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World J Gastroenterol 2005;11(39):6159-6164
World Journal of Gastroenterology ISSN 1007-9327
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• *BASIC RESEARCH* •

Receptor-binding domain of SARS-Cov spike protein: Soluble expression in *E.coli*, purification and functional characterization

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Received: 2005-03-22 Accepted: 2005-04-11

Abstract

AIM: To find a soluble and functional recombinant receptor-binding domain of severe acute respiratory syndrome-associated coronavirus (SARS-Cov), and to analyze its receptor binding ability.

METHODS: Three fusion tags (glutathione *S*-transferase, GST; thioredoxin, Trx; maltose-binding protein, MBP), which preferably contributes to increasing solubility and to facilitating the proper folding of heteroprotein, were used to acquire the soluble and functional expression of RBD protein in *Escherichia coli* (BL21(DE3) and Rosetta-gamiB (DE3) strains). The receptor binding ability of the purified soluble RBD protein was then detected by ELISA and flow cytometry assay.

RESULTS: RBD of SARS-Cov spike protein was expressed as inclusion body when fused as TrxA tag form in both BL21 (DE3) and Rosetta-gamiB (DE3) under many different cultures and induction conditions. And there was no visible expression band on SDS-PAGE when RBD was expressed as MBP tagged form. Only GST tagged RBD was soluble expressed in BL21(DE3), and the protein was purified by ÄKTA Prime Chromatography system. The ELISA data showed that GST•RBD antigen had positive reaction with anti-RBD mouse monoclonal antibody 1A5. Further flow cytometry assay demonstrated the high efficiency of RBD's binding ability to ACE2 (angiotensin-converting enzyme 2) positive Vero E6 cell. And ACE2 was proved as a cellular receptor that meditated an initial-affinity interaction with SARS-Cov spike protein. The geometrical mean of GST and GST•RBD binding to Vero E6 cells were 77.08 and 352.73 respectively.

CONCLUSION: In this paper, we get sufficient soluble N terminal GST tagged RBD protein expressed in *E.coli* BL21 (DE3); data from ELISA and flow cytometry assay demonstrate that the recombinant protein is functional and binding to ACE2 positive Vero E6 cell efficiently. And the recombinant RBD derived from *E.coli* can be used to developing subunit vaccine to block S protein binding with receptor and to neutralizing SARS-Cov infection.

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Key words: Receptor-binding domain; SARS-Cov; Spike protein expression; *E.coli*

Chen J, Miao L, Li JM, Li YY, Zhu QY, Zhou CL, Fang HQ, Chen HP. Receptor-binding domain of SARS-Cov Spike protein: Soluble expression in *E.coli*, purification and functional characterization. *World J Gastroenterol* 2005; 11(39): 6159-6164
http://www.wjgnet.com/1007-9327/11/6159.asp

INTRODUCTION

Severe acute respiratory syndrome (SARS), a newly emerging infectious disease, is caused by a SARS-associated coronavirus (SARS-Cov)[1,2]. SARS-Cov can infect African green monkey kidney cells (Vero E6) and cause a similar disease in *Cynomolgus macaques* (*Macaca fascicularis*)[3,4]. The SARS viral genome comprises approximately 30 000 nucleotides, which are organized into approximately 13-15 open reading frames (ORFs), taking into consideration only those exceeding 50 amino acids in translational capacity[5,6]. Sequence comparison with corresponding ORFs of other known coronaviruses reveals a similar pattern of gene organization typical of coronaviruses[7]. SARS-Cov contains a single-strand plus-sense RNA genome about 30 kb in length and has a 5'-cap structure and a 3'-polyadenylation tract. And the genomic organization is typical of coronaviruses, having five major ORFs that encode the replicase polyproteins: the spike (S), envelope (E), and membrane (M) glycoproteins. The full-length genome sequence of SARS-Cov has been elucidated within few weeks after the identification of this novel pathogen[8-10].

When coronavirus enters the cells, the 50-region of viral genome is translated into a large poly-peptide that is cleaved by viral-encoded proteases to release RNA-dependent RNA polymerase and adenosine triphosphatase/helicase. These proteins, in turn, are responsible for replicating the viral genome as well as generating nested transcripts that are used in the synthesis of viral proteins. Viral membrane proteins, including S and M, are inserted into the endoplasmic reticulum (ER), while RNA genome assembles with the N

protein. This RNA-protein complex then associates with M proteins and buds into the lumen of the ER. The virus particles then migrate through the Golgi complex and exist in the cells by exocytosis[6,11]. The first step *in viral* infection is the binding of viral proteins to certain cellular receptors. So far, the S protein of coronavirus is considered as the site of viral attachment to the host cells. It has been shown that a cellular metallopeptidase, angiotensin-converting enzyme 2 (ACE2), could bind to S1 domain of SARS-Cov' S protein and support viral replication[12]. And a 193-amino-acid fragment of the S protein (residues 318-510) is a receptor binding domain (RBD) of SARS-Cov S protein[13]. Here we reported the soluble expression, purification, and functional characterization of RBD expressed in *Escherichia coli*.

MATERIALS AND METHODS

Materials

E.coli DH5α competent cells, *E.coli* BL21(DE3) competent cells, *E.coli* Rosetta-gamiB (DE3) competent cells, pET22b, TrxATag (Thioredoxin) expression vector pET32a were products of Novagen Inc. The genotype of Rosetta-gamiB (DE3): △*ara-leu 7697* △*lacX74* △*phoAPvuII phoR araD139 ahpC gale galK rspL* F'[*lac*+(*lacIq*)*pro*] *gor522*::Tn*10* (TcR) *trxB*:: kan (DE3) pRARE (CmR). GSTag (glutathione *S*-transferase) expression vector pGEX-4t-2 was purchased from Invitrogen Inc. Chromatography media Q Sepharose Fast Flow, Q Sepharose High Performance, Butyl Sepharose Fast Flow, Superdex 75, goat anti-GST polyclone antibody were purchased from Amersham Inc. FITC-labeled rabbit anti-goat IgG, goat anti-mouse IgG conjugated with HRP were products of Beijing Xin Jing Ke Biotechnology Co., Ltd. The primers with restriction enzyme sites *Bam*HI and *Xho*I to amplify RBD gene were synthesized by Beijing Bioasia Inc. pET-S plasmid containing the full length gene of S protein of SRAS-Cov (isolate BJ01, GenBank accession number AY278488) was kindly provided by Beijing Hua Da Gene Research Center. Anti-RBD of S protein mouse monoclonal antibody 1A5 was provided by the Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences. pET-MBP was constructed by inserting maltose-binding protein (MBP) gene derived from *E.coli* JM109 strain into pET22b between *nde*I and *Bam*HI restriction enzyme sites, conserved by the Institute of Biotechnology, Academy of Military Medical Sciences.

Plasmids construction and engineering bacteria

A pair of primers was used in PCR reaction to amplify RBD gene (N terminal 318-510 amino acid fragments) of S protein from pET-S plasmid (EC1: 5'-CGGGATCCGACG-ATGACGATAAGAATATTACAAACTTGTGTCCT-3', EC2: 5'-CCG CTCGAGTTAAACCGTGGCCGGT-GGATTTAA-3'). The amplified RBD gene fragment was purified on a 10 g/L low-melting agarose gel, utilizing the PCR purification system (Q Biogene Inc.) The purified PCR products were directly ligated into expression vector pET32a, pETGEX-4t-2, and pET-MBP respectively. The progress of vector construction follows the example of pET32-RBD according to the clone strategy from TaKaRa Biotechnology Co., Ltd. (Figure 1). After transformation of the *E.coli* strain DH5α competent cells, the clone was selected on ampicillin containing (100 mg/L) plates for mini-preparation and insert evaluation was by enzyme digestion and DNA sequencing. Bacterial transformation for expressing RBD was carried out by incubating bacterial strain BL21(DE3) (Novagen) with pET32-RBD, pGEX-RBD, and pET-MBP-RBD plasmid DNAs on ice for 30 min and heat-shock treatment for 90 s as per the manufacturer's instruction, so did transforming pET32-RBD into bacterial strain Rosetta-gamiB(DE3) (Novagen) but ampicillin (100 mg/L), chloramphenicol (34 mg/L), kanamycin (12 mg/L), tetracycline (12.5 mg/L) selection. Hence expression of four engineering bacteria was performed (Table 1).

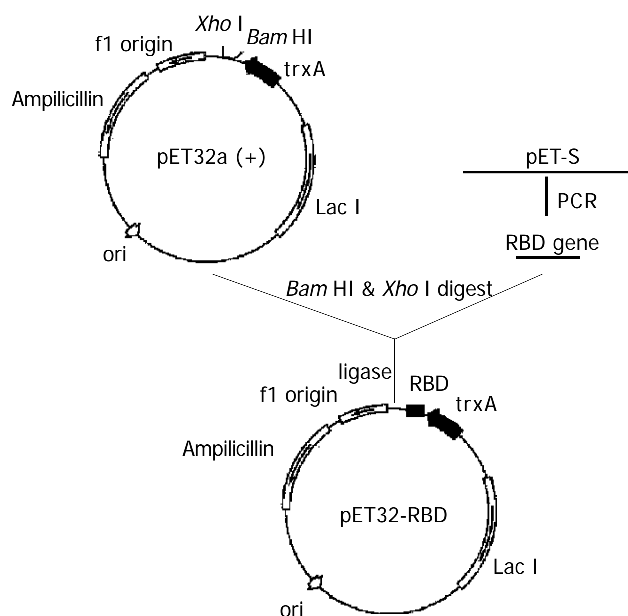

Figure 1 Schematic illustration of the constructs of expression vector pET32-RBD.

Optimization of protein expression and solubility analysis in *E.coli*

Each single colony on the LB plate was inoculated into

Table 1 Construction of recombinant plasmid and expression of engineering bacterium

Engineering bacterium	Recombinant plasmid	Expression host (*E.coli* strain)	Expression vector used	Fusion tag
E.coli BL-TB	pET32-RBD	BL21(DE3)	pET32a	TrxA·tag S·tag
E.coli R-TB	pET32-RBD	Rosetta-gamiB(DE3)	pET32a	TrxA·tag S·tag
E.coli BL-GB	pGEX-RBD	BL21(DE3)	pGEX-4t-2	GST·tag
E.coli BL-MB	pET-MBP-RBD	BL21(DE3)	pET-MBP	MBP·tag

5 mL of LB broth containing 100 μg/mL ampicillin (chloramphenicol (34 mg/L), kanamycin (12 mg/L), tetracycline (12.5 mg/L) additional for *E.coli* R-TB) and incubated overnight at 37 ℃ in a bacterial shaker. One hundred microliters of fresh engineering bacterium preparation was added to 20 mL of antibiotic containing LB broth. The bacteria were induced with 0.25-5 mmol/L IPTG when the optical density of the bacteria reached between 0.5 and 0.6 $A_{600\ nm}$, then shaked for an additional 1-8 h in a bacterial shaker at 20 or 37 ℃. One milliliter of the bacteria was taken out at 2, 4, 6, and 8 h, centrifuged, and lysed. Four microliters of cell lysate, after centrifuging the soluble and insoluble fragments, was electrophoresed on a 150 g/L SDS-PAGE for evaluation of protein expression. The expression of engineering bacteria derived from BL21(DE3) also performed by FML medium which is enriched in LB broth (7 g/L K_2HPO_4, 3 g/L NaH_2PO_4, 0.5 g/L $MgSO_4$, 2 g/L glucose, 2 mmol/L lactose) and target protein was induced by lactose. The protein expression has been evaluated after being cultured 9 h later as the same way.

Purification of the recombinant RBD protein

The *E.coli* BL-GB pellet from the 1 L culture was washed with 20 mmol/L Tris-HCl, pH 7.4, 5 mmol/L sodium EDTA, and 200 g/L sucrose. The pellet was resuspended with sonication buffer (20 mmol/L Tris-HCl, pH 8.0, 5 mmol/L EDTA) at the ratio of 1:40. The suspension was sonicated for lysing cells cooled in ice between sonication pulses. The lysate was centrifuged at 12 000 *g* for 30 min at 4 ℃ to separate out the debris. Then supernatant containing the recombinant RBD protein was purified by ÄKTA Prime Chromatography system using Q Sepharose FastFlow, Q Sepharose High Performance, Butyl Sepharose FastFlow, Superdex 75 gel exclusion Columns (Amersham-Pharmacia) respectively. The purified protein which conserved in 1×PBS was electrophoresed on a 150 g/L SDS-PAGE gel and stained with Coomassie blue. The gel was destained with 70 mL/L acetic acid and 50 mL/L methanol. The protein appeared to be induced quite well and purified to single band entities, demonstrating high purity of the protein products. The protein migrated at approximately 48 ku as expected.

ELISA

Purified GST·RBD protein was blocked to Maxisorp ELISA plate in 1×PBS at 1 μg/well. Anti-RBD of SARS-Cov spike protein mouse monoclonal antibody 1A5, then goat anti-mouse IgG conjugated with HRP was incubated with different ratios of dilution in each well at 37 ℃ for 2 h. Wells were washed with buffer then $A_{450\ nm}$ was measured.

Flow cytometry assay

A Becton Dickinson FACSCalibur cytofluorometer, which was in the Institute of Biotechnology, Academy of Military Medical Sciences, was used for flow cytometry. SARS-Cov susceptible Vero E6 cell was cultured in 12-well plate (5×10^6 cells/well). Cells were incubated at 37 ℃ for 3 h with 0.4 mg/mL purified GST protein and GST·RBD protein respectively, then washed thrice with fluorescence-activated cell sorting (FACS) buffer (PBS containing 20 g/L bovine serum)[14-16]. After being trypsinized, the cells were washed thrice with FACS buffer and then centrifuged at 2 000 r/min for 4 min in a 1.5 mL eppendorf tube for collecting cells. The prime antibody goat anti-GST polyclone antibody diluted in PBS containing 50 g/L bovine serum albumin at the ratio 1:2 000 were adsorbed to 5×10^6 cells in suspension for 1 h at room temperature. Then the secondary antibody was also adsorbed. Control, which was negative, was Vero E6 cells alone.

RESULTS

Soluble expression of recombinant RBD protein

TrxA tagged RBD protein expressed in two engineering bacteria (*E.coli* BL-TB and R-TB) were inclusion bodies. Under various cultures and induction conditions, *E.coli* BL-TB expressed RBD protein as inclusion body, including reduced IPTG induction concentration, shortened induction time and lowered induction temperature (data not shown). *E.coli* R-TB yielded high target protein expression, with level approaching approximately 40% of the total cellular protein but inclusion body. Especially, bacterial strain Rosetta-gamiB carries the thioredoxin reductase (*TrxB*) and glutathione reductase (*gor*) mutations which have the potential to enhance disulfide bond formation and ultimately solubility and activity, meanwhile it carries pRare plasmid which directs the rare tRNA genes synthesis that can enhance the expression of target protein. But the predominance of Rosetta-gamiB (DE3) for protein soluble expression was not clear. Maybe the overexpression of heteroprotein greatly counteract the helpfulness of host's *TrxB* and *gor* double mutations to the proper folding of the protein . Or the nature RBD folding form does not depend upon the disulfide bond formed by two cysteines (Figure 2A).

There was no visible expression in *E.coli* BL-MB in which RBD was MBP fused according to SDS-PAGE analysis. Some secondary structure might emerge at the upstream of ribosome binding domain of cloned gene in the pET-MBP-RBD. Or there was very little protein expressed and needed detection by Western blot. Among all four engineering bacteria, only *E.coli* BL-GB expressed soluble RBD protein GST tagged, and its expressional capacity was greater in FML medium than in LB broth. The protein migrated on SDS-GAGE at approximately 48 ku as expected (Figure 2B). So engineering bacterium BL-GB was selected to generate recombinant GST·RBD for further research (Figure 2C).

ELISA

Data from ELISA showed that the purified GST·RBD antigen had positive reaction with anti RBD mouse monoclonal antibody 1A5. Test result primarily indicated that the recombinant protein was by nature a three-dimensional structure (Figure 3).

Flow cytometry assay

To better detect the receptor binding ability of GST·RBD and then prove it was of dimensional structure and functional, flow cytometric analysis was performed (Figure 4). Because Vero E6 cell is SARS-Cov susceptible, the functional recombinant RBD should bind to ACE2 existing at the cell membrane of Vero E6 cell, after prime antibody and fluorescence-

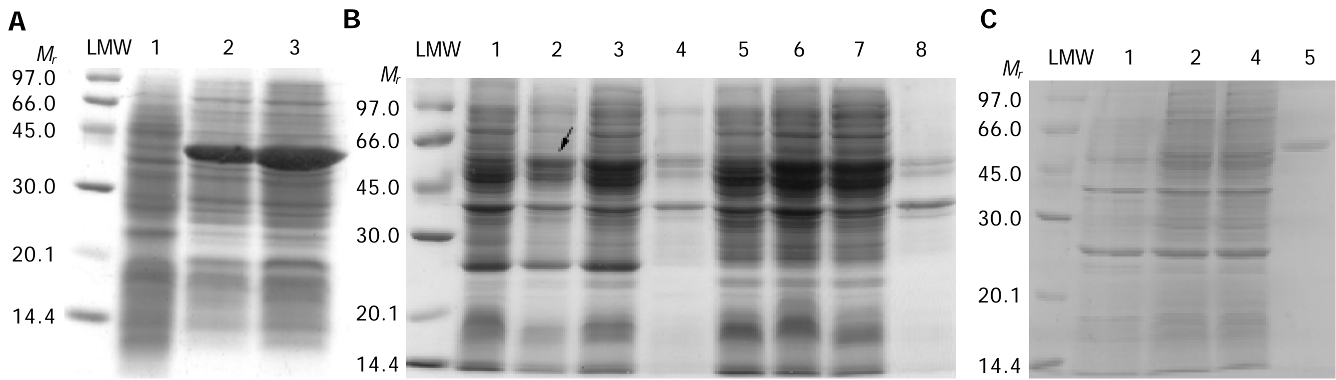

Figure 2 **A:** RBD expressed in *E.coli* R-TB as inclusion body form. LMW: protein low molecule weight marker (Amersham-Pharmacia); lane 1: *E.coli* BL21(DE3) negative control; lane 2: *E.coli* R-TB 5 mmol/L IPTG induced 4 h later; lane 3: *E.coli* R-TB 5 mmol/L IPTG induced 6 h later; **B:** *E.coli* BL-GB and BL-MB expression and solubility analysis. LMW: protein low molecule weight marker (Amersham-Pharmacia); lane 1: *E.coli* BL21(DE3) negative control; lane 2: *E.coli* BL-GB in FML medium; lane 3: soluble fraction; lane 4: insoluble fraction; lane 5: *E.coli* BL21(DE3) negative control; lane 6: *E.coli* BL-MB induced at 5 mmol/L IPTG 6 h later; lane 7: soluble fraction; lane 8: insoluble fraction; **C:** The purified recombinant RBD protein expressed in *E.coli* BL-GB. LMW: protein low molecule weight marker (Amersham-Pharmacia); lane 1: *E.coli* BL21(DE3) negative control; lane 2: IPTG induced *E.coli* BL-GB; lane 2: soluble fraction; lane 4: purified RBD protein.

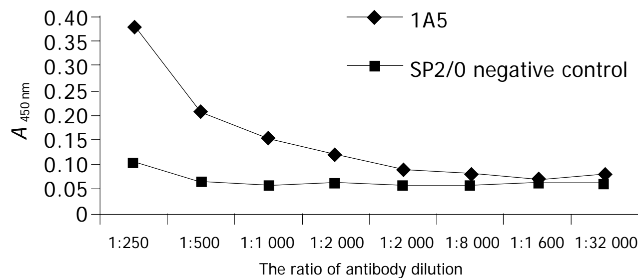

Figure 3 ELISA of GST·RBD antigen.

labeled secondary antibody was absorbed, it can be subjected to flow cytometry assay. Another negative control was set (Vero E6 cells with purified GST protein) to exclude the interference of GST tag and antibodies binding to cells. It has been seen that GST·RBD bound Vero E6 cells effectively. It also proved that the binding progress was decided by three-dimensional structure attachment because no side chain glycosylatio was formed when heteroprotein expressed in *E.coli*.

DISCUSSION

During the pandemic outbreak of SARS in 2002/2003, despite the lack of effective and specific therapy, most SARS patients survived after the acute illness, and neutralizing antibodies were detectable in the sera of convalescent SARS patients[17,18]. Many researchers devoted themselves into developing anti SARS-Cov drugs. Currently, one candidate vaccine using inactivated SARS-Cov is in a phase I clinical trial in China. Although the inactivated SARS-Cov may be effective in protecting animals from challenge by the virus, its efficacy in human is unclear. There has been a serious safety concern since some antigens in the virions may elicit antibodies that did not neutralize, but rather infected. Some viral proteins may induce harmful immune and inflammatory response, a potential cause of SARS pathogenesis[19]. Now a number of vaccine candidates are of preclinical study, including inactivated vaccines, DNA vaccines, and attenuated viruses encoding SARS-Cov S protein[20-23]. Especially, vaccine blocking SARS-Cov entry into susceptible cell is a good way to fight against the virus[24].

The S proteins of coronaviruses are large type I membrane glycoprotein projections from viral envelope[25]. S proteins are responsible for both binding to receptors on host cells and for membrane fusion[11,12]. S proteins also contain important virus-neutralizing epitopes that elicit neutralizing

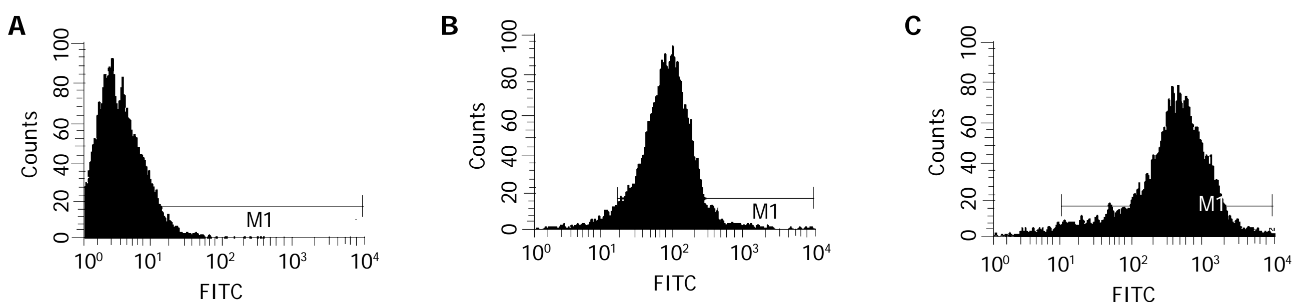

Figure 4 Cell binding test preformed by flow cytometry assay. **A:** Vero E6 cells alone, geometrical mean 3.3; **B:** Vero E6 cells+GST protein+two antibodies, geometrical mean 77.08; **C:** Vero E6 cells+GST·RBD+two antibodies, geometrical mean 352.73.

antibody in the host species. Furthermore, mutations in this gene dramatically affect the virulence, pathogenesis, and host cell tropism. The S1 domain of all characterized coronaviruses, including that of SARS-Cov, meditated an initial-affinity interaction with a cellular receptor[26-28]. In respect of that mutations in S protein' gene dramatically affect the virulence, pathogenesis, and host cell tropism, at the same time S proteins also contain important virus-neutralizing epitopes that elicit neutralizing antibody in the host species, so much work about antigenicity and receptor-binding ability of full length or fragments were done to discover the potent neutralization of SARS-Cov infection[29-32]. It has been proved that a cellular metallopeptidase, ACE2, could bind to S1 domain of SARS-Cov S protein and support viral replication[20]. Recent reports showed that a 193-amino-acid fragment of the S protein (residues 318-510) bound ACE2 more efficiently than did the full S1 domain and should be RBD of S protein of SARS-Cov, and rabbit antisera directed against RBD (cell expressed) were effective in binding to RBD on the S1 domain of SARS-Cov S protein and blocking RBD binding to soluble and cell-expressed ACE2. These data confirmed that the RBD of S protein has the potential to be developed as a subunit vaccine since it induces highly potent antibodies to block S protein binding to ACE2 and to neutralize SARS-Cov infection and has lower level of risk comparing with inactivated viruses. In these researches, the RBD protein was all expressed in mammalian cells and still in research pipeline.

These results suggested that RBD of S protein is a good candidate for vaccine because neutralizing antibodies are directed against S protein. Moreover, RBD of S protein is also a good target for antiviral therapies because blockade of binding of S protein to cellular receptor can prevent virus entry.

To research the receptor-binding ability of RBD, we chose *E.coli* as an expression host to get recombinant protein. According to the database from Internet there is 88.2% possibility of the recombinant RBD expressed in *E.coli* as inclusion body (www.biotech.ou.edu), at the same time, the gene of RBD contains lots of codon rarely used in *E.coli*, the expressional capacity should be little. In order to acquire soluble and functional recombinant protein, different fusion vectors, *E.coli* strain, culture, and induction conditions were considered. In this paper we adopted three fusion tags (GST, TrxA, MBP) which preferably contributes to increasing solubility and to the proper folding of heteroprotein when expressed in *E.coli*. At the same time we chose two *E.coli* strains: BL21 (DE3) and Rosetta-gamiB(DE3). Unexpectedly, TrxA tagged RBD protein expressed in Rosetta-gamiB(DE3) was inclusion body still, nature RBD folding form might not depend upon the disulfide bond formed by two cysteines. At last we got soluble expressed recombinant RBD protein as GST fusion form.

The recombinant GST·RBD protein was purified, and then proved positive reaction with anti RBD of S protein monoclonal antibody by ELISA and to have effective binding ability to ACE2 positive Vero E6 cell by flow cytometry assay. After removing the fusion tag, the recombinant protein can be directly used to develop subunit vaccine for blocking S protein binding with receptor and to neutralize SARS-Cov infection.

REFERENCES

1 **Lee N**, Hui D, Wu A, Chan P, Cameron P, Joynt GM, Ahuja A, Yung MY, Leung CB, To KF, Lui SF, Szeto CC, Chung S, Sung JJY. A major outbreak of severe acute respiratory syndrome in Hong Kong. *N Engl J Med* 2003; **348**: 1986-1994

2 **Peiris JS**, Lai ST, Poon LL, Guan Y, Yam LY, Lim W, Nicholls J, Yee WK, Yan WW, Cheung MT, Cheng VC, Chan KH, Tsang DN, Yung RW, Ng TK, Yuen KY. SARS study group. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 2003, **361**: 1319-1325

3 **Fouchier RA**, Kuiken T, Schutten M, van Amerongen G, van Doornum GJ, van de Hoogen BG, Peiris M, Lim W, Stöhr K, Osterhaus AD. Aetiology: Koch's postulates fulfilled for SARS virus. *Nature* 2003; **423**: 240

4 **Ksiazek TG**, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE, Dowell SF, Ling AE, Humphrey CD, Shieh WJ, Guarner J, Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes JM, LeDuc JW, Bellini WJ, Anderson LJ. SARS Working Group. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med* 2003; **348**: 1953-1966

5 **Rota PA**, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle JP, Penaranda S, Bankamp B, Maher K, Chen MH, Tong S, Tamin A, Lowe L, Frace M, DeRisi JL, Chen Q, Wang D, Erdman DD, Peret TC, Burns C, Ksiazek TG, Rollin PE, Sanchez A, Liffick S, Holloway B, Limor J, McCaustland K, Olsen-Rasmussen M, Fouchier R, Gunther S, Osterhaus AD, Drosten C, Pallansch MA, Anderson LJ, Bellini WJ. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 2003; **300**: 1394-1399

6 **Marra MA**, Jones SJ, Astell CR, Holt RA, Brooks-Wilson A, Butterfield YS, Khattra J, Asano JK, Barber SA, Chan SY, Cloutier A, Coughlin SM, Freeman D, Girn N, Griffith OL, Leach SR, Mayo M, McDonald H, Montgomery SB, Pandoh PK, Petrescu AS, Robertson AG, Schein JE, Siddiqui A, Smailus DE, Stott JM, Yang GS, Plummer F, Andonov A, Artsob H, Bastien N, Bernard K, Booth TF, Bowness D, Czub M, Drebot M, Fernando L, Flick R, Garbutt M, Gray M, Grolla A, Jones S, Feldmann H, Meyers A, Kabani A, Li Y, Normand S, Stroher U, Tipples GA, Tyler S, Vogrig R, Ward D, Watson B, Brunham RC, Krajden M, Petric M, Skowronski DM, Upton C, Roper RL. The Genome sequence of the SARS-associated coronavirus. *Science* 2003; **300**: 1399-1404

7 **Drosten C**, Günther S, Preiser W, Van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnikova L, Fouchier RAM, Berger A, Burguière AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Müller S, Rickerts V, Stürmer M, Vieth S, Klenk HD, Osterhaus ADME, Schmitz H, Doerr HW. Identification of a Novel Coronavirus in patients with Severe Acute Respiratory Syndrome. *N Engl J Med* 2003; **348**: 1967-1976

8 **Holmes KV**. SARS-Associated Coronavirus. *N Engl J Med* 2003; **348**: 1948-1951

9 **Tsui SK**, Chim SS, Lo YM. Chinese university of Hong Kong molecular SARS Research Group. Coronavirus genomic-sequence variations and the epidemiology of the severe acute respiratory syndrome. *N Engl J Med* 2003; **349**: 187-188

10 **Ying W**, Hao Y, Zhang Y, Peng W, Qin E, Cai Y, Wei K, Wang J, Chang G, Sun W, Dai S, Li X, Zhu Y, Li J, Wu S, Guo L, Dai J, Wang J, Wan P, Chen T, Du C, Li D, Wan J, Kuai X, Li W, Shi R, Wei H, Cao C, Yu M, Liu H, Dong F, Wang D, Zhang X, Qian X, Zhu Q, He F. Proteomic analysis on structural proteins of Severe Acute Respiratory Syndrome coronavirus. *Proteomics* 2004; **4**: 492-504

11 **Simmons G**, Reeves JD, Rennekamp AJ, Amberg SM, Piefer AJ, Bates P. Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. *Proc Natl Acad Sci USA* 2004; **101**: 4240-4245

12 **Li W**, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. Angiotensin- converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 2003; **426**: 450-454

13 **Wong SK**, Li W, Moore MJ, Choe H, Farzan M. A 193-amino-acid fragment of the SARS coronavirus S protein efficiently binds angiotensin- converting enzyme 2. *J Biol Chem* 2004; **279**: 3197-3201

14 **Jeffers SA**, Tusell SM, Gillim-Ross L, Hemmila EM, Achenbach JE, Babcock GJ, Thomas WD Jr, Thackray LB, Young MD, Mason RJ, Ambrosino DM, Wentworth DE, DeMartini JC, Holmes KV. CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. *Proc Natl Acad Sci USA* 2004; **101**: 15748-15753

15 **Wang P**, Chen J, Zheng A, Nie Y, Shi X, Wang W, Wang G, Luo M, Liu H, Tan L, Song X, Wang Z, Yin X, Qu X, Wang X, Qing T, Ding M, Deng H. Expression cloning of functional receptor used by SARS coronavirus. *Biochem Biophys Res Commun* 2004; **315**: 439-444

16 **Chang MS**, Lu YT, Ho ST, Wu CC, Wei TY, Chen CJ, Hsu YT, Chu PC, Chen CH, Chu JM, Jan YL, Hung CC, Fan CC, Yanga YC. Antibody detection of SARS-CoV spike and nucleocapsid protein. *Biochem Biophys Res Commun* 2004; **314**: 931-936

17 **Zheng BJ**, Guan Y, Wong KH, Zhou J, Wong KL, Young BW, Lu LW, Lee SS. SARS-related virus predating SARS outbreak, Hong Kong. *Emerg Infec Dis* 2004; **10**: 176-178

18 **Nie QH**, Luo XD, Zhang JZ, Su Q. Current status of severe acute respiratory syndrome in China. *World J Gastroenterol* 2003; **9**: 1635-1645

19 **He Y**, Zhou Y, Liu S, Kou Z, Li W, Farzan M, Jiang S. Receptor-binding domain of SARS-CoV spike protein induces highly potent neutralizing antibodies: implication for developing subunit vaccine. *Biochem Biophys Res Commun* 2004; **324**: 773-781

20 **Zeng F**, Chow KY, Hon CC, Law KM, Yip CW, Chan KH, Peiris JS, Leung FC. Characterization of humoral responses in mice immunized with plasmid DNAs encoding SARS-CoV spike gene fragments. *Biochem Biophys Res Commun* 2004; **315**: 1134-1139

21 **Gao W**, Tamin A, Soloff A, D'Aiuto L, Nwanegbo E, Robbins PD, Bellini WJ, Barratt-Boyes S, Gambotto A. Effects of a SARS-associated coronavirus vaccine in monkeys. *Lancet* 2003; **362**: 1895-1896

22 **Sui J**, Li W, Murakami A, Tamin A, Matthews LJ, Wong SK, Moore MJ, Tallarico AS, Olurinde M, Choe H, Anderson LJ, Bellini WJ, Farzan M, Marasco WA. Potent neutralization of severe acute respiratory syndrome (SARS) coronavirus by a human mAb to S1 protein that blocks receptor association. *Proc Natl Acad Sci USA* 2004**; 101**: 2536-2541

23 **Bisht H**, Roberts A, Vogel L, Bukreyev A, Collins PL, Murphy BR, Subbarao K, Moss B. Severe acute respiratory syndrome coronavirus spike protein expressed by attenuated vaccinia virus protectively immunizes mice. *Proc Natl Acad Sci USA* 2004; **101**: 6641-6646

24 **Yang ZY**, Kong WP, Huang Y, Roberts A, Murphy BR, Subbarao K, Nabel GJ. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. *Nature* 2004; **428**: 561-564

25 **Ho Y**, Lin PH, Liu CY, Lee SP, Chao YC. Assembly of human severe acute respiratory syndrome coronavirus-like particles. *Biochem Biophys Res Commu* 2004; **318**: 833-838

26 **Liu S**, Xiao G, Chen Y, He Y, Niu J, Escalante CR, Xiong H, Farmar J, Debnath AK, Tien P, Jiang S. Interaction between heptad repeat 1 and 2 regions in spike protein of SARS-associated coronavirus: implications for virus fusogenic mechanism and identification of fusion inhibitors. *Lancet* 2004; **363**: 938-947

27 **Bosch BJ**, Martina BE, Van der Zee R, Lepault J, Haijema BJ, Versluis C, Heck AJ, Groot R, Osterhaus AD, Rottier PJ. Severe acute respiratory syndrome coronavirus (SARS-CoV) infection inhibition using spike protein heptad repeat-derived peptides. *Proc Natl Acad Sci USA* 2004; **101**: 8455-8460

28 **Spiga O**, Bernini A, Ciutti A, Chiellini S, Menciassi N, Finetti F, Causarono V, Anselmi F, Prischi F, Niccolaia N. Molecular modelling of S1 and S2 subunits of SARS coronavirus spike glycoprotein. *Biochem Biophys Res Commun* 2003; **310**: 78-83

29 **Ho TY**, Wu SL, Cheng SE, Wei YC, Huang SP, Hsiangc CY. Antigenicity and receptor-binding ability of recombinant SARS coronavirus spike protein. *Biochem Biophy Res Commun* 2004; **313**: 938-947

30 **Xiao X**, Chakraborti S, Dimitrov AS, Gramatikoff K, Dimitrov DS. The SARS-CoV S glycoprotein: expression and functional characterization. *Biochem Biophys Res Commun* 2003; **312**: 1159-1164

31 **Huang LR**, Chiu CM, Yeh SH, Huang WH, Hsueh PR, Yang WZ, Yang JY, Su IJ, Chang SC, Chen PJ. Evaluation of antibody responses against SARS coronaviral nucleocapsid or spike proteins by immunoblotting or ELISA. *J Med Viro* 2004; **73**: 338-346

Science Editor Guo SY **Language Editor** Elsevier HK