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Original article

Expression of *Aspergillus niger* N5-5 in *E. coli* and purification and identification of products

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ABSTRACT

Due to the feature of high hydrolysis, tannase is widely used in food, beverage, brewing and other fields. However, high cost in producing natural tannase makes it difficult to apply tannase to industry in a large-scale. Microbial expression systems can be used for preparing numerous amount of enzyme at low cost, so in this paper *Aspergillus niger* N5-5 was expressed using *E. coli* system. Specific primers were designed based on the *Aspergillus niger* N5-5 sequence N3 (GenBank, No.: KP677552), and tannase gene *tan* was promoted to carry 6 His tag and enzyme cutting site which contains NdeI/HindIII using PCR amplification. Then, tannase gene *tan* was connected to expression vector by NdeI/HindIII enzyme cutting. In this way, recombinant expression vector *tan*-pET43.1a was formed. Then, the expression vector pET43.1a by NdeI/HindIII enzyme cutting was transformed into *E. coli* BL21 (DE3) to induce expression of *Aspergillus niger* N5-5. When the induced fungi were disrupted by the ultrasonic wave, the crude enzyme was extracted and purified by using the IMAC, and then the activity of the crude enzyme and pure enzyme was determined. According to the results of determination of the tannase activity, the tannase activity of the crude enzyme was greatly improved after the crude enzyme was purified, and the specific activity of the pure enzyme was about 8 times of that of the crude enzyme. The results of SDS-PAGE of the pure enzyme showed that the molecular mass of the pure enzyme was about 65 kDa/64–65 kDa, which was consistent with the expected result (64.2 kDa), It can be concluded that the crude enzyme solution was purified successfully. The results of pure enzyme's protein identification by Western Blotting showed that clear protein bands *pro*-3 were observed. Molecular mass of clear protein bands pro-3 was about 65 kDa, which was in line with the expected results (64.2 kDa). It can be seen that the aforementioned expression protein could be specifically combined with His tag. It proved expression protein to be a recombinant fusion protein with 6 His tag.
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1. Introduction

Tannase (EC, 3.1.1.20) is an induced enzyme that widely exists in microorganisms and plants, and can be produced massively especially in filamentous fungi which use tannic acid as an inducer. Tannase belongs to the cell membrane bound enzyme that can be secreted into the extracellular environment (Aguilar and Gutiér rez-sánchez, 2001; Vania and Gabriela, 2007; Hong et al., 2017). Tannase can specifically hydrolyze tannins such as gallotannins, ellagitannins, ester bonds and depside bonds in alkyl gallate, so as to produce gallic acid, glucose, HHDP, alcohol and so on (Chávez-gonzález et al., 2012; Lekha and Lonsane, 1997; Battestin et al., 2008; Li et al., 2017). Due to the feature of high hydrolysis of tannins, tannase is widely applied to food, beverage, brewing, medicine, chemicals and other sectors (Hong et al., 2013; Mesut, 2013; Zhang et al., 2015) . *Aspergillus niger* N5-5 is a fungal tannase with a molecular mass of 64.2 kD. *Aspergillus niger* N5-5 can be obtained from fermentated Aspergillus niger 5-5 of solid state. High production cost makes it difficult to realize large-scale application of natural tannase to industry.

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Microbial expression systems can be used for preparing enzyme in a large-scale with convenience in purifying, so during commercial enzyme preparations, most enzymes used are recombinant ones expressed by foreign genes through microbial expression systems. However, so far, in commercial sector, natural enzyme (Govindarajan et al., 2016; Bassi and Kaur, 2017; Li and Cheng, 2016; Sofulu et al., 2017) obtained through microbial fermentation are still used, thus how to obtain recombinant tannase through microbial expression systems has become the key problem needed to solved in the current research.

As the genetic background and gene expression regulation mechanism of *E. coli* are very clear, and *E. coli* has many vectors and hosts that can adapt to different expressions, the *E. coli* expression system is an exogenous expression system that can be studied thoroughly and is the first choice for expression system in the laboratory. In addition to prokaryotic proteins, eukaryotic proteins with a molecular mass of less than 70 kDa, which is not required for post-translational modifications, are usually expressed using the *E. coli* expression system (Rosano and Ceccarelli, 2014; Vincentelli and Romier, 2013; Gao et al., 2017). At present, the *E. coli* expression system is the most commonly used expression system for the microbial expression of tannase. Noguchi et al. (2007) cloned a S., lugdunensis TP415 *tanA* with a molecular mass of 50 kDa, which was expressed using *E. coli* DH5α expression system, and encoded a protein sequence of 613 amino acids. Iwamoto et al. (2008) cloned a *L. plantarum* ATCC 14917 ^{T} *tanLpl* with a molecular mass of 50 kDa, which was expressed using *E. coli* DH5α expression system, and encoded a protein sequence of 469 amino acids. Later, Ueda et al. (2014) cloned a *L. Paraplantarum* NSO120 *tanLpa* and *L. Pentosus* 21A-3 *tanLpe,* and compared their encoded proteins with *tanLpl*. It was found that the consistency rates of amino acids were 88% and 72%, respectively. Subsequently, the author transformed the *tanLpl, tanLpa and tanLpe* using the expression tannase of *B. Subtilis* RIK 1285 and made further comparison. It was found that the three recombinant tannases varied in substrate specificity. In addition, Wu et al. (2013) also cloned a *L. Plantarum* tannase, which was induced for expression using *E. coli* BL21-AI expression system. The molecular mass of the recombinant tannase is about 50.8 kDa, which is basically the same as the research results from Noguchi. Moreover, Jimenez et al. (2014) cloned a *A. Parvulum* tannase, which was induced for expression using *E. coli* BL21 (DE3) expression system.

In this paper, specific primers were designed based on the *Aspergillus niger* N5-5 sequence N3 (GenBank, No.: KP677552), and tannase gene *tan* was promoted to carry 6 His tag and enzyme cutting site which contains NdeI/HindIII using PCR amplification. Then, tannase gene tan was connected to expression vector pET43.1a by NdeI/HindIII enzyme cutting. In this way, recombinant expression vector *tan*-pET43.1a was formed. Then, the expression vector pET43.1a by NdeI/HindIII enzyme cutting was transformed into *E. coli* BL21 (DE3) so as to induce expression of *Aspergillus niger* N5-5. When the induced fungi were disrupted by the ultrasonic wave, the crude enzyme was extracted and purified by using the IMAC. The activity of crude enzyme was determined. Finally, the pure enzyme's protein was identified by Western Blotting. In previous studies, bacterial tannase was expressed using *E. coli* expression system, while in this study fungal tannase was expressed using *E. coli* expression system for the first time.

2. Materials and instruments

2.1. Experimental materials

Aspergillus niger N5-5 sequence N3 (cloned by the Laboratory of Food Chemistry, South China Agricultural University, 1749 bp, GenBank Accession No.: KP677552, see http://www.ncbi.nlm.nih.gov/nuccore/KP677552).

E. coli BL21 (DE3), expression host; pET43.1a, expression vector carrying 6 His tag, a fusion tag, for purifying and identificating targeted protein, which was purchased from Takara in Japan.

2.2. Chemicals and reagents

LB Agar, LB Broth; Ampicillin (Amp), Kanamycin (Kan), Isopropyl-β-D-Thiogalactoside (IPTG), Buffer Tango (Thermo Fisher Scientific (China) Co., Ltd.); Citrate, Sodium Citrate; Dimethyl Sulfoxide, Sodium Dodecyl Sulfate (SDS), Acrylamide, Bis-Acrylamide, Ammonium Persulfate, Tetramethylethylenediamine (TEMED), Bromophenol Blue, Glycine, Imidazole, Coomassie Brilliant Blue G-250, Trishydroxymethylaminomethane (Tris), Bovine Serum Albumin (BSA), Polyvinylidene Fluoride Membrane (PVDF Membrane); Tween 20, Ponceau S; Yili Skimmed Milk Powder (purchased from supermarkets); NdeI Enzyme, HindIII Enzyme (Fermentas in Canada); T4 DNA Ligation Buffer, T4 DNA Ligase, Binding Buffer (Shanghai Generay Biotech Co., Ltd.); DNA Marker, SDS-PAGE Protein Marker, Western Blotting Protein Marker, Pfu Master Mix (Takara from Japan); Mouse Anti-6 His tag Monoclonal Antibody (Primary Antibody), Goat Anti-Mouse IgG (H + L)-HRP (Secondary Antibody) (American Bio-Rad); DAB Chromogenic Kit (Beijing Solarbio Science & Technology Co., Ltd.) and; Gallic Acid, Propyl Gallate.

2.3. Experimental instruments and equipment

SW-CJ-2FD Superoclean Bench (Suzhou Purifying Equipment Co., Ltd.); DHZ-C Thermostatic Oscillator (Taicang Qiangle Laboratory Equipment Co., Ltd.); U-3010 UV–Vis Spectrophotometer (HITACHI from Japan); Gel Doc XR + Gel Imaging Analysis System, Powerpac HV 164-5056 Electrophoresis Apparatus, Electrophoresis Tank (American Bio-Rad); 5417C High Speed Refrigerated Centrifuge (Eppendorf from Germany); TDL-16C High Speed Tabletop Centrifuge (Shanghai Anheng Scientific Instruments Factory); BD-EPS100 Nucleic Acid Electrophoresis Apparatus (Suzhou Bozhao Scientific Instrument Co., Ltd.); LDZM-40KCS Autoclave (Shanghai Shenan Medical Instrument Factory); JYD-900 Ultrasonic Cell Disruption System (Shanghai Zhi Sun Instrument Co., Ltd.); His-Trap ^{TM} Nickel Chelate Chromatography Column (Amersham Pharmacia Biotech); Amicon Ultra-15 Centrifugal Ultrafiltration Tube (MWCO 30 kDa) (Millipore); FD-1PF Freezing Dryer (Beijing Detianyou Science Technology Development Co., Ltd.); R204B3 Rotary Evaporator (Shanghai SENCO Technology Co., Ltd.).

3. Experimental methods

3.1. Preparation for reagents

3.1.1. Citrate-sodium citrate buffer

Citrate of an exact amount of 21.01 g was measured, and distilled water was placed into the volumetric flask. The total dissolution was 1000 mL (Buffer A, 0.1 mol/L); sodium citrate of an exact amount of 29.41 g was measured, and distilled water was placed into the volumetric flask. The total dissolution was 1000 mL (Buffer A, 0.1 mol/L) . The Buffer A and Buffer B were mixed at a volume ratio of 1:2 to obtain citrate-sodium citrate buffer (hereinafter referred to as buffer).

3.1.2. Preparation for related reagents of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

30% polyacrylamide solution: the ratio was 29.2 g acrylamide to 0.8 g bis-acrylamide and then, both acrylamide and bis-acrylamide

were fully dissolved in ddH_2O. ddH_2O was placed into the volumetric flask to reach 100 mL. The solution was reserved in a dark place at the constant temperature of 4 °C.

Separating gel buffer (1.5 mol/L Tris-HCl, pH 8.8): 18.15 g Tris was measured and dissolved in 4 mL hydrochloric acid. The pH of the mixture was adjusted to be 8.8, and then hydrochloric acid was placed into the volumetric flask. The total solution was 100 mL.

Stacking gel buffer (1.5 mol/L Tris–HCl, pH 6.8): 6.0 g Tris was measured and dissolved in 8 mL hydrochloric acid. The pH of the mixture was adjusted to be 6.8. Then, hydrochloric acid was placed into the volumetric flask. The total solution was 100 mL.

10 % SDS: 10 g SDS was measured and dissolved, and then the solvent was placed into the volumetric flask. The total solution was 100 mL. The solution was reserved at room temperature.

10 % ammonium persulfate: 10 g ammonium persulfate was measured and dissolved, and then the solvent was placed into the volumetric flask. The total solution was 100 mL.

5× sample buffer: 0.6 mL 1 mol/L Tris-HCl (pH 6.8), 5 mL 50% glycerol, 2 mL 10% SDS, 1 mL 1 % bromophenol blue and 0.9 mL distilled water were measured, and then mixed together.

Electrode buffer: 3.0 g Tris, 14.4 g glycine and 1.0 g SDS were measured and dissolved, and then the solvent was placed into the volumetric flask. The total solution was 1000 mL (pH was about 8.3).

Staining solution: 0.5 g coomassie brilliant blue G-250 was measured and dissolved in 225 mL methanol + 50 mL glacial acetic acid, and then the solvent was dropped into the volumetric flask. The total solution was 500 mL. The solution was reserved at room temperature.

Destaining solution: ration was 75 mL glacial acetic acid and 50 mL methanol. Then the solvent was placed into the volumetric flask. The total solution was 1000 mL. The dissolution was reserved at room temperature.

SDS-PAGE separating gel (5 mL): the ratio was ddH_2O, 1.0 mL; 1.5 mol/L Tris-HCl (pH 6.8), 1.9 mL; 30% gel reserving solution (A rc:Bis = 29:1), 2.0 mL; 10 % SDS, 0.05 mL; 10% ammonium persulfate, 0.05 mL, and; TEMED, 0.015 mL.

SDS-PAGE stacking gel (3 mL): ddH2O, 2 mL; 1.5 mol/L Tris–HCl (pH 6.8), 0.38 mL; 30% gel reserving solution (Arc:Bis = 29:1), 0.5 mL; 10% SDS, 0.03 mL; 10% ammonium persulfate, 0.03 mL; TEMED, 0.007 mL.

3.1.3. Preparation for related reagents for Western Blotting

Transferring buffer: 5.8 g Tris, 2.9 g glycine, 0.37 g SDS and 200 mL methanol were measured and dissolved in ddH_2O. The ddH_2O was placed into the volumetric flask. The total solution reached 1000 mL. Upon just being prepared, the solution was used immediately.

TBST buffer: 8.78 g NaCl and 2.42 g Tris were measured and dissolved in 800 mL ddH_2O. The pH of the mixture was adjusted to be 7.4 with concentrated hydrochloric acid, and then the ddH_2O was placed into the volumetric flask. The total solution reached 1000 mL, and then 0.5 mL Tween 20 was added to the solution. The solution was reserved at room temperature.

Ponceau S staining solution: 1 g Ponceau S was measured and dissolved in 50 mL glacial acetic acid. Then, the ddH_2O was placed into the volumetric flask. The solution reached 1000 mL and was reserved in a dark place at 4 °C.

Sealing buffer: 1 g skimmed milk powder was measured and dissolved in 20 mL TBST buffer,. At that moment, the concentration of the skimmed milk powder was 5% (m/v), and then the mixture was shaken up. Upon just being prepared, the solution was used immediately. The solution was kept at room temperature.

3.2. SDS-PAGE

The method by Hatch et al. (2006) was consulted and then improved. (1) A mixture was prepared according to the separating gel reagent formula in 3.1.2, and poured into the gap between the two vertical glass plates of electrophoresis tank until about 2 cm away from the top edge of the glass plates. The separating gel surface was flattened with distilled water. After about 10 min when the separating gel became solidified, the water was abandoned. Then, the mixture prepared according to the stacking gel reagent formula in 3.1.2 was placed with a comb being inserted. After the stacking gel became solidified after about 60 min, the comb was taken out carefully. At that moment, several grooves in the stacking gel for sample injection can be observed. (2) All samples were mixed with equal amount of sample buffer, then the mixture was placed in boiling water for 3–5 min (for sufficient protein denaturation), and then cooled down to room temperature. An approximately 400 mL electrode buffer was added to the electrophoresis tank, and the marker and sample with amount of 20 μL and 10 μL respectively were placed into the grooves. (3) After the voltage of the electrophoresis apparatus adjusted to 60 V, the sample passed through the stacking gel. The voltage of the electrophoresis apparatus adjusted to 110 V after the sample entered the separating gel. After the bromophenol blue moved to the bottom of the separating gel, the electrophoresis completed. (4) After the electrophoresis completed, the gel was taken out, and the stacking gel was cut and discarded. The separating gel was placed in the staining solution for 4 h at room temperature. (5) The stained gel was placed in the destaining solution at room temperature until clear protein bands can be observed.

3.3. Determination of protein content

The method of coomassie brilliant blue G-250 (Bradford method) was adopted (Bradford, 1976). After the chemical action of BSA, in the same way as the standard protein, coomassie brilliant blue G-250 formed. The absorbance value A_{595} at 595 nm wavelength was measured, and the A595-BSA concentration standard curve was established. The curve regression equation was: $Y = 0.00801X - 0.02823$, and the adjusted determination coefficient was: Adj. $R^2 = 0.99348$. The above equation showed that the curve fitting was good, so the equation can be used as an equation model for calculating protein content in samples. The protein content in samples can be calculated based on standard curve.

3.4. Determination of tannase activity

The method by Lin et al. (2010) was consulted and then improved. Five tubes with 2 mL Eppendorf (EP), one control tube, one blank tube and three measuring tubes were taken and added with 0.2 mL enzyme liquid. Then, each tube was under metal bath for 45 °C constant temperature for 10 min. Then, each measuring tube was added with PG standard solution for 0.2 mL, the blank tube, with 0.2 mL buffer, and the control tube, with 0.6 mL absolute ethanol for enzymatic reaction for 20 min. Then, the measuring tubes and the blank tube were added with 0.6 mL absolute ethanol respectively to terminate the enzymatic reaction. The control tube was added with PG standard solution of 0.2 mL. After cooling, the solution in each tube was diluted for 10 times.

Definition of enzyme activity: it was defined as one enzyme activity unit (U) in the case where the absorbance value decreased by 0.001 per minute as a result of hydrolyzating PG standard solution using 1 mL enzyme solution. According to this definition, the formula of enzyme activity can be obtained as follows: enzyme activity $(U/mL) = (A_{\text{control}} - A_{\text{measuring}}) \times 2.5 \times 10^3$, where the A

$_{control}$ and A $_{measuring}$ were the absorbance value of the control tube and measuring tubes, respectively.

3.5. Construction of recombinant expression vector

3.5.1. Designing primers

Specific primers were designed based on the *Aspergillus niger* N5-5 sequence N3 using Primer Premier 6.0 (Singh et al., 1998). Protection base sequence ACTTA was introduced to Primer 5′ end, NdeI enzyme cutting site sequence CATATG was introduced to F primer, and HindIII enzyme cutting site sequence AAGCTT was introduced to R primer. 6 His base sequence was introduced to R primer. The primer sequence was as follows: F3, 5′-ACTTACA TATGCGCTCCAAGATGAGCCTTCTGGCC-3′; R3, 5′- ACTTAAAGCTTT CATTAATGGTGATGGTGATGGTGATACAGCGGCAGCTCGTAAGCATC -3′. The primer sequence was synthesized by Shanghai Generay Biotech Co., Ltd., and purified by SDS-PAGE.

3.5.2. PCR amplification

The reaction system was as follows: 2 × Pfu Master Mix (25 μL), F primer (2 μL), R primer (2 μL), and ddH_2O (added to be 50 μL).

PCR amplification program:

(1) Initial denaturation: 94 °C, 5 min;

(2) 26 cycles of "denaturation-annealing-elongation" were carried out:

① Denaturation: 94 °C, 30 s; ② Annealing: 55 °C, 30 s; ③ Elongation: 72 °C, 30 s;

(3) Last elongation: 72 °C, 5 min.

After the reaction, the PCR products were analyzed using agarose gel electrophoresis.

3.5.3. Enzyme cutting of expression vector

Enzyme cutting system

Expression vector waiting for enzyme cutting pET43.1a (30 μL, 150 ng/μL), double enzyme: NdeI (10 U, 1 μL) and HindIII (10 U, 1 μL), 2 × Buffer Tango (5 μL), and ddH_2O (added to be 50 μL). All of double enzymes were placed in the biochemical incubator at a constant temperature of 37 °C for 2–3 h, and the target bands were recycled for backup.

3.5.4. Connecting target gene fragment and expression vector by enzyme cutting

Connection system

Amplified target gene fragment (2 μL), expression vector by enzyme cutting pET43.1a (1 μL), T4 DNA Ligase (5 U), T4 DNA Ligation Buffer (1.5 μL), and ddH_2O (added to be 15 μL). After 1 h of connection at 22 °C, agarose gel electrophoresis was used for connecting liquid to identify whether the recombinant expression vector tan-pET43.1a (*tan* was target gene) was successfully formed. Finally, the connection liquid was directly transformed into the host *E. coli* BL21 (DE3).

3.6. Screening of positive clone

(1) The transformed *E. coli* DH5α (LB plate) was placed overnight for about 12–14 h;
(2) The monoclonal antibodies were picked out and inoculated in a 2 mL LB liquid culture medium which contained Kan + resistance (resistance concentration of 100 ng/mL) according to the growing speed of plate, and a total of 10 monoclonal antibodies were picked out (to prevent false positive);
(3) Shaking culture was carried out for 12–14 h at 37 °C, with 220 r/min;
(4) The plasmids were extracted using SDS alkaline lysis method, and the plasmid DNAs were extracted by a TaKaRa MiniBEST Plasmid Purification Kit Ver.4.0 in a small amount.
(5) The extracted plasmids were identified using enzyme cutting method, and then the positive clones were screened and identified by Shanghai Generay Biotech Co., Ltd.

3.7. Culture of seed liquid

The positive clone transformants were picked out and inoculated in a 100 mL LB liquid culture medium which contained 100 mg/L Amp, and culture was shaken for 12–14 h at 37 °C with 180 r/min. Then, the A_{600} value was detected to 2.0.

3.8. Induced expression

10 mL seed liquid was taken and inoculated in fresh LB liquid culture medium of 1000 mL, and culture was shaken for 3 h at 37 °C with 250 r/min. When the A_{600} value was detected to 0.6–0.8, each bottle of fungal liquid was added until IPTG reached the concentration of 0.5 mmol/L. Then, the liquid was induced for culture for 5-6h. Then, the A_{600} value was detected to 3.0.

3.9. Expression and identification of target protein

The culture medium of the induced fungi was taken out and centrifuged at 6000 r/min for 10 min. Then, the fungi were collected. They were added with 30 mL fungi re-suspended with citrate buffer and centrifuged for 10 min with 6000 r/min, and then the supernatant was discarded; the precipitate was added with 30 mL fungi re-suspended with citrate buffer, and placed in an ice bath. The induced fungi were disrupted by the ultrasonic wave at 300 W for 5 s, and then paused for 2 s. The whole test lasted for 14 min in total. After centrifuged at 4 °C for 20 min with 12,000 r/min, the supernatant was sampled to go through SDS-PAGE. Meanwhile, the same operation was carried out for the non-induced fungi as the control group to analyze whether the target protein was expressed.

3.10. Extraction and purification of the crude enzyme solution and determination of tannase activity

100 mL culture medium of the induced fungi was taken, and the fungi were disrupted by the ultrasonic wave. After being centrifuged, the collected supernatant was crude enzyme solution, which was then purified by using the Immobilized Metal Affinity Chromatography (IMAC) as referred to by Wang et al. (2013). First, 10 column volumes of nickel chelate chromatography column (i.e. Ni column) were balanced out by Binding Buffer (balanced solution), then 2 mL crude enzyme solution was loaded to columns with a flow rate of 1 mL/min. After being loaded, the crude enzyme solution reached the baseline with a balanced solution of 10 column volumes, then mixed with 20 mmol/L Tris–HCl buffer which contained 0.5 mol/L NaCl and 50 mmol/L imidazole to remove non-specific adsorptive proteins. Then, the specific binding proteins were eluted with 20 mmol/L Tris-HCl buffer of 0.5 mol/L NaCl and 250 mmol/L imidazole. Then, the samples were collected, and dialyzed at 4 °C for 48 h (the Tris-HCl buffer was replaced for 3–4 times during the period) to remove the imidazole. Then, the pure enzyme solution was obtained by ultrafiltration (MWCO 30 kDa) and concentration. SDS-PAGE was used to obtain the pure

enzyme solution to detect the quality of purification. Finally, the overall activity of tannase, protein content of the pure enzyme and crude enzyme and specific activity of the tannase were determined respectively.

3.11. Protein identification by Western Blotting

Western Blotting, also known as Protein Immunoblot, is an immunological identification technique for the detection of protein expression level. The basic principle is: The protein samples separated by electrophoresis are transferred to a solid phase carrier (NC membrane, PVDF membrane, etc.), which adsorbs proteins in non-covalent bond form, and keeps the polypeptides separated by electrophoresis and biological activities unchanged. The proteins or polypeptides on the solid phase carrier, as antigens, first react with the corresponding antibodies, and then with enzymes or isotope labeled secondary antibodies. In this way, the protein component expressed by the specific target gene separated by electrophoresis by means of substrate coloration or autoradiography (Kurien and Scofield, 2003; Mahmood and Yang, 2012) can be detected.

After the SDS-PAGE electrophoresis bands of the pure enzyme were obtained, protein was identified by Western Blotting as referred to by Wisdom (1994). (1) In the transfer buffer, the expression proteins in the electrophoresis gel were transferred into the PVDF membrane (first electrotransferred at 300 mA for 1.5 h, and then electrotransferred at 150 mA for 0.5 h); (2) The membrane was stained with Ponceau S for 5–10 s, and then red protein bands can be observed on the membrane. The Ponceau S was washed off with TBST buffer (washed 3 times, 10 min each time); (3) The PVDF membrane was immersed in a sealing liquid of 5% skimmed milk powder, and sealed after being gently shaken at room temperature for 1 h; (4) After being sealed, the membrane was rinsed with TBST with the purpose of removing the remaining skimmed milk powder (rinsed 3 times, 3 min each time), and then immersing membrane in primary antibody (Mouse Anti-6 His tag Monoclonal Antibody) diluent (ratio of 1:1000) for incubating overnight at 4 °C; (5) The blotting membrane was rinsed with TBST to remove the remaining secondary antibody (rinsed 3 times, 10 min each time), and then immersed in secondary antibody (Goat Anti-Mouse IgG (H + L)-HRP) diluent (1:2000) for incubating at room temperature for 1 h; (6) The blotting membrane was rinsed with TBST to remove the remaining secondary antibody (rinsed 3 times, 10 min each time), and then the membrane (protein surface) was added with an appropriate amount of DAB colour-developing filling liquid for incubating at room temperature in a dark place for 15 min for forming color. At appropriate time, the DAB colour-developing filling liquid was washed off by water. The color development reaction terminated. After being dried, the membrane was reserved in a dark place.

4. Results and analysis

4.1. Formation of recombinant expression vector

The results of agarose gel electrophoresis of the target gene *tan* after the PCR amplification are shown in Fig. 1. From Fig. 1, it can be seen that the bands of the target gene *tan* were consistent with the expected results (1749 bp). The results show that the PCR amplification was successful. The results of agarose gel electrophoresis of the recombinant expression vector *tan*-pET43.1a formed by expression vector using enzyme cutting pET43.1a and *tan* are shown in Fig. 2. From Fig. 2, it can be seen that the bands of the target gene were in line with the expected results, showing that the recombinant expression vector *tan*-pET43.1a was formed successfully.

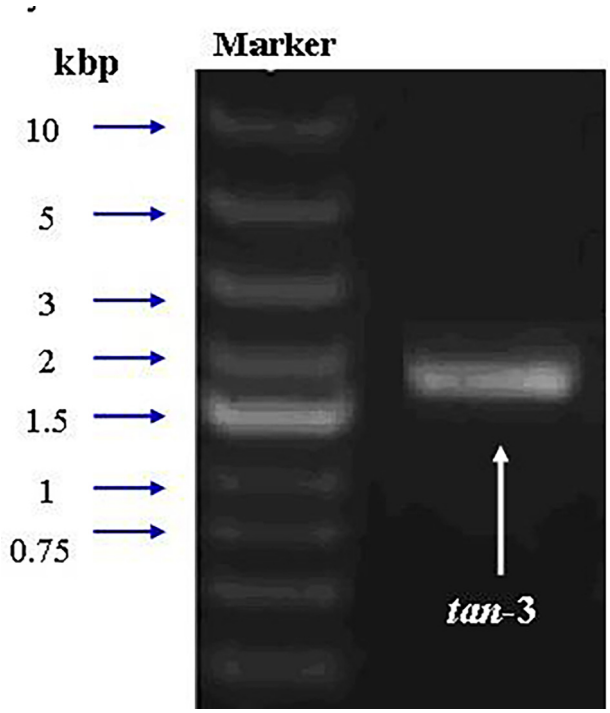

Fig. 1. Agarose gel electrophoresis of the PCR products.

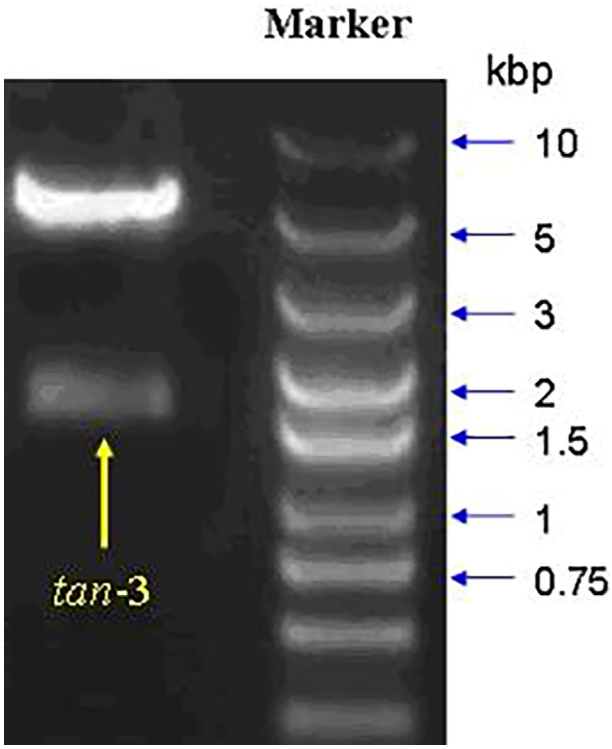

Fig. 2. Agarose gel electrophoresis of the recombinant expression vector tan-pET43.1a using enzyme cutting.

4.2. Expression and identification of target protein

The complete protein SDS-PAGE of the induced fungi and control fungi is shown in Fig. 3. By comparison, it can be seen that the molecular mass of the target protein was about 64–65 kDa

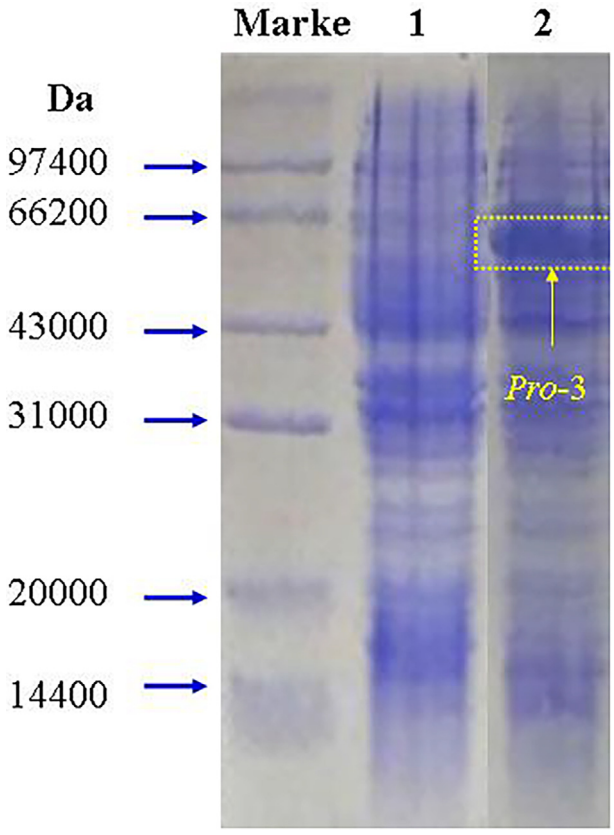

Fig. 3. Complete protein SDS-PAGE of the induced fungi. Note: Lane 1 shows complete protein SDS-PAGE of the control fungi (non-induced); Lane 2 shows complete protein SDS-PAGE of the induced fungi, and *pro*-3 was target protein.

(including the molecular mass of 6 His tag). The mass was in line with the expected results (64.2 kDa), showing that the target protein was obviously expressed in the fungal cells after being added with inducer IPTG. Based on the gel imaging analysis system, the expression quantity of the target protein was about 30%.

4.3. SDS-PAGE analysis of the pure enzyme and determination of tannase activity

The results of SDS-PAGE of the pure enzyme are shown in Fig. 4. Fig. 4 shows that clear pure enzyme band *pro*-3 was observed. The molecular mass of clear pure enzyme band *pro*-3 was about 65 kDa, which was consistent with the expected result (64.2 kDa). The results indicated that the crude enzyme solution was purified successfully. Based on the gray analysis using gel imaging analysis system, the purity of the target protein was >90%.

The results on determination of the tannase activity of the pure and crude enzyme are shown in Table 1. From Table 1 it can be seen that the tannase activity of the crude enzyme was greatly improved after the crude enzyme was purified. The specific activity of the pure enzyme was about 8 times of that of the crude enzyme.

4.4. Protein identification by Western Blotting

After SDS-PAGE, the pure enzyme was transferred to the PVDF membrane. The results of its protein identification by Western Blotting are shown in Fig. 5. From Fig. 5, clear protein band *pro*-3 can be observed. The molecular mass was about 65 kDa, which was in line with the expected results (64.2 kDa). It can be shown that the expression protein could be specifically combined with

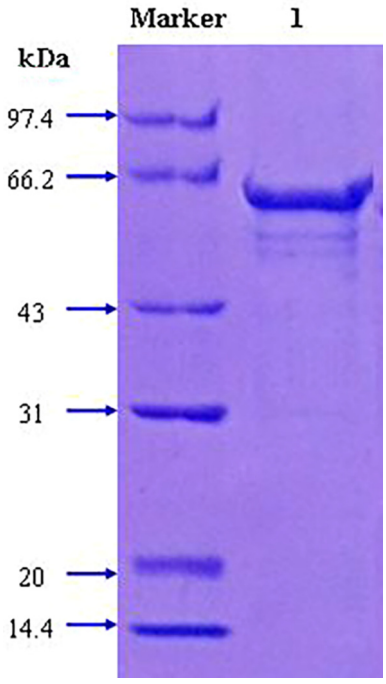

Fig. 4. SDS-PAGE analysis of the purified enzyme. Note: Lane 1 was pure enzyme band *pro*-3.

Table 1
Tannase activity of the pure enzyme and crude enzyme.

Enzyme	Activity/(U/mL)	Protein content/(μg/mL)	Specific activity/(U/μg)
Pure enzyme	1468.55	458.24	3.20
Crude enzyme	155.75	388.46	0.401

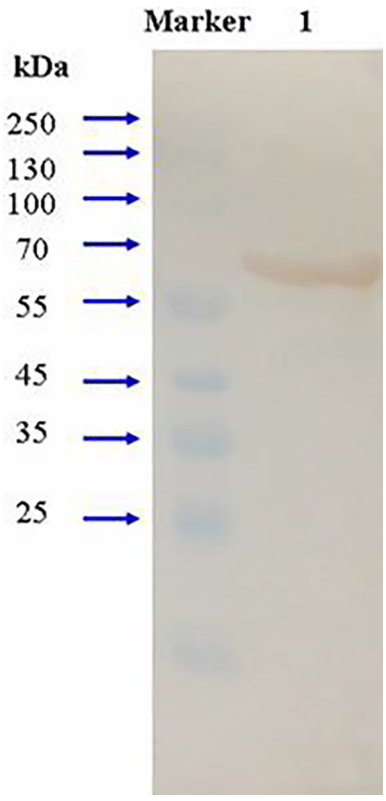

Fig. 5. Protein identification by Western Blotting. Note: Lane 1 shows pure enzyme band *pro*-3.

His tag. Thus, expression protein was proved to be a recombinant fusion protein with 6 His tag.

5. Conclusion

In this paper, specific primers were designed based on the *Aspergillus niger* N5-5 sequence N3 (GenBank, No.: KP677552). Tannase gene *tan* carried 6 His tag and enzyme cutting site which contained NdeI/HindIII using PCR amplification, and was connected with expression vector pET43.1a using NdeI/HindIII enzyme cutting. The nucleic acid electrophoresis analysis showed that the recombinant expression vector *tan*-pET43.1a was formed successfully. Then, the expression vector *tan*-pET43.1a by NdeI/HindIII enzyme cutting was transformed into *E. coli* BL21 (DE3) to induce expression of *Aspergillus niger* N5-5. The positive clone transformants were picked out and inoculated in a LB liquid culture medium for culturing seed liquid and were induced for expression by IPTG. The complete protein SDS-PAGE of the induced fungi and control fungi showed that the molecular weight of the target protein was about 64–65 kDa, which was in line with the expected size (64.2 kDa). Such an analysis result showed that the target protein was successfully expressed in the fungal cells. After the induced fungi were disrupted by the ultrasonic wave, the crude enzyme was extracted and purified using the IMAC. Further, the activity of crude enzyme was determined. The results on determination of the tannase activity of the pure enzyme and crude enzyme showed that the tannase activity of the crude enzyme was greatly improved after the crude enzyme was purified, and the specific activity of the pure enzyme was about 8 times of that of the crude enzyme. The results of SDS-PAGE of the pure enzyme showed that the molecular mass of the pure enzyme was about 65 kDa, which was in line with the expected size (64.2 kDa). Such a mass showed that the crude enzyme solution was purified successfully. The results for identifying pure enzyme's protein by Western Blotting showed that clear protein band *pro*-3 was observed. The molecular mass of clear protein band *pro*-3 was about 65 kDa, which was in line with the expected size (64.2 kDa). Thus, it can be said that the expression protein could be specifically combined with His tag. Thus, the expression protein was proved to be a recombinant fusion protein with 6 His tag.

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