High-yield expression of recombinant SARS coronavirus nucleocapsid protein in methylotrophic yeast *Pichia pastoris*

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AIM: Nucleocapsid (N) protein plays an important role in reproduction and pathological reaction of severe acute respiratory syndrome (SARS) coronavirus (SCoV), the antigenicity of the protein is better than spike (S) protein. This study was to find a highly specific and antigenic recombinant SCoV nucleocapsid (rSCoVN) protein, and to provide a basis for further researches on early diagnosis of SARS.

METHODS: Full length cDNA of SCoV nucleocapsid (SCoVN) protein was amplified through polymerase chain reaction (PCR) and cloned into yeast expression vector pPIC3.5K to construct plasmid of pPIC3.5K-SCoVN. The plasmid was linearized and then transformed into Pichia pastoris (P. pastoris) GS115 (*His*+ *Mut*−) by electroporation. *His*+ *Mut*− recombinant strains were identified by PCR and cultivated on MM/MD plates. The influence of different factors on biomass and rSCoVN protein production during induction phase, such as various induction media, dissolved oxygen (DO) and different final concentrations of methanol, was subsequently studied. The expression level and activation were detected by SDS-PAGE and Western-blot respectively.

RESULTS: All of the recombinants were *His*+ *Mut*− after transformation of *P. pastoris* with linearized plasmids. The BMMY medium was optimal for recombinant SCoVN (rSCoVN) protein expression and growth of the recombinant strains. The final optimal concentration of methanol was 20 mL/L, the DO had a significant effect on rSCoVN protein expression and growth of recombinant strains. The rSCoVN protein expressed in recombinant strains was about 8% of the total cell protein, 520 mg/L of rSCoVN protein was achieved, and a maximum cell A at 600 nm of 62 was achieved in shake flask culture. The rSCoVN protein had a high specificity against mouse-anti-SARS-CoV-mAb and SARS positive sera, but had no cross-reaction with normal human serum. The biological activity of rSCoVN expressed in *P. pastoris* was about 4-fold higher than that expressed in *E. coli* when the same rSCoVN protein quantity was used.

CONCLUSION: Active recombinant severe acute respiratory syndrome (SARS) coronavirus nucleocapsid (rSCoVN) protein can be successfully expressed in recombinant methylotrophic yeast *P. pastoris* GS115. The rSCoVN protein has a high specificity against SARS-CoV-mAb and SARS positive sera, but has no cross-reaction with normal human serum. This provides a basis for further researches on the early diagnosis of SARS and the mechanism of SCoV.

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INTRODUCTION

The etiologic agent of SARS has been recently identified as a novel coronavirus (SCoV, SARS-CoV) causing respiratory and enteric diseases in humans and other animals[1-3]. The SCoV is a group of large, enveloped, positive single stranded RNA (ssRNA) viruses[4]. Sequence analysis has revealed that the phylogeny of SCoV has most of the characteristic features of a coronavirus, but it belongs to a new group different from all known coronaviruses[5]. The genome size is 29,725 kb in full-length, has 11 open reading frames (ORFs)[6]. The SCoV is the largest virus found in any of the RNA viruses, encoding 23 putative proteins, including four major structural proteins, nucleocapsid (N), spike (S), membrane (M), and small envelope (E). The S, M, E and N mature proteins all contribute to generating the host immune response as seen in transmissible gastroenteritis coronavirus[7], infectious bronchitis virus[8,9], pig respiratory coronavirus[10], and mouse hepatitis virus[11].

SARS is an infectious disease with a high potential for transmission due to close contacts. The outbreak of SARS over 25 countries around the world, such as China, Singapore, Canada, threatened people’s health throughout the world. Within a very short time, the disease became pandemic with new cases appearing in the rest of the world. However, it is not easy to differentiate SARS from other causes of pneumonia. Laboratory tests that can confirm a diagnosis of SCoV infection early in the course of the illness are therefore a critical clinical need[12]. Serology is a sensitive and specific diagnostic approach in the early stage of the disease. N protein plays an important role in reproduction and pathology reaction of SCoV. Moreover, the antigenicity of N protein is better than S protein[13,14,15]. To find an effective serologic diagnostic method, rSCoVN may be a perfect antigen.

*Pichia pastoris* (*P. pastoris*), a methylotrophic yeast, is an efficient host for recombinant protein production. The increasing popularity of this particular expression system can be attributed to several factors[16-19], such as the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris*, the ability of *P. pastoris* to produce foreign proteins at high levels, many eukaryotic posttranslational modifications, and the commercially available expression system.

Up to now, rSCoVN has been expressed in *E. coli*. However, the biological activity of the rSCoVN protein expressed in *E. coli* is not perfectly understood. High-yield intracellular
expression in *P. pastoris* and activity analysis were investigated in this report. Western blot showed that the rSCoVN expressed in *P. pastoris* had a high specificity to mouse-anti-SCoVN-mAb and patient sera. The biological activity of rSCoVN expressed in *P. pastoris* was about 4-fold higher than that expressed in *E. coli* when the same rSCoVN protein quantity was used.

**MATERIALS AND METHODS**

**Materials**

*E. coli* strains used for cloning the gene were TOP10F' [proAB, lacI^Q^, lacZ^ΔM15^, Tn10 (Tet^R^)] and TGL1 (supE Δ hisG4Δ thiA1658lacZΔM15). These two strains were cultivated in LB medium (5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl). *P. pastoris* GS115 (his^mut^-) used as expression host was purchased from Invitrogen (California, USA). The following media were employed in cultivation of expression host was purchased from Invitrogen (California, USA). The following media were employed in cultivation of expression host was purchased from Invitrogen (California, USA).

**Methods**

**Construction of pPIC3.5K-SCoVN expression plasmid** The standard recombinant DNA technologies were used. The coding sequence of SCoVN protein was amplified from the *T. saccharolyticus* genomic DNA using the upstream primer (SCoVNFP: 5'-GAATTCTTATGCCTGAGTT [Image 61x200 to 71x211] H I site, in addition to a Kozak consensus sequence, and 3' primer (SCoVNRP: 5'-GAATTCTTATGCCTGAGTT [Image 61x200 to 71x211] H I site, in addition to a Kozak consensus sequence, and 3' primer (SCoVNRP: 5'-GAATTCTTATGCCTGAGTT [Image 61x200 to 71x211] H I site, in addition to a Kozak consensus sequence, and 3' primer (SCoVNRP: 5'-GAATTCTTATGCCTGAGTT [Image 61x200 to 71x211] H I site, in addition to a Kozak consensus sequence, and 3' primer ([Image 235x200 to 245x211]) leading to targeting his^+^Mut^+^ locus. Before transformation, the linearized DNA was desalted by gel electrophoresis cuvette. Transformation was performed using a BioRad GenePulser II. Parameters used were 7.5 kV/cm, 50 µF and 400 Ω. After pulsed, 1.0 mL of ice-cold RDB liquid media was immediately added to the cuvette, and then incubated at 29 °C for 60 min in a shaking incubator (250-300 rpm). Two aliquots of 400 µL each were plated on RDB-geneticin agar plates, the plates were incubated at 29 °C for 3-4 d. Using sterile toothpicks, the His^+^ and genetical resistant transformants were picked in a regular pattern on MM and MD plates and incubated at 29 °C for 2-3 d. The His^+^Mut^-^ (methanol utilization slow) transformants were differentiated from his^+^Mut^-^ (methanol utilization plus) via comparison of patch growth rate on MM and MD plates.

**PCR analysis of *P. pastoris* recombinant transformants** The primers used were as follows. 5' AOX1: 5'-GACTTGTCTACATGAAACGC-3'; 3' AOX1: 5'-GCAAATGCGACTGCATCC-3'. The genomic DNA was isolated as described in multi-mopy *Pichia* expression kit. PCR amplification was performed as follows: initial denaturation at 95 °C for 4 min, followed by 34 cycles, (each at 94 °C for 60 s, at 55 °C for 60 s, at 72 °C for 150 s) and a final extension at 72 °C for 10 min. The genomic DNAs isolated from recombinant *P. pastoris* transformed with parent plasmid and *P. pastoris* GS115 were used as control for PCR.

**Shake-flask cultivation of *P. pastoris* and intracellular expression of rSCoVN** The recombinant *P. pastoris* strains judged by PCR analysis were grown in 15 mL BMGY medium at 29 °C, until the final cell OD at 600 nm reached 2-6 (log-phase growth). The cells were harvested by centrifuging at 1500 r/min for 10 min, and the supernate was applied to SDS-PAGE, and separated by 10% SDS-PAGE. Western blot showed that the rSCoVN expressed when the same rSCoVN protein quantity was used. **Optimization of culture and induction protocol in shake flasks** The highly expressed recombinant strains judged by BMGY/BMMY media firstly were grown in different media, and then induced in relevant medium. At each time during induction, samples were withdrawn to analyse expression level, activity and to detect A at 600 nm.

**Lysis of cells and detection of proteins** Yeast cells induced to express rSCoVN protein were harvested, the cells were washed with sterile water, and then harvested and stored at -80 °C. Frozen yeast cells were removed from storage and thawed for approximately 3 h at room temperature, breaking buffer (25 mmol/L sodium phosphate, 150 mmol/L sodium chloride, 1 mmol/L EDTA, 1 mmol/L DTT) was added, the cells (6 mL breaking buffer/g wet cells) were resuspended and then stirred for 15 min, followed by approximately 18 h at 4 °C, then disrupted by four passes through a sanitized APV Gaulin 30 CD homogenizer at chamber pressures of 12 000 to 14 000 psi, resulting in 95% cell disruption. About 10 µL breaking sample mixed with 10 µL ddiH₂O and 10 µL SDS-PAGE sample buffer [Image 441x584 to 451x594] were boiled for 5-10 min, then centrifuged at 12 000 g for 10 min at room temperature, and 10 µL of the supernate was applied to SDS-PAGE, and separated on 100 g/L polyacrylamide gels with 50 g/L stacking gel. For protein estimations, Coomassie-stained SDS-gels were analyzed by densitometry using UVI.

**Western blot analysis of rSCoVN protein** After equilibration of gels and nitrocellulose membranes in transfer buffer (25 mmol/L Tris-HCl, 192 mmol/L glycine, 3.5 mmol/L SDS, 200 mL/L methanol), the protein was electroblotted onto a membrane (Amersham Pharmacia Biotech Hoefer TE 70 Series Semiphor Semi-Dry transfer Units, 0.8 mm/cm² 30 min). After the membrane (50 g/L fat free milk/TN buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 8.0) was blocked, it was probed with a...
mouse-anti-SCoVN-mAb (dilution 1:1 000, 50 g/L fat free milk/TN buffer or human serum (dilution 1:100 in 5% fat free milk/TN buffer; 20-25 °C, 1 h), followed by a sheep-anti-mouse or sheep-anti-human IgG coupled to alkaline phosphatase (dilution: 1:10 000 in 50 g/L fat-free milk/TN buffer; 20-25 °C, 1 h). Bound antibodies were detected using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro-blue tetrazolium (NBT) in 100 mmol/L NaCl, 5 mmol/L MgCl₂, 100 mmol/L Tris. HCl buffer, pH 9.5 as substrates.

Quantitative activity analysis of rSCoVN proteins expressed in *P. pastoris* and *E. coli*  The biological activity of rSCoVN proteins expressed in *P. pastoris* and *E. coli* was compared using Western blot. The purified protein expressed in *P. pastoris* was diluted by 4-fold gradient, and 12, 3, 0.75, 0.18 μg proteins were added to each well of SDS-PAGE respectively, then probed with a mouse-anti-SCoVN-mAb.

RESULTS

Construction of pPIC3.5K-SCoVN expression plasmid

The coding sequences for SCoVN gene were amplified from T-SCoVN plasmid by PCR using primers (SCoVNFP and SCoVNRP) incorporating 5′ *BamHI* and 3′ *EcoR I*, and subcloned into the *BamHI* and *EcoR I* sites of the pPIC3.5K expression plasmid (Figure 1). Then the constructed expression plasmid was sequenced and proved to be correct. The SCoVN protein coding sequence was under the control of the AOX1 promoter.

![Figure 1](image1.png)

**Figure 1** Construct of expression plasmid pPIC3.5K-SCoVN under the control of AOX1 promoter (5′ AOX1: promoter fragment; 3′ AOX1 (TT): transcription termination, HIS4 ORF: a select marker, SCoVN was inserted between 5′ AOX1 and 3′ AOX1 (TT), Kanamycin: multi-copy select marker).

Screening of transformant phenotype

pPIC3.5K-SCoVN or pPIC3.5K was digested with *SacI* to linearize the plasmids, and then electroporated into *P. pastoris* GS115 (*his·mut·r*) respectively. Twenty geneticin-resistant colonies were cultivated on MD and MM plates. They were all *His·Mut·r*, because they all had the same growth rate. The geneticin-resistant colonies were grown in YPED for 24 h; then the genomic DNA was purified, PCR amplification of SCoVN gene was carried out with 5′ *AOX1* primer and 3′ *AOX1* primer. Twenty transformants each had the alcohol oxidase gene and SCoVN gene. The result demonstrated that all geneticin-resistant colonies were *His·Mut·r*, identical to the result identified on MD and MM plates. The SCoVN protein coding sequence was correctly integrated into the *P. pastoris* genome in the positive recombinants via a single crossover (Figure 2: lanes 5 and 6).

Expression of rSCoVN protein in shake flasks and activity assay

A single recombinant colony (*His·Mut·r*) was used for expression study in shake flask expression[21]. After one day of cell growth on BMGY medium at 30 °C, 250 rpm, then the BMGY medium was changed to BMMY medium. The cells were fed with 100 mL/L methanol to 5 mL/L (final concentration) every day. The strains transformed by parent plasmid pPIC3.5K were taken as control.

Samples were withdrawn after 60 h. SDS-PAGE showed that heterologous rSCoVN protein was successfully expressed in *P. pastoris*, the rSCoVN protein expression level was about 6% of the total cell protein (Figure 3), a 45 ku protein band could be seen in the positive recombinant and there was no 45 ku protein band in control. To confirm the 45 ku protein was the SCoVN protein, 45 ku protein was detected by Western blot with mouse-anti-SCoVN protein mAb, SARS positive serum and negative serum. The results showed that a single positive reaction band at about 45 ku (Figure 4) could be seen in the nitrocellulose membrane. The band of the same molecular mass could not be detected in the induced recombinant strain transformed pPIC3.5K vector by Western blot analysis. The results showed that the recombinant protein was successfully expressed and had a high specificity and good-antigenicity against SCoVN-Ab and SARS positive serum, but had no reaction with normal human serum.

![Figure 2](image2.png)

**Figure 2** Integration of SCoVN gene into the *P. pastoris* GS115 confirmed by PCR using 5′ *AOX1* primer and 3′ *AOX1* primer (lane 1: DNA ladder, lane 2: PCR product of pPIC3.5K, lane 3: PCR product of pPIC3.5K-SCoVN, lane 4: PCR product of *P. pastoris* GS115 without transformation, lane 5: PCR product of control strains, lanes 6 and 7: PCR product of positive recombinants).

![Figure 3](image3.png)

**Figure 3** Expression analysis of intracellular SCoVN protein from recombinant yeasts on SDS-PAGE: lane M: protein molecular mass marker; lane 1: protein from control strain; lane 2: proteins from the best strain; lane 3: purified rSCoVN protein; Lane 4: purified SCoVN protein.

![Figure 4](image4.png)

**Figure 4** Western blot analysis of intracellular SCoVN protein from recombinant yeasts. A: Western blot analysis against mouse-anti-SCoVN-mAb; B: Western blot analysis against SARS positive serum; C: Western blot analysis against SARS negative serum: lane M: protein molecular-mass marker; lane 1: protein from control strain; lane 2: proteins from the best strain; lane 3: purified rSCoVN protein.
Comparison of biologic activity of rSCoVN in P. pastoris and E.coli

The biologic activities of rSCoVN protein expressed in P. pastoris and E.coli were compared using Western-blot. As shown in Figure 5, both rSCoV proteins were able to react with mouse-anti-SARSN-mAb. However, the rSCoVN protein expressed in P. pastoris appeared to have a more potent activity than that in E.coli. Dose-response studies demonstrated that the biological activity of rSCoVN expressed in P. pastoris was approximately 4-fold higher than that in E.coli when the same rSCoVN protein quantity was used. But the Western blot and SDS-PAGE demonstrated that there was no glycosylation in the rSCoVN protein expressed in P. pastoris, because the rSCoVN protein expressed in P. pastoris had the same molecular weight as in E.coli. So rSCoVN protein expressed in P. pastoris had more application potential as a diagnostic agent.

Effect of DO on yeast growth and rSCoVN protein expression

The effect of DO in media on growth and rSCoVN protein expression of P. pastoris was also investigated. Different volume media (10, 20, 30 and 50 mL) were loaded in 100 mL flasks. The different volume of the media loaded in flasks meant different DO. The more the medium was loaded, the less the DO was, and it should be noted that the shape was identical. Figure 6 C, D show that the DO had an obvious effect on the yeast growth and expression of rSCoVN. The rSCoVN protein expression level was the same in the flask loaded 10 mL or 20 mL medium, because the DO was efficient for growth and rSCoVN protein expression of the recombinant strains in the flask, so the growth and rSCoVN expression level of the recombinant protein were very high, over 20% higher than others. When the volume of medium was increased(above 20 mL), the DO decreased, and the growth and expression level were decreased. So the DO was an important factor for the growth and rSCoV protein expression of the recombinants.

Effect of the final concentration of methanol during induction on yeast growth and rSCoVN protein expression

To find an optimum methanol feeding protocol, different volumes of 100% methanol were added to the media twice a day instead of once a day to decrease the resulting concentration shifts in the BMMY medium. Figure 6 E, F show that when the

Figure 5 Determination of biological activity of rSCoVN protein expressed in P. pastoris and E.coli. SDS-PAGE (left) and Western blot (right). Lanes 1, 5: sample containing 12 µg rSCoVN, lanes 2, 6: sample containing 3 µg rSCoVN, lanes 3, 7: sample containing 0.75 µg rSCoVN, lanes 4, 8: sample containing 0.18 µg rSCoVN. Lanes 1, 2, 3, 4: sample expressed in P. pastoris and lanes 5, 6, 7, 8: sample expressed in E.coli.

Figure 6 Effects of different media, DO (dissolved oxygen) and methanol amount on the growth and expression of rSCoVN. A, C, E: Growth curves during induction phase by different media, DO and methanol amount, B, D, F: rSCoVN expression and induction time by different media, DO and methanol amount.
feeding strategy. In the present study, active rSCoVN was highly expressed in the perfect antigen. N protein plays an important role in transmission due to close contacts. So it is critical to find a perfect diagnostic agent. N protein expression level was examined. The results are shown in Figure 7. After the optimal conditions for yeast growth and rSCoVN protein expression were found, the kinetics of the growth and expression were examined. The results are shown in Figure 7. The rSCoVN protein could be detected readily by SDS-PAGE after 12 h of induction (the inserted panel). The kinetics of growth and expression were evident when cells entered the logarithmic growth state and peaked when cell growth reached a steady state and became more or less constant thereafter. The magnitude of expression level reached 526 mg/L. The rSCoVN protein expression was about 8% of the total cell protein, and the maximum cell OD at 600 nm of 62 was achieved. So the optimal harvesting time of cells was 48–60 h after induction. The insert in the panel showed the accumulation of rSCoVN protein by SDS-PAGE during the course of the 84 h experiment.

Figure 7 Kinetics of biomass and rSCoVN protein expression using SDS-PAGE over a span of 84 h. The insert shows rSCoVN protein expression level using SDS-PAGE during the course of experiment.

DISCUSSION

The P. pastoris expression system has gained acceptance as an important host organism for the production of foreign proteins as illustrated by the fact that a number of proteins synthesized in P. pastoris are being used in biopharmaceuticals. IGF-1 is administered to patients as a treatment for amyotrophic lateral sclerosis and human serum albumin (HSA) in a serum replacement product have passed clinical trials. Another protein, hepatitis B surface antigen, has been currently available on the market as a subunit vaccine against hepatitis B virus [24,25]

The etiologic agent of severe acute respiratory syndrome (SARS), has been recently identified as a novel coronavirus causing respiratory and enteric diseases in humans and other animals [3]. SARS is an infectious disease with a high potential for transmission due to close contacts. So it is critical to find a perfect diagnostic agent. N protein plays an important role in reproduction and pathologic reaction of SCoV, and its antigenicity is better than spike protein. So N protein may be the perfect antigen.

In the present study, active rSCoVN was highly expressed in P. pastoris. Compared with various media, the BMMY medium was optimal for rSCoVN expression. A 2-fold difference in biomass and 3.5-fold difference in expression level were noticeable between BMMY and MM media. Difference in biomass and expression was also presented between BMMY and BMM media. The result also revealed that phosphate, yeast extracts and yeast pH had prominent effects on the growth and expression of recombinant P. pastoris. The buffer medium could maintain a stable pH value, and benefit the absorption and use of the nutrients. Moreover, yeast extracts, peptone are rich in peptides, amino acids, vitamins and trace elements. These compounds could enhance the biomass and energy for foreign protein synthesis [25].

The effect of DO on the growth and expression of rSCoVN revealed that DO was an important factor for yeast growth and expression of recombinant protein. While microorganisms growing on carbohydrates use molecular oxygen mainly for respiration. Oxygen availability could influence the production of proteins, both in prokaryotic cells [26] and in yeast [27]. Yeasts growing on methanol might also require a substantial amount of oxygen for the initial oxidation of methanol to formate [28]. All methanol taken up by the cells is oxidized to formaldehyde in a coupled reaction involving alcohol oxidase (AOX) and catalase (CAT) in peroxisomes. These reactions use molecular oxygen as an ultimate electron acceptor. Foreign protein synthesis needs abundant energy, so it is important to maintain a relatively high DO for expressing foreign protein in Pichia pastoris.

For the expression of protein using AOX1 promoter, it is important to keep the methanol level within a relatively narrow range. In bioreactors it could be achieved by different methods [29]. In shake flasks, a technique for online monitoring of methanol concentration was introduced [30]. The ordinary methanol feeding protocol without any complicated devices, still has not been reported. So we established an empirically feeding protocol for our recombinant strains, which worked without any measuring devices. A methanol feeding of 20 mL/L (final concentration) was optimal for yeast growth and expression of SCoVN protein. Slightly reduced growth and expression while methanol feeding below or above 20 mL/L was probably due to the limited carbon or the toxic effect of accumulated methanol [30].

Western blot demonstrated that rSCoVN protein had a high-specificity and good-antigenicity against SCoV-Ab and SARS positive serum, and no reaction with normal human serum. The biological activity of rSCoVN expressed in P. pastoris was about 4-fold higher than that expressed in E. coli when the same rSCoVN protein quantity was used. The preliminary results indicate that the conformation of rSCoVN protein expressed in P. pastoris is almost the same as the natural SCoV protein, and the rSCoVN protein produced may be suitable for the detection of anti-SCoV in diagnostic assay.

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