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An Efficient Method for Extraction of Secreted Proteins of a Filamentous Fungus, *Cryphonectria parasitica*

Jinzi Wang, Fangzhen Wang, Jinjie Shang and Baoshan Chen*

State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, College of Life Science and Technology, Guangxi University, Nanning 530004, China

Abstract

Three different methods were evaluated for extraction of secreted proteins from a filamentous fungus, *Cryphonectria parasitica,* cultured in liquid medium. Protein samples prepared with each method were subjected to SDS-PAGE and two-dimensional gel electrophoresis (2-DE). It was found that ultrafiltration was not suitable for extraction of the secreted proteins. Protein samples prepared by chloroform treatment followed by ammonium sulfate precipitation were acceptable for 2-DE separation. The modified sevage method was the best to extract secreted proteins with highest yield and best quality. About 300 protein spots could be identified on a 2-DE gel using protein samples prepared with this method from 3 day-old culture. Successful isolation of high quality secreted proteins is the pre-request for the analysis of fungal secreted proteome. The new method may be applied to other filamentous fungi, particularly the pathogenic ones..

Keywords: Filamentous fungi; Secreted protein; *Cryphonectria parasitica*

Introduction

Cryphonectria parasitica is the causal agent responsible for the chestnut blight, a well-known forest disease [1,2]. Many aspects of this fungus, such as etiology, diversity in vegetative incapability and interaction with its endogenous viruses had been investigated [3-5]. Recent researches on the transcriptome revealed that a wide range of genes may be involved in the pathogenesis [6,7]. Secreted proteins have been implied to be involved in pathgenesis in many pathogenic bacteria and a few fungi [8-10]. Although the values of secreted proteome of pathogenic fungi are appreciated, progress in this field has been in slow pace, with only a few species, e.g. *Kluyveromyces lactis* [11], partially due to the difficulties in preparation of high quality secreted proteins.

In this short communication, we report the development of an efficient method for preparation of high quality secreted proteins of *C. parasitica*, which were suitable for 2-DE separation and mass spectrum identification.

Materials and Methods

Fungal strains and culture conditions

C. parasitica strains used in this work were virus-free strain EP155 (ATCC 38755) and virus-infect strain EP713 (ATCC 52571). The fungal strains were cultured on solid potato dextrose agar (PDA) medium for 3 days at room temperature (24-26°C). For liquid culture, mycelia of 0.25g were harvested from PDA and inoculated into a 500-mL flask containing 200 mL EP complete liquid medium [12] and cultured without shaking for another 3 days at room temperature. These cultures were then used as seeds for a further culture in EP complete medium with a ratio of 10 mL seed culture to 200 mL fresh EP medium and cultured for the desired time from one to seven days.

Extraction and purification methods

Ultrafiltration: Amicon Ultra-15 tube (Millipore, USA) was used to concentrate the mycelia-free culture medium by gauze filtration. The concentrated protein samples were further purified by 2-D Clean-Up kit (GE Healthcare, USA).

Ammonium sulfate precipitation: Ammonium sulfate solution with varied concentration was used to precipitate proteins. Mycelia-free liquid medium was mixed with a half volume of chloroform to denature proteins. The interface phase was collected and washed with -20°C cold acetone containing 0.07% β-mercaptoethanol and re-dissolved in lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 1% DTT, 0.5% cocktail of protease inhibitors). Ammonium sulfate was added to the sample (20%, w/v) and the preparation was centrifuged at 18000 *g* for 20 min at 4°C to collect the precipitant. More ammonium sulfate was added to the supernatant at concentration intervals of 40%, 60% and 80% sequentially. At each of these concentrations, centrifugation was performed to collect precipitant proteins. Pellets were washed twice with -20°C cold acetone containing 0.07% β-mercaptoethanol and dried at room temperature for 10 min. The dried protein pellets were solubilized in 100 µL lysis buffer and clarified by centrifugation at 18000 *g* at 4°C for 20 min. The supernatant was transferred to a fresh tube and stored at -80°C.

The modified sevage method: The mycelia-free culture medium was centrifuged at 8500 g for 20 min at 4°C. A half volume of chloroform/butanol (4:1) was added to the supernatant and mixed thoroughly. The protein-containing interface phase was transferred into a fresh 50-mL Corning tube. The residual upper phase was treated the same way again. This process could be repeated 3-4 times to increase protein yield. The pooled interface phase collection was centrifuged at 4000 g for 10 min and transferred to a 2-mL tube. The interface precipitant together with the upper aquatic phase was centrifuged at 10000 *g* for 5 min. After removing the supernatant, the pellet was washed 3 times with the washing buffer (0.3 M guanidine hydrochloride in 95% ethanol),

***Corresponding author:** Baoshan Chen, College of Life Science and Technology, Guangxi University, Nanning, 530004, PR China Tel: +86 771 3239566; Fax: +86 771 3237873; E-mail: chenyaoj@gxu.edu.cn

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with each wash followed by centrifugation at 7500 g for 5 min at 4°C. One milliliter of ethanol (100%) was then added to the protein pellet and centrifuge at 7500 g for 5 min at 4°C. The pellet was dissolved in 200 µL of lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 1% DTT, 0.5% cocktail of protease inhibitors) and kept for 30 min at 4°C to allow resuspension. To remove insoluble substance, the preparation was centrifuged at 18000 *g* at 4°C for 20 min. The proteins in the supernatant were precipitated by adding 800 µL of pre-cold acetone (-20°C) containing 10% TCA and 0.07% β-mercaptoethanol and further incubated at -20°C for 30 min. The precipitate was pelleted by centrifugation at 18000 *g* at 4°C for 20 min. The pellet was washed twice with 1 mL of cold acetone containing 0.07% β-mercaptoethanol and dried at room temperature to allow evaporation of residual acetone. The dried protein pellet was solubilized in 100 µL of lysis buffer.

Quantity and quality analysis

Protein quantification: The protein concentration of the samples were determined by Bradford assay using BSA as standard [13].

SDS-PAGE analysis: The protein samples were mixed with equal volume of 2×SDS-PAGE loading buffer, boiled for 5 min and separated on 12.5% polyacrylamide gels in a Hofer™ MiniVE vertical electrophoresis system (GE Healthcare, USA).

Two-dimensional electrophoresis: Samples each containing 200µg of proteins were rehydrated in the rehydration buffer. The IPG buffer pH 3-10 NL was added to a final concentration of 2% (v/v). The samples were applied to a non-linear pH 3-10 IEF strip (Immobiline™ DryStrip, GE Healthcare, USA). IEF was carried out at 20°C with a constant current of 50 µA per strip on a IPGphor (GE Healthcare, USA) using the following parameters: 30 V, 6 h; 60 V, 6 h; 500 V, 1 h; 1000 V, 1 h; 1000-6000 V, 4 h; and 6000 V, 120000 Vh.

The focused IEF strips were subjected to reduction and alkylation procedures followed by equilibrated for 15 min in 15 mL of equilibration buffer containing 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% glycerol, 2% SDS and 2% DTT, and for an additional 15 min in the same buffer containing 2.5% iodoacetamide in place of the DTT. After equilibration the strips were mounted onto 12.5% polyacrylamide gels for second dimension electrophoresis with constant current of 10 mA per gel for 30 min and then with the current raised to 20 mA per gel.

Protein staining

The silver staining was compatible with mass spectrometry with modification [14]. The gels from SDS-PAGE and 2-DE were fixed in 40% methanol and 10% acetic acid for 30 min then incubated in 0.02% sodium thiosulfate, 30% methanol and 6.8% NaAc for 30 min. The gels were rinsed with deionized water for three times, each for 5 min, and then incubated in 0.25% $AgNO_3$ for 20 min. After rinsed twice with deionized water, each for 1 min, the gels were developed in 2.5% Na_2CO_3 and 0.04% formaldehyde for 5-10 min until the protein spots displayed clearly. The gels were fixed in 12% acetic acid for 30 min and stored in deionized water.

In-gel tryptic digestion of protein and mass spectrometry

In-gel digestion of protein was done according to the established protocol [15]. Gel pieces excised from the 2-D gels were washed twice in 200 µL of Milli-Q water. Then 50 µL of washing solution (15 mmol/L $K_3Fe(CN)_6$, 50 mmol/L $Na_2S_2O_3$) was added. When the gel pieces turned to the same color as the solution, 400 µL of Milli-Q water was added to dilute the solution. Washed the gel pieces with 200 µL of Milli-Q water once and 200 µL of 40 mM ammonium bicarbonate twice. Dehydrated gel pieces in 150 µL of acetonitrile for 3 min, repeated this step once and dried gel pieces in SpeedVac for 3 min at room temperature. Added 10 µL of the trypsin solution (trypsin 10 ng/µL) to the gel pieces and rehydrated on ice for 30 min. Removed excess trypsin solution and added 10 µL of 40 mM ammonium bicarbonate to the gel pieces. After incubation 14 hours at 37°C, 20 µL of extraction solution (0.1% trifluoroacetic acid, 50% acetonitrile) was added to stop digestion and further incubated 20 min at room temperature. The supernatant was transfer to a fresh 1.5-mL tube. This process could be repeated three times. The supernatant pool was concentrated to 1-3 µL by evaporation.

The peptides solution was mixed with 1µL of matrix solution (0.1% TFA and 50% acetonitrile saturated with CHCA) and analyzed on ABI 4800 plus Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, USA) in the m/z range 800-3500. The combined

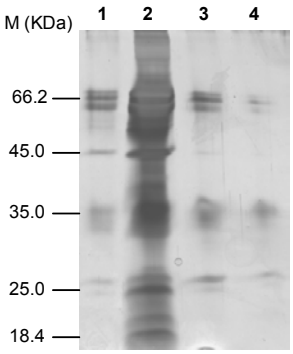

Figure 1: SDS-PAGE analysis of secreted proteins prepared with ammonium sulfate precipitation. Protein standards were marked beside the gel (Molecular masses in kDa). Sample volumes were 10µl. The gel was treated by sliver staining. Lane 1 corresponded to the proteins came from the concentration of ammonium sulfate under 20%. Lane 2 corresponded to the proteins came from the concentration of ammonium sulfate between 20-40%. Lane 3 corresponded to the proteins came from the concentration of ammonium sulfate between 40-60%. Lane 4 corresponded to the proteins came from the concentration of ammonium sulfate between 60-80%.

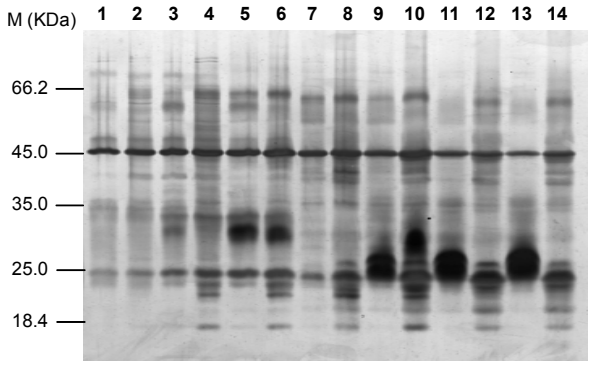

Figure 2: SDS-PAGE analysis of secreted proteins prepared with modified sevage method. Protein standards were marked beside the gel (Molecular masses in kDa). Each lane load 8 µg proteins measuring by Bradford assay. The gel was sliver stained. Lane1, lane3, lane5, lane7, lane9, lane11 and lane13 represented the secreted proteins from EP155 and the culture time was one to seven days with 24-hour interval. Lane2, lane4, lane6, lane8, lane10, lane12 and lane14 represented the secreted proteins from EP713 and the culture time was one to seven days with 24-hour interval.

PMF search (MS plus MS/MS) was carried out using GPS Explorer™ software with the MASCOT search engine (Matrix Science, London, UK) against *Cryphonectria parasitica* data base from JGI (http://genomeportal.jgi-psf.org/Crypa1/Crypa1.download.ftp.html) in a local server.

Results and Discussion

Ultrafiltration is the most common method used to prepare secreted proteins for bacterial and cultured cells [16]. However, the filter membrane was easily blocked by substances in the liquid medium in our tries with *C. parasitica* cultured in EP complete medium, making it impossible to proceed to the following steps of protein preparation.

Ammonium sulfate precipitation could pulled down secreted proteins by concentration gradients [17,18]. The samples prepared with this method were suitable for analysis by SDS-PAGE with the highest protein yield when the concentration of ammonium sulfate was between 20-40% (Figure 1). Although most proteins precipitated at 40% of ammonium sulfate, there were still some proteins which were not completely precipitated.

Protein samples prepared with the modified sevage method were excellent when analyzed on SDS-PAGE (Figure 2). As could be seen in a 2-DE image, more and clearer protein spots appeared with the sample prepared by modified sevage method than with the sample prepared by the ammonium sulfate precipitation (Figure 3). It was observed that most secreted proteins distributed between pH 3 to 8 and abundance of proteins appeared at the acidic end of the strips.

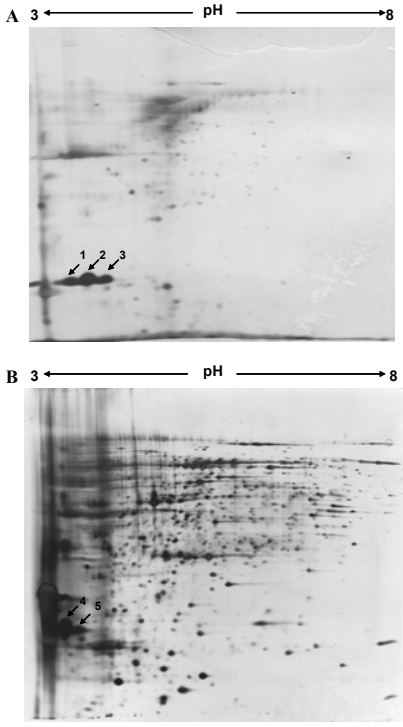

Figure 3: 2-DE of secreted proteins. (A) The secreted proteins extracted by Ammonium sulfate precipitation. (B) The secreted proteins extracted by modified sevage method. The protein samples from both methods were performed on IPG trips with pH range 3-10 for first dimension and 12.5% polyacrylamide gels for second dimension. The proteins were visualized using sliver staining. Protein spots which were marked by arrows were chosen for mass spectrum analysis and identified as 22kDa glycoprotein (*Cryphonectria parasitica* database accession number 100735).

Time course analysis showed that some highly secreted proteins appeared at a later time of culturing and there were some differences in the patterns of secreted proteins between the wild type strain EP155 and the virus-infected EP713 (Figure 2).

2D-quant kit (GE Healthcare, USA) was found not to be suitable for measuring the protein concentration of secreted proteins in our study, due to its large deviation, particularly with the increase culture time. Bradford method seemed to be relatively accurate as verified by SDS-PAGE.

To demonstrate the usefulness of proteins separated, five spots from 2-D gels (Figure 3) were chosen for mass spectrum analysis. These spots were identified to be the same protein, 22-kDa glycoprotein (*Cryphonectria parasitica* database accession number 100735). These protein spots appeared at the same position on 2-DE in protein samples prepared by both ammonium sulfate precipitation and modified sevage method.

Sevage method was used previously for the extraction and purification of polysaccharides. In this study we added chloroform to denature proteins so that they would precipitate in the interface phase while polysaccharides remain in the aqueous phase. The use of low concentrations of guanidine hydrochloride effectively got rid of the interference components including residual chloroform. The establishment of an efficient method for preparation of *C. parasitica* secreted proteins opens the door of systemic analysis of the secreted proteins of this important tree fungus.

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References

1. Sun L, Nuss DL, Suzuki N (2006) Synergism between a mycoreovirus and a hypovirus mediated by the papain-like protease p29 of the prototypic hypovirus CHV1-EP713. J Gen Virol 87: 3703-3714.
2. Anagnostakis SL (1982) Biological control of chestnut blight. Science 215: 466-471.
3. Lin H, Lan X, Liao H, Parsley TB, Nuss DL, et al. (2007) Genome sequence, full-length infectious cDNA clone, and mapping of viral double-stranded RNA accumulation determinant of hypovirus CHV1-EP721. J Virol 81: 1813-1820.
4. Chen B, Choi GH, Nuss DL (1994) Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. Science 264: 1762-1764.
5. Chen B, Nuss DL (1999) Infectious cDNA clone of hypovirus CHV1-Euro7: a comparative virology approach to investigate virus-mediated hypovirulence of the chestnut blight fungus Cryphonectria parasitica. J Virol 73: 985-992.
6. Shang J, Wu X, Lan X, Fan Y, Dong H, et al. (2008) Large-scale expressed sequence tag analysis for the chestnut blight fungus Cryphonectria parasitica. Fungal Genet Biol 45: 319-327.
7. Nuss DL (2005) Hypovirulence: mycoviruses at the fungal-plant interface. Nat Rev Microbiol 3: 632-642.
8. Poiatti VA, Dalmas FR, Astarita LV (2009) Defense mechanisms of Solanum tuberosum L. in response to attack by plant-pathogenic bacteria. Biol Res 42: 205-215.
9. Abbas A, Koc H, Liu F, Tien M (2005) Fungal degradation of wood: initial proteomic analysis of extracellular proteins of Phanerochaete chrysosporium grown on oak substrate. Curr Genet 47: 49-56.
10. Thomas DP, Bachmann SP, Lopez-Ribot JL (2006) Proteomics for the analysis of the Candida albicans biofilm lifestyle. Proteomics 6: 5795-5804.

11. Swaim CL, Anton BP, Sharma SS, Taron CH, Benner JS (2008) Physical and computational analysis of the yeast Kluyveromyces lactis secreted proteome. Proteomics 8: 2714-2723.

12. Puhalla JE, Anagnostakis SL (1971) Genetics and Nutritional Requirements of Endothia parasitica. Phytopathology 61: 169-173.

13. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

14. Yan JX, Wait R, Berkelman T, Harry RA, Westbrook JA, et al. (2000) A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. Electrophoresis 21: 3666-3672.

15. Gharahdaghi F, Weinberg CR, Meagher DA, Imai BS, Mische SM (1999) Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. Electrophoresis 20: 601-605.

16. Kazemi-Pour N, Condemine G, Hugouvieux-Cotte-Pattat N (2004) The secretome of the plant pathogenic bacterium Erwinia chrysanthemi. Proteomics 4: 3177-3186.

17. Lee EY, Bang JY, Park GW, Choi DS, Kang JS, et al. (2007) Global proteomic profiling of native outer membrane vesicles derived from Escherichia coli. Proteomics 7: 3143-3153.

18. Wingfield P (2001) Protein precipitation using ammonium sulfate. Curr Protoc Protein Sci 3: 3F.