Protein Expression and Purification 90 (2013) 178–185

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

High-level expression of *Staphylococcal* Protein A in *Pichia pastoris* and purification and characterization of the recombinant protein

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ARTICLE INFO

Article history:
Received 23 March 2013
and in revised form 30 May 2013
Available online 13 June 2013

Keywords:
Pichia pastoris
Protein purification
Secretory expression
Staphylococcal Protein A

ABSTRACT

Staphylococcal Protein A (SPA), a cell wall protein of *Staphylococcus aureus*, is in high demand because of its ability to bind immunoglobulins. Much of the SPA that we use today is recombinant SPA (rSPA), which is produced in *Escherichia coli*. As rSPA is obtained by expressing SPA as an intracellular protein, its purification is tedious and time consuming. In order to obtain a large amount of highly purified rSPA with relative ease, we expressed SPA as a secretory form in the yeast *Pichia pastoris*. To increase the expression level of SPA and repress its proteolysis during fermentation, the cell density (OD_{600}), temperature and pH at which SPA expression was induced as well as the induction time were optimized. The final yield of SPA obtained was about 8.8 g per liter of culture, which under the optimized fermentation condition, accounted for 80% of the total protein in the culture supernatant. The expressed SPA was purified from the culture supernatant by DEAE ion-exchange chromatography (IEC) after the supernatant was subjected to a desalting step. The purified SPA was resolved as a single band by SDS–PAGE and as a single peak by HPLC. Its identity was confirmed by MALDI-TOF MS and western-blot. Moreover, the protein also exhibited excellent affinity for IgG when tested with human IgG. The production and purification of SPA described in this study offers a new method for obtaining high level of SPA in relatively pure form that is suitable for practical application.

Introduction

Staphylococcal Protein A (SPA)[1] is a cell wall protein of *Staphylococcus aureus* [1]. SPA represents about 1.7% of the total protein of *S. aureus.* Due to its high affinity for immumoglobuins, SPA has been widely used in the purification and detection of antibody [2,3], rapid diagnosis of pathogen [4] and immunological analysis [5]. In 1998, Immunosorba adsorption column, made with SPA as the affinity ligand, was certificated by the FDA for the treatment of autoimmune diseases. Since then, mass production of SPA has become an important resource for the medical industry as well as an important tool for biological research. Currently, most of the SPA that is in used is recombinant SPA (rSPA), which is expressed in *Escherichia coli* [6,7]. To separate and purify rSPA from the mixture of complex intracellular proteins and DNA, the *E. coli* cells must first be disrupted and the DNA precipitated from the cell lysate. During these processes, the structure of SPA might be destroyed and part of it might be lost. Moreover, when a recombinant protein is expressed inside the cell, a multiple-step purification process is often needed to purify the target protein from the intracellular proteins of the host after the cell is lysed. An example of such method that has been used for the large scale production rSPA is the method recommended by Repligen Corporation [8], which involves heating the rSPA-containing extract for 30 min at 80 °C to remove most of the host proteins, followed by filtration, ethanol precipitation and ion-exchange chromatography. Alternatively, rSPA can be purified by IgG-affinity chromatography, which is a single step. However, it is difficult to turn this method into a widely-used method for large scale production of rSPA due to the high cost of IgG affinity column. Furthermore, the IgG molecules of these columns are also not very stable, and may become detached from the column resin, thereby contaminate the final product.

Compared to intracellular expression, extracellular expression of SPA is another way to produce rSPA, since this would greatly facilitate its subsequent purification. Until now, extracellular expression of SPA has only been reported in protease-deficient *Bacillus sabtilis*, where an expression level of about 3 g/L has been achieved [9]. No other study on extracellular expression of SPA has been published. Thus a new technology to produce large-scale SPA based on extracellular expression is needed.

Besides *E. coli*, yeast is another common host for expressing target proteins through genetic engineering [10]. The advantage of yeast, such as *P. pastoris*, is that the target proteins can also be

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[1] *Abbreviations used:* SPA, *Staphylococcal* Protein A; IEC, ion-exchange chromatography; DADPA, diaminodipropylamine.

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http://dx.doi.org/10.1016/j.pep.2013.06.005

expressed as secretory forms [11]. Moreover, the production of target proteins can be increased by high-cell-density fermentation. The cell biomass could reach as high as 600 g (wet cell weight) per liter of culture, which is nearly 10 times higher than the biomass achieved by *E. coli*. Furthermore, as a yeast cell, *P. pastoris* can express proteins for clinic application without contamination by endotoxins [12]. Contamination of the purified recombinant protein by endotoxin can be a serious problem for the *E. coli* expression system. So far, *P. pastoris* has been used for overexpressing many different proteins [13]. To the best of our knowledge, only one study has reported the expression SPA in *P. pastoris* [14]. However, that particular study only concerns with the expression of the IgG-binding domain of SPA, which is fused to EGFP. It managed to achieve an expression level of 115 mg per liter of culture. Furthermore, the application of the fusion protein is limited to immunodetection. Thus so far, no study concerning with the expression of intact and non-fusion SPA in *P. pastoris* has been reported. In order to simplify the purification process for SPA and to decrease the purification cost of SPA, we expressed SPA as a secretory protein in *P. pastoris* GS115 under the control of the *AOX1* (*alcohol oxidase I*) promoter [15]. To decrease the proteolysis of the expressed SPA, reduce pigments secretion and increase SPA production during fermentation, several parameters, including the cell density (OD_{600}), temperature, and pH at which SPA was induced were optimized. A purification method for the expressed SPA was described, with emphasis given to the removal of complex pigments and the prevention of hydrolysis that could lead to fragmented SPA.

Material and methods

Reagents, media and host cells

P. pastoris GS115 and pPIC9K vector used for SPA expression were obtained from Invitrogen (USA). *S. aureus* ATCC6538 was donated by Dalian Municipal Center for Disease Control and Prevention. Commercial SPA was purchased from Repligen (Waltham, USA) and Sepharose CL-4B was purchased from GE China-Healthcare (Beijing). *E. coli* strain DH5α was purchased from Takara (Dalian, China). Restriction endonucleases, T4 DNA ligase, DNA and Protein Markers, PCR kit, Agarose Gel DNA Fragment Recovery Kit Ver.2.0, MiniBEST Plasmid Purification Kit Ver.2.0, Lysis Buffer for Microoganism to Direct PCR and TaKaRa LA Taq Kit were all purchased from Takara (Dalian, China).

Construction of SPA expression vector

Genomic DNA isolated from *S. aureus* ATCC6538 was used as template for PCR amplification of the SPA gene using the forward primer 5′-GCTGCAGAATTCGCGCAACACGATGAAG-3′ (underlined bases indicates *Eco*R I site) and reverse primer 5'-AGGTTTGTT GGCGGCCGCTTATTTTGGT-3′ (underlined bases indicate *Not* I site). The PCR amplification consisted of 35 cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 2 min, and followed by a final extension step at 72 °C for 5 min. The PCR product was cloned into the expression vector pPIC9K, downstream of the *AOX1* promoter and the α-factor signal sequence. The recombinant vector, pPIC9K-spa, was verified by PCR, digestion by restriction endonucleases, and DNA sequencing (Takara Dalian, China).

Transformation of P. pastoris and screening of recombinants

pPIC9K-spa was linearized by SacI and then introduced into competent *P. pastoris* GS115 cells by electroporation. The transformation was performed under standard method as described previously [16]. Recombinant GS115 clones were selected on MD plates and MM plates to confirm that they were positive with respect to methanol-utilizing phenotype (Mut+). Eight clones were identified by TaKaRa LA Taq Kit using the AOX1 primers, which could detect the integration of the target gene into the genome of *P. pastoris*. After cultivation in BMGY (1% w/v yeast extract, 2% w/v peptone, 1.34% w/v yeast nitrogen base, 4×10^{-5}% w/v biotin, 1% v/v glycerol, 100 mM potassium phosphate, pH 6.0) and induction in BMMY (1% w/v yeast extract, 2% w/v peptone, 1.34% w/v yeast nitrogen base, 4×10^{-5}% w/v biotin, 1% v/v methanol, 100 mM potassium phosphate pH6.0), the clone that showed the highest level of SPA expression (as detected by SDS–PAGE) [17] was used to determine the optimum condition for SPA expression by fermentation.

Optimization of SPA expression

Fermentation was performed according to the protocol described by Tolner et al. [18]. To increase the expression level and repress the proteolysis of SPA (during expression), protein expression was induced at different cell densities (OD_{600} 300, 400, 480), temperatures (30 °C, 25 °C, 22 °C), pHs (from 2.5 to 4.5 with 0.5 pH intervals) and for different periods of time. Protein expression was carried out using BSM medium supplemented with PTM1 trace-salt solution [19], and monitored by SDS–PAGE. The expression of SPA was measured by quantifying the relevant band in the gel using by a computerized image processing system (Scion Image).

Western blot analysis

Proteins resolved by 15% SDS–PAGE gel were first transferred to polyvinylidene difluoride (PVDF) membrane using a semi-dry electro-blotting apparatus (Bio-Rad, USA). The transfer was carried out for 2 h at 18 V. After that, the membrane was blocked with TBST (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween) containing 5% non-fat milk for 1 h, and then incubated with a 1:10,000 dilution of goat anti-Protein A (HRP) antibody (Abcam, UK) at room temperature for 1 h. The membrane was then washed six times with TBST (10 min per wash), and positive bands in the membrane were detected by ECL reagent.

IgG affinity Detection of SPA

The affinity of the crude rSPA for IgG was investigated by IgG-affinity column. After ultrafiltration, the clear culture supernatant (from shake-flask fermentation conducted in BMMY at 22 °C and pH 5.5) was loaded onto an IgG affinity column pre-equilibrated with buffer A (25 mM Tris–HCl, pH 8.0). The column was washed with buffer A until the absorbance of the eluent at 214 nm reached a steady baseline. Protein bound to the column was eluted with 25 mM citrate buffer (pH 2.3) and analyzed by SDS–PAGE.

Pigments removal

Several methods were tried to get rid of the pigments in the clear culture supernatant obtained after centrifugation at 13000*g* for 10 min at 4 °C. In the first method, the supernatant was subjected to $(NH_4)_2SO_4$ or ethanol precipitation and the precipitated protein pellet was re-suspended in 25 mM Tris–HCl (pH 8.0). The protein sample was treated with resin-based activated carbon spheres (average diameter of 0.8–1.2 mm and pore size of about 2 nm). In the second method, the clear supernatant (7 mL) was directly loaded onto a desalting column (HiPrep 26/10, GE Healthcare) pre-equilibrated with buffer A. The column was connected to an AKTA explorer (ÄKTA purifier 100, GE Healthcare). The column