Different Strategies for Preparation of Non-tagged rV270 Protein and Its Efficacy against *Yersinia Pestis* Challenge[1]

WANG WANG[*], ZHI-ZHEN QI[#], QING-WEN ZHANG[#], BEN-CHUAN WU[+], ZI-WEN ZHU[*], YONG-HAI YANG[#], BAI-ZHONG CUI[#], RUI-XIA DAI[#], YE-FENG QIU[*], ZU-YUN WANG[#], ZHAO-BIAO GUO[*], TAO-XING SHI[*], HU WANG[#], RUI-FU YANG[*], AND XIAO-YI WANG[*,2]

[*]*Laboratory of Analytical Microbiology, State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of icrobiology and Epidemiology, Beijing 100071, China;* [#]*Qinghai Institute for Endemic Disease Prevention and Control of Qinghai Province, Xining, 811602, China;* [+]*Beijing Institute of Bioengneering, Beijing 100071, China*

Objective LcrV is an important component for the development of a subunit vaccine against plague. To reduce immunosuppressive activity of LcrV, a recombinant LcrV variant lacking amino acids 271 to 326 (rV270) was prepared by different methods in this study. **Methods** A new strategy that produced non-tagged or authentic rV270 protein was designed by insertion of rV270-thrombin-hexahistidine fusion gene into the vector pET24a, or by insertion of hexahistidine-enterokinase-rV270 or hexahistitine-factor Xa-rV270 fusion gene into the vector pET32a. After Co^{2+} affinity chromatography, a purification strategy was developed by cleavage of His tag on column, following Sephacryl S-200HR column filtration chromatography. **Results** Removal of His tag by thrombin, enterokinase and factor Xa displayed a yield of 99.5%, 32.4% and 15.3%, respectively. Following Sephacryl S-200HR column filtration chromatography, above 97% purity of rV270 protein was obtained. Purified rV270 that was adsorbed to 25% (v/v) $Al(OH)_3$ adjuvant in phosphate-buffered saline (PBS) induced very high titers of antibody to rV270 in BALB/c mice and protected them (100% survival) against subcutaneous challenge with 10^6 CFU of *Y. pestis* virulent strain 141. **Conclusion** The completely authentic rV270 protein can be prepared by using enterokinase or factor Xa, but they exhibited extremely low cleavage activity to the corresponding recognition site. Thrombin cleavage is an efficient strategy to prepare non-tagged rV270 protein and can be easily operated in a large scale due to its relatively low cost and high cleavage efficacy. The recombinant rV270 can be used as a key component to develop a subunit vaccine of plague.

Key words: *Yersinia pestis*; rV270 antigen; Purification; Protection; Plague

INTRODUCTION

Plague is a zoonotic disease caused by the Gram-negative bacterium *Y. pestis*, which is usually transmitted to humans from infected rodents via the bite of an infected flea[1]. Historically plague has been an awful infectious disease afflicting human populations, leading to millions of deaths. Recently plague has attracted a considerable attention because of its potential use as an agent of biological warfare or bioterrorism[2].

It is believed that a subunit vaccine based on F1 and LcrV antigens is likely to offer the best near-term solution to the provision of a vaccine that protects against both bubonic and pneumonic plague[3]. LcrV antigen is an important component for the development of a subunit vaccine against plague. However, LcrV antigen displays immunosuppressive properties besides its role in protective immunity. Recently, an LcrV antigen variant lacking amino acid residues 271 to 300 (rV10) elicited immune responses that protected mice against a lethal challenge with *Y. pestis*, and displayed a reduced immunosuppressive activities comparing with full-length LcrV-antigen[4-5].

In this study, another LcrV variant (rV270) deleting amino acids 271 to 326 of LcrV was prepared in *E. coli* BL21 cells. To prepare authentic rV270 protein and simplify purification of recombinant proteins, a hexahistidine tag that has a strong affinity for matrices containing metal-ions like Ni^{2+} or Co^{2+} [6] and a protease cleavage site specific to thrombin, factor Xa or enterokinase were inserted into the vectors pET24a and pET32arespectively.

[1]This research was supported by the National Key Program for Infectious Diseases of China (2009ZX10004-4001).

[2]Correspondence should be addressed to Xiao-Yi WANG. Tel: 86-10-63815689. Fax: 86-10-63815689. E-mail: wgenome@yahoo.com.cn; or to Rui-Fu YANG. Tel: 86-10-63815689. Fax: 86-10-63815689. E-mail: ruifuyang@gmail.com

Biographical note of the first author: Wang WANG, male, born in 1984, master, majoring in vaccine research.

0895-3988/2010
CN 11-2816/Q

After Co^{2+} affinity chromatography and removal of hexahistidine tag on column by thrombin, enterokinase or factor Xa, following Sephacryl S-200HR column filtration chromatography, purified rV270 protein was obtained, and its immunogenicity and protective efficacy against *Y. pestis* challenge were evaluated in mouse model.

MATERIALS AND METHODS

Reagents

The expression vectors pET-24a (+) and pET-32a (+) and their host strain *E. coli* BL21 (DE3) were purchased from Novagen, and thrombin, factor Xa and enterokinase from Novagen. Co^{2+} affinity chromatography column and Sephacryl S-200HR were from Amersham Biosciences. Aluminum hydroxide adjuvant was obtained from the Lanzhou Institute of Biological Products (LIBP), China. All the other reagents were analytical grade.

Construction of Expression Vectors pET24a-rV270-TB, pET32a-Xa-rV270 and pET32a- EK-rV270

For the construction of vectors pET24a-rV270-TB, pET32a-Xa-rV270 and pET32a-EK-rV270, three pairs of primers FP1-RP1, FP2-RP2 and FP3-RP3 were designed for the amplification of rV270 gene. The PCR products were referred to as rV270-TB (rV270-Thrombin), Xa-rV270 (factor Xa-rV270) and EK-rV270 (Enterokinase-rV270) respectively. *Nde*I site (underlined) was added to the 5′ end of the FP1 (5′-GGAATTCCATATGAT TAGAGCCTACGAAC-3′); *Hin*dIII site (underlined) and thrombin cleavage site (shown in bold) were added on the 5′ end of RP1 (5′-CCCAAGCTT **AGAACCGCGTGGCACCAG**GGCAAAGTGAG A TAATTC-3′). *Kpn*I site (underlined) and factor Xa coding sequence (shown in bold) were added to the 5′ end of the FP2 (5′-CGGGGTACC**ATCGAAGG TCGT**ATGATT AGAGCCTACGAAC-3′); *Eco*RI site (underlined) and extra termination codon (shown in bold) were added to the 5′ end of the RP2 (5′-CGGAATTC**TTA**GGCAA AGTGAGATAATTC-3′). *Kpn*I restriction site (underlined) and enterokinase coding sequence (shown in bold) were added to the 5′ end of the FP3 (5′-CGGGGTACC **GACGACGACGACAAG**ATGATTAGAGCCTAC GAAC-3′); *Eco*RI site and end codon (shown in bold) were added to the 5′ end of the RP3 (5′-CGGAATTC**TTA**GG CAAAGTGAGATAATTC-3′).

The PCR products rV270-TB, Xa-rV270 and EK-rV270 were purified by DNA quick purification kits, digested with the corresponding restriction enzymes and then cloned into the multiple cloning site of the corresponding vectors digested with the restriction enzymes. *E. coli* BL21 competent cells were transformed with the recombinant vectors and cultured at 37 ℃ on LB medium with kanamycin (50 μg/mL) or ampicillin (100 μg/mL) for selection of recombinants. The resulting plasmids were referred to as pET24a-rV270-TB, pET32a-Xa-rV270 and pET32a-EK-rV270, which were confirmed by PCR and DNA sequencing.

*Expression and Purification of the R*ecombinant *rV270 Proteins*

The *E. coli* BL21/pET24a-rV270-TB, *E. coli* BL21/pET32a-Xa-rV270 or *E. coli* BL21/pET32a-EK-rV270 cells were grown in the Luria-Bertani (LB) medium supplemented with 50 μg/mL kanamycin or 100μg/mL ampicillin at 37 ℃ with 175 rpm shaking until the OD_{600} reached 0.6~0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mmol/mL, and the incubation was prolonged for an additional 4 h. The cells were harvested by centrifugation at 6 000 *g* for 10 min, washed thoroughly with PBS, and the pellet was resuspended in ice-cold lysis buffer, and then was disrupted by sonication (Bandelin UW 2 000). The homogenate was centrifuged at 12 000 g for 20 min at 4 ℃ to remove the cell debris. 20 μL of supernatant was analyzed by SDS-PAGE and stained by Coomassie brilliant blue R-250. The remaining supernatant was diluted to 200 mL, loaded onto a Co^{2+} affinity chromatography column equilibrated with 0.05 mol/L Tris-HCl buffer containing 0.5 mol/L NaCl (pH 8.0), and eluted with 200 mL of wash buffer (0.05 mol/L Tris-HCl buffer containing 0.5 mol/L NaCl and 0.02 mol/L imidazole, pH8.0) at a rate of 1-2 mL/min, and then with the cleavage buffer. Afterwards, thrombin, factor Xa or enterokinase diluted with the corresponding dilution buffer in the kits was loaded onto the Co^{2+} affinity chromatography column bond with fusion proteins. Cleavage reaction was performed at room temperature for about 16 h. After the column was eluted with 0.05 mol/L Tris-HCl buffer containing 0.5 mol/L NaCl, the target protein was collected. The crude protein was loaded onto a Sephacryl S-200 HR column equilibrated with 0.01 mol/L PBS (pH 7.2), eluted with 0.01 mol/L PBS (pH 7.2). The purified rV270 proteins were collected and stored at -80 ℃.

SDS-PAGE, Purity Determination and Western Blotting

The protein samples and rabbit phosphorylase B

were loaded on 12% polyacrylamide gel, and electrophoresed at 150 V for 1.5 h, staining with Coomassie brilliant blue R-250. The molecular size of the purified rV270 protein was determined by SDS-PAGE and the stained protein was quantified based on the area and the intensity of bands by Gel Imaging Analysis System BINTA 2020D using Gel Pol 32 software. Protein purity was determined by the method of bicinchoninic acid assay.

The samples separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membrane by a Pharmacia Biotech apparatus. Blots were incubated in diluted mouse monoclonal antibody (diluted at 1:500 with 3% BSA to a final concentration of 44 μg/mL) specific for the rV270 antigen for 1 h at room temperature with gentle agitation. Blots were washed three times with TBST, and then incubated with HRP-conjugated goat anti-mouse IgG. After the blots were washed four times in TBST, 3, 3'-diaminobenzidine tetra hydrochloride (Sigma) substrate was in 25 mL of PBS buffer with 10 μL of H_2O_2 (30%) was added for 2-5 min at room temperature. Finally, the reaction was stopped by adding distilled water to strips.

Identification of the Protein by Mass Spectrometry

Purified rV270 protein was digested by trypsin at 37 ℃ for 18h, and the digestion mixture was desalted on ZipTip microcolumn according to manufacturer's instruction (Millipore). The matrix solution was mixed in equal volumes with the sample solution. One microliter of the mixture was spotted on the MALDI plate and was allowed to be air-dried at room temperature. The peptide mass fingerprint was determined by Ultraflex III TOF/TOF Mass Spectrum and used to search NCBI nonredundant protein sequence database using Mascot tool from Matrix Science (http://www.matrixscience.com).

N-terminal Sequence and C-terminal Sequence Analysis

N-terminal sequencing was performed by Edman degradation using a protein sequenator (AB491, Applied Blosystem, USA). C-terminal sequences were determined by using a Q-TOF2 (Waters, UK) equipped with a CapLC NanoFlow / NanoSpray ion source.

Animal Immunizations

Groups of 10 female BALB/c mice were immunized with 10 μg of rV270 antigen adsorbed to 25% (v/v) aluminum hydroxide in PBS buffer. Each animal was injected *i.m*. in the two hind limbs with a total volume of 0.1 mL and the control mice received the same volume of aluminum hydroxide (25%, v/v). After primary immunization, on day 21 the animals were boosted with an identical dose at the same injection sites. Blood samples were collected from the tail veins of the immunized and control animals on the day 42, 56, and 70 after primary immunization.

ELISA for Determining IgG Titer in the Immunized Animal Sera

Sera collected from immunized mice throughout the study were assayed for the presence of rV270-specific IgG by a modified ELISA[7-8]. The titre of specific antibody was estimated as the maximum dilution of the corresponding serum giving an OD reading of 0.2 units over background. Background values were obtained from serum samples collected from the animals only receiving aluminum hydroxide. Antibody endpoint titre per immunization group is presented as the geometric mean endpoint titre to rV270 antigens.

Challenge with Y. pestis

Challenge experiments were carried out with the *Y. pestis* 141 strain, which was isolated from *Marmota himalayana* in Qinghai-Tibet plateau and had a median lethal dose (MLD) of 5.6 colony-forming units (CFU) for BALB/c mice by the subcutaneous route. The animals from the immunized and control groups were challenged 10 weeks after the primary immunization with 10^6 CFU by the subcutaneous route, and then closely observed for 14 days. On the day 14, the survival animals were sacrificed humanely for a post-mortem examination. Hearts, livers, spleens, lungs and lymph nodes were removed to confirm if *Y. pestis* was presented in these organs.

Statistical Analysis

The difference of protective efficacy between treatment groups was analyzed by log-rank test with SARS 8.0 software, and the difference of antibody titres among groups was compared by analysis of variance (ANOVA) with SARS 8.0 software. The probability values of $P<0.05$ were taken as significant.

RESULTS

Construction of the Recombinant Plasmids

The recombinant vector pET24a-rV270-TB, pET32a-Xa-rV270 or pET32a-EK-rV270 was identified by PCR and DNA sequencing, indicating that the rV270 gene and the corresponding cleavage site were correctly inserted into pET-24a (+) or pET-32a (+) vector. The pET-24a (+) expression vector provided 6×His tag at the carboxyl terminus

for recombinant rV270-TB fusion protein, which could be removed by thrombin after the V270-TB fusion protein was purified by using Co^{2+} affinity chromatography. The pET-32a (+) expression vector provided 6×His tag at both N- and C- terminus for recombinant Xa-rV270 or EK-rV270 fusion protein, therefore extra termination codon TTA was respectively added to the 5′ end of the RP2 or RP3 to make sure that non-tagged rV270 protein was correctly prepared from the Xa-V270 or EK-V270 fusion protein by factor Xa or enterokinase.

Expression and Purification of rV270 Protein

Three fusion proteins rV270-TB (31.7 kD), EK-rV270 (46.8 kD) and Xa-rV270 (46.8 kD) were all expressed in a soluble form, and could be purified from the supernatant of the bacteria lysates (Fig. 1 A). The fusion protein containing a 6×His tag was easily isolated by Co^{2+} affinity chromatography in an initiative purification step, and then the tag was removed by proteases. After cleavage reaction was performed by loading thrombin, enterokinase or factor Xa onto the Co^{2+} affinity chromatography column bond with the corresponding fusion protein, proteins were collected and analyzed by SDA-PAGE (Fig. 1 B). When thrombin was added to a final protease to substrate ratio of 1:1 (U/mg) according to specification of thrombin kit and on-column digestion was carried out at room temperature for 24 h, about 99.5% fusion protein was cleaved into rV270 (designated as rV270-1), indicating that thrombin had a high efficiency for cleavage of the rV270-TB-His fusion protein. After digestion with 1 U of entero kinase/μg of protein in accordance with specification of enterokinase kit at room temperature for 24 h, approximately 32.4% of the rV270 protein (designated as rV270-2) was recovered from the His-EK-rV270 fusion protein. When His-Xa-rV270 fusion protein was digested by using 1 U of factor Xa/μg of fusion protein according to specification of factor Xa kit at room temperature for 24 h, about 15.3% of the rV270 protein and 16.8 % of non-specific protein recovered from the His-Xa-rV270 fusion protein, indicating that factor Xa might cleave at non-specific sequences besides the specific site. Finally, the rV270 protein was prepared by cleaving rV270-TB-His or His-EK-rV270, and further purified with Sephacryl S-200 HR column as the final purification step. Western blotting was employed to determine the immunogenicity of the purified rV270 protein by using specific McAb against rV270 antigen (Fig. 1 C). The summary of the purification process was presented in Table 1. The final yield of purified

TABLE 1

Purification of rV270-1 or rV270-2 from *E.coli* BL21/pET24a-rV270-TB or *E.coli* BL21/pET32a-EK-rV270 Cells (2L LB Medium)

Expression Strains	Purification Step	Total Volume (mL)	Total Protein (mg)	Yield (%)	rV270 Protein (mg)	Purity (%)
E.coli BL21/ pET24a-rV270-TB	Cell lysate	200	2157	100		
	Co^{2+} and thrombin	60	40.3	1.9	38.2	94.8
	Sephacryl S-200 HR	60	34.8	86.4	34.2	98.3
E.coli BL21/ pET32a-EK-rV270	Clarified lysate	200	2297.4	100		
	Co^{2+} and enterokinase	60	14.2	0.62	13.3	93.7
	Sephacryl S-200 HR	60	11.8	83.1	11.5	97.5

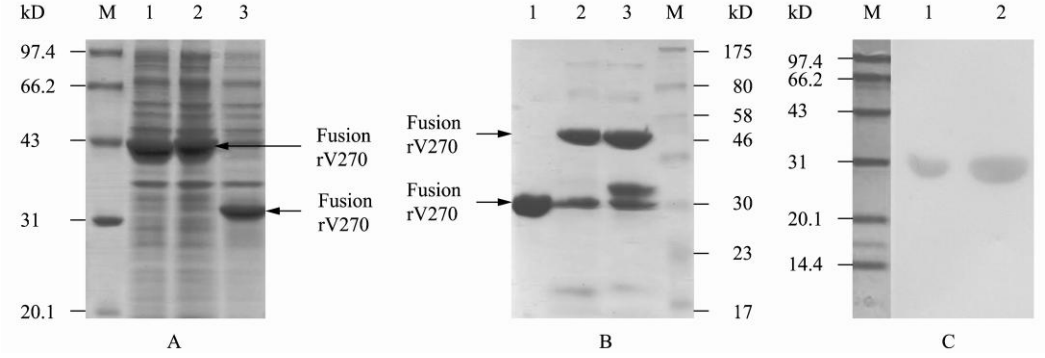

FIG. 1. A. The fusion proteins were identified by SDS-PAGE gradient gels. Lane M: low molecular mass standards; Lane 1: His-EK-rV270 fusion protein; Lane 2: His-Xa-rV270 fusion protein; Lane 3: rV270-TB-His fusion protein. B. The fusion proteins were cleaved by using thrombin, enterokinase or factor Xa. Lane 1: rV270-TB-His fusion protein was digested by thrombin; Lane 2: His-EK-rV270 fusion protein was cleaved by enterokinase; Lane 3: His-Xa-rV270 fusion protein was cut by factor Xa; Lane M: low molecular mass standards. C. The purified rV270 proteins were identified by western blotting. Lane M: molecular weight marker proteins; Lane 1: rV270-1; Lane 2: rV270-2.

rV270 protein from 2 liters of *E. coli* BL21/pET24a-rV270-TB overnight culture and *E. coli* BL21/pET32a-EK-rV270 culture was about 34.2 and 11.5 mg, respectively.

Characterization of the Purified Protein

The purified rV270-1 and -2 proteins were respectively identified by peptide mass fingerprints (Fig. 2 A, 2 B) and by N- and C-terminal sequence analysis. The N-terminal sequences of rV270-1 and -2 antigens were respectively determined as: MIRAYEQNPQHFIED and MIRAYEQNPQ. The C-terminal sequences of rV270-1 and -2 antigens were respectively identified as: RPVLAFHSLENND and AFHSLENNDKNYSYSN. Putative rV270-1 and -2 antigen sequences were respectively shown in Fig. 3. These results correctly identified the purified proteins as rV270-1 and -2 antigens, respectively.

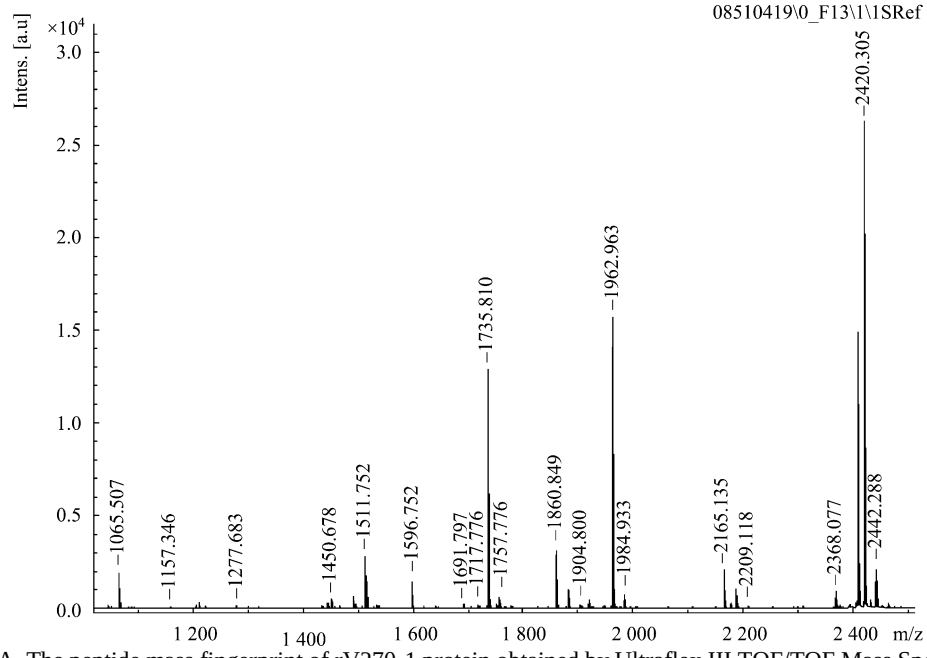

FIG. 2 A. The peptide mass fingerprint of rV270-1 protein obtained by Ultraflex III TOF/TOF Mass Spectrum.

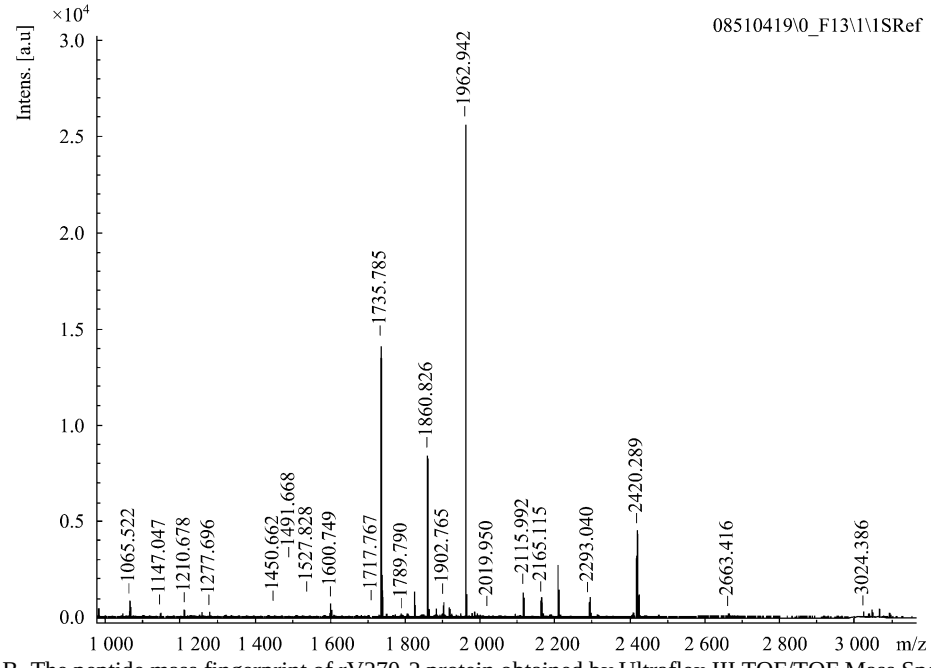

FIG. 2 B. The peptide mass fingerprint of rV270-2 protein obtained by Ultraflex III TOF/TOF Mass Spectrum.

MIRAYEQNPQHFIEDLEKVRVEQLTGHGSSVLEELVQLVKDKNIDISIKYDPRK
DSEVFANRVITDDIELLKKILAYFLPEDAILKGGHYDNQLQNGIKRVKEFLESSP
NTQWELRAFMAVMHFSLTADRIDDDILKVIVDSMNHHGDARSKLREELAELT
AELKIYSVIQAEINKHLSSSGTINIHDKSINLMDKNLYGYTDEEIFKASAEYKIL
EKMPQTTIQVDGSEKKIVSIKDFLGSENKRTGALGNLKNSYSYNK**DNNELSHF**
ALVPR

FIG. 3 A. N- and C-terminal sequence analysis of rV270-1 protein. The whole amino acid sequence was the rV270-1 protein. The N-terminal sequence identified by amino acid sequencing was underlined, and the C-terminal sequence of rV270-1 protein was underlined and shown in bold.

MIRAYEQNPQHFIEDLEKVRVEQLTGHGSSVLEELVQLVKDKNIDISIKYDPRK
DSEVFANRVITDDIELLKKILAYFLPEDAILKGGHYDNQLQNGIKRVKEFLESSP
NTQWELRAFMAVMHFSLTADRIDDDILKVIVDSMNHHGDARSKLREELAELT
AELKIYSVIQAEINKHLSSSGTINIHDKSINLMDKNLYGYTDEEIFKASAEYKIL
EKMPQTTIQVDGSEKKIVSIKDFLGSENKRTGALGNLK**NSYSYNKDNNELSH**
FA

FIG. 3 B. N- and C-terminal sequence analysis of rV270-2 protein.The whole amino acid sequence was the rV270-2 protein. The N-terminal sequence identified by amino acid sequencing was underlined, and the C-terminal sequence was underlined and shown in bold.

Antibody Responses and Protection against Challenge

Mice from rV270-1 or -2 immunized groups developed very high serum titers of rV270-specific IgG. Statistical analysis showed that there was no significant difference between the two immunized groups of mice (F = 0.15, P=0.7027), and sera from the control mice had negligible reactivity to the rV270 antigen (Fig. 4). Complete protection was observed for two groups of animals immunized with purified rV270-1 and -2 antigens by challenging *s.c.* with 10^6 CFU of *Y. pestis* strain 141. In contrast, the control mice immunized with aluminum hydroxide

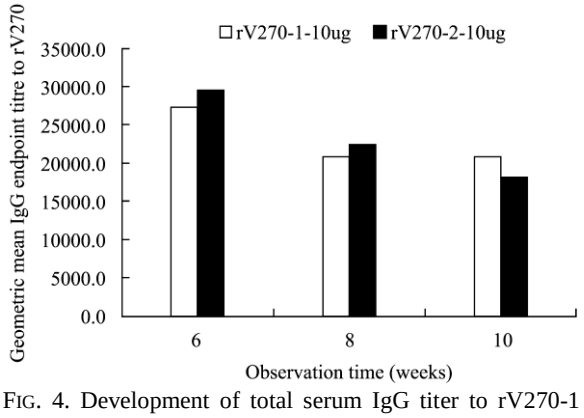

FIG. 4. Development of total serum IgG titer to rV270-1 and rV270-2 varies with time. Geometric mean values of IgG endpoint titer were derived from 10 serum samples per group. Standard deviation (SD) corresponding to each treatment group was as follows: 1.5 (rV270-1, 6 weeks), 2.0 (rV270-2, 6 weeks), 1.6 (rV270-1, 8 weeks), 1.6 (rV270-2, 8 weeks), 1.6 (rV270-1, 10 weeks), 1.8 (rV270-2, 10 weeks).

succumbed to the same dose of *Y. pestis* 141 challenge with a mean time to death (TTD) of 3±0 days. The result of protective efficacy was presented in (Table 2). The difference in protective efficacy between the treatment group and the control group was significant (*Chi-Square*=20.46, P<0.0001).

TABLE 2

Protective Efficacy of rV270-1 or rV270-2 against a Lethal *Y. pestis* 141 Strain Subcutaneous Challenge in Female Mice

Treatment Group	Challenge Dosage (CFU)	Survival/Total
10 μg rV270-1+Alum.	10^6	10/10
10 μg rV270-2+Alum.	10^6	10/10
Alum. Alone	10^6	0/10

A post-mortem was carried out on all the control animals who succumbed to the challenge in the 14 days post-challenge observation period, and *Y. pestis* were recovered from the hearts, livers, spleens, lungs and lymph nodes of all the control animals. This result indicated that the death of animals was caused by the systemic infection of *Y. pestis*. At day 14 post-challenge, two immunized groups of survivors were killed humanely and autopsied for post-mortem analysis. Microbiological analysis did not isolate *Y. pestis* from hearts, livers, spleens, lungs and lymph nodes of the immunized animals, indicating that *Y. pestis* had been cleared from the animals.

DISCUSSION

To prepare non-tagged rV270 protein, thrombin cleavage site was artificially fused to the C-terminus of the rV270 protein with a cleavage site between the chromatography, thrombin was used to process the fusion protein on column, displaying a high efficiency for cleavage of the rV270-TB-His fusion protein. However, thrombin is a site-specific protease that has a six-amino-acid recognition site (Leu-Val-Pro-Arg-▼-Gly-Ser) whose cleavage occurs between the Arg and Gly[9], resulting in a residual Leu-Val-Pro-Arg sequence at the C-terminus of the rV270 protein. Factor Xa (Ile-Glu-Gly-Arg▼) and Enterokinase (Asp-Asp-Asp-Asp-Lys▼) cleave target on the C-terminal side of the recognition sequence allowing complete removal of affinity tag sequence[10-11]. Factor Xa or enterokinase cleavage site was artificially inserted at the N-terminus with a cleavage site between His-tag and the rV270 protein. When enterokinase was employed to cleave His-EK-rV270 on column, a lower yield of rV270 protein was obtained, whereas factor Xa might cleave at non-specific sequences besides the predicted sites. Although the completely authentic rV270 protein was prepared by using enterokinase protease, it exhibited

extremely low cleavage activity to the corresponding recognition site. Thrombin cleavage is an efficient strategy to prepare non-tagged rV270 protein and can be easily operated in a large scale due to its relatively low cost and high cleavage efficacy, but it is nevertheless not so attractive since it can not yield an authentic protein. Enterokinase displays a low cleavage efficiency to the fusion rV270 protein, but it is a useful tool at a small laboratory scale application for the isolation of small quantities of an authentic rV270 protein, whereas factor Xa is not suited for the preparation of non-tagged rV270 protein because of its non-specific cleavage.

It is generally assumed that histidine tag does not interfere with the biological activity of fusion proteins, but a previous study has demonstrated that introduction of histidine affinity tags to tumor necrosis factor alpha (TNF-alpha) resulted in a remarkable decrease in biological activity[12]. In this study, the rV270-1 protein prepared by using thrombin contained no His-tag, but still had four-amino-acid residues of thrombin site on its C-terminus. The data herein demonstrated that two groups of mice immunized with the rV270-1 or rV270-2 protein prepared by TB or EK developed very high IgG titers specific to the rV270 protein and did not show any signs of disease, and deaths were not observed by the end of the experiment when they were challenged with 10^6 CFU doses of *Y. pestis* 141. The statistical analysis results also revealed that there were no antibody titer difference and protection difference between the authentic rV270-2 and unauthentic rV270-1 proteins. This result demonstrated that four-amino-acid residues on the C-terminus of rV270 protein did not interfere with its immunogenicity or protection activity. Elimination of 26 amino acids from the C-terminus of LcrV antigen comprising 326 amino acids still had highly protective activity. This finding was consistent with the previous conclusion that the protective epitopes of LcrV were found between amino acids 2 and 275[13]. Therefore, the rV270 antigen can be used as an important component for the development of a subunit vaccine against plague caused by F1 negative *Y. pestis* strains.

Complete protection against challenging with 10^6 CFU of *Y. pestis* strain 141 was observed for mice immunized with the rV270-1 or rV270-2 protein and post-mortem analysis did not isolate *Y. pestis* from hearts, livers, spleens, lungs or lymph nodes of the immunized mice, indicating that *Y. pestis* had been eliminated from the animals by the end of the experiment. Macrophages are supposed to play a crucial role in host immunosurveillance against invading pathogens by phagocytosis. It has been demonstrated that LcrV and YopB inhibite LPS induced TNF-α and NO production by disrupting the signal transduction cascade in macrophages. TNF-α, a proinflammatory cytokine, is primarily released by activated macrophages and plays a crucial role in limiting the severity of the bacterial infection and NO is considered to be one of the principal molecules involved in macrophage-mediated cytotoxicity[14].

Unlike the role of F1 antigen in blocking uptake by macrophage[15], LcrV antigen and YopB inhibit LPS mediated activation of macrophages and LPS induced TNF-α and NO production in macrophages. Another previous report has also demonstrated that anti-LcrV antibody promotes phagocytosis with consequent inhibition of Yops production, such as YopB, rather than by directly blocking the delivery of Yops[16]. *Y. pestis* can survive and replicate within macrophages for a limited time[17], cytotoxic T lymphocyte activity and enhanced innate responses may be important for achieving sterilizing immunity. The rV270 protein elicits robust humoral immunity, but its capacity to prime effective cellular immunity has not been demonstrated. A previous study has suggested that antibodies help phagocytes internalize *Y. pestis*, while cytokine products of T cells enable phagocytes to *Y. pestis* encounters and kill internalized bacilli. An ideal vaccine eliciting both humoral and cellular immunity may confer optimal defense against pneumonic plague[18-19]. Death or survival is dependent not only on antibody to rV270 antigen, but also on cell-mediated immunity responses, because circulating antibody specific to rV270 antigen promotes phagocytosis, whereas once the bacteria have established an intracellular infection, cell-mediated immunity is required for efficient eradication of *Y. pestis*.

ACKNOWLEDGEMENTS

This study is financially supported by the National Key Program for Infectious Diseases of China (2009ZX10004-4001).

REFERENCES

1. Perry R D, Fetherston J D (1997). Yersinia pestis--etiologic agent of plague. *Clin Microbiol Rev* **10** (1), 35-66.
2. Riedel S (2005). Plague: from natural disease to bioterrorism. *Proc (Bayl Univ Med Cent)* **18** (2), 116-124.
3. Titball R W, Williamson E D (2004). Yersinia pestis (plague) vaccines. *Expert Opin Biol Ther* **4** (6), 965-973.
4. Overheim K A, Depaolo R W, Debord K L, *et al*. (2005). LcrV plague vaccine with altered immunomodulatory properties. *Infect Immun* **73** (8), 5152-5159.
5. DeBord K L, Anderson D M, Marketon M M, *et al*. (2006). Immunogenicity and protective immunity against bubonic plague and pneumonic plague by immunization of mice with the recombinant V10 antigen, a variant of LcrV. *Infect Immun*

74 (8), 4910-4914.
6. Terpe K (2003). Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol* **60**, 523-533.
7. Rasoamanana B, Leroy F, Boisier P, *et al*. (1997). Field evaluation of an immunoglobulin G anti-F1 enzyme-linked immunosorbent assay for serodiagnosis of human plague in Madagascar. *Clin Diagn Lab Immunol* **4** (5), 587-591.
8. Williamson E D, Stagg A J, Eley S M, *et al*. (2007). Kinetics of the immune response to the (F1+V) vaccine in models of bubonic and pneumonic plague. *Vaccine* **25** (6), 1142-1148.
9. Dakshinamurthy R, Koteshwara A, Paul D, *et al*. (2008). A method for the prevention of thrombin-induced degradation of recombinant proteins. *Aanl Biochem* **375**, 361-363.
10. Nagai K, Thogersen H C (1984). Generation of beta-globin by sequence-specific proteolysis of a hybrid protein produced in Escherichia coli. *Nature* **309** (5971), 810-812.
11. Kim T J, Chauhan S, Motin V L, *et al*. (2007). Direct transcriptional control of the plasminogen activator gene of Yersinia pestis by the cyclic AMP receptor protein. *J Bacteriol* **189** (24), 8890-8900.
12. Kenig M, Peternel S, Gaberc-Porekar V, *et al*. (2006). Influence of the protein oligomericity on final yield after affinity tag removal in purification of recombinant proteins. *J Chromatogr A* **1101** (1-2), 293-306.
13. Hill J, Leary S E, Griffin K F, *et al*. (1997). Regions of Yersinia pestis V antigen that contribute to protection against plague identified by passive and active immunization. *Infect Immun* **65** (11), 4476-4482.
14. Sodhi A, Sharma R K, Batra H V, *et al*. (2004). Mechanism of rLcrV and rYopB mediated Immunosuppression in murine peritoneal macrophages. *Mol Immunol* **41** (8), 767-774.
15. Du Y, Rosqvist R, Forsberg A (2002). Role of fraction 1 antigen of Yersinia pestis in inhibition of phagocytosis. *Infect Immun* **70** (3), 1453-1460.
16. Cowan C, Philipovskiy A V, Wulff-Strobel C R, *et al*. (2005). Anti-LcrV antibody inhibits delivery of Yops by Yersinia pestis KIM5 by directly promoting phagocytosis. *Infect Immun* **73** (9), 6127-6137.
17. Pujol C, Bliska J B (2005). Turning Yersinia pathogenesis outside in: subversion of macrophage function by intracellular yersiniae. *Clin Immunol* **114** (3), 216-226.
18. Philipovskiy A V, Smiley S T (2007). Vaccination with Live Yersinia pestis Primes CD4 and CD8 T Cells That Synergistically Protect against Lethal Pulmonary Y. pestis Infection. *Infect Immun* **75** (2), 878-885.
19. Smiley S T (2008). Immune defense against pneumonic plague. *Immunological Reviews* **225**, 256-271.

(*Received January* 12, 2010 *Accepted August* 20, 2010)