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Full Length Research Paper

New purification process of fungal immunomodulatory protein, FIP-*fve* from *Flammulina velutipes* via filtration and column chromatography

Yuequn Cao[1#], Chongyang Liang[2#], Yandong Zhang[3], Jianlin Lin[2] and Fei Sun[2]*

[1]Changchun Normal University, China.
[2]Institute of Frontier Medical Science of Jilin University, 1163 Xinmin Street, Changchun 130021, People's Republic China, Jilin 130021, China.
3 Department of rheumatism immunity, the first hospital of Jilin University, Changchun 130021, China.

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Microfiltration, ultrafiltration, cation exchange and size-exclusion chromatography were used to isolate FIP-*fve* from *Flammulina velutipes* fruiting body in the pilot scale. At the sample preparing stage, the homogenate of mushroom is usually hard to be resolved hence microfiltration was used to clarify the homogenate of *F. velutipes* efficiently. Next ultrafiltration is applied to concentrating supernatant and removal of small molecules. The samples from ultrafiltration can be directly used for the chromatography stage, and in simplifying the entire sample preparation process, all technical parameters can be amplified linearly. Concentrated supernatant from ultrafiltration was further separated with two-step chromatographic purification of strong cation-exchange and size-exclusion, purified product shows as single band at 13 kDa in SDS-PAGE gel, the analysis result of reversed phase HPLC C4 column also indicated that purified product has a great purity (95% or more). The purified sample was identified as FIP-*fve* (sequence coverage, more than 95%) by MALDI TOF/TOF, and its molecular weight was 12 740Da. The purification process is reproducible, robust and well suited for scaling-up to an industrial scale operation.

Key words: *Flammulina velutipes*, ultrafiltration, FIP-*fve.*

INTRODUCTION

The fungal immunomodulatory proteins (FIPs) are a new family of bioactive proteins, isolated from *Flammulina velutipes*, *Ganoderma* and several other species of medical and edible mushrooms. The FIPs resemble lectins in terms of structure and immunomodulating activities. Up to now, only four FIPs have been found and identified, and internationally recognized from *Ganoderma lucidum* (LZ-8), *Ganoderma tsugae* (FIP-*gts*), *F. velutipes* (FIP-*fve*), Volvariella volvacea (FIP-*vvo*), respectively (Kino et al., 1989, 1990; Ko et al., 1995, 1997; Hsu et al., 1997; Liao et al., 2006, 2007; Lin et al., 1997). These FIPs consist of 110 to 114 amino acid residues with molecular weights of 12.4 to 13.0 kDa and share high homology ranging from 57 to 100% in the peptide sequence (Murasugi et al., 1991; Tanaka et al., 1989). The amino acid composition and primary structure of these FIPs have been elucidated, a 1.7 Å structure of FIP-*fve* and a 1.8 Å structure of LZ-8 solved by single anomalous diffraction of NaBr-soaked crystal has been also illustrated (Seow et al., 2003; Paaventhan et al., 2003). FIPs also are mitogens to human peripheral blood lymphocytes, neutrophils and mouse splenocytes *in vitro* (Kino et al., 1989; Ko et al., 1997). Not only could they prevent systemic anaphylaxis and footpad edema during the Arthus reaction, but also inhibit the growth of tumors, induce an autophagic cell death in tumor cell SGC-7901 (Liang et al., 2012).

FIP-*fve*, which is characterized from *F. velutipes* is able to prevent food-allergic reaction induced by ovalbumin and airway hyper responsiveness caused by Dp2 in mice

*Corresponding author. E-mail: s.fei1958@gmail.com.

Share first co-authorship.

(Hsieh et al., 2003; Liu et al., 2003). Moreover, it could lead to eosinophil apoptosis, which indicates its possible therapeutic effect on eosinophil-related allergic inflammation (Hsieh et al., 2007). FIP-*fve* has potent adjuvant properties that enhance T helper type 1 antigen-specific humoral and cellular immune responses which confer strong antitumour effects (Ding et al., 2009). The biological activity of the protein has become research focus in the field of immunology recent years.

The *F. velutipes* contains a lot of FIP-*fve*, the mushroom is so cheap, so it is suggested that FIP-*fve* that is the strong pharmacodynamics activity protein can be obtained through large-scale separation and extraction process. Lin et al. (1995) reported that FIP-*fve* has been isolated and purified from *F. velutipes* using DEAE-cellulose column and Mono S HR 5/5 column, but the ability of the sample handling in above purification process is difficult to enlarge, which may be a technical bottleneck hindering the industrialization process of the FIP-*fve*. Here, we used microfiltration, ultrafiltration, and column technology, and have established a set of large-scale separation process of the FIP-*fve*, from Flammulin, and revealed FIP-*fve*'s pI.

MATERIALS AND METHODS

Reagents and instrument

Fresh *F. velutipes* fruiting bodies 5 kg. Sodium acetate, glacial acetic acid, Tris (hydroxymethyl) aminomethane (Tris), concentrated hydrochloric acid, acrylamide and N, N'-Methylene Bisacrylamide (chromatography grade, sigma, SDS for analysis are purchased from Beijing chemical plant. Proteinase inhibitors (Roche, Germany), sheep's blood are from Jilin Province the People's Hospital. Human's blood is from healthy volunteers. ÄKTA purifier 10 (GE Healthcare, US), Mass Spectrometry (PerSeptive Biosystems, Framingham, MA, USA), Hydroextractor (Thermo Fisher Scientific, US), pH meter (Mettler Toledo, Switzerland), electronic analytical balance (Mettler Toledo, Switzerland), soybean milk machine, Hollow Fiber Cartridge (#UFP-5-C-6A, #CFP-2-E-8A, GE, USA), vertical electrophoresis machine (BIO-RAD, USA), decolorize table concentrator, gel formatter (Tanon, Shanghai), High Performance Liquid Chromatography (SHIMADZU, Japan), C4 chromatography column, cation exchange SP Sepharose™ XL, anion exchange Q Sepharose™ XL, gel chromatography Superdex™ 75 prep grade, all from GE lifescience.

Upstream clarification of *Flammulina velutipes* fruiting bodies homogenate

Fresh *F. velutipes* fruiting bodies 5 kg was mixed and homogenized with 10 L HAc-NaAc buffer (50 mM, pH 3.2). The 0.2 μM pore size microfiltration membrane was selected for the clarification of *F. velutipes* fruiting bodies homogenate. The flow rate of Inlet was set at 10-13L/min, the pressure sum of P1 and P2 always controlled at below 25 psi, temperature: 4-12°C (39 to 53.6°F). The system control strategy is as follows: when the pressure is too high, increase the pump flow rate of pump 1, and reduce the flow rate of pump 2; when the pressure is too low, increase the pump flow rate of pump 2, and reduce the flow rate of pump 1. The sample was collected from every step of preparation process for polyacrylamide gel electrophoresis (SDS-PAGE). The practice is shown in the following Figure 1.

Downstream protein purification by ultrafiltration concentration

The clear liquor from clarification process was mixed into reflux pool. The 5000 NMWC ultrafiltration membrane was used for concentration and diafiltration. The flow rate of Inlet was set at 4 to 8.5 L/min, the pressure sum of P1 and P2 always controlled at below 25 psi, temperature: 4 to 12°C (39 to 53.6°F). We set the pressure as restrictive parameters; adjust the flow rate of pump 1 and 2. The system controlling strategies of the process are as follows: When the pressure is too high, increase the pump flow rate of pump 1, and reduce the flow rate of pump 2; when the pressure is too low, increase the pump flow rate of pump 2, and reduce the flow rate of pump 1. Keep the liquid content of reflux pool more than 30 L, after 2 h, and gradually reduce buffer amount of liquid that is filled into reflux pool, and finally concentrated to 0.5 L, remove concentrated liquid. 2 L HAc-NaAc buffer (50 mM, pH 3.2) were added into reflux pool, the samples from every step of preparation process was collected for polyacrylamide gel electrophoresis (SDS-PAGE). The practice is shown in Figure 2.

Chromatographic separation

The clear liquor from clarification process (1 L) was applied to a BPG column (140 × 500 mm) containing 2.5 L SP Sepharose™ XL which was previously equilibrated with 50 mM HAc-NaAc buffer pH 4.5 using flow rate 80 ml/min. The ultraviolet absorption monitoring was set at 280 and 254 nm. The column was washed with 40 L equilibration buffer and then linearly eluted with 50 L 0 to 1.5 M NaCl in 50 mM HAc-NaAc buffer pH 4.5, the fractions was detected with SDS-PAGE assay. The positive fractions were further purified by application to a XK column (26 mm width, 1000 mm height) containing 500 ml Superdex 75 prep grade which was equilibrated and eluted with 50 mM HAc-NaAc + 150 mM NaCl, the fractions was detected with SDS-PAGE assay. The positive fractions were collected, approx. 3500 mg purified FIP-*fve* was obtained.

Reversed-phase high-performance liquid chromatography and isoelectric focusing electrophoresis (IEF)

Use C4 reversed-phase HPLC to detect the purity of the purified product from peak of S75. Phase A: 0.1% TFA, ddH_2O. Mobile phase B: 0.055% TFA, 70% acetonitrile. Equilibrate the column with 10 column volumes. Inject the sample and linearly elute 20 column volumes. The flow rate was 0.5 ml/min; ultraviolet absorption was set at 280 nm. Accurately weigh out 20 to 25 mg of pancreatin enzyme concentrate (PEC) or pancreatin enzyme concentrate-high lipase (PEC-HL) and transfer to a 2.0 ml micro centrifuge tube. Add 1.0 ml 50% glycerol and vortex vigorously for 5 min. Centrifuge samples at 2000 *g* (8,000 rpm on Beckman Microfuge™ 11) for 10 min. Dilute 90 μL supernatant with 10 μL IEF sample buffer (50% glycerol).

Mass spectrography

All MALDI-ToF-MS data were acquired on a Voyager DE-STR biospectrometry workstation using a nitrogen laser (337 nm). The protein samples were prepared using a conventional dried droplet protocol in which sinapinic acid (SA) was used as the matrix. The SA matrix was prepared as a saturated, aqueous solution that contained 60% acetonitrile and 0.3% TFA. 1 μL of the sample was mixed with 20 μL of SA matrix, before depositing 1.2 μL of the sample matrix mixture on the MALDI sample stage.

Hemagglutination assay

Human blood was obtained from healthy volunteers who approved

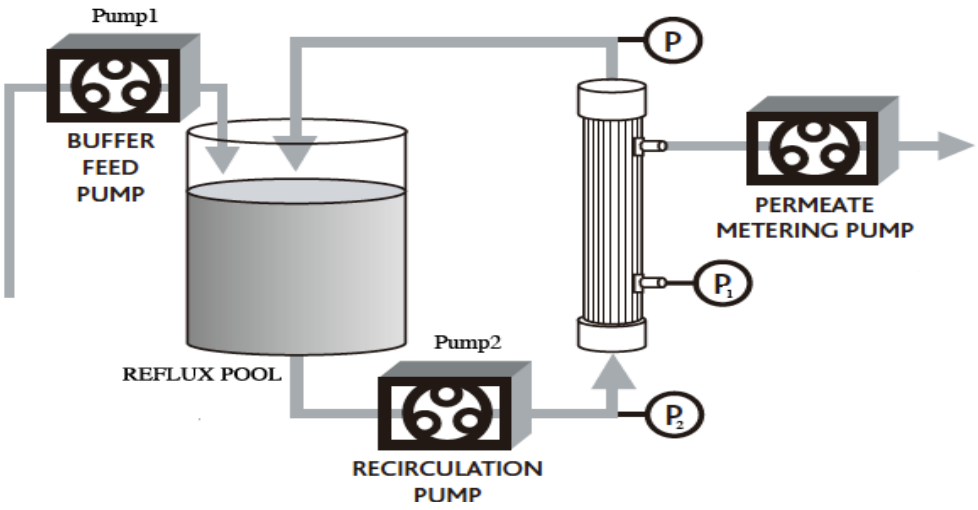

Figure 1. Upstream clarification of the homogenate. Arrow indicates the direction of fluid flow.

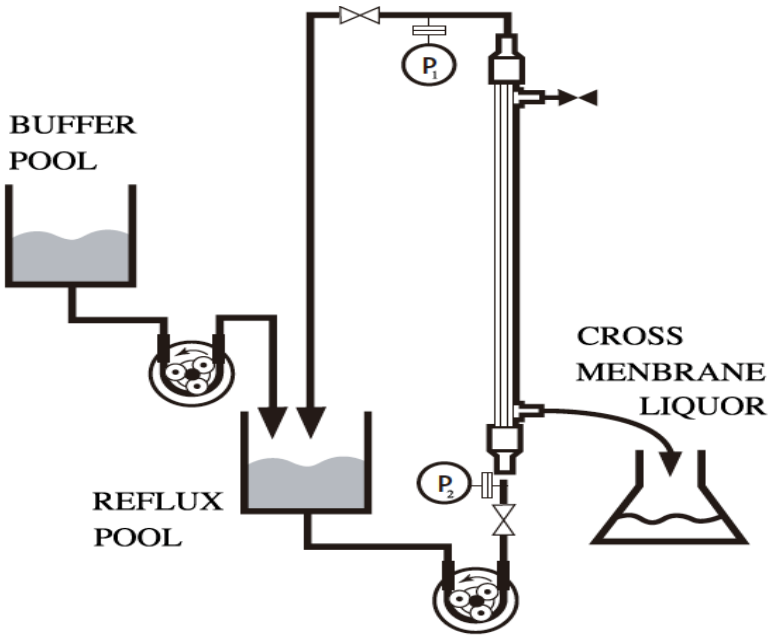

Figure 2. Desalting and concentration of the homogenate. Arrow indicates the direction of fluid flow.

the use of their blood for this study. Sheep whole blood (Alsever's solution) was obtained from Jilin University Medical Center and was used within 1 month after being received. 5 ml of whole blood (human or sheep) were centrifuged at 1200 *g* for 10 min. The plasma was removed and 2 ml of packed red blood cells were collected from the bottom of the tubes. The cells were washed five times with 10 ml of PBS and centrifugation at 1200 *g* for 10 min and then suspended to 0.5% and 1.0% (v/v) with PBS. Suspensions of 25 pl of purified FIP-*fve* and rLZ-8 (30 ng/ml to 50 pg/ml final concentration) in PBS, 75 pl of 0.2% gelatin in PBS, and 25 p1 of 0.5% and 1.0% red blood cells placed in 96-well microtiter plates (round bottom, Nunc) were shaken for 30 s at room temperature and then incubated at 37°C. The plates were examined for hemagglutination after 2, 6 and 24 h.

RESULTS AND DISCUSSION

Upstream clarification, desalting and concentration of the homogenate

Unlike cell harvesting or any other simple particulate concentration, clarification of a target molecule from a solution containing particulates requires more attention to process equilibrium. Since protein passage is of paramount concern in clarification processes, open pore size microfiltration membranes, such as 0.2 to 0.8 µM pore size, are typically used, especially for larger recombinant

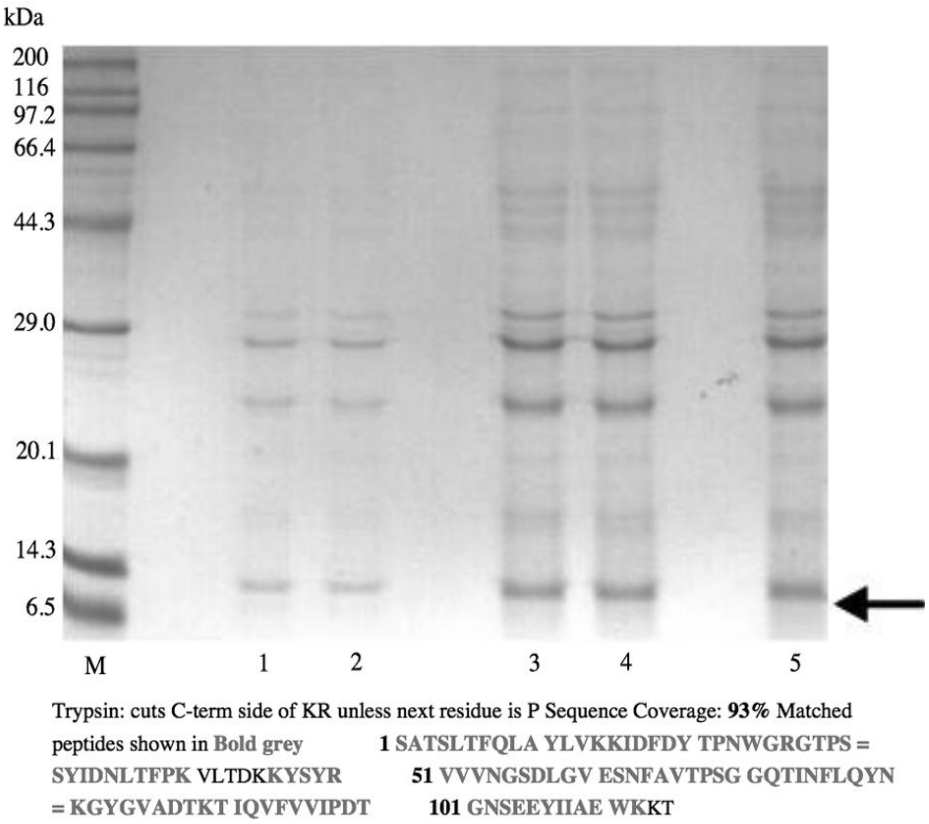

Figure 3. SDS-PAGE analysis of FIP-*fve* from Flammulina velutipes. Lane M, protein marker (kDa); lane 1 and lane 2 : washing membrane solution; from lane 3 to lane 5: the concentrated solution from ultrafiltration. The position of FIP-*fve* (about 15 kDa) is indicated by a black arrow.

proteins and monoclonal antibodies. In general, choose a membrane pore size that is at least 10 times larger than the target protein to pass through the membrane. FIP-*fve* is a non-covalently linked homodimer, each subunit being 114 amino acid residues in length. Hence the true molecular size of FIP-*fve* is equivalent to a 26 kDa MW protein, 0.2 μM pore size is very suitable for FIP-*fve* passage, and a smaller pore size can reduce the probability of target protein to plug holes. To note here is that operators must exert more deliberate control of transmembrane pressure and more careful timing of concentration and cell washing to promote protein passage. Even though retentate backpressure is almost certainly reduced or even absent, further steps can also be taken to reduce transmembrane pressure to prevent premature fouling of the membrane. The whole process took a total of 30 min, the volume of the homogenate is close to 10 L, add the 40 L HAc-NaAc buffer, clear liquor was a total of 43 L for the next step of ultrafiltration.

Downstream protein purification by ultrafiltration concentration

The most widely used applications for cross flow ultrafiltration membranes are downstream: Concentration and diafiltration of the previously clarified biomolecule as it is loaded onto and eluted from the chromatography columns. In general, choose a membrane that is 3 to 5 times smaller in NMWC rating than the molecular weight of the target protein. Here, as FIP-*fve* is an approximate 26 kDa dimer, we decided to choose 5000 NMWC ultrafiltration membranes for concentration and diafiltration of clear liquor. Protein concentration and diafiltration processing is usually suited to smaller lumen diameter fibers, such as 0.5 mm fibers. This is feasible because protein solutions being processed are typically well-clarified. Considering clear liquor containing FIP-*fve* is very viscous, it may contains some high-viscosity component such as glycerol or sucrose and our laboratory condition for FIP-*fve*'s purification is accompanied by cold-room processing (4°C). Large number of practical results indicated that 1.0 mm fiber avoid rapidly increasing inlet pressures as our solution reaches final concentration; it is the most critical parameter in the ultrafiltration. The clear liquor was concentrated to about 0.5 L finally, while collecting 1.5 L washing membrane solution, the concentrated clear liquor and washing membrane solution were used for the next step of strong cation exchange purification process. The Mascot result of MS/MS showed that the band (black arrow) in Figure 3 is FIP-*fve*.

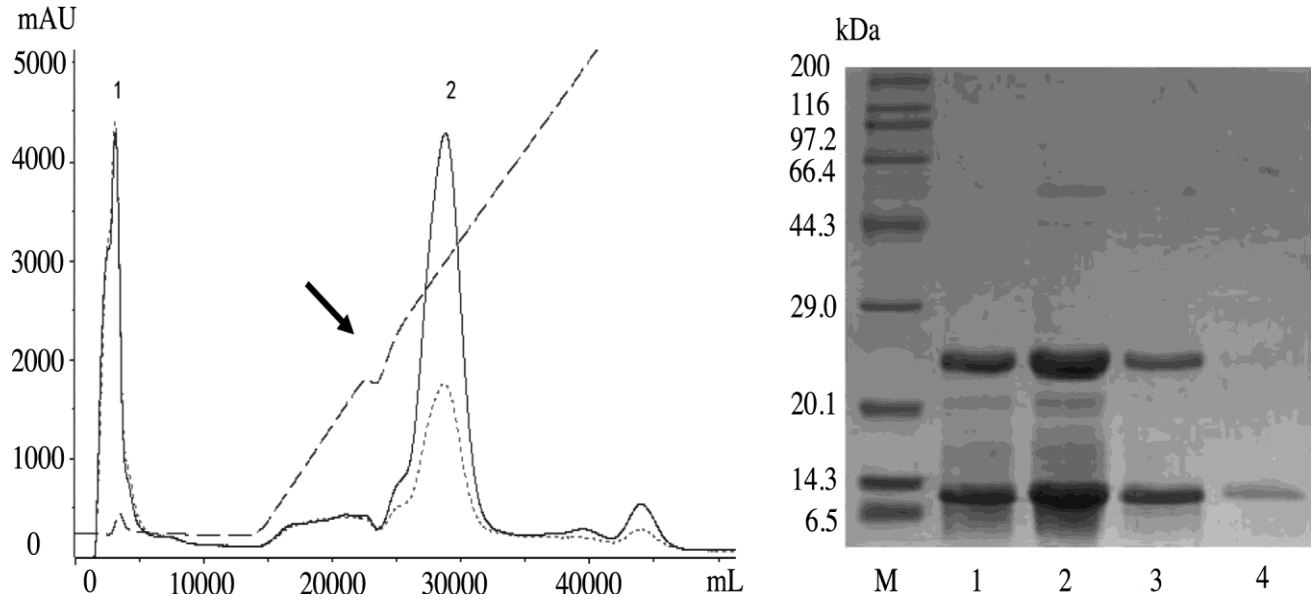

Figure 4. Cation exchange chromatography column purification of FIP-*fve*. An elution profile of FIP-*fve* from a BPG column containing 2.5L SP Sepharose^{TM} XL. Protein fractions (Peak 1 and Peak 2) according to absorbance at 280 nm were obtained from auto-fractions. SDS-PAGE analysis of FIP-*fve* purified from *Flammulina velutipes*. Lane M, protein marker (kDa); lane 1 – 4 the different parts of Peak 2.

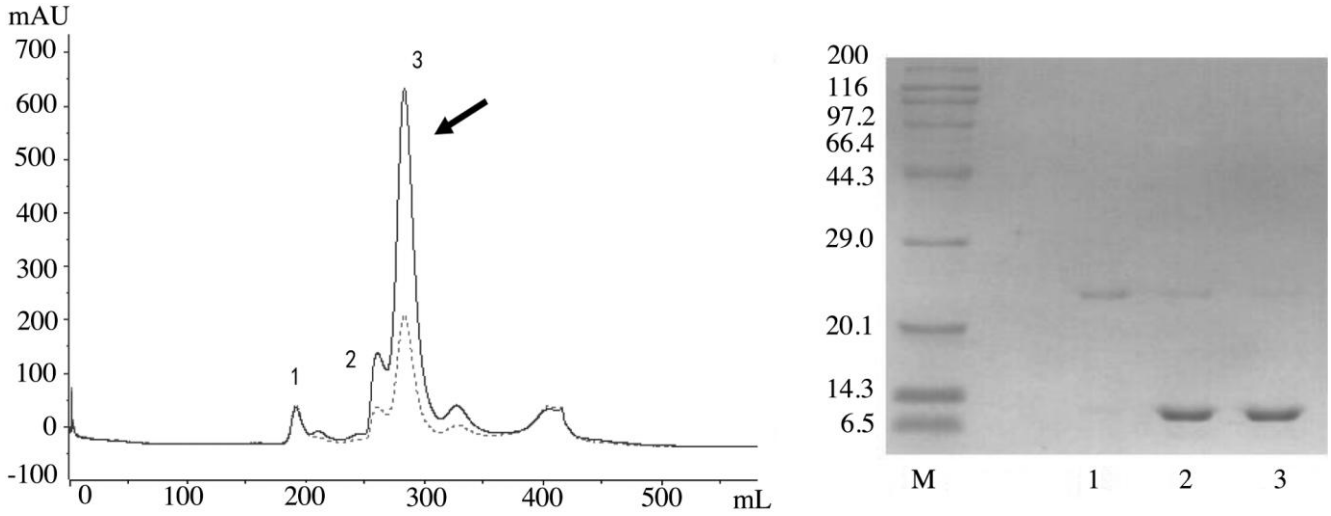

Figure 5. Size-exclusion chromatography column purification of FIP-*fve*. An elution profile of FIP-*fve* from a XK 26/100 column containing 500mL Superdex^{TM} 75. Protein fractions (Peak 1, Peak 2 and Peak 3) according to absorbance at 280 nm were obtained from auto-fractions. SDS-PAGE analysis of FIP-*fve* purified from *Flammulina velutipes*. Lane M, protein marker (kDa); lane 1 – 3 the different parts of Peak 3.

Chromatographic process

The isoelectric point of FIP-fve is 6.1, so we choose the chromatographic condition: 50 mM HAc-NaAc buffer pH 4.5. As shown in Figure 4, the fraction 1 (Peak 1) has absorption peaks at 280 and 254 nm, but the result of SDS-PAGE showed that F1 contained no proteins, it may be some nucleic acid of *F. velutipes*. When the linear elution process to electrical conductivity of 19.2 ms/cm, a peak (Peak 2) at 280 nm appeared. In Figure 5, the different parts of Peak 2 were exhibited in lane 1 to 4, most of the impurities have been removed, and only two distinguished protein bands still displayed in SDS-PAGE gel, their molecular weight respectively were 24 and 13 kDa. Then we applied size-exclusion chromatography to further purify the fraction of ion-exchange column. As

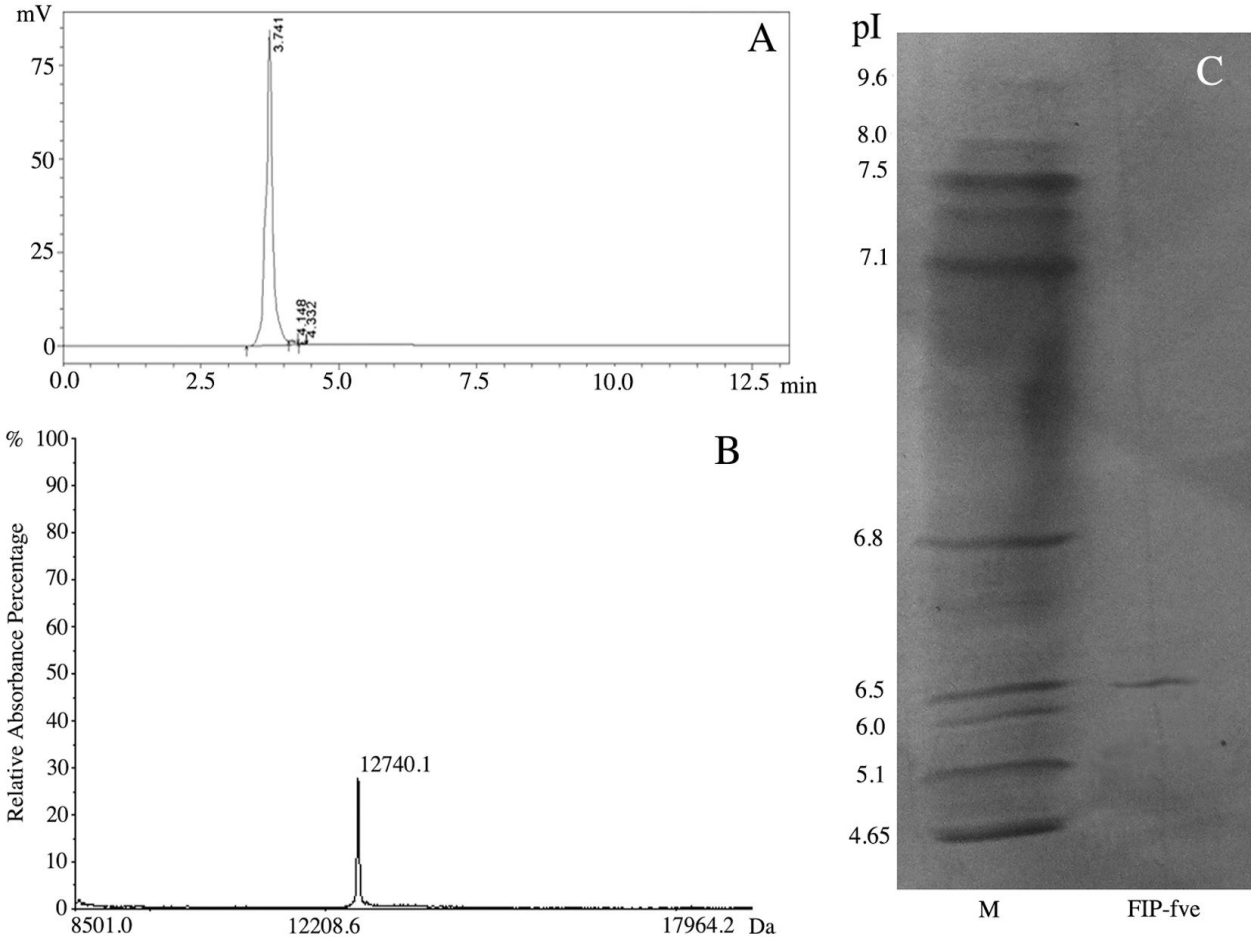

Figure 6. Purity, Molecular Weight and isoelectric point identification of FIP-*fve*. A: use C4 reverse-phase liquid chromatography to do purity analysis with the product; B: the relative molecular weight of purified product is 12 704 Da assayed by MALDI-TOF; C: isoelectric point (*p*I) of FIP-*fve* was identified by IEF assay.

shown in Figure 3, the latter half of F3 (Peak 3) showed only a distinguished band that has 13 kDa molecular weight on the lane 3 of SDS-PAGE gel, which indicates that the sample from the latter half of F3 (Peak 3) has great purity. In the study, the purity of purified FIP-*fve* was identified by C4 reversed phase chromatography and isoelectric focusing electrophoresis.

The purity of prepared FIP-fve and IEF identification

We get 3 mg sample, and dissolve it in 1 ml purified water. Add 20 µL of the sample to the column. A C4 reverse-phase liquid chromatography column was used to analyze the purity of the product. The purity is greater than or equal to 90% (Figure 6A). The purification spectrum of a graph is shown in Figure 6B. As shown in IEF (Figure 6C), isoelectric point (*p*I) of FIP-*fve* has the same position with IEF standard human carbonic anhydrase (pI: 6.5), and no other bands were seen in lane FIP-*fve* of the native IEF gel. The results of C4 HPLC suggested that purified FIP-*fve* has a great purity.

Mass chromatographic analysis for the MW of FIP-fve

The relative molecular weight of purified product is 12 704 Da assayed by laser desorption ionization time of flight mass spectrometry. The result basically agreed with that calculated by computer software.

Hemagglutination activity of FIP-fve

Using human red blood cells and sheep red blood cells, the hemagglutination activity of FIP-*fve* and LZ-8 (as a control, it comes from Ganoderma Lucidum) was examined (Figure 7). No aggregation was observed between any sheep red blood cells in the presence of purified FIP-*fve* within the concentration range of 1.56 to 100 µg/ml. However, with human red blood cells, aggregation was seen in the presence of FIP-*fve* at concentrations greater than 12.5 µg/ml. For a control, the opposite phenomenon was observed in the presence of rLZ-8 with human red blood cells or sheep red blood cells, at concentration greater than 6.25 µg/ml.

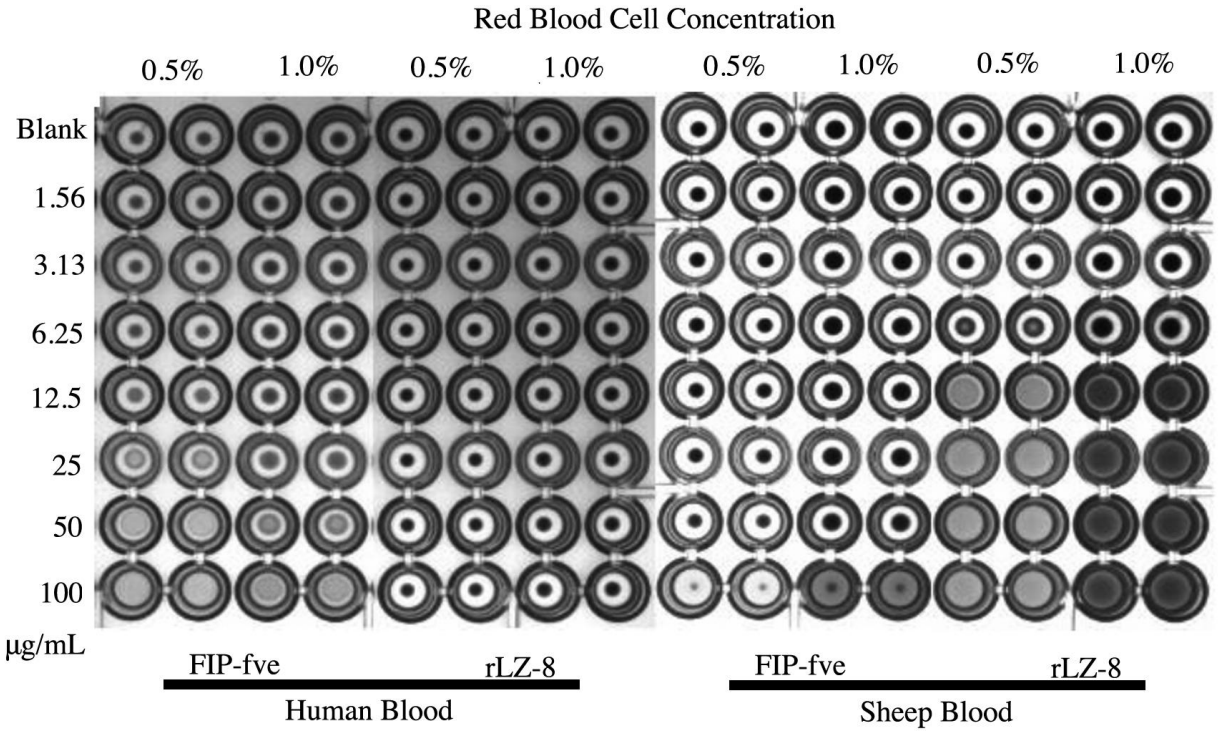

Figure 7. Hemagglutination activity of FIP-*fve*. Hemagglutination activity toward human red blood cells and sheep red blood cells were determined as described under “materials and methods”.

Conclusion

A three-step procedure has been devised for purifying FIP-*fve* from *F. velutipes* fruiting bodies in large scale. The downstream purification process is reproducible, robust and well suited for scaling-up to an industrial scale operation. The characteristics of the ultra-filterable and Chromatographic media are easily restored by a standard CIP procedure making it possible to use these media for several cycles of adsorption and de-sorption processes, thereby reducing significantly the material and operational costs for the entire downstream purification process.

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