# Overproduction of the bleomycin-binding proteins from bleomycin-producing *Streptomyces verticillus* and a methicillin-resistant *Staphylococcus aureus* in *Escherichia coli* and their immunological characterisation

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**Abstract The bleomycin-binding proteins designated BLMA and BLMS, which confer resistance to bleomycin (Bm), from Bm-producing *Streptomyces verticillus* ATCC15003 and a methicillin-resistant *Staphylococcus aureus* B-26, respectively, were overexpressed in *Escherichia coli*. The present study showed that both BLMA and BLMS quench the antibacterial activity of Bm by the binding to the drug. To immuno-chracterize the Bm-binding proteins, we constructed a monoclonal antibody against BLMA. The antibody, designated 893-12, did not cross react to BLMS and another Bm-binding protein from tallysomycin-producing *Streptoalloteichus hindustanus*. Although the ability of Bm to cleavage DNA was eliminated by a binding of BLMA to Bm, as shown by Sugiyama et al. [Gene 151 (1994) 11–15], the Bm-induced DNA degradation was restored by pre-incubation of BLMA with the anti-BLMA monoclonal antibody.**

*Key words:* Bleomycin; Bleomycin-binding protein; Methicillin-resistant *Staphylococcus aureus*; Monoclonal antibody; Polymerase chain reaction; *Streptomyces verticillus*

## 1. Introduction

Bleomycin (Bm) and the related antibiotic, phleomycin and tallysomycin inhibit DNA synthesis in bacterial and mammalian cells [1]. The antibiotic-producing microorganisms must be protected from the lethal effect of their own product. We have cloned and sequenced two independent genes, designated *blmA* and *blmB*, encoding Bm resistance determinants from Bm-producing *Streptomyces verticillus* ATCC15003 [2]. The genes *blmA* and *blmB* were shown to encode a Bm-binding protein and a Bm *N*-acetyltransferase [2–3], respectively. A gene designated Sh*ble*, which confers resistance to Bm, phleomycin and tallysomycin, has been cloned from tallysomycin-producing *Streptoalloteichus hindustanus* belong to a family Actinoplanaceae and sequenced [4]. The Sh*ble* gene encoded a small acidic protein ($M_r$ = 13,665) which was a binding protein with a strong affinity for Bm [5].

Almost all strains of methicillin-resistant *Staphylococcus aureus* (MRSA), isolated in Hiroshima University Hospital from October 1990 to April 1992, were resistant to Bm, although the drug has been not used as an antibacterial agent. We have cloned and sequenced a gene encoding a Bm resistance determinant from the chromosomal DNA of MRSA B-26 isolated clinically in the hospital [6]. The sequence analysis revealed that the gene, designated as *blmS* in the present study, was identical to that located on the staphylococcal plasmid pUB110 [6,7]. Interestingly, an inverted repeat, designated IR-r in an insertion sequence IS*431*mec which possibly mediated the transposition of *blmS* into the chromosome from pUB110, was located upstream from *blmS* of MRSA B-26 [6]. A transposon Tn*5* also carries a Bm-resistance determinant [8]. Although these Bm-resistance genes from MRSA B-26, pUB110 and Tn*5* have been sequenced [6–9], the characterisation of the gene products and the biochemical mechanism of resistance has not yet been elucidated in detail.

The aim of the present study is to overproduce and to characterize the *blmA* and *blmS* products, designated BLMA and BLMS, respectively, in *Escherichia coli* host. These are the first purification of the bleomycin-binding proteins. Moreover, this paper report on the successful generation of the anti-BLMA monoclonal antibody for the immunological characterisation of BLMA and BLMS.

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*Abbreviations:* Ap, ampicillin; Bm, bleomycin; *blmA*, a gene encoding Bm-binding protein from *Streptomyces verticillus*; BLMA, a protein encoded by *blmA*; *blmS*, a gene encoding bleomycin-binding protein from a methicillin resistant *Staphylococcus aureus*; BLMS, a protein by *blmS*; IPTG, isopropyl-β-D-thiogalactoside; *malE*, a gene encoding MBP; MBP, maltose-binding protein, MBP/BLMA, a fusion protein between MBP and BLMA; MRSA, methicillin resistant *Staphylococcus aureus*; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

*E. coli* TG1 harboring the pUC18-based p181EB1 plasmid [2] carries *blmA* cloned from the chromosomal DNA of Bm-producing *S. verticillus* ATCC15003. *E. coli* HB101 and pKK*trp* plasmid were used to overexpress *blmS* from MRSA B-26.

### 2.2. Construction of plasmids to overexpress BLMS and BLMA in E. coli

To amplify the *blmA* structural gene (369 bp) having *Eco*RI and


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*Bam*HI sites at the 5′- and 3′- adjacent regions, respectively (Fig. 1), a sense- and an antisense-oligonucleotide PCR primers were designed and chemically syntheized, based on the nucleotide sequence of *blmA* [2]. The plasmid p181EB1 was used as a template for amplification of *blmA* by PCR. The amplified DNA fragment was inserted into the *Eco*RI-and *Bam*HI-digested pUC18 to generate pUAB10. The pUAB10 plasmid was digested with *Eco*RI and *Pst*I and the small fragment was subcloned into pKK223–3 [10] (Pharmacia LKB Biotechnology product) digested with the same restriction enzymes, to generate pUAB20. The *Eco*RI–*Pst*I fragment from pUAB20 was inserted into the *Eco*RI–*Pst*I digested pMALc-2 (New England Biolabs) to create pMALB10. *E. coli* host harboring pMALB10 was resistant even to 1,000 μg Bm/ml.

To overexpress BLMS under the control of *trp* prpmoter, the *blmS* structural gene having *Eco*RI and *Bam*HI sites at the 5′ and 3′-adjacent regions [2], respectively, was inserted into the same enzymes-digested pKK*trp* plasmid to generate pKK*trp*S and introduced in *E. coli* HB101. The transformed cells were also resistant to over 1,000 μg Bm/ml.

*2.3. Isolation of BLMA and BLMS*

*E. coli* TB1 harboring pMALB10 was grown in LB medium containing 200 μg Ap/ml. To induce the expression of fusion gene, IPTG was added at a final concentration of 1 mM. The cells were grown at 37°C until stationary phase and sonicated. The cell extract was subjected to the column (1.0 × 10 cm) of amylose resin according to the instruction manual of a Protein Fusion and Purification System Kit (New England Biolabs) based on the previous report [11]. The column was washed with buffer I (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA-$Na_3$ and 10 mM 2-mercaptoethanol). The elution was done with buffer I containing 10 mM maltose. After the fusion protein (MBP/BLMA) was digested with blood coagulation factor $X_a$ protease, the digests were subjected to a hydroxyapatite column chromatography to remove maltose and then applied to the amylose resin column. BLMA was purified to homogeneity away from MBP by the affinity chromatography step.

*E. coli* HB101 harboring pKK*trp*S was cultivated in M9-casamino acid medium [12] containing 100 μg Ap/ml. To induce expression of the *trp* promoter-controlled *blmS* gene, 3-indoleacrylic acid was added at a final concentration of 0.13 mM during the exponential phase of growth. The harvested cells were ground with quartz sands and centrifuged at 18,000 × *g* for 20 min. Solid ammonium sulfate was added to the supernatant fluid at 20% saturation and the resulting precipitate was removed by centrifugation. BLMS was precipitated by addition of ammonium sulfate at 40% saturation, dissolved in the small amount of buffer III (5 mM Tris-HCl (pH 7.2), 0.5 mM EDTA and 5% glycerol) and subjected to DEAE-Sepharose CL-6B column (1 cm × 20 cm) equilibrated with 10 mM Tris-HCl (pH 7.2) and eluted with the same buffer containing 0–1 M NaCl (linear gradient of concentration). One of four fractions eluted with approximately 0.5 M NaCl contained BLMS together with the very insignificant impurities.

*2.4. N-termial amino acid sequence*

The N-terminal amino acid sequences of BLMA and BLMS were determined by Edman degradation method using the autosequencer.

*2.5. Generation of anti-BLMA monoclonal antibody*

The purifed MBP/BLMA (50 μg) in saline (50 μl) was mixed with Freund's complete adjuvant (50 μl) and injected intraperitoneally to BALB/c mice. At 12, 18 and 31 days, respectively, they were boosted with 100 μg of the MBP/BLMA. The spleen cells from the immunized mouse, harvested at 3 days after the last injection, were fused with P3/X63-Ag-8.U1 murine myeloma cells. Hybridomas-secreting anti-MBP/BLMA antibodies were screened by ELISA: that is, 96-wells immunoplates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with MBP/BLMA, BLMA or MBP (1 μg/ml, 100 ml/well) in PBS (76 mM phosphate, 0.45% NaCl, pH 6.4) at 37°C for 1 h, washed with the deionized water and then blocked with 0.5% bovine serum albumin in PBS at 25°C for 2 h. The antigen-coated wells were incubated with the culture supernatants from hybridomas for 1 h at room temperature, followed by the incubation with horseradish peroxidase(HRP)-labeled anti-mouse immunoglobulins (Dako-Japan) for 1 h at 25°C. After washing with saline containing 0.05% Tween 20, the captured HRP activities were measured by the method described in [13]. The hybridomas in the selected wells were cloned by limiting dilution method, and reactivities of the candidates were confirmed by ELISA again.

*2.6. SDS-PAGE and Western-blotting*

Tricine-SDS-PAGE system [14] was employed for the resolution of small proteins such as BLMA at lower acrylamide (10%) concentrations than in glycine-SDS-PAGE system [15] which have an insufficient resolving power below 10 kDa. The protein was electrophoretically transferred to a nitrocellulose membrane and then detected by enzyme-antibody method using the specific antibody.

## 3. Results and discussion

*3.1. Overexpressions of BLMA and BLMS in* E. coli

Since we could not obtain BLMA from *E. coli* harboring p181EB1 because of the less amount of production, we tried to overproduce BLMA as a protein fused to the maltose-binding protein (MBP) [11]. The plasmid pMALB10 was used to produce a fusion protein between BLMA and MBP proteins. Since the fusion protein (Fig. 2A, lane 2) contains a recognition site digested with blood coagulation factor $X_a$ protease, it was cleavaged into the two domains (Fig. 2A, lane 3). The BLMA was purified to homogeneity by affinity chromatrography to a closslinked amylose-resin (Fig. 2A, lane 5). Although the open reading frame of *blmA* consisting of 366 bp, is predicted to encode a protein of $M_r$ = 13,179 [2], BLMA always migrated on Tricine-SDS-PAGE at about 15.5 kDa, which is larger than expected. We confirmed that the first 20 amino acid sequence from the N-terminus was Ile-Ser-Glu-Phe-Val-Val-Lys-Phe-Leu-Gly-Ala-Val-Pro-Val-Leu-Thr-Ala-Val-Asp-Val and identical with that deduced from the nucleotide sequence of *blmA* [2] except the additional Ile-Ser-Glu-Phe sequence at the N-terminus (Fig. 1). We also sequenced the N-terminal amino acid of BLMA expressed directly under the control of *trp* promoter. The N-terminal was Met-Val-Lys-Phe-Leu-Gly-Ala-Val-Pro-Val-Leu-Thr-Ala-Val-Asp, indicating that the initiator GTG codon for *blmA* is read as Met but not Val, and the N-terminal Met is not processed by the N-terminal Met-aminopeptidase in *E. coli*.

The isoelectric point (pI) of the purified BLMA, which was measured with PAGE containing carrier ampholites (pH 3–5, Bio-Lyte, Bio-Rad Laboratories, California, USA), was 4.67 (data not shown). The theoritical pI calculated from the

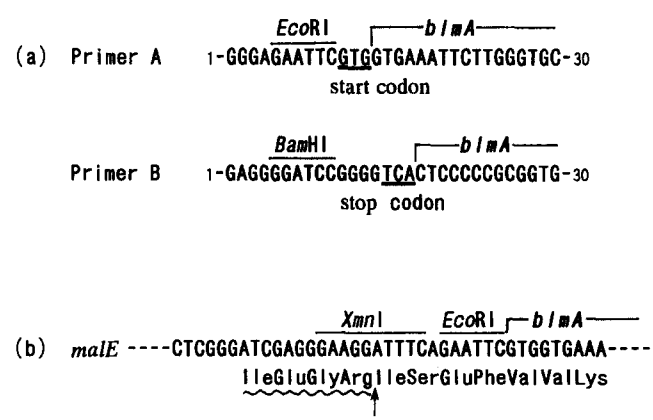


Fig. 1. Design of PCR primers to antisense- (primer A) and sense (primer B)-DNAs to amplify the artificial *blmA* gene (a), and the nucleotide and amino acid sequences of 5′-end of *blmA* fused to *malE* having the recognition (⌒) and cleavage sites (↑) for blood coagulation factor $X_a$ protease between the two domains (b).

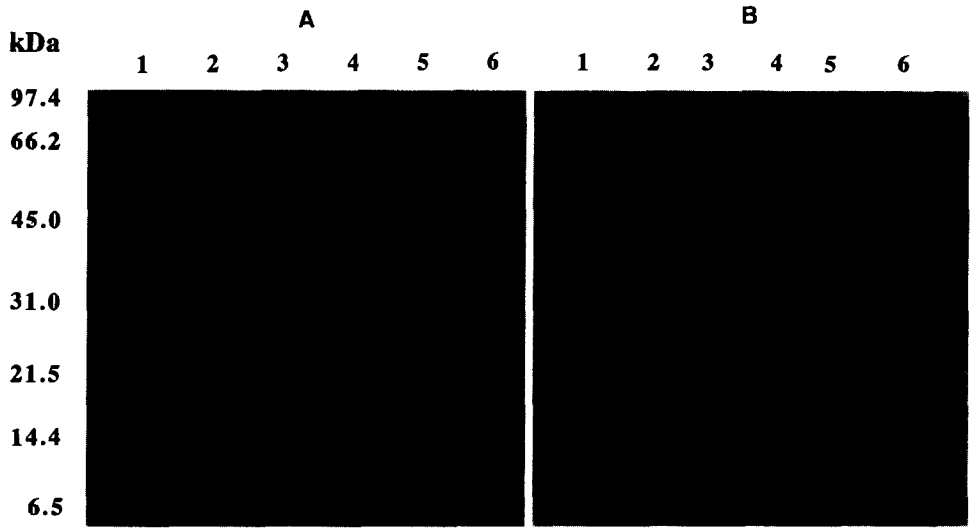


Fig. 2. Tricine-SDS-PAGE of BLMA purified from *E. coli* harboring pMALB10 (A) and Western blot analysis of anti-BLMA monoclonal antibody to BLMA (B). (A) Gel stained with Coomassie brilliant blue. Lanes: 1, standard proteins; 2, MBP/BLMA (1 μg) purified from *E. coli* TB1 harboring pMALB10; 3, the sample (4 μg) cleavaged between MBP and BLMA with factor $X_a$ protease; 4, MBP (1 μg); 5, the purified BLMA (1 μg); 6, Sh*ble* protein (1 μg, purchased from CYRLA, Toulouse, France). Molecular weights are indicated on the left side. (B) Gel stained with Vectastain *Elite* ABC Kit containing the biotinated-anti-mouse IgG (Vector Laboratories, Inc., CA, USA) using anti-BLMA monoclonal antibody 839–12 for immuno blot analysis. lanes: 1 and 3, BLMA (1 ng); 2, Sh*ble* protein (100 ng); 4, MBP (100 ng); 5, the sample (50 ng) cleavaged between MBP and BLMA; 6, MBP/BLMA (10 ng).

deduced amino acid sequence of BLMA having additional Ile-Ser-Glu-Phe at the N-terminus was 4.38, indicating that BLMA is an acidic protein similar to the Sh*ble* protein which has a very low theoritical pI of 3.78 [9].

We have previously suggested that BLMA prevents the Bm-induced DNA breakage in vitro, and that the prevention is likely to occur without enzymatic modification of the drug [2]. The present study shows that the antibacterial activity of Bm disappeared when incubated with BLMA (data not shown).

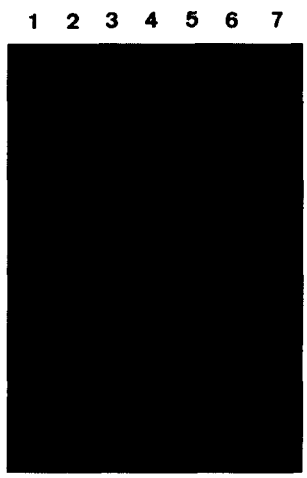


Fig. 3. Agarose gel electrophoresis of DNA treated with Bm alone, in combinations with BLMS. Bleomycin (Bm) $A_2$ sulfate was used in the present study. After BLMS was incubated with Bm (0.5 μM), for 5 min at room temperature, 100 mM $FeSO_4$, 1 mM dithiothreitol and the linearized-pBR322 plasmid obtained by digestion with *Eco*RI (0.2 μg), were added and kept for 5 min at room temperature. The reaction mixtures were stopped by addition of 40 mM EDTA and subjected to agarose gel (0.8%) electrophoresis. Lanes: 1 and 7, DNA; 2–5, Bm + DNA + BLMS (1.5, 3.0, 4.5 and 6.0 μM, respectively); 6, Bm + DNA.

The antibacterial activities of pepleomycin and phleomycin was also quenched during incubation with BLMA. However, heat-treatment in a boiling water for 10 min or proteinase K-digestion at 37°C for 60 min of Bm/BLMA complex significantly restored the antibacterial activity of the antibiotic (data not shown).

BLMS was directly overproduced under the control of *trp* promoter and practically obtained as a single protein (data not shown). We confirmed that the first 15 amino acid sequence from the N-terminus was Met-Leu-Gln-Ser-Ile-Pro-Ala-Leu-Pro-Val-Gly-Asp-Ile-Lys-Lys and identical with that deduced from the nucleotide sequence of *blmS*. The N-terminal Met of

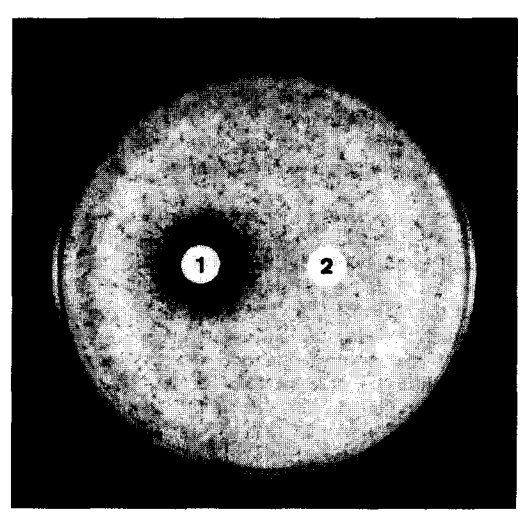


Fig. 4. Assay for antibacterial activity of Bm by incubation with BLMS. *Bacillus subtilis* IFO3134 was used as a test organism. Bm (5 μg) dissolved in 9 mM Tris-HCl (pH 7.5) was incubated in the absence (1) or presence (2) of BLMS (220 μg) in the same buffer for 5 min at room temperature and applied on a paper disk.

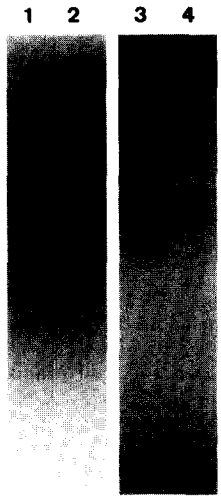


Fig. 5. The profile of electrophoretic migration of BLMA or BLMS incubated with Bm. BLMA (3.75 μg) or BLMS (8.5 μg) was incubated with Bm (5 μg) for 3 h at 16°C and subjected to the native-PAGE containing 15% polyacrylamide. Lanes: 1, BLMA without Bm; 2, BLMA with Bm; 3, BLMS without Bm; 4, BLMS with Bm.

BLMS is also not processed in *E. coli*. BLMS prevents the Bm-induced DNA breakage in vitro without enzymatic modification of the drug (Fig. 3, lanes 4 and 5). The antibacterial activity of Bm disappeared when incubated with BLMS (Fig. 4). The theoritical molecular weight and pI calculated from the deduced amino acid sequence of BLMS were 14, 926 and 4.68, respectively.

We observed that BLMA or BLMS incubated with Bm, respectively, migrated more slowly in native-PAGE than those without Bm (Fig. 5), indicating that Bm, a cationic antibiotic, forms an electrostatic complex by the binding to the acidic BLMA or BLMS.

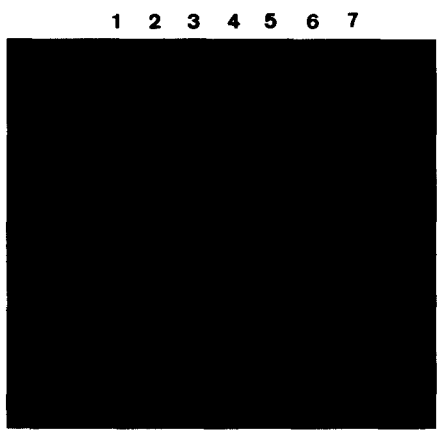


Fig. 6. Agarose gel electrophoresis of DNA treated with Bm alone, in combinations with BLMA or anti-BLMA antibody. After BLMA was incubated with or without anti-BLMA antibody for 5 min at room temperature, Bm (0.5 μM), 100 mM $FeSO_4$, 1 mM dithiothreitol and the linearized-pBR322 plasmid obtained by digestion with *Eco*RI (0.2 μg), were added and kept for 5 min at room temperature. The reaction mixtures were stopped by addition of 40 mM EDTA and subjected to agarose gel (0.8%) electrophoresis. Lanes: 1 and 7, DNA; 2, Bm + DNA; 3, Bm + DNA + BLMA (1.15 μg); 4–6, Bm + DNA + BLMA pre-incubated with anti-BLMA antibody (5.78 μg , 11.56 μg and 17.34 μg as proteins, respectively).

### 3.2. *Specific binding of anti-BLMA monoclonal antibody to BLMA*

For generation of anti-BLMA monoclonal antibodies, we used the MBP/BLMA fusion protein to enhance the immunogenecity of BLMA, a low molecular weight protein. Seven candidates of anti-BLMA antibodies which showed both anti-MBP/BLMA and anti-BLMA activities but not anti-MBP activity were selected by ELISA. In these candidates, an antibody, designated 893–12, showed the highest reactivity. Western-blot analysis revealed that 893-12 cross reacts with BLMA having molecular weight of 14 kDa (Fig. 2B, lane 1 and 3), but not with MBP of 43 kDa (Fig. 2B, lane 4), suggesting strongly that 893–12 is a monoclonal antibody which recognizes BLMA but not MBP.

When incubated with BLMA, Bm lost the ability to degrade DNA (Fig. 6, lane 3). On the other hand, although the Bm-induced DNA cleavage was protected when BLMA was pre-incubated with the less amounts of 893–12 antibody (Fig. 6, lane 4), the ability of Bm to degrade DNA was restored by increasing the amounts of the anti-BLMA antibody against BLMA (Fig. 6, lanes 5 and 6), suggesting that Bm no longer bind to the BLMA which formed the complex with the anti-BLMA antibody.

### 3.3. *Immunological properties of BLMA*

We have previously compared these translated protein sequences from the genes encoding Bm-resistance determinants from transposon Tn5, pUB110, MRSA B-26 [3] and a tallysomycin-producing microorganism with that from *blmA*, and shown that the homology with the Sh*ble* protein from tallysomycin producer was 60% [2], but were 25–32% with the other Bm-resistance determinants.

In the present experiments, we analyzed for immunological homology between the BLMA and the Sh*ble* protein. A polyclonal antibody raised against Sh*ble* protein in rabbit did not cross react with BLMA (Fig. 7B, lane 2). In addition, Sh*ble* protein did not cross react with the monoclonal antibody 893-12 against BLMA, even when applied at the concentration of

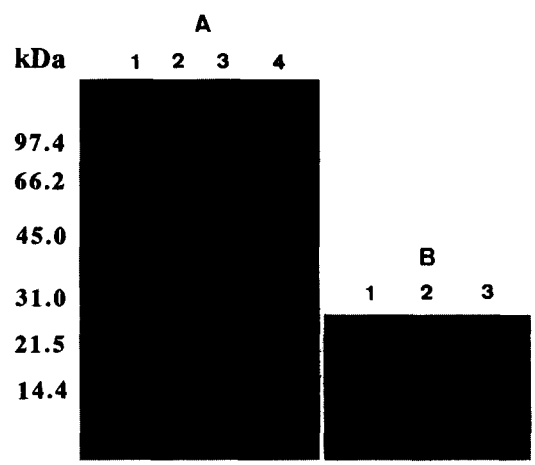


Fig. 7. Western blot analysis of BLMA and Sh*ble* proteins by anti-Sh*ble* protein antibody. (A) Gel stained with Coomassie brilliant blue. Lanes: 1, standard proteins; 2 and 4, Sh*ble* protein (1 μg); 3, BLMA (1 μg). Molecular weights are indicated on the left side. (B), gel stained with Vectastain Elite ABC kit using polyclonal antibody raised against Sh*ble* protein in rabbit for imuno blot analysis. Lanes: 1 and 3, Sh*ble* protein (10 ng); 2, BLMA (10 ng).

100-fold of BLMA (Fig. 2B, lane 4). In addition, the anti-BLMA monoclonal antibody also did not cross react with BLMS (data not shown). Thus, these Bm-binding proteins are functionally the same between Bm-producing and Bm-resistant microorganisms, whereas not immunologically.

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