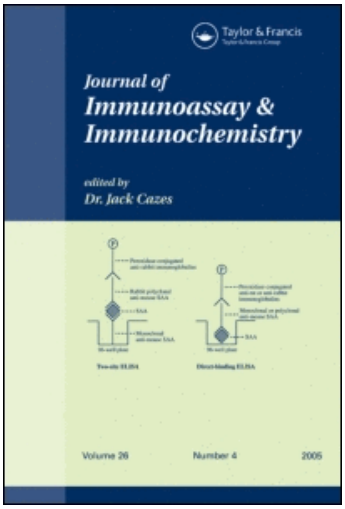

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[a] Bacteriology and Parasitology Division, National Veterinary Research Institute, National Veterinary Research & Quarantine Service, Anyang, Kyounggi, Republic of Korea [b] Foreign Infectious Disease Division, National Veterinary Research Institute, National Veterinary Research & Quarantine Service, Anyang, Kyounggi, Republic of Korea

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Definition of Purified Enzyme-Linked Immunosorbent Assay Antigens from the Culture Filtrate Protein of Mycobacterium bovis by Proteomic Analysis

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[1]Bacteriology and Parasitology Division, National Veterinary Research Institute, National Veterinary Research & Quarantine Service, Anyang, Kyounggi, Republic of Korea

[2]Foreign Infectious Disease Division, National Veterinary Research Institute, National Veterinary Research & Quarantine Service, Anyang, Kyounggi, Republic of Korea

Abstract: Enzyme-linked immunosorbent assay (ELISA) has been developed as the ancillary diagnosis of bovine tuberculosis at ante-mortem to overcome the disadvantages of intradermal skin test. In this study, the antigenic proteins were purified, applied to bTB ELISA, and identified through proteomic analysis. Culture filtrate protein of *Mycobacterium bovis* was fractionated by MonoQ® column chromatography, and examined the antigenicity by immunoblotting. The antigenic 20 kDa protein was in-gel digested and identified the antigenome by LTQ® mass spectrometer and peptide match fingerprinting, which were MPB64, MPB70, MPB83, Fas, Smc, Nrp, RpoC, Transposase, LeuA, and MtbE. The 20 kDa protein exhibited the highest antigenicity to bTB positive cattle in ELISA and would be useful for bTB serological diagnosis.

Address correspondence to Yun Sang Cho, Bacteriology & Parasitology Division, National Veterinary Research Institute, National Veterinary Research & Quarantine Service, Mannan-gu, Anyang 480, Gyeonggi-do 430-824, Republic of Korea. E-mail: choys@nvrqs.go.kr

Keywords: Antigenicity, Antigenome, Bovine tuberculosis, Enzyme-linked immunosorbent assay (ELISA), Proteomic analysis

INTRODUCTION

Mycobacterium bovis is the major causative bacteria of bovine tuberculosis (bTB) and belongs to *M. tuberculosis* complex together with *M. tuberculosis, M. africanum, M. pinnipedii, M. microti, M. caprae*, and *M. bovis*.[1,2] *M. bovis* has the broadest host range of the *M. tuberculosis* complex, and is able to infect humans as well as animals.[3,4]

Moreover, human immunodeficient virus (HIV) infection has been increased worldwide, and the mortality rate of people coinfected with HIV and mycobacteria has also increased.[3] The approximate number of worldwide deaths caused by human tuberculosis (TB) was recently estimated to be 3 million people annually.[3,5] Western and Eastern Europe, the United States, Canada, Latin America, Australia, New Zealand, Africa, India, Israel, Taiwan, and Turkey have reported the occurrence of human tuberculosis caused by *M. bovis*, which may imply that these human infections originated from animals.[4]

Bovine TB has been reported in cattle industry worldwide and it is estimated that over 50 million cattle have been infected by *M. bovis*.[6,7] The major routes of infection of TB from cattle to humans include the oral route via raw milk and unpasteurillized milk products and the respiratory tract via inhalation of droplets containing tubercle bacilli nuclei.[4] The contact with TB-infected cattle has also resulted in the infection crossing from cattle to human. Therefore, the control of bTB is important to protect public health.

Bovine TB is officially detected by IST and can be further tested by histopathological findings, identification of *M. bovis*-specific gene, and isolation of tubercle bacilli.[8,9] An enzyme-linked immunosorbent assay (ELISA) has been newly developed for the ante mortem diagnosis of bTB that possesses economical advantages to the cattle industry.[8] The economical advantages of diagnosis by ELISA include the fact that the sera used for the bTB ELISA can also be used to test for other diseases and a large number of samples can be detected simultaneously.[9] Consequently, research into the serological diagnosis of bTB has focused on identifying antigens that could improve the specificity and sensitivity of the ELISA.

Previously, antigens were identified by their resolving rate on one dimensional gel electrophoresis and their size was subsequently compared with a commercial size marker.[10–14] Proteomics has been applied to define biomarkers of human life threatening disease such as cancers and infectious diseases;[15–17] therefore, this technique can be also applied to define the antigenome in *M. bovis*. Information from the proteomic

analysis of bTB would be useful for the development of bTB diagnostics. The antigenome in *M. bovis* was defined by the purification from an *M. bovis* culture, the confirmation of antigenic proteins by immunoblotting, the application of the purified antigens to ELISA, and the proteomic analysis through in-gel digestion, mass spectrometry, and peptide match fingerprinting (PMF).

EXPERIMENTAL

Culture and Protein Precipitation

M. bovis AN5 (ATCC 35726) was used for the preparation of culture supernatant, which is the standard strain of IST diagnostics in animals.[1] *M. bovis* was cultured in Sauton broth at 37°C for 9 weeks, heated at 100°C for 3 hours, filtered and centrifuged to harvest the culture supernatant. Eighty five per cent ammonium sulfate (Sigma Chemical, St. Louis, MO, USA) was added to the culture supernatant to maximize the protein precipitation in culture supernatant. The precipitates were pelleted by centrifugation at 10,000 × g for 10 min and dialyzed against 0.01 M PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, D.W. to 1,000 mL, pH 7.4). The concentration of precipitated proteins was calculated by BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL, USA).

Purification and Immunoblotting

The ammonium sulfate precipitates, also called as culture filtrate proteins (CFP), were fractionated by fast performance liquid chromatography (FPLC) using a MonoQ® HR 5/5 column (GE Healthcare, Piscataway, NJ, USA) for anion exchange chromatography.[12] CFP was loaded onto the MonoQ® HR 5/5 column and fractionated by 1.0 mL, with a linear gradient of salt concentration from 0 to 2.0 M NaCl using buffer A (30 mM Tris-HCl w/2% butanol, pH 8.8) and buffer B (buffer A w/2.0 M NaCl) at 1.0 mL flow rate. The protein concentration represented by optical density (OD) at 280 nm, salt concentration, and fraction number were recorded. The fractions that were above 1.0 OD at 280 nm were further analyzed by BCA protein assay (Pierce, Rockford, IL, USA), SDS-PAGE and immunoblotting. The protein fractions (10 μg per lane) were separated by SDS-PAGE and transferred onto nitrocellulose (NC) membrane soaked in 600 mL transfer buffer (15.6 mM Tris, 120 mM glycine) plus 150 mL methanol at 100 V, 400 mA for 1 h.[18] The membranes were then incubated in *M. bovis* infected cattle serum diluted 1:500 in 0.01 M PBS at 37°C for 1 h, and then in anti-bovine IgG horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories

Inc., Gaithersburg, MD, USA) diluted at 1:1000 in 0.01 M PBS at 37°C for 1 h. After washing with 0.01 M PBS (pH 7.4), the membranes were incubated in 3,3′-diaminobenzidine (Sigma Chemical, St. Louis, MO, USA) for 5 min at room temperature (22 ± 2°C).

In-Gel Digestion and Protein Identification

MonoQ® fractions of CFP were analyzed by SDS-PAGE and stained with Coomassie brilliant blue. The antigenic 20 kDa protein confirmed by immunoblotting was cut and digested with trypsin (Roche, Basel, Switzerland). Trypsinized peptides were analyzed and identified at the protein level by LTQ® mass spectrometer (Thermo Electron, USA) and MASCOT search (http://www.matrixscience.com/).[15,16] NanoLC-MS/MS analysis was performed on an agilent 1100 Series nano-LC and LTQ® mass spectrometer (Thermo Electron, USA). The capillary column used for LC-MS/MS analysis (150 mm × 0.075 mm) was obtained from Proxeon (Odense M, Denmark) and slurry packed in house with 5 μm, 100 μm pore size Magic C18 stationary phase (Michrom Bioresources, Auburn, CA, USA). The mobile phase A for the LC separation was 0.1% formic acid in deionized water and the mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was set up to give a linear increase from 5% B to 35% B in 50 min, from 40% B to 60% B in 20 min and from 60% B to 80% B in 5 min. The flow rate was maintained at 300 nL/min after splitting. Mass spectra were acquired using data-dependent acquisition with full mass scan (400–1800 m/z) followed by MS/MS scans. Each MS/MS scan acquired was an average of one microscan on the LTQ®. The temperature of the ion transfer tube was maintained at 200°C and the spray was 1.5.0–2.0 kV. The normalized collision energy was set at 35% for MS/MS. SEQUEST® software (http://fields.scripps.edu/sequest/) was used to identify the peptide sequences. For high confidence results, deltaCn ≥ 0.1, Rsp ≤ 4 and Xcorr ≥ 1.5 with charge state 1+, Xcorr ≥ 2.0 with charge state 2+, and Xcorr ≥ 2.5 with charge state 3+, peptide probability > 0.1, were used as cutoff values for protein identification. Peptides were allowed to be variably oxidized at methionine residues and to be variably carboxyamidomethylated and carboxymethylated at cystein. Highly probable peptides that were identified by MASCOT were fully matched with the *M. bovis* database by BLAST (http://www.ncbi.nih.gov/BLAST) searches by PMF.

ELISA and S/N Ratio

Bovine TB ELISA by the antigenic 20 kDa protein was compared with that of PPD and recombinant MPB70.[11] Each antigen was resuspended

in coating buffer (Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, NaN_3 0.2 g, D.W. 1,000 mL, pH 9.6) at the optimal concentration and was used to coat 96 well ELISA plates at 2–8°C for 18 h. The solutions that were used for the ELISA included PBS w/0.05 % Tween 20 (Tween-PBS; NaCl 8.5 g, KH_2PO_4 0.22 g, Na_2HPO_4 1.15 g, KCl 0.2 g, Tween 20 0.5 mL, D.W. 1,000 ml, pH 7.4), citrate buffer (citric acid solution (1.92 g/100 ml) 24.3 mL, Na_2HPO_4 solution (2.84 g/100 mL) 25.7 mL, D.W. 50 ml, pH 5.0), stock solution of 2,2-azino-di(3-ethylbenzothiazoline-6-sulphonate) (ABTS; Sigma Chemical, St. Louis, MO, USA) (ABTS 0.54 g, D.W. 25 mL), and 0.5 M H_2O_2; these solutions were equilibrated at room temperature (RT) for 2–3 h before the ELISA was performed. The sera were diluted to 1:200 with PBS (NaCl 8.5 g, KH_2PO_4 0.22 g, Na_2HPO_4 1.15 g, KCl 0.2 g, D.W. 1,000 mL, pH 7.4) prior to use. Tween-PBS containing 1% gelatin was cooled to RT and used as the blocking solution by incubating at 37°C for 1 h. Duplicate wells (100 μL) of each serum, plus positive and negative control sera were added to each plate and the plates were incubated at 37°C for 1 h. After washing with Tween-PBS, each well was injected with 100 μL of rabbit anti-bovine IgG-conjugated horseradish peroxidase (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) diluted 1:2000 with Tween-PBS and then incubated for 30 min at RT. The substrate solution (0.1 mL 0.5 M H_2O_2, 0.25 mL stock ABTS, and 24.65 mL citrate buffer) was made just before use; 100 μL were injected into each well and the plates were incubated for 30 min at RT. The reaction was stopped by the injection of 50 μL 1.0 M H_2O_2 and the OD at 405 nm was calculated for each well within 10 min using an ELISA reader (TECAN, USA). The antibody titer was described as the S/N ratio [the sample serum OD was divided by the negative serum OD]. The cattle serum from a TB-free farm with no evidence of bTB infection was provided as a negative control serum.

RESULTS

One milliliter fractions were collected from fraction no. 1 to 37 at a 1.0 mL/min flow rate. The fractions containing flow through were from no. 1 to 6, those obtained when the salt gradient was between 0 to 1.0 M were from no. 7 to 26, and those obtained when the salt gradient was between 1.0 to 2.0 M were from no. 27 to 31. Most proteins were fractionated by the 0.5 to 1.0 M salt gradient; these exhibited four major peaks (Fig. 1). Proteins were not fractionated further after the 1.0 M gradient. The protein concentrations of fraction no. 18, 19, 20, and 21 were 65, 348, 131, and 57 μg/mL, respectively. The protein size in fraction no. 18 were 20 kDa, those in fraction no. 19 were 10, 20, and 25 kDa, those in fraction no. 20 were 20 and 25 kDa, and those in fraction no. 21 were 10 kDa (Fig. 2).

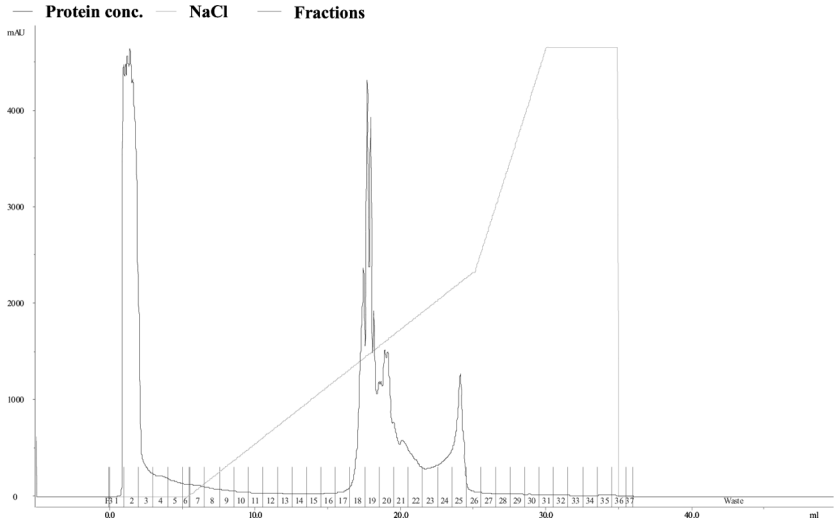

Figure 1. Chromatogram of antigen fractions by MonoQ® HR 5/5 column chromatography. Culture filtrate protein (CFP) were fractionated from no. 1 to 37 by anion exchange chromatography. Linear salt gradient was obtained using a NaCl concentration slope with buffer A and buffer B (buffer A w/2.0 M NaCl). Fractions from no. 1 to 6 were flow through before the salt gradient, those from no. 6 to 26 corresponded to 0 to 1.0 M NaCl concentration after the salt gradient, and those from no. 27 to 31 corresponded to 1.0 to 2.0 M NaCl concentration. The first peak after the salt gradient was collected in fraciton no. 18, the second peak in no. 19, and the third peak in no. 20. Conc; concentration. AU; absorbance unit.

The proteins in fraction no. 18, 19, 20, and 21 were analyzed by immunoblotting using bTB positive cattle serum. The 20 kDa protein was the only protein to exhibit antigenicity; no antigenicity was detected with the 10 and 25 kDa proteins in fraction no. 19, 20, and 21 (Fig. 2). A higher protein concentration was detected in fraction no. 20 (131 μg/mL) than fraction no. 18 (65 μg/mL); however, fraction no. 18 contained more of the 20 kDa protein than fraction no. 20. Therefore, the intensity of the 20 kDa protein band by immunoblotting in fraction no. 18 was greater than that in fraction no. 20. The intensity of the 20 kDa band in fraction no. 19 was greater than that in fraction no. 18 (Fig. 2b). The antigenic protein was eluted in fraction no. 18, 19, and 20, corresponding to the 0.5 and 0.7 M salt gradient (Figs. 1 and 2). The 20 kDa protein was first fractionated as shown in fraction no. 18, followed by the elution of 10 and 25 kDa proteins in fraction no. 19 and 20.

The 20 kDa protein in fraction no. 18 and 19 was identified by in-gel digestion and mass spectrometry; 11 proteins were identified by proteomic analysis in fraction no. 18 and 17 proteins were identified in fraction

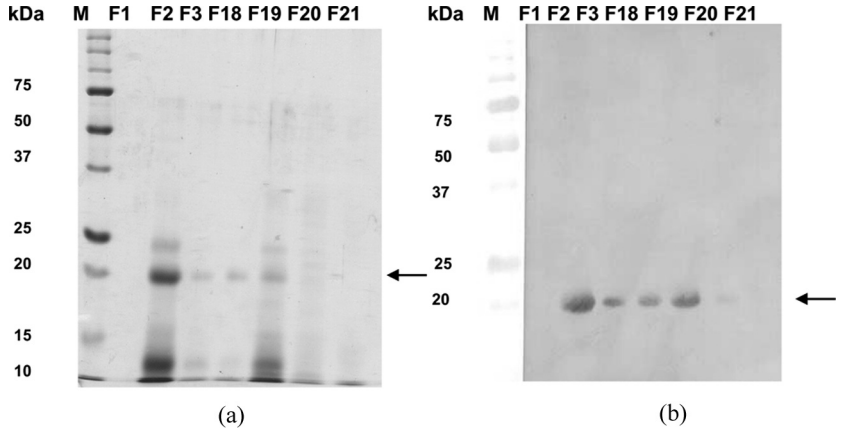

Figure 2. SDS-PAGE (a) and immunoblotting (b) analysis of MonoQ® HR 5/5 fractions showed protein contents represented by OD at 280 nm. (a) Each lane was loaded with 10 μg of each fraction. Fraction no. 18 exhibited one band at 20 kDa, fraction no. 19 possessed three bands at 10, 20, and 25 kDa, and fraction no. 20 had one band at 20 kDa. (b) The 20 kDa protein showed antigenicity to *M. bovis* infected cattle serum by immunoblotting. Fraction no. 18, 19, and 20 all contained the antigenic 20 kDa protein. The intensity of the 20 kDa protein was highest in fraction no. 19 and lowest in fraction no. 20. Arrow (←) indicates the antigenic 20 kDa protein.

no. 19 (Table 1). After the antigenic 20 kDa protein was digested with trypsin, 137 probable peptides were identified from fraction no. 18 and 386 probable peptides were identified from fraction no. 19. MPB70, MPB83, MPT64, Fas, and RpoC were identified in both fractions (Table 1). The theoretical 20 kDa size matched proteins in fraction no. 18 and 19 were MPB70, MPB83, and MPB64 (Table 1). In addition, fraction no. 19 contained the theoretical size matched proteins CFP21, PpiA, LppX, GreA, and Mb3713c. The proteins in fraction no. 18 other than MPB70, MPB83, and MPB64 were identified Fas, RpoC, Smc, Nrp, Transposase, LeuA, and MbtE. In the pI ranges of fraction no. 18 and 19 were from 4.5 to 6.1 and from 4.3 to 7.0, respectively. MPB70, MPB83, and MPB64 were eluted first, followed by CFP21, PpiA, LppX, GreA, and Mb3713c (Fig. 2 and Table 1).

MonoQ® fraction no. 18 showed the highest sensitivity and specificity to bTB skin positive reactors (TB+; $n = 15$), false positive reactors (TB±; $n = 8$), and negative reactors (TB−; $n = 7$) in ELISA among purified protein derivative tuberculin, recombinant MPB70, MonoQ® fraction no. 18 and 19. The S/N ratios of fraction no. 18 to TB+, TB±, and TB− were 2.7, 2.5, and 0.6, respectively (Fig. 3). The S/N ratios of fraction no. 19 to TB+. TB±, and TB- were 1.9, 1.9, and

Table 1. Protein identification of MonoQ® antigenic fraction by proteomic analysis

Fraction no.	Protein name (Mb No.)	Function	Identified peptides in 18/19*	Score by MASCOT in 18/19	Theoretical size (kDa)**	pI
18, 19	MPB70 (Mb2900)	Immunogenic protein	24/26	186/264	19	4.5
18, 19	MPB83 (Mb2898)	Cell surface lipoprotein	13/22	222/336	22	4.7
18, 19	MPT64 (Mb2002c)	Immunogenic protein	16/24	201/338	25	4.6
18, 19	Fas (Mb2553c)	Fatty acid synthase	22/23	167/163	326	4.8
18, 19	RpoC (Mb0687)	RNA polymerase beta subunit	8/9	140/146	147	6.1
18	Smc (Mb2946c)	Chromosome partition protein	15/–	164/–	131	4.9
18	Nrp (Mb0104)	Peptide synthase	4/–	156/–	269	5.6
18	Transposase (Mb2814c)	Transposase	7/–	129/–	51	6.1
18	LeuA (Mb3737)	2-isopropyl-amalate synthase	10/–	120/–	76	4.9
18	MbtE (Mb2401c)	Peptide synthase	18/–	120/–	183	5.1
19	CFP21 (Mb2006c)	Cutinase	–/40	–/240	22	5.9
19	Mb0973c	Chorismate mutase	–/74	–/426	12	7.0
19	GroEL1 (Mb3451c)	Chaperonin	–/44	–/337	56	4.7
19	PepA (Mb0130)	Serine protease	–/18	–/292	35	4.9
19	FixB (Mb3054c)	Electron transfer flavoprotein	–/10	–/276	32	4.4
19	PpiA (Mb0009)	Iron-regulated peptidyl-prolyl cis-trans isomerase	–/17	–/257	19	6.2
19	LppX (Mb2970c)	Lipoprotein	–/12	–/222	24	4.8
19	GreA (Mb1109c)	Transcription elongation factor	–/20	–/212	18	4.6
19	CFP10 (Mb3904)	10 kDa culture filtrate protein	–/20	–/200	11	4.3
19	MPT32 (Mb1891)	Fibronectin attachment protein	–/8	–/186	33	4.7
19	EsxN (Mb1821)	Esat-6 like protein	–/7	–/163	10	4.6
19	Mb3713c	Unknown	–/12	–/148	17	5.1

*Peptides corresponded to Rank number one of fraction no. 18 and 19, which means that it is high probable peptide in each fraction.
**Refer to BoviList (http://genolist.pasteur.fr/BoviList/)

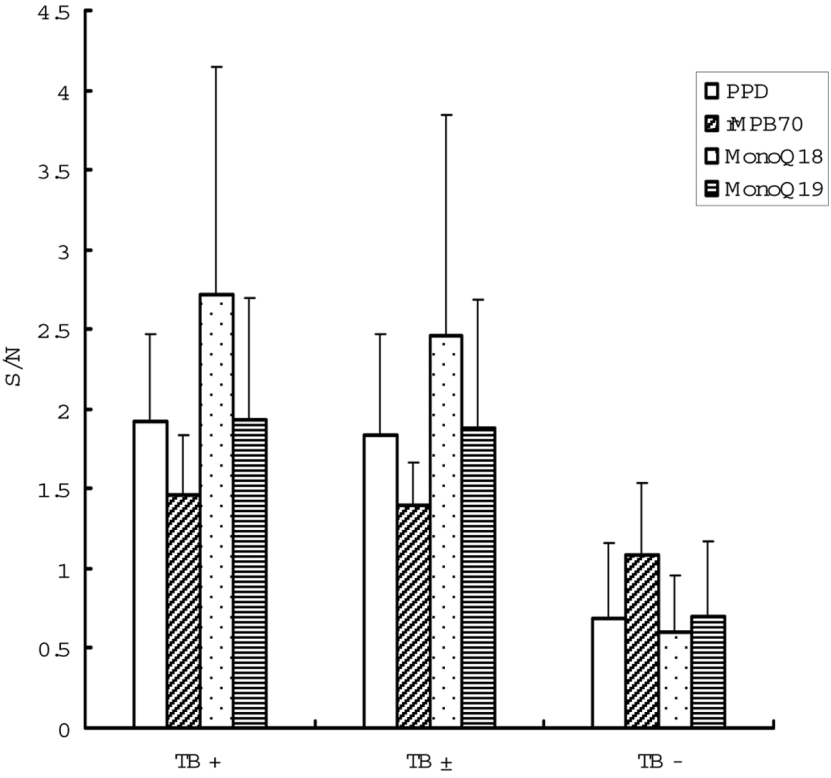

Figure 3. Comparison of PPD, recombinant MPB70, and MonoQ® fractions in ELISA. MonoQ® fraction no. 18 exhibited the highest postivity to an intradermal skin test (IST) positive reactors ($n = 15$) and false positive reactors ($n = 8$), and the lowest negativity to IST negative reactors ($n = 7$). TB+, bTB skin positive reactors; TB±, bTB skin false positive reactors; TB−, bTB skin negative reactors.

0.7, respectively, and those of PPD were similar to those of fraction no. 19 which contained 10 and 25 kDa as well as 20 kDa protein (Fig. 3).

DISCUSSION

These findings indicate that the first protein fraction obtained from the MonoQ® HR 5/5 column chromatography was highly antigenic and exhibited the greatest sensitivity and specificity to bTB positive cattle. The antigenome in this fraction was identified as MPB64, MPB70, MPB83, Fas, Smc, Nrp, RpoC, Transposase, LeuA, and MbtE.

Previously, many researchers have tried to identify B cell antigens to facilitate bTB diagnosis and the development of a vaccine. Purified protein derivative (PPD) has been previously used as an ELISA

antigen.[7,19] CFP precipitated with ammonium sulfate was used for the purification of B cell antigen in this study because it is a whole protein in *M. bovis*. Chromatofocusing,[7] anion-exchange chromatography,[7,11,12] gel filtration,[7,11,12] hydrophobic interaction or reverse phase chromatography,[14] and lectin-affinity chromatography on concanavalin A – Sepharose[12] have been used for the purification of *M. bovis* antigens. We used the MonoQ® HR 5/5 column as anion-exchange chromatography without further purification steps, which can result in the loss of dominant proteins. The purification step in this study was simplified, and the loss of antigenic proteins was reduced. The fraction no. 18 had only 20 kDa protein on SDS-PAGE (Fig. 3); this fraction exhibited the highest specificity and sensitivity in the bTB ELISA, compared with PPD, recombinant MPB70, and proteins purified by MonoQ® (Fig. 3).

We defined the antigens in the MonoQ® fraction of *M. bovis* CFP by state-of-the-art proteomic analysis, which included in-gel digestion, mass spectrometry and PMF by database search. Of those, MPB64, MPB70, and MPB83 were identified in fraction no. 18; these are the same proteins as the known dominant B cell antigens in *M. bovis*.[1,2,10,19,20–27] In addition, 7 proteins were newly identified as B cell antigens in fraction no. 18; Fas, Smc, Nrp, RpoC, Transposase, LeuA, and MbtE (Table 1). Proteomic analysis has been developed to identify biomarker that can be used for the diagnosis and vaccine development.[28,29] The definition of the *M. bovis* proteome through proteomic analysis cannot be performed until whole genome sequencing of *M. bovis* is completed.[6,15–17] Previously reported antigens were identified by their size or resolving rate by SDS-PAGE and Western blot analysis using positive control serum and defined monoclonal antibodies;[10,19,21–23] these proteins can be now defined by proteomic analysis.[5,7,9–11,19,21–24,27] The newly identified antigens in fraction no. 18 did not theoretically match the 20 kDa protein (Table 1). This might be due to degradation caused by heat treatment step after culture. We confirmed the antigenicity of 20 kDa protein by immunoblotting using cattle serum infected with *M. bovis* (Fig. 2). Once the antigens in *M. bovis* were identified by serological diagnostics, they were further produced as recombinant forms and B-cell epitopes of those were identified at the peptide level.[1,2,15,20,23–27] In the same way, the newly identified antigens in this study could be produced in their recombinant forms and used in the future for serological diagnostics of bTB.

Bovine TB has been diagnosed at ante mortem by IST, in which cattle were injected intradermally with PPD prepared from an *M. bovis* AN5 culture.[2,26] In addition, γ-IFN detection in the blood after the stimulation of specific *M. bovis* antigens, *M. bovis*-specific gene detection by polymerase chain reaction (PCR) and *M. bovis* isolation from milk, nasal discharge, feces, and urine were also used for bTB ante mortem diagnosis;[30] however, these diagnosis are time-consuming and laborious.

The serological diagnosis of bTB has been intensively researched because it has several advantages over IST. For example, it is an economical test because it can be performed in a high-throughput manner, serum can be shared with other serological diagnosis and it produces the objective data.[2,20,26,27] Cell-mediated immunity (CMI) can be used to diagnose bTB at an early stage because it increases at early and middle stages of bTB infection; however, it decreases at late and terminal stages of bTB infection at which point cattle may be in anergy status to CMI-based diagnosis.[8] Humoral immunity (HI) increases after the middle stage of bTB infection and peaks at late stages of infection.[8] Bovine TB diagnosis detecting HI can therefore be used to compensate for those detecting CMI, such as IST and γ-IFN assay. When serological diagnosis of bTB is used as ancillary diagnosis, the sensitivity of detection of bTB tuberculosis will increase.

CONCLUSIONS

In this study, 20 kDa protein was fractionated and analyzed through peptide match fingerprinting. The 20 kDa protein was purified using a MonoQ® HR 5/5 column as anion exchange chromatography and applied to ELISA, resulting in the improvement of sensitivity and specificity of the bTB ELISA. MPB64, MPB70, and MPB83 were known as major B cell antigens in *M. bovis* CFP, and also identified in this study. Nineteen proteins in addition to MPB64, MPB70, and MPB83 identified in fraction no. 18 and 19 and the identified proteins could be produced in their recombinant forms and evaluated for use as bTB ELISA antigens in the future.

ABBREVIATIONS

bTB, bovine tuberculosis; IST, intradermal skin test; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; PMF, peptide match fingerprinting; BCA, bicinchoninic acid; CFP, culture filtrate protein; FPLC, fast performance liquid chromatography; NC, nitrocellulose; ABTS, 2,2-azino-di(3-ethylbenzothiazoline-6-sulphonate); RT, room temperature; OD, optical density; PPD, purified protein derivative; PCR, polymerase chain reaction; CMI, cell-mediated immunity; HI, humoral immunity.

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