPTG; 5: the precipitation of pET28a-TransBL21 after induced by IPTG; 6: the supernatant of Visfatin-pET28a-Transetta after induced by IPTG; 7: the precipitation of Visfatin-pET28a-Transetta after induced by IPTG; 8: the supernatant of Visfatin-pET30a-TransBL21 after induced by IPTG; 9: the precipitation of Visfatin-pET30a-TransBL21 after induced by IPTG; 10: the supernatant of Visfatin-pET30a-Transetta after induced by IPTG; 11: the precipitation of Visfatin-pET30a-Transetta after induced by IPTG; 12: the supernatant of pET30a-TransBL21 after induced by IPTG; 13: the precipitation of pET30a-TransBL21 after induced by IPTG; 14: the supernatant of pET30a-Transetta after induced by IPTG.

Fig. 4: Large-scale expression of Visfatin-pET28a-Transetta strain

a. Protein purification elution peaks

b. SDS-PAGE analysis of protein purification

M: Protein Marker; 1~13 Separately indicate: The supernatant, precipitation, solution that has been combined with a nickel column of Visfatin-pET28a-Transetta after induced by IPTG and the 10th, 11th, 12th, 13th, 16th, 18th, 23rd, 31st, 33rd and 37th tubes sampled according to protein elution peak of Visfatin-pET28a-Transetta strain.

Fig. 5: Large-scale expression of Visfatin-pET30a-Transetta strain

a. Protein purification elution peaks

b. SDS-PAGE analysis of protein purification

M: Protein Marker; 1~14 Separately indicate: The ultrafiltration concentrated protein of Visfatin-pET28a-Transetta, the supernatant, precipitation, solution that has been combined with a nickel column of Visfatin-pET30a-Transetta after induced by IPTG and the 19th, 20th, 21st, 29th, 30th, 32nd, 33rd, 34th, 35th tubes sampled according to protein elution peak of Visfatin-pET30a-Transetta strain, the ultrafiltration concentrated protein of Visfatin-pET28a-Transetta.

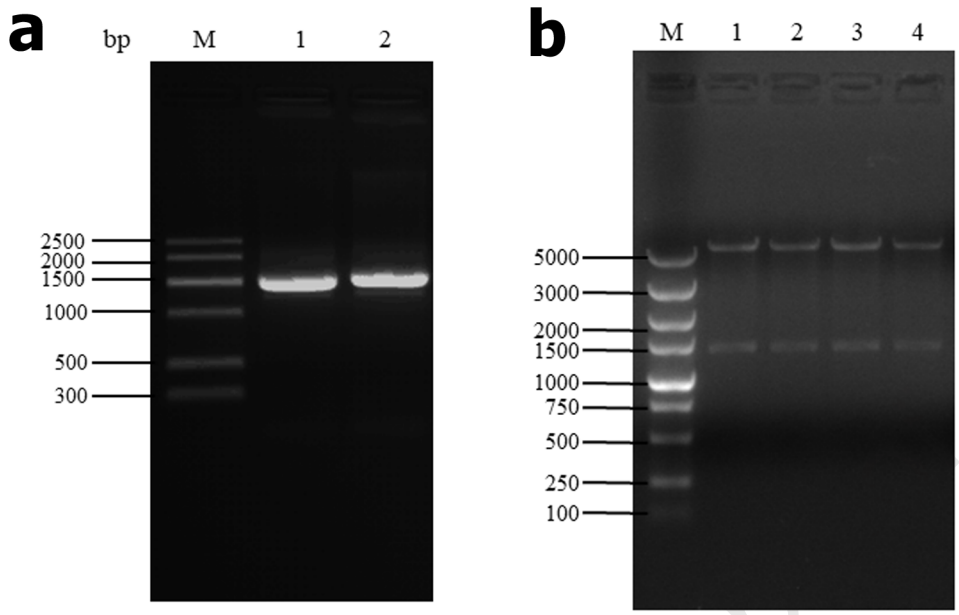
Fig. 6: Detection of endotoxin removal effect and effect of endotoxin-free recombinant visfatin

a. Endotoxin removal effect b. Effect of endotoxin-free recombinant visfatin on the survival of Raw264.7 cells

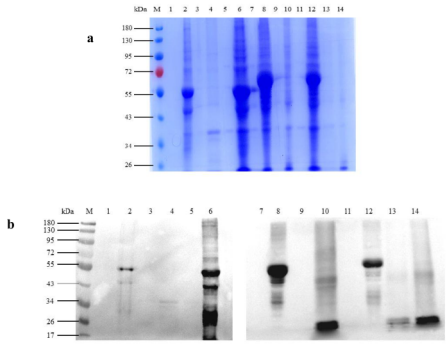
Fig. 7: Detection of inflammatory factors by RT-qPCR and ELISA

a-c. Expression levels of IL-1β, TNF-α and MCP-1 by RT-qPCR, d-f. Expression levels of IL-1β, TNF-α and MCP-1 by ELISA

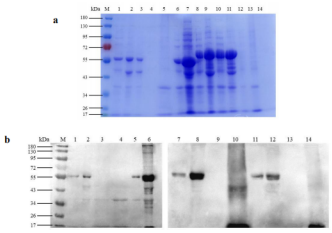
Journal Pre-proof



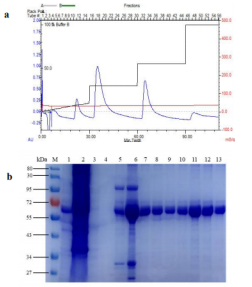
Journal Pre-proof



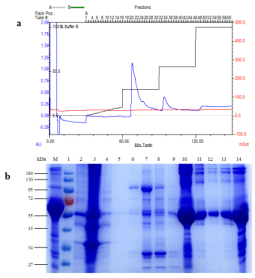
Journal Pre-proof



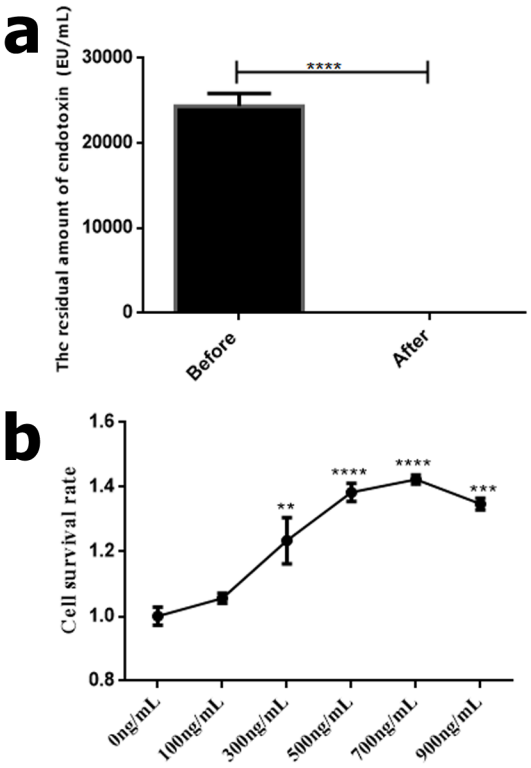
Journal Pre-proof



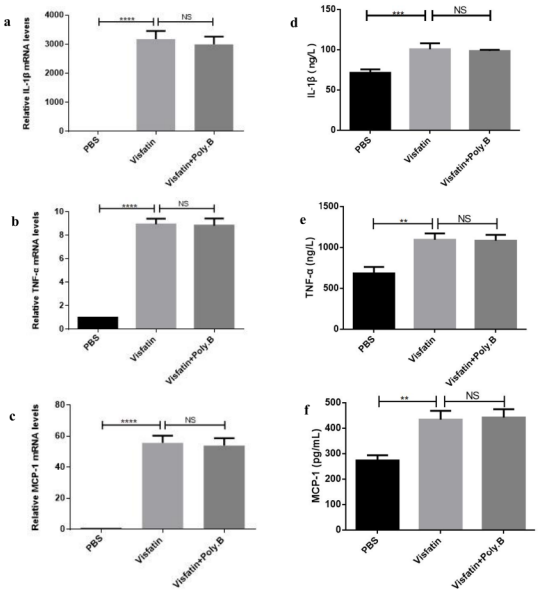
Journal Pre-proof



Journal Pre-proof



Journal Pre-proof



Journal Pre-proof

Journal Pre-proof

Highlights

- Four different prokaryotic expression strains were constructed successfully
- Then prepared a large number of porcine recombinant visfatin with low endotoxin content and biological activity
- Porcine recombinant visfatin promoted the inflammatory activity of RAW264.7 cells
- Moreover, endotoxin did not affect the above results, hence indicated the biological activity of porcine recombinant visfatin

Journal Pre-proof