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Expression of the C-terminal of 34kDa protein of *Mycobacterium paratuberculosis*

Doo Kim, Hyung-wook Park

Department of Veterinary Medicine, Kangwon National University
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*Mycobacterium paratuberculosis*의 34kDa C-terminal 단백질의 발현

김 두 · 박 형 욱

강원대학교 수의학과
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Abstract : Paratuberculosis (Johne's disease), a chronic enteritis produced by *Mycobacterium paratuberculosis* , affects a large proportion of ruminants in all continents and causes important economic losses. The identification of well-characterized and species-specific components of *M paratuberculosis* would provide the means to improve the specificity and sensitivity of immunodiagnostic assays for Johne's disease. The aims of this study were to express the recombinant C-terminal of 34kDa protein (rC34P) of *M paratuberculosis* in *E coli* and to investigate the effectiveness of this protein in detecting antibodies to the native protein in sera from paratuberculosis infected cattle. The C-terminal of the gene encoding the 34kDa protein was amplified by polymerase chain reaction from the chromosomal DNA of *M paratuberculosis* (ATCC 19698) and cloned into vector pGEX-4T-2. Then, cloned plasmid was transformed into *E coli* DH5α and the rC34P was overexpressed. The rC34P was purified by affinity chromatography and gel filtration. The rC34P was examined antigenicity by Western blot. The rC34P was reactive with culture positive bovine serum and hyperimmune rabbit anti-*M paratuberculosis* serum but was not reactive with culture negative bovine serum and tuberculin positive bovine serum in Western blot. In conclusion, the rC34P produced in this study is expected as a useful candidate for antigen in serological diagnosis of Johne's disease.

Key words : Johne's disease, *M paratuberculosis* , C-terminal of 34kDa protein.

Address reprint requests to Dr. Doo Kim, Department of Veterinary Medicine, Kangwon National University, Chunchon 200-701, Republic of Korea.

Introduction

Paratuberculosis (Johne's disease), a chronic enteritis produced by *Mycobacterium paratuberculosis*, affects a large proportion of ruminants in all continents and causes important economic losses[1,2].

The most common route of exposure of calves to *M paratuberculosis* is by ingestion of contaminated feces on the surface of the dam's mammary gland during suckling[3]. Clinically affected animals usually excrete between 1.3×10^5 and 5.9×10^6 *M paratuberculosis* organisms per gram of feces, which are considered the most important source of environmental contamination and animal infection[4]. Since infected cattle at an early stage of paratuberculosis outnumber those at a late stage, early diagnosis is crucial to identify potential bacterial shedders and to avoid the propagation of infection[5,6].

Several serologic tests have been developed for detecting antibodies in sera of cattle experimentally or naturally exposed to *M paratuberculosis*, but many of these tests have not been widely used under field condition. Comparison of an ELISA, a complement-fixation test and an agar gel immunodiffusion test revealed ELISA to be the most sensitive serologic tests for use in herds in which paratuberculosis persists[7-9]. At present there is no specific therapy, effective control or vaccination programs against Johne's disease. Although good management to control this disease can reduce incidence, eradication is dependent on detection and culling of infected animals as early as possible. Eradication program and management limitations for paratuberculosis have been hampered by the lack of simple and specific diagnostic tests for the disease in subclinical infected animals[1,10,11].

For the identification of subclinical paratuberculosis, most of the research efforts have been directed towards the development and application of new and improved specific serologic tests as well as nucleic acid probes and polymerase chain reaction (PCR) assays[5,12,13]. The identification of well-characterized and species-specific components of *M paratuberculosis* would provide the means to improve the specificity and sensitivity of immunodiagnostic assays for Johne's disease. De Kesel *et al*[5,14] and Gilot *et al*[15] identified a novel 34kDa protein and characterized a peptide bearing a species-specific epitope of *M paratuberculosis*. The 34kDa protein consists of two regions; a strongly hydrophobic portion and a highly hydrophilic part. Such a peculiar feature is compatible with a peripheral location of the 34kDa protein within the mycobacterial cell, the hydrophobic NH_2 moiety being buried within the envelope and the hydrophilic COOH part (carboxyl-terminal) being exposed at the cell surface. This view was supported by the observation that B epitopes localized in the carboxyl-terminal part of the protein were found by immune electron microscopy to be present at the cell surface[5]. Serodiagnosis with this peptide permitted diagnosis of Johne's disease in infected animals at all stages of the disease[5].

In Korea, Lee *et al*[16] reported the occurrence of Johne's disease by clinical and pathological survey for the first time, and Jeon *et al*[17] isolated *M paratuberculosis* from bovine feces of symptomatic Hereford cattle. Kim *et al*[18] reported about an outbreak of paratuberculosis in dairy and Korean native cattles by immunological methods (enzyme linked immunosorbent assay, complement fixation test, agar gel immunodiffusion test and intradermal skin test). However, to date, definitive serological diagnosis of Johne's disease was not achieved because of the lack of species-specific antigens.

The aims of this study were to express the recombinant C-terminal of 34kDa protein which was species-specific B epitope of *M paratuberculosis* and to investigate the effectiveness of this protein in detecting antibodies to the native protein in sera from paratuberculosis infected cattle.

Materials and Methods

Oraganisms : *Mycobacterium paratuberculosis* (ATCC 19698) was purchased commercially and maintained in Herrold's egg yolk medium (HEYM). *E coli* DH5α(Pharmacia Biotech, Uppsala, Sweden) was purchased commercially and maintained in Luria-Bertani (LB) agar.

Preparation of chromosomal DNA : Chromosomal DNA from *M paratuberculosis* was prepared from 12 weeks grown bacterial cells on HEYM. At first, bacterial pellet was wash-

ed with 500μl of TEN buffer [50mM Tris-HCl, 100mM EDTA, 150mM NaCl(pH 8.0)] three times. And then the pellet was suspended in 175μl of TEN containing protease (10mg/ml of final concentration, Sigma, St Louis, MO). After incubation for 3 hours at 37℃, 250μl of lysozyme(10mg/ml, Sigma) was added. After incubation for 2 hours at 37℃, 175μl of proteinase K(12.9mg/ml, Sigma) and 75μl of 10% sodium dodecyl sulfate (SDS ; wt/vol) were added and the mixture was incubated overnight at 55℃. The lysate was treated with RNase (Sigma) at a final concentration of 50μl/ml and incubated further 30 min at 37℃. The DNA was extracted twice with equal volume of phenol. After another extraction with equal volume of chloroform, the DNA was precipitated with 1/10 volume of 2.5M sodium acetate and 2 volumes of 100% cold ethanol. And then, the DNA was pelleted by centrifugation (13,000rpm, 15 min at 4℃), washed with 70% cold ethanol, and dried with vacuum. Finally the DNA was dissolved in TE buffer (1M Tris pH 8.0, 0.5M EDTA pH 8.0). The DNA concentration was determined spectrophotometrically at 260nm. The purified DNA was stored at 4℃.

Cloning and sequencing of the C-terminal of 34kDa protein gene : The recombinant techniques described by Kim *et al* [19] were employed for cloning and expression with minor modification. At first, the C-terminal of 34kDa protein gene was amplified from *M paratuberculosis* chromosomal DNA by polymerase chain reaction (PCR) using thermal cycler. The primers used in PCR amplification were designed from DNA sequences of the gene encoding the C-terminal of 34kDa protein in the Gene Bank (accession No. X68102). One forward primer was a 21-mer (5'-GGTCAG-CCCGGATCCCAGCCG-3') and the other reverse primer was a 21-mer (5'-CACGTTGTCGACTAGGCGCGA-3'), located at positions 1,321-1,341 and 1,648-1,668 nucleotides, respectively, of the 34kDa protein gene of *M paratuberculosis*. The restriction enzyme sites of *BamH* I and *Sal* I (underlined) were inserted in each primer, respectively. Each PCR mixture contained 100μl of a master mix consisting of distilled water, $MgCl_2$ (25mM), four deoxynucleotide triphosphates (200μM, dATP, dCTP, dGTP, dTTP), two primers (100pM), 5 units of *Taq* DNA polymerase (Promega, Madison, WI), 10X reaction buffer (Promega), 100ng template DNA. The cycling conditions in the thermal cycler were an initial incubation for 10 min at 94℃, followed by 35 cycles each of 1 min of denaturation at 94℃, 1.5 min of annealing at 55℃, and 1.5 min of extention at 74℃. And then, the tube kept at 74℃ for 15 min. After reaction, 10μl of reaction mixture was electrophoresed on a 0.7% agarose gel, and stained with ethidium bromide. The PCR product (*pC34P*) was purified by DNA Purification System (Bio Rad, Hercules, CA). The purified PCR product and expression vector plasmid, pGEX-4T-2 (Pharmacia Biotech) were digested with *BamH* I and *Sal* I, and the 331bp digested PCR product was inserted into vector by ligation. The competent cell of *E coli* DH5α was then tranformed with C-terminal : pGEX-4T-2 plasmid (*pC34P/GEX*), and transformants were selected on LB agar containing 12.5μg/ml of ampicillin. To identify the *pC34P*, the *pC34P/GEX* plasmid was purified by alkali-lysis method and digested with *BamH* I and *Sal* I and digested plasmids were electrophoresed on a 0.7% agarose gel. And then, the inserted *pC34P* gene was sequenced using the 5' pGEX sequencing primer and the 3' pGEX sequencing primer (Pharmacia Biotech) by primer extention dideoxy termination method of Sanger *et al* [20]. The 5' pGEX sequencing primer was 5'-GGGCTGGCAAGCCACGTTTGGTG-3' which bound to nucleotides 869~891 on the pGEX-4T-2. The 3' pGEX sequencing primer was 5'-CCGGGAGCTGCATGTGTCA-GAGG-3' which bound to 1,020~1,042 on the pGEX-4T-2.

Expression and purification of the recombinant C-terminal protein (rC34P) : For expression of recombinant C-terminal protein fused with glutathione S-transferase (rC34P : GST), the positive clone (pKD1) was grown until OD_{600} = 1 with LB broth contained ampicillin at 37℃ in a shaking incubator and induced with final concentration of 1mM isopropyl thiogalactosidase (IPTG) for 3 hours. The cells were harvested by centrifugation (7,000rpm, 10 min) and the pellet was resuspended in PBS (pH 7.4, 0.3M NaCl, 0.002M NaH_2PO_4, 0.008M Na_2HPO_4). Then the cells were ruptured at 16,000psi with French press(Spectronic Instruments, Rochester, NY). The lysate was centrifuged to remove the cell debris and unruptured cells. The supernatant was collected and mixed with Ni^+-NTA resin (Pharmacia Biotech). The rC

34P-GST was eluted with elution buffer (Pharmacia Biotech). And then, eluted rC34P was digested with thrombin and purified by gel filtration with sephadex 75. The purified rC 34P was examined by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with 0.25% coomassie brilliant blue solution for the presence of rC34P and for its purity.

Serum samples : 1) Rabbit hyperimmune anti-*M paratuberculosis* serum. Rabbit hyperimmune antiserum was produced by repeated subcutaneous and intravenous immunization with *M paratuberculosis* sonicate[13]. Briefly, the *M paratuberculosis* was grown on HEYM, washed with PBS, suspended in PBS (0.5g of bacilli/ml), and sonicated with Ultrasonic Disintegrator Soniprep 150 (MSE, Houston, TX) in ice-water bath until translucent. A dose of clarified sonicate, containing 0.5mg mycobacterial proteins, was emulsified in incomplete Freund's adjuvant [1 : 1 ratio (vol : vol); Sigma] and administered weekly subcutaneous in rabbit. At the end of the 6th week, the animal was immunized again intravenously with 500μg of mycobacterial protein in 500μl PBS (pH 7.4). On the 8th week of immunization, the rabbit was exsanguinated and the antiserum was stored at -70℃. 2) Serums from cattle with clinical paratuberculosis were obtained from College of Veterinary Medicine, Cornell University, Ithaca, NY.

Immunoblot analysis : To identify the antigenicity of the recombinant C-terminal protein, the purified rC34P was electrophoresed by SDS-PAGE, then transferred to nitrocellulose membrane (Trans-Blot® Transfer Medium, Bio-Rad). After transfer, transblotted nitrocellulose membrane was incubated with 3% gelatin in tris buffered saline with tween 20 (TBST, pH 7.5, 20mM Tris Base, 0.9% NaCl, 0.05% Tween 20, 1 : 10,000 merthiolate) for blocking and washed with TBST buffer (pH 10.0, 10mM Tris base, 0.9% NaCl, 0.05% Tween 20). Then the membrane was reacted for 1 hour with diluted serum (1 : 30) containing 1% gelatin in TBST (pH 7.5). After repeated washings with TBST buffer (pH 10.0), the membrane was incubated for 1 hour with horse radish peroxidase-antirabbit or antibovine IgG [1 : 500 diluted with TBS buffer (pH 7.5, 20mM Tris base, 500mM NaCl), Sigma], followed by washing with TBST and TBS buffer. A color reaction was developed by addition of substrate (4-chloro-1-napthol, Sigma) in the presence of 30% hydrogen peroxidase. The color reaction was stopped by washing the membrane with distilled water.

Results

Cloning and sequencing of C-terminal of 34kDa protein (*pC34P*) gene : In order to clone the *pC34P* gene into vector pGEX-4T-2, PCR amplified *pC34P* gene from *M paratuberculosis* (ATCC 19698) and pGEX-4T-2 were digested with *BamH* I and *Sal* I, and the *pC34P* gene was ligated into pGEX-4T-2 (*pC34P/GEX*). And then, *E coli* DH 5α was transformed with *pC34P/GEX* (pKD1). To confirm the *pC34P/GEX* plasmid, the recombinant plasmid was digested with *BamH* I and *Sal* I, and electrophoresed on a 0.7% agarose gel. The 331bp gene was inserted correctly (Fig 1). The insert was sequenced with the 5' pGEX sequencing primer and the 3' pGEX sequencing primer. Fig 2 shows the DNA sequence and deduced amino acid sequence of the *pC34P* gene including enzyme sites which were matched with the DNA sequence of the C-terminal of 34kDa protein gene of pre-

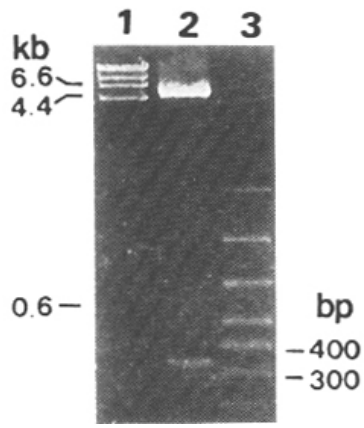

Fig 1. Electrophoresis of pKD1 clone digested with *BamH* I and *Sal* I. To check the C-terminal of 34kDa protein gene inserted into vector pGEX-4T-2 plasmid, the purified plasmid DNA from pKD1 clone was digested with *BamH* I and *Sal* I. The 331bp gene was inserted into vector pGEX-4T-2 correctly. Lane 1, DNA size standard (high range, Bio-Rad); lane 2, 331bp band of C-terminal of 34kDa protein gene and 4,970bp band of pGEX-4T-2; lane 3, DNA size standard(low range, Bio-Rad).

GGA TCC CAG CCG GGT GGT CAG CAG CAT TCG CCG CAG GGC TAC GGG TCG CAG TAC GGC GGT 60
Gly Gly Gln Pro Gly Gly Gln Gln His Ser Pro Gln Gly Tyr Gly Ser Gln Tyr Gly Gly

TAC GGC CAG GGC GGC GCT CCG ACC GGC GGT TTC GGT GCC CAG CCG TCG CCG CAG TCC GGC 120
Tyr Gly Gln Gly Gly Ala Pro Thr Gly Gly Phe Gly Ala Gln Pro Ser Pro Gln Ser Gly

CCG CAA CAG TCC GCG CAG CAG CAG GGC CCG TCC ACA CCG CCC ACC GGC TTC CCC AGC TTC 180
Pro Gln Gln Ser Ala Gln Gln Gln Gly Pro Ser Thr Pro Pro Thr Gly Phe Pro Ser Phe

AGC CCG CCG CCC AAC GTC GGC GGG GGA TCG GAC TCC GGT TCG GCG ACC TCT AAT TAC TCC 240
Ser Pro Pro Pro Asn Val Gly Gly Gly Ser Asp Ser Gly Ser Ala Thr Ser Asn Tyr Ser

GAG CAG GCC GGT GGC CAG CAG TCC TAC GGC CAG GAG CCT TCT TCA CCG TCT GGC CCG ACG 300
Gln Gln Ala Gly Gly Gln Gln Ser Tyr Gly Gln Glu Pro Ser Ser Pro Ser Gly Pro Thr

CCC GCC TAA CGT GCC CTG TCG CGC CTA GTC GAC
Pro Ala

Fig 2. Nucleotide sequence and deduced amino acid sequence of the *pC34P*. The restriction enzyme sites were underlined in each end of the open reading frame of the C-terminal of 34kDa protein gene. *; the changed nucleotide from previously published sequence[15]. ***; stop codon.

viously published nucleotide sequence except 2bp difference[15].

Expression and purification of the recombinant C-terminal of 34kDa protein (rC34P) : The recombinant rC34P : GST protein (41kDa) was expressed from the pKD1 clone. The recombinant C-terminal of 34kDa protein (15kDa) was fused with GST (26kDa) and could be purified by affinity chromatography using GST Purification Module. The purified recombinant protein was examined by SDS-PAGE (Fig 3). To purify the rC34P, the recombinant rC34P : GST protein was digested with thrombin and purified by gel fil-

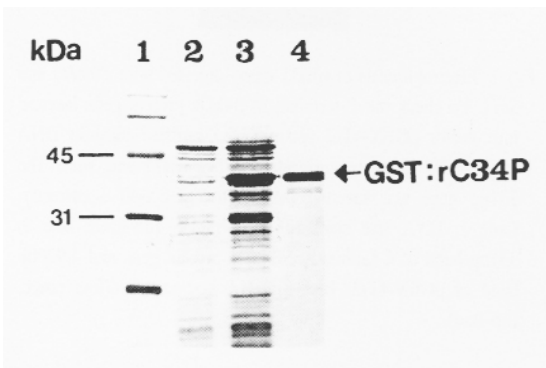

Fig 3. SDS-PAGE of the rC34P:GST. Lane 1. SDS-PAGE molecular weight standards (low range, Bio-Rad); lane 2, before the induction of rC34P:GST; lane 3, 3 hours after the induction of rC34P:GST; lane 4, purified rC34P:GST. The gel was visualized by 0.25% coomassie brilliant blue.

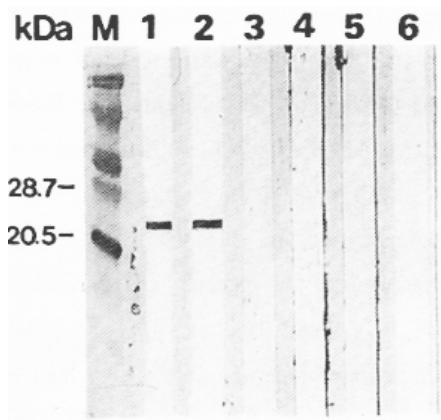

Fig 4. Reactivity of the rC34P with serum from culture positive bovine serum (lane 1), rabbit hyperimmune anti-*M paratuberculosis* serum (lane 2), culture negative bovine serums (lane 3~4) and tuberculin positive bovine serums (lane 5~6) in western blotting. Lane M was the prestained SDS-PAGE standards (low range, Bio-Rad).

tration with Sephadex 75 column. The purified rC34P was identified by western blot.

Western blot analysis : In order to determine antigenicity of rC34P, western blot was performed with the purified C-terminal of 34kDa protein as an antigen. The rC34P was reactive with culture positive bovine serum and hyperimmune rabbit anti-*M paratuberculosis* serum. However, the rC34P was not reactive with culture negative bovine serums and tuberculin positive bovine serums (Fig 4). The result confirmed that the rC34P retained the antigenicity of the native C-terminal of 34kDa protein.

Discussion

Paratuberculosis is a continuing enigma for cattle producers and veterinarians. Attempts to control or eradicate the disease from herds have been based on two practices; separation of newborn calves from older cattle and slaughter of cattle with positive test results. Separation of newborn calves from older cows has undoubtedly helped to lower the incidence and prevalence of paratuberculosis by reducing the potential for horizontal spread of disease. However, *in utero* transmission does take place and will perpetuate the disease in herds[21].

Efforts to control paratuberculosis in cattle and other species have been limited by the lack of rapid and reliable diagnostic tests for identifying all *M paratuberculosis*-infected animals. Though several serologic tests have been developed for detecting antibodies in sera of cattle exposed to *M paratuberculosis*, the specificities of these tests have been poor due to antigenic cross-reactivity between *M paratuberculosis* and other mycobacterial species and nonmycobacterial organisms[1,10,22-25].

Like other mycobacteria, it is difficult to analyze the antigenic structure of *M paratuberculosis* due to its extremely slow growth and cross-reactivity with other mycobacterial and nonmycobacterial species[1,22,23]. These problems are common to the serologic diagnosis of all mycobacterial infection[22].

In mycobacterial infection, it is not known how many antigens or epitopes are involved in the development of disease or in protective immunity[22]. *M paratuberculosis* contains a major antigen complex called A36 which is highly recognized by sera from cattle infected with *M paratuberculosis*. The 34kDa protein of the A36 complex is immunodominant and contains B epitopes specific for *M paratuberculosis*[14,26]. The carboxyl-terminal (C-terminal) of 34kDa protein is exposed at the cell surface and B epitopes was localized in the C-terminal part of the 34kDa protein. Therefore, recombinant C-terminal of 34kDa protein could be used for the development of a specific serological test for paratuberculosis.

The molecular weight of the recombinant C-terminal of 34kDa protein (rC34P) produced in this study was 22,500 Da which is about 7,500Da larger than the theoretical molecular mass (14,976Da). Such a discrepancy might be due to the high proline content of the protein (16.7%), which reduces the mobility of the protein in the SDS-PAGE fractionation, thus reading to an overestimation of its size. This phenomenon was previously observed[15,27,28].

There is also a discordance between the molecular mass determination by electrophoresis under denaturing conditions and the theoretical molecular mass. As a matter of fact, the sum of the molecular masses of the amino acids encoded by the nucleotide sequence of open reading frame of the rC34P is about 15kDa.

The sequence analysis of the C-terminal of 34kDa protein gene insert in the expression vector pGEX-4T-2 revealed that the DNA sequence of the cloned gene was matched with those of previously published nucleotide sequence except 2bp difference at position 229 (G→T) and 231 (C→T)[15]. Such difference of 2bp nucleotides caused to change of amino acid from alanine to serine. Although one amino acid was changed, the antigenicity of rC34P was not affected.

Most dyes tend to be attracted by positively charged amino acid groups (lysine, arginine and histidine) on the proteins; consequently proteins with higher proportions of these-generally more basic proteins tend to be stained more strongly.

Indeed, some acidic polypeptides have escaped detection because they bind so little dye[29]. The rC34P produced in this study was not stained with 0.25% coomassie brilliant blue solution or silver stain. The rC34P was composed of 102 amino acids, which contained 22 acidic amino acids (1

aspartic acid, 2 asparagines, 1 glutamic acid and 18 glutamines) and 1 basic amino acid (histidine). Some of above reasons explained why the rC34P was not stained.

The rC34P showed positive immune reaction with culture positive bovine serum and hyperimmune rabbit anti-*M paratuberculosis* serum, but was not reactive with culture negative bovine serum in western blot analysis. And the rC 34P was also not reactive with serum from tuberculin positive cattle in western blotting. Hence, this recombinant C-terminal of 34kDa protein is expected as a species-specific antigen in serological diagnosis of Johne's disease.

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