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Cloning, Expression, and Identification of a Novel Extracellular Cold-Adapted Alkaline Protease Gene of the Marine Bacterium Strain YS-80-122

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Abstract As one of the most important groups of industrial enzymes, cold-adapted protease has been studied widely. An extracellular cold-adapted alkaline protease metalloproteinase (MP), produced by a marine bacterium strain YS-80-122, has been purified. The NH_2-amino acid sequence of the purified alkaline protease MP was ANGTSSAFTQ, which was identical to that of the serralysin from *Pseudomonas* sp. "*TAC II 18*". The MP structural gene (*lupA* gene) was cloned by inverse PCR, and the open reading frame of 1,443 bp encoded a 463 amino acid protein (without signal peptide). Sequence alignment reveals that the alkaline protease MP belongs to the serralysin-type metalloproteases. The recombinant protein LupA was expressed in *Escherichia coli*, and Western blotting confirmed that the LupA was homologous to the cold-adapted alkaline protease MP.

Keywords Cold-adapted · Alkaline protease · Metalloprotease · *lupA* · Serralysin

Introduction

In recent years, there has been a growing interest in enzymes from psychrophilic microorganisms, for these special enzymes can serve as models in studies on thermal stability and molecular adaptation of proteins, as well as be potential candidates for biotechnological applications [1]. When compared with their mesophilic counterparts, the cold-adapted enzymes are usually characterized by a higher specific activity at a low temperature and also by a lower stability versus temperature and denaturing agents [2–5].

Proteases represent one of the three largest groups of industrial enzymes and account for 60% of the total worldwide sale of industrial enzymes [6]. Proteases are the most widely used enzymes in the detergent industry because of the nonenzymatic detergents' inefficiency of removing proteins from textile fibers [7]. The increased usage of cold-adapted proteases as detergent additives is mainly due to their excellent cleaning

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capabilities in an environment friendly manner and compatibility with nonphosphate detergents. In addition to high washing efficiency, the use of enzymes allows lower washing temperatures and shorter washing time, which directly results in saving energy [8].

At present, knowledge of full nucleotide sequences of the enzyme genes has facilitated the deduction of the primary structure of the encoded enzymes and, in many cases, identification of various functional regions [9]. These sequences also serve as the basis for predicting the secondary structure of proteins and help in the study of structure–function relationships of the enzyme, which can further expand its application fields.

Life in marine environments requires physiological and biochemical adaptations of microorganisms [2] which usually produce low-temperature enzymes [10]. Recently, a marine bacterium strain YS-80-122 was isolated from the Yellow Sea sediment in China. The cold-adapted alkaline protease metalloproteinase (MP) produced by this bacterium exhibits a maximum activity at 30 °C, stability at pH values between 8 and 11, and is insensitive to phenylmethanesulfonyl fluoride. In addition, we found that MP is stable and active in the presence of surfactants (such as Tween 80 and Tween 40) and bleaching agents (such as H_2O_2) [11]. All these results indicate that the cold-adapted alkaline protease MP has potential application as a detergent additive. Therefore, in this study, the gene *lupA* encoding MP was cloned, characterized, and expressed in *Escherichia coli*. Furthermore, the recombinant protein LupA was identified by Western blotting.

Material and Methods

Strains and Media

The marine bacterium strainYS-80-122 was isolated from Yellow Sea sediment. *E. coli* DH5α[F^-(*Φ80d lacZ ΔM15*) Δ(*lacZYA-argF*) U169 *recA1 endA hsdR17* (*rk*, *mk*) *supE44 thil gyrA relA1*] was used to transform plasmids carrying the cloned gene. *E. coli* BL21 (DE3) was a host strain for expressing recombinant LupA. Strain YS-80-122 was grown in GB medium (peptone 1.2%, yeast extract 2.4%, glycero 0.4%, NaCl 2.5%) at 18 °C. All *E. coli* strains were grown in Luria-Bertani broth (LB) medium [12] at 37 °C. DNA polymerases, restriction endonucleases, and DNA ligase were from Fermentas (China). Appropriate antibiotics were supplemented at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml.

Plasmids

The pGMT vector was purchased from Tiangen Company in China. The protein expression vector pET28a came from Novagen. The plasmid pETLUPA was constructed as follows: *lupA* was amplified by PCR with primer UAF1/UAR1, and the 1460 bp product was ligated to the pGMT vector. After digestion of pGMT-lupA with NdeI and XhoI, the *lupA* DNA fragment was inserted into pET28a.

Enzyme Purification from Strain YS-80-122

The strain YS-80-122 was grown in GB medium for 48 h at 18 °C on a shaker at 200 rpm. The culture was centrifuged at10,000*g* for 20 min at 4 °C. The supernatant was precipitated by adding filtered ammonium sulfate slowly up to 65% saturation with gentle stirring and left for 4 h at 4 °C for complete precipitation. The precipitate was collected after

centrifugation at 12,000 rpm for 20 min at 4 °C, air-dried, and re-dissolved in a minimum amount of Tris buffer (10 mM Tris, 100 mM NaCl, pH 8.0). The crude enzyme was dialyzed against the same Tris buffer for 24 h with 6–8 changes. Finally, purification to homogeneity was achieved by fast protein liquid chromatography (FPLC) using Superdex 200 column HR10/30 (10*300~310 mm, Amersham Pharmacia Biotech, Uppsala, Sweden) which had been equilibrated with Tris buffer described above and then eluted with the same buffer. The peak with protease activity was collected and used for SDS-PAGE analysis.

N-terminal Sequencing

To determine the N-terminal sequence, protein bands from SDS gels were transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was stained with 0.1% (*w*/*v*) Ponceau S in 2% (*v*/*v*) acetic acid, and the stained bands were cut out and subjected to an N-terminal sequence analysis with a model PPSQ-10S protein sequencer (Shimadzu).

DNA Manipulation

The genomic DNA was prepared according to the method reported by Ausubel et al. [13]. Bacterial plasmid DNA was purified by using Perfectprep plasmid minikits (OMEGA). Restriction endonuclease digestions and DNA ligations were performed according to the manufacturer's recommendations. *E. coli* was transformed with plasmid DNA according to Sambrook et al. [12].

Cloning of Partial MP Gene

PCR was used for partial extracellular alkaline protease DNA amplification. Genomic DNA was prepared as described above. Degenerate primers 5F1(5′-GCNAAYGGNACN WSNWSNGCN-3′) and 5R1(5′-NCCNGGRTGNSWNARNCC-3′) were designed based on the N-terminal sequence of the MP protein and the conserved sequence of the other alkaline protease. The PCR program was as follows using the Taq DNA polymerase at 95 °C 5 min, 95 °C 30 s, 45 °C to 55 °C 1 min, 72 °C 1 min for 30 cycles, followed by extension for 6 min at 72 °C. The recovered PCR product and pGMT vector were circulated by T4 DNA ligase at 25 °C for 2 h and transformed into *E. coli* DH5α competent cells. The transformants were selected on plates with ampicillin. The plasmids in the transformant cells were extracted by using the methods described above and sequenced by Shanghai Sangon Company.

Full-Length MP Gene Cloning by Inverse PCR

Inverse PCR was performed according to the methods described by Triglia et al. [14]. The genomic DNA was first cleaved with Sau3AI for 1 h at 37 °C. Afterwards, the enzyme was heat-inactivated at 75 °C for 10 min. The diluted DNA was then ligated overnight and used as PCR templates. The primers for inverse PCR were designed according to the partial sequence encoding MP as described above. The forward primer was5F2 (5′-GCGTTGCTGGCGTTTCCATAGGCT-3′) and the reverse primer was 5R2 (5′-TTCTTCTCGCTCCAGTAA-3′). PCR conditions were 95 °C 5 min; 95 °C 30 s, 45 °C to55°C 1 min, 72 °C 3 min; 30 cycles, final extension at 72 °C for 6 min. The PCR products were checked and separated by agarose gel electrophoresis and sequenced as described above. The cloned full-length MP gene was named *lupA*.

Nucleotide Sequence Accession Number and Sequence Analysis

Nucleotide and amino acid sequence searching was conducted using the BLAST and ORF Finder programs at the National Center for Biotechnology Information (NCBI). The nucleotide sequence of *lupA* has been deposited in the GenBank database under accession no. GU084389.

Expression of Recombinant LupA in *E. coli*

The primers for amplification of the *lupA* gene were designed to express recombinant protein in *E. coli*. The forward primer was UAF1 5′-CGCGCATATGTCGAAACTTAAA GAG-3′ (underlined bases encoding NdeI site), and the reverse primer was 5′-UAR1CTCGAGCGCGACGATGTCGTAAGTT-3′ (underlined bases encoding XhoI site). The plasmid pETLUPA was constructed as described above. Then, the recombinant plasmid was transformed into the *E. coli* strain BL21 (DE3) which was a most widely used host having the advantage of being deficient in both *lon* and *ompT* proteases. After reaching an optical density of 0.6~0.8 at 600 nm, the production of target protein from BL21 (DE3)/pETLUPA was induced by the addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were induced for 12 h at 20 °C and were harvested by centrifugation (15,000*g*, 20 min at 4 °C). The cells were lysed with 8 M urea and subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western Blot Assay for Identifying the Recombinant Protein

Specific alkaline protease antibodies were generated as follows: the Wistar rat was immunized with 50–100 μg of purified MP three times with about 1-month intervals. The titers of the antibodies were tested by usual method (Peng [15]). The recombinant protein and control samples were subjected to SDS-PAGE. The proteins were stained with Coomassie brilliant blue G-250 or electrophoretically transferred to a nitrocellulose sheet (Millipore). The blots were blocked by soaking them in 5% nonfat dry milk in phosphate-buffered saline (PBS) for 1 h and then incubated with anti-MP antiserum (diluted 1:1,500) for 1 h. The nitrocellulose sheet signals were detected with horseradish peroxidase-conjugated anti-rat immunoglobulin G (diluted 1:2,000) and the enhanced chemiluminescence system.

Results and Discussion

Purification of the Cold-Adapted Alkaline Protease MP

The cold-adapted alkaline protease MP was partially purified to homogeneity by precipitation with ammonium sulfate (65%). After dialysis, the crude enzyme was further purified by FPLC using Superdex 200 column. The collected samples were run through SDS-PAGE to detect proteolytic activity. One peak showing proteolytic activity was obtained. SDS-PAGE was done by the method of Zhao Bosheng et al. [16]. Separating gels contained 15% acrylamide. When analyzed by SDS-PAGE, the sample from peak 1exhibited a single band with a molecular mass of about 49 kDa in the gel stained with Coomassie blue (Fig. 1). And this result was confirmed by gel filtration chromatography, suggesting that the enzyme is monomeric. The NH_2-amino acid sequence of the purified

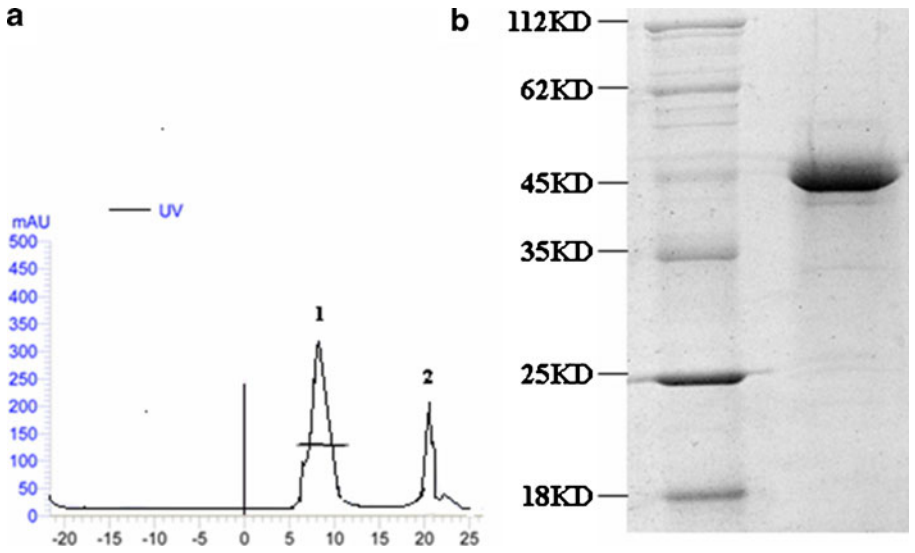

Fig. 1 **a** Superdex 200 chromatography of the alkaline protease MP. The sample from peak 1 was collected. **b** SDS-PAGE analysis of purified MP from YS-80-122 strain. Molecular mass (MW) standards (in kilodaltons) are shown in the *left-hand lane*; the purified MP from peak 1 is shown in the *right-hand lane*

alkaline protease was ANGTSSAFTQ, which is identical to that of the serralysin from *Pseudomonas* sp. "*TAC II 18*" [2].

Full-Length MP Gene Cloning and Sequence Analysis

The degenerate primers 5F1 and 5R1 were synthesized on the basis of the NH_2-amino acid sequence of the purified MP and the consensus amino acid sequences of serralysins. The PCR-generated fragments were sequenced. Analysis of the sequence by BLAST program indicated that the fragment (550 bp) was a partial gene of the putative metalloprotease gene. To further obtain the full-length metalloprotease gene, the new primers were designed according to the sequenced fragment and used for PCR amplification from the genomic DNA of strain YS-80-122 by inverse PCR as described in Materials and Methods. Finally, the inverse PCR products with 1,482 bp were obtained and sequenced. The MP gene, designated *lupA*, consisted of a 1,443-bp ORF, which corresponds to a protein of 463 amino acid residues (without signal peptide). There is a typical Shine-Dalgarno consensus sequence (AGGAAC) seven nucleotides upstream from the start codon ATG. Sequence alignment revealed that the *lupA* displayed the highest percentage identity (84% at the DNA level; 91% at the deduced amino acid level) to *Pseudomonas* sp. "*TAC II 18*" PAP (Fig. 2), with identity at the amino acid level to serralysin-type metalloproteases of other strains, *Pseudomonas fluorescens* Pf-5 and *Dickeya zeae* Ech1591 at 66% and 53%, respectively. The results indicated the alkaline protease MP belongs to the serralysin-type metalloproteases.

The alignment of Conserved Domain Database (CDD) of MP from strain YS-80-122 at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed several conserved regions and active sites in the serralysin-like superfamily (Fig. 3). Most metalloproteases are zinc-containing proteins. Zinc is an integral component of many proteins which are involved in

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MP    MSKLKEKAALSVNPTFAANGTSSAFTQVDNFSHFYDRGNHLVNGKPSFTVDQAADQLTRS
PAP   MSKLKEKAALSVNQTFAANGTSSAFTQIDNFSHFYDRGEHLVNGKPSFTVDQVADQLTRS
      ************ ************.********** ************ *******

MP    GASWYDLNGDGVINLSYTFLTSPPPGYASRGLGTFSSFSGLQKEQAKLSLESWADVAKVT
PAP   GASWHDLNNDGVINLTYTFLTAPPVGYASRGLGTFSQFSALQKEQAKLSLESWADVAKVT
      **** ***.******.*****.** ********** ** ********************

MP    FTEGPAARGDDGHMTFANFSASNGGAAFAYLPNSSRKGESWYLINKDYDVNKTPGEGNYG
PAP   FTEGPAARGDDGHMTFANFSASNGGAAFAYLPNSSRKGESWYLINKDYQVNKTPGEGNYG
      ************************************************ ***********

MP    RQTLTHEIGHTLGLSHPGDYNAGNGNPSYRDAVYGEDTRAYSVMSYWSEKNTGQVFTKTG
PAP   RQTLTHEIGHTLGLSHPGDYNAGNGNPTYRDAVYAEDTRAYSVMSYWSEKNTGQVFTKTG
      ****************************.****** *************************

MP    EGAYASAPLLDDIAAVQKLYGANMETRAGDTVYGFNSTADRDYYSATSATDKLIFSVWDG
PAP   EGAYASAPLLDDIAAVQKLYGANLETRADDTVYGFNSTADRDFYSATSSTDKLIFSVWDG
      ************************.**** *************.*****.***********

MP    GGNDTLDFSGFSQNQKINLAAGSFSDVGGMTGNVSIAQGVTIENAIGGSGNDLLLGNAAS
PAP   GGNDTLDFSGFSQNQKINLTAGSFSDVGGMTGNVSIAQGVTIENAIGGSGNDLLIGNDAA
      ********************.************************************.** *.

MP    NILKGGAGNDIIYGGGGADKLWGGSGSDTFVYREVSDSTPKAADTLMDFQTGLDKIDLTG
PAP   NVLKGGAGNDIIYGGGGADVLWGGTGSDTFVFGAVSDSTPKAADIIKDFQSGFDKIDLTA
      *.****************** ****.******.  ********** .  ***.* ******

MP    ITHLSGLNFVNAFTGQAGDAVVSYNQASNAGSLQVDFSGHGVADFLITTVGQVATYDIVA
PAP   ITKLGGLNFVDAFTGHAGDAIVSYHQASNAGSLQVDFSGQGVADFLVTTVGQVATYDIVA
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Fig. 2 Amino acid alignment of alkaline metalloprotease MP and PAP. The signal peptide is *bold* and *italic*; the NH2-amino acid sequence of the purified alkaline protease MP was *underlined*; zinc is coordinated by the consensus sequence HEIGHTLGLSH which was *boxed*; four continuous calcium-binding domains GGXGXD were *shaded in gray*

virtually all aspects of metabolism of the different species of all phyla. X-ray crystallographic analyses of several zinc-containing proteins have defined the features of the catalytic and structural zinc-binding sites [17, 18]. In the serralysin subgroup, the zinc is coordinated by the consensus sequence HEXXH+H, the three histidines being involved in a Zn^{2+} coordination. The equivalent sequence HEIGHTLGLSH (Fig. 2) was found in the alkaline protease MP. In addition, serralysins and related proteases have a calcium-binding domain C-terminal to the metalloprotease domain, which contains multiple tandem repeats of a nine-residue motif including the pattern GGXGXD, which forms a parallel beta roll that may be involved in the translocation mechanism and/or substrate binding [19]. In the C-terminal of the protease MP, there are four continuous calcium-binding domains which increase the thermal stability of MP. In subtilisin [20] as well as in the case of the cold-

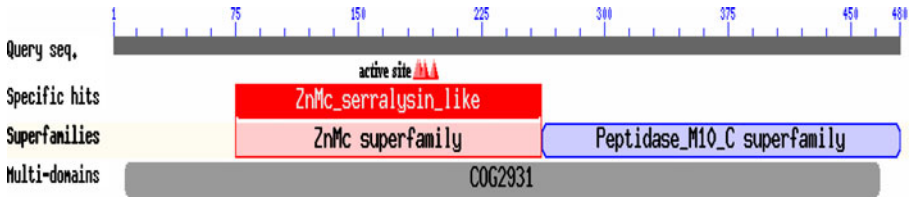

Fig. 3 The alignment of Conserved Domain Database (CDD) of MP from strain YS-80-122 at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The *red rectangle* and *triangle*, respectively represent several conserved regions and active sites in the serralysin-like superfamily

adapted K-amylase [21], a lower affinity constant for Ca^{2+} is observed. MP also displays a greater sensitivity towards moderate EGTA concentrations (5 mM) indicating that the global affinity for Ca^{2+} is lower and could contribute to increasing the flexibility of the molecular structure.

Expression of Recombinant LupA in *E. coli*

PCR amplification was carried out with primer UAF1 and UAR1 as described above. Plasmid DNA containing the mature LupA clone in pGMT was digested with NdeI and XhoI and cloned into the pET28a expression vector digested with the same enzymes. Then, the recombinant plasmid Petlupa was sequenced and transformed to competent *E. coli* BL21 (DE3) cells for overexpression, which were plated on LB/kanamycin plates, and individual colonies were screened by PCR method for the presence of the *lupA* gene. Miniprep plasmid DNA from a LupA-positive colony was subsequently used. Expression was induced by the addition of IPTG (final concentration 0.4 Mm) to a 500-ml culture of transformed *E. coli* BL21 (DE3), and the cells were pelleted after 12 h of continued shaking at 20 °C. Meanwhile, a pET28a expression plasmid vector without *lupA* gene was manipulated by the same methods. Then, the induced cells were lysed as described above for SDS-PAGE. The recombinant protein LupA has significant expression compared with the control (Fig. 4). The molecular mass of the recombinant protein LupA is consistent with the estimated mass of the purified metalloprotease from the supernatant of the strain YS-80-122 culture. Though we tried several methods to induce the recombinant protein, the recombinant protein LupA was still insoluble and accumulated as inclusion bodies without proteolytic activities (data not shown). Probably because of the specific secretion apparatus of alkaline metalloprotease MP, the recombinant protein could not be folded normally in *E. coli* cells.

Western Blot Assay for Identifying the Recombinant Protein

Because the proteolytic activities of the recombinant protein LupA were not detected, it was necessary to identify the recombinant protein LupA by a Western blot assay. The

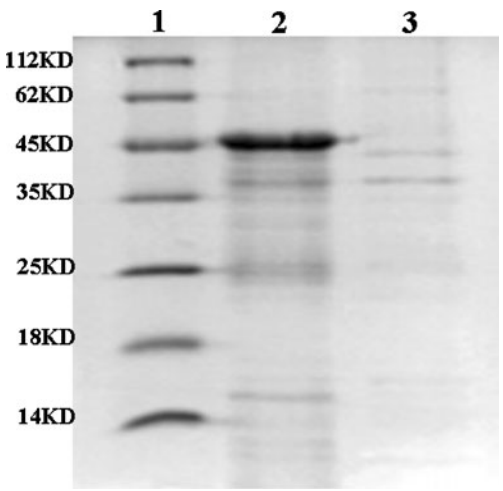

Fig. 4 SDS-PAGE analysis of the recombinant protein. *Lane 1*, molecular standard markers; *lane 2*, the recombinant protein LupA; *lane 3*, negative control (BL21 strain with pET28a plasmid)

primary antibody was obtained by immunizing Wistar rat using purified MP. The titers of the antibody were 1,500 times compared with control (data not shown). Then, the recombinant proteins, positive and negative controls, were subjected to SDS-PAGE. The positive control was purified MP, and the negative control was serine protease DegQ (Wei-wei [22]). After SDS-PAGE, one half of the protein gel was stained with Coomassie brilliant blue G-250, and the other was electrophoretically transferred to a nitrocellulose sheet at the same time. The immunoblotting was carried out using the method described above. Figure 5 shows that the recombinant protein LupA can specifically recognize the anti-MP and displayed the band, which was consensus to the positive control on the nitrocellulose sheet. The results confirmed that the recombinant protein LupA was homologous to the alkaline protease MP.

Summary

The alkaline protease MP produced by marine bacterium YS-80-122 has a high specific activity at low temperature and is stable and active in the presence of some surfactants and bleaching agents. These properties are extremely useful in detergent additive applications. In this paper, we purified the MP protein and obtained the NH_2-amino acid sequence "ANGTSSAFTQ"; the full-length MP gene was cloned by inverse PCR. Sequence alignments reveal that the alkaline protease MP belongs to the serralysin-type metalloproteases and has the highest percentage identity with the psychrophilic enzyme PAP [2]; the recombinant protein LupA accumulated as inclusion bodies in *E. coli*. Western blotting confirmed that the LupA was homologous to MP protein. In order to obtain a recombinant protein with proteolytic activity, we are planning to use other systems to express the cold-adapted alkaline protease MP.

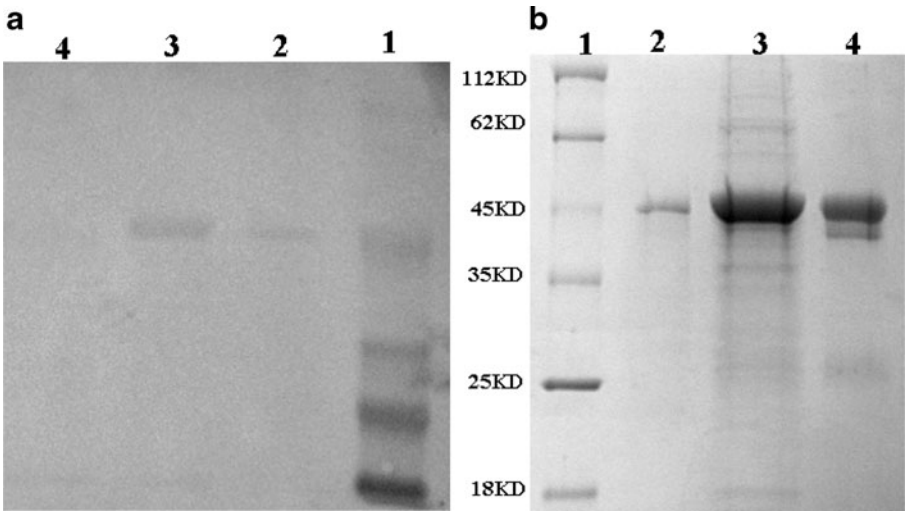

Fig. 5 **a** Western blotting. **b** SDS-PAGE. *Lane 1*, molecular standard markers; *lane 2*, the MP protein (positive control); *lane 3*, the recombinant protein LupA; *lane 4*, serine protease DegQ (negative control)

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