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ORIGINAL PAPER

# Expression and purification of two major outer membrane proteins from *Vibrio alginolyticus*

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**Abstract** *Vibrio alginolyticus*, a Gram-negative bacterium, is one of *Vibrio* pathogens common to human and marine animals. Outer membrane proteins of bacteria play an important role during infection and induction of host immune response. In present research, two outer membrane protein genes (*OmpK* and *OmpW*) of *V. alginolyticus* were cloned and expressed. The open reading frames of *OmpK* and *OmpW* contain 846 bp and 645 bp, respectively, the mature proteins consist of 261 and 193 amino acid residues. At the signal peptides positions –3 to –1, the amino acids were V-M-A in *OmpK* and V-F-A in *OmpW*, which consistented with the observed sequence V-X-A of the signal peptides of transmembrane OMP. The alignment analysis indicated that both proteins were highly conserved, which could serve as surface antigens for vaccine candidates. SDS-PAGE indicated two genes over-expressed in *E. coli* BL21 (DE3). By affinity chromatography on $Ni^{2+}$-nitriloaceate resin, the recombinant proteins were purified from inclusion bodies. Western blot analysis revealed that both proteins had immunoreactivity, which provided a base for further study on the evaluation of diagnostication and vaccine candidates.

**Keywords** Characterization · Expression · Outer membrane proteins · Purification · *Vibrio alginolyticus*

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## Introduction

In Gram-negative pathogenic bacteria, the outer membrane plays an important role in the infection and pathogenicity to host (Tsolis 2002). In the outer membrane, membrane proteins have a crucial role during many cellular and physiological processes. They are basically composed of protein, lipid and sugar, which could be easily recognized as foreign substances by immunological defence systems of hosts. Among these components, outer membrane proteins (OMPs) are particularly attractive for the development of vaccine candidates and diagnostic kits (Ding et al. 1996; Pizza et al. 2000).

Among OMPs of bacteria, OmpK and its homologues are widely distributed among *Vibrio* and *Photobacterium*, it was shown to be the receptor of KVP40, a broad-host-range vibriophage in *V. parahaemolyticus* (Inoue et al. 1995). Another OMP, OmpW, belongs to a family of small OMPs which are widespread in Gram-negative bacteria, it forms an 8-stranded beta-barrel with a long and narrow hydrophobic channel which involve in osmoregulation(Xu et al. 2004, 2005) and transportation of small hydrophobic molecules across the bacterial outer membrane (Hong et al. 2006; Albrecht et al. 2006). OmpW is a major antigen in bacterial infections and host immune response, it has been demonstrated to be immunogenic in *V. cholera* (Nandi et al. 2005; Stevenson et al. 1985).

However, little information of OMPs has been focused on *V. alginolyticus*, which is a common pathogen to human and sea animals (McLaughlin et al. 1999). This organism mainly causes such clinical syndromes as wound infections, otitis media, food-intoxication, gastroenteritis and septicemia (Hlady and Klontz 1996; Morris and Black 1985; Daniels et al. 2000). Furthermore, it is one main *Vibrio* pathogen which causes serious episode to marine animals, such as marine fish, shrimp and shellfish, and leads to large economic damage (Lee et al. 1996; Zorrilla et al. 2003).

In present study, two major membrane protein genes (*OmpK* and *OmpW*) were cloned and expressed from *V. alginolyticus*, the recombinant proteins were purified by immobilized nickel affinity chromatography, which provided a base for further study on the evaluation of vaccine candidates and diagnostication.

## Materials and methods

### Bacterial strains and plasmids

*V. alginolyticus* strain ZJ04107 was isolated from pathogenic large yellow croaker (*Pseudosciaena crocea*) cultured in marine cage in Xiangshan Bay of Zhejiang Province, China. Strain ZJ04107 was cultured on thiosulfate–citrate–bile salt–sucrose (TCBS) agar plate (Tianhe, Hangzhou, China), then inoculated into ZoBell2216E broth (Yeast extract 1.0 g, Bacto-Tryptone 5.0 g and $Fe_2(PO_4)_3$ 0.01 g lysed in 1L seawater, pH 7.5) and maintained in Zobell2216E broth containing 15% glycerol at –70 °C. *E. coli* strains DH5α and BL21(DE3) (Novagen) were used to subclone and express the *OmpK* and *OmpW* genes. Plasmid pGEM-T-Easy vector (Promega) was used to conserve the cloned genes for sequencing. pET-30a(+) (Novagen) with His-tags was used to construct recombinant expression plamids.

### Genomic DNA extraction

The overnight cultures of *V. alginolyticus* strain ZJ04107 were centrifuged at 6,000 rpm for 5 min, the cells washed twice with sterile distilled water and centrifuged at 6,000 rpm for 5 min. After added 50 μL double distilled water ($ddH_2O$) and 50 μL TZ solutions (5.0% TritonX-100, 5.0 μg/mL $NaN_3$), the pellets were placed at –20 °C for 30 min, then boiled for 10 min, iced for 10 min, centrifuged at 10,000 rpm for 2 min. The collected supernatants which could be used as genomic DNA template for PCR were stored at –20 °C.

### Cloning of the *OmpK* and *OmpW* genes

The primers PK1/PK2 and PW1/PW2 (Table 1[t1]) were designed according to the sequences of the most conserved regions of other *Vibrios*' *OmpK* and *OmpW* genes in GenBank (Fig. 1), they were used to acquire the full-length open reading frames (ORF) of these two genes. Polymerase chain reaction (PCR) system(50 μL): 10 × PCR Buffer 5 μL, $MgCl_2$ (25 mM) 5 μL, dNTP (10 mM) 2 μL, PK1/PK2 or PW1/PW2 (200 mM) 2 μL, respectively, Taq DNA Polymerase (5 U/μL) 1 μL, DNA Template 2 μL, $ddH_2O$ 31 μL. PCR was performed with initial denaturation at 95 °C for 3 min; 32 cycles with denaturation for 45 s at 94 °C, annealing for 30 s at 54.5 °C and extension for 3 min at 72 °C; the reaction was completed by incubation at 72 °C for 10 min. The PCR product was analyzed by electrophoresis on a 1.0% agarose gel, and purified by PCR purification kit (Sangon, Shanghai, China). The purified PCR products were subcloned into the pGEM-T-Easy vector (Promega). The recombinant plasmids were identified by restriction enzyme analysis and sequenced at an automatic ABI prism 3730 sequencer (Invitrogen). Cloning, restriction endonuclease digestion, and DNA ligation were carried out according to standard protocols (Sambrook et al. 2000). Restriction enzymes and DNA ligation kits (version 2) were purchased from Takara.

### Construction of recombinant expression plasmids

In order to amplify the encoding sequences of the mature OmpK and OmpW proteins, the signal peptide sequences of the two ORFs were deleted, and two pairs of primer PK3/PK4 and PW3/PW4 with specific restriction sites (*Bam*HI in the forward primers and *Xho*I in the reverse primers) (Table 1) were respectively designed from the cloned nucleotide sequences. The PCR was carried out on a thermocycler 9700 (Applied Biosystems, Norwalk, CT, USA) with Taq DNA polymerase (Promega, Madison, WI, USA). The reaction procedures as follows: initial denaturation at 94 °C for 3 min; 30 cycles of 35 s at 94 °C, 30 s at 60.5 °C and 1.5 min at 72 °C; followed by an additional 10 min at 72 °C. The PCR products digested with *Bam*HI/*Xho*I were purified with DNA gel extraction Kit (Sangon, Shanghai, China), and subcloned into the expression vector pET-30a(+) digested with the same enzymes, then transformed into *E. coli* DH5α, and cultured in LB plate with 50 μg/mL kanamycin. The positive recombinant cells were picked to culture in LB medium with 50 μg/mL kanamycin. The recombinant expression plasmids detected by restriction enzyme analysis and sequencing were named pET-OmpK and pET-OmpW.

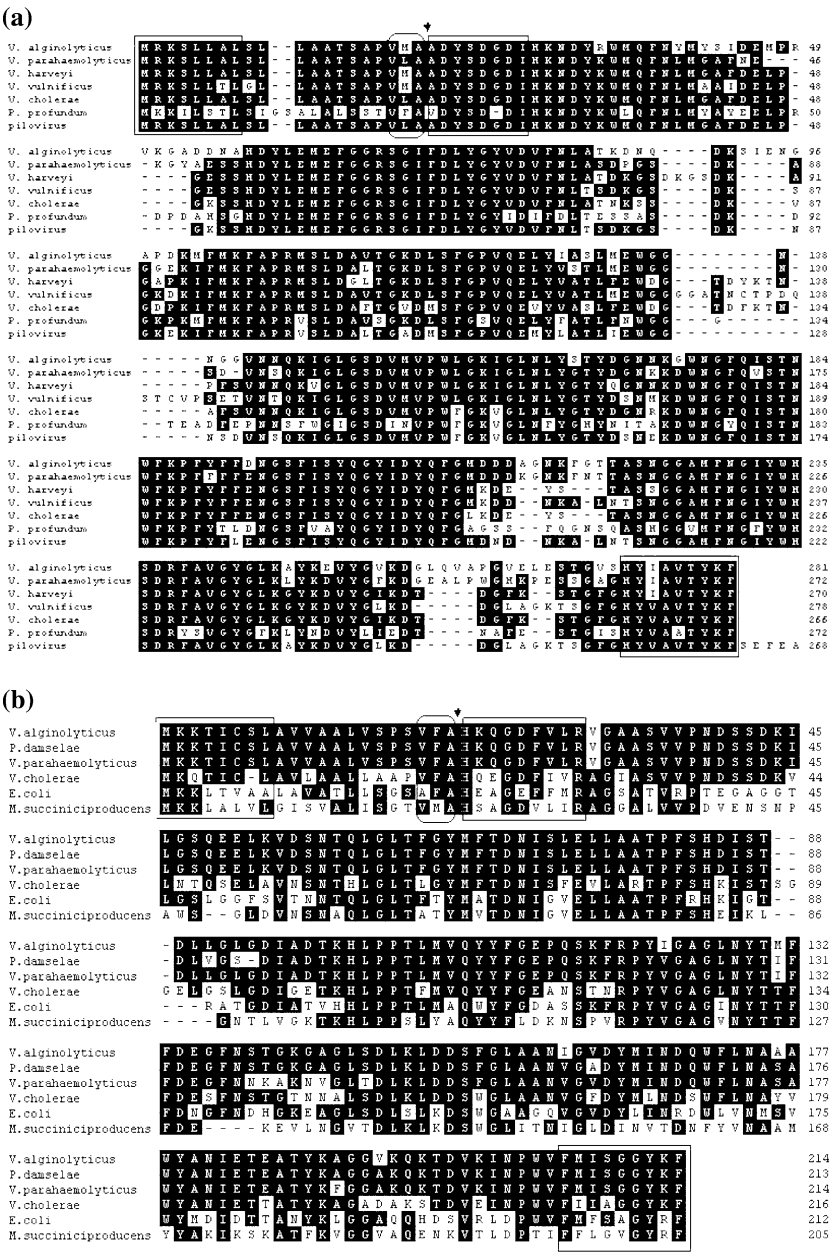


**Fig. 1** Alignment of OmpK (**a**) and OmpW (**b**) sequences from *V. alginolyticus* and other strains. The amino acid sequences were aligned using the Clustal W program from the European Bioinformatics Institute (Cambridge, United Kingdom). Identical amino acid residues presented were boxed in black, and boxes in gray indicated the location of similar residues. Alignment gaps were denolted by (–). The arrow indicated signal-sequence peptide cleavage site, V-X-A were indicated with an elliptic bracket. The regions used to design primers were indicated with the vertical brackets

Expression and purification of recombinant proteins

The overnight cultures of *E. coli* BL21 (DE3) harboring recombinant plasmids pET-OmpK, pET-OmpW and pET-30a(+) as a negative control were respectively diluted 1:100 (v/v) in fresh LB with Kanamycin (50 μg/mL), then incubated at 37 °C until the absorbance at 600 nm ($OD_{600}$) reached 0.6. The expression was induced with 1 mM isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) (BBI) for 5 h at 37 °C after the optimization of expression conditions.

Bacterial cells were harvested by centrifugation at 10,000 *g* for 20 min at 4 °C, then the whole cell pellets were washed in the solution containing 50 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl (pH 8.0), resuspended in 50 mM (pH 8.0) sodium phosphate buffer containing 10 mM imidazole and 100 mM NaCl, then incubated 30 min in ice bath. The cell suspension was disrupted by sonication in ice bath (350 W, 10 min × 3), then centrifuged at 10,000 *g* for 20 min at 4 °C, the denatured proteins from inclusion bodies were completely spin down. The aggregated proteins were washed in order with buffer I (50 mM Tris–HCl pH 8.0, 2 mM EDTA, 100 mM NaCl, 0.5% Triton X-100 (V/V), 4 M Urea), buffer II (50 mM Tris–HCl pH 8.0, 2 mM EDTA, 100 mM NaCl, 3% Triton

**Table 1** The primers aimed for cloning and constructing recombinant plasmids of ompK and *ompW* genes

| Primers | Sequences (5′ to 3′) | Aim for | Restriction site |
|---|---|---|---|
| PK1 | ATGCGTAAATCACTTTTAGCTCTTAG | cloning | |
| PK2 | TTAGAACTTGTAAGTTACTGCG ATGTAG | cloning | |
| PK3 | GCGAATTCGCGGCTACTTCAGCTCCAGTTATG | express | *Ban*HI |
| PK4 | GCCTCGAGTTAGAACTTGTAAGTTACTGCGATGTAGT | express | *Xol*I |
| PW1 | ATGAAAAAAACAATCTGCAGTC | cloning | |
| PW2 | TTAGAACTTGTAACCGCCGCTG | cloning | |
| PW3 | GCGAATTCGCAGTGGTTGCTGCACTCGT | express | *Ban*HI |
| PW4 | CGCTCGAGTTAGAACTTGTAACCGCCGCT | express | *Xol*I |

Notes: P, primer; K, *ompK*; W, *ompW*; ATG, the start site; TTA , the end site; GAATTC, the restriction site of *Ban*HI ; CTCGAG, the restriction site of *Xol*I

X-100) and buffer III (50 mM Tris–HCl pH 8.0, 2 mM EDTA, 100 mM NaCl, 0.5%Triton X-100, 2 M carbamidine hydrochloride). The washed proteins were dissolved in lysis buffer (50 mM Tris–HCl, 0.3% Sodium lauryl sulfate (SLS), 0.1 mM Dithiothreitol) at 4 °C for 15 h. Then the proteins were renatured in the buffer (50 mM Tris–HCl, 0.1 mM Dithiothreitol). The clear supernatants were loaded into a column packed with $Ni^{2+}$-nitriloaceate, which was charged with 50 mM $NiSO_4$, and purified by affinity chromatography on Ni-NTA Superflow resin (Pierce) according to the manufacturer's instructions. The eluted fractions were collected and analyzed by SDS-PAGE with 12% polyacrylamide gel. The protein concentration was determined by the Lowry method (Lowry et al. 1951). The protein pellets were stored at –40 °C before use.

## Antisera screening of recombinant proteins

Rabbit sera were used for detecting of the recombinant proteins. Two male adult New Zealand white rabbits were respectively immunized intramuscularly with the purified OmpK and OmpW recombinant proteins (300 μg each rabbit) emulsified with Freund's complete adjuvant. Four weeks later, the rabbits were boost respectively with the purified proteins in Freund's incomplete adjuvant (100 μg of proteins per dose). Two weeks later, the blood was collected from the rabbits, and clotted at room temperature for 1 h , stored at 4 °C overnight. The separated antisera by centrifugation were stored at –70 °C. The immunized rabbit sera were diluted 1:3000 and added to each well of recombinant samples for 15 h of incubation. After washing six times with 1 × PBS plus 0.5% Tween 20, the samples were incubated with 1:1000 secondary antibody goat anti-rabbit antibody conjugated with HRP for 15 h. After substrate development, the plate was read with a plate reader.

## SDS-PAGE and Western blot analysis

The expression of recombinant OmpK and OmpW proteins were examined by SDS-PAGE. The immunoactivity was analyzed by Western blot according to the standard protocol (Sambrook et al. 2000). Fifty microliters expressed protein samples were mixed with an equal amount of 2 × SDS loading buffer, and boiled for 10 min. 12% SDS polyacrylamide gel was used to analyze the recombinant proteins. SDS-PAGE was performed in the Protein Electrophoresis System Electrophoresis Power Supply EPS 601(Amersham). After electro-phoresis, the gel was stained with Coomassie brilliant blue R250 to visualize the protein bands.

After SDS-PAGE, protein bands were transferred onto a nitrocellulose membrane (Hybond-C, Amersham), the membrane was treated with blocking solution (5% non-fat milk, 20 mM Tris–HCl [pH 7.5], 10 mM NaCl, 0.02% $NaN_3$) for 2 h. After washed three times (15 min each time) with TBST (TBS with 0.05% Tween-20), the membrane was then incubated with the rabbit anti-OMPs (OmpK or OmpW) sera diluted 1:800 with gentle agitation for 1 h, and furthermore incubated for 1 h with horse radish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G antibodies (Roche Diagnostics) diluted 1:1,000. After thorough washing with TBST, the membrane was dipped in the HRP TMB (tetramethylbenzidine) Liquid Membrane Substrate (AMRESCO, Solon, USA) for color development.

## Bioinformatics analysis

The OmpK and OmpW genes were blastn in the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). The primers were designed with DNAStar and Primerpremier 5.0. Sequence alignment was performed with CLUSTAL W (Thompson et al. 1994). The signal-sequence peptide cleavage site was performed using SignalP 3.0 program on

the web (http://www.cbs.dtu.dk/services/SignalP-3.0/). All experiments were repeated at least three times.

## Results and discussion

### Cloning of the *OmpK* and *OmpW* genes

Based on the designed primers (PW1/PW2 and PK1/PK2) (Table 1), by PCR and sequencing, 645 bp and 846 bp flagments were respectively obtained from the prepared genome DNA of *V. alginolyticus* strain ZJ04107. The results of blastn in GenBank showed that the two flagments were the full-length ORFs of *OmpW* and *OmpK* genes respectively. The deduced amino acid sequences of two genes consisted of 214 and 281 amino acid residues with predicted molecular weight of 23.3 kDa and 31.3 kDa, respectively. (GenBank accession no. are DQ075316 for OmpW and DQ063588 for OmpK).

### Sequence characterization

Blastn in GenBank, the OmpK of *V. alginolyticus* was found to be high similarity with other *Vibrio* spp., amino acids identities as follows: 81.2% with *V. parahaemolyticus*, 78.1% with *V. harveyi*, 75.2% with *V. vulnificus*, 73.7% with *V. cholerae*. It also had high identity with the uncultured pilovirus(75%). For OmpW, amino acids identities were 96.2% with *P. damselae*, 94.4% with *V. parahaemolyticus*, 70.6% with *V. cholerae*, 57.1% with *E. coli*, which indicated that both OmpK and OmpW proteins were highly conserved. The results of alignment analysis also indicated that they were highly conserved (Fig. 1) and serve as surface antigens for good vaccine candidates. In the C-terminal, the OmpK protein had a phenylalanine, a characteristic common to many outer membrane proteins (Struywe et al. 1991).

Analyzed by the SignalP 3.0, the ORFs of OmpK and OmpW respectively included 20 and 21 amino acids signal peptide in the N-terminal domain, which had common characteristics to bacterial signal peptides (Inoue et al. 1995). Signal peptides possess a consensus cleavage site for a signal peptidase, at positions –3 to –1, A (G/S/L/T/V/C)-X-A is the most commonly observed sequence (von Heijne 1985, 1986). In Fig. 1, the arrow indicated signal-sequence peptide cleavage site, the amino acids at positions –3 to –1 were V-M(L/F)-A for OmpK and V(A)-F(M)-A for OmpW, which consistented with the observed sequence V-X-A of the signal peptides of transmembrane OMP. Characterized with a $\beta$-barrel structure, the transmembrane OMPs can translocate across the cytoplasmic membrane by the secretion machinery, cleave their signal peptides by leader peptidase (Lep) and arrive at the outer membrane (Schulz 2002; Eicken et al. 2002). The $\beta$-barrel which consists of antiparallel $\beta$-sheets is able to traverse the outer membrane since the hydrophobic residues of the amphipathic $\beta$-sheets are exposed on the exterior of the molecule, while the hydrophilic residues are concealed within the interior of the structure. OmpW forms an 8-stranded beta-barrel with a long and narrow hydrophobic channel which could be involved in the transportation of small hydrophobic molecules across the bacterial outer membrane (Hong et al. 2006; Albrecht et al. 2006).

### Expression and measurement of the recombinant OmpK and OmpW proteins

After identification, the constructed recombinant expression plasmids pET-OmpK and pET-OmpW were introduced into *E. coli* BL21 (DE3), and the negative control was cells transformed with pET-30a(+) vector alone. The host cells were cultured overnight in LB medium with 50 μg/mL kanamycin, then were diluted in the ratio 1:100 into fresh LB medium with 50 μg/ml kanamycin and cultured for another 3–4 h until $OD_{600}$ measurements reached 0.6–0.8 U. Then, the cell cultures of each sample were induced with 1 mM isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) at 37 °C. The preliminary experiments showed the induced recombinant cells started expression of protein at 2.5 h and reached a maximum level at 4–6 h. Thus, optimal time for the protein expression was 5 h after the chemical induction. The recombinant proteins were analyzed by SDS-PAGE with 12% gel, the results showed that both OmpK and OmpW recombinant proteins gained overexpression in *E. coli* BL21 (DE3) (Fig. 2a). By further Western blot analysis, the antigenicity of the recombinant fusion proteins were demonstrated. The immunized sera of rabbit anti-OMPs recognized the same sizes as both OmpK and OmpW recombinant fusion proteins with His-Tags (Fig. 2b).

### Purification of recombinant proteins

The recombinant cells containing two target proteins were respectively aggregated. The inclusion bodies were disrupted by sonication. Through lysis, denature and renature, about 350 μg of OMP per mg of bacterial dry weight were routinely obtained. Finally, the OMP supernatants were loaded into a Chelating nickel affinity column, purified by affinity chromatography on $Ni^{2+}$-nitriloaceate (Ni-NTA) resin. The OmpK and OmpW proteins were eluted with 0.5 M imidazole. SDS-PAGE analysis showed that the OmpK and OmpW His-tagged proteins were homogeneously purified, only a single band was detected from each purified protein sample (Fig. 3). The purification efficiency reached to 95%.

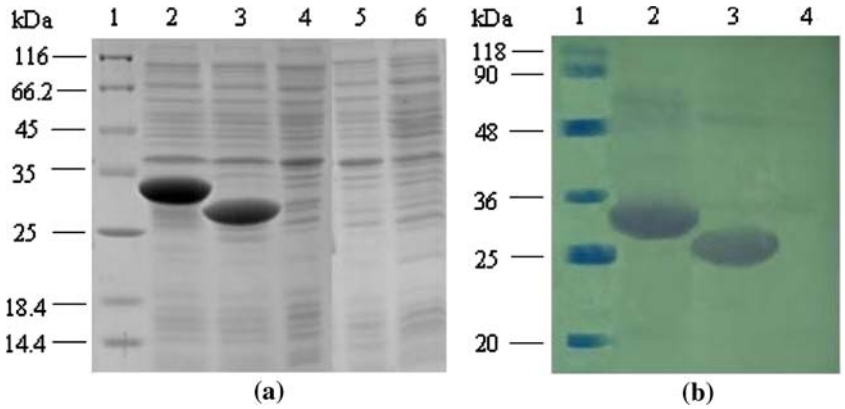


**Fig. 2** SDS-PAGE (**a**) and Western blot (**b**) analysis of pET-OmpK and pET-OmpW expression in *E. coli* BL21(DE3). The vectors or recombinant plasmids transferred into *E. coli* BL21(DE3) were cultured in LB medium with 50 μg/mL Kanamycin and induced with 1 mM IPTG. (**a**) Lane 1, protein molecular marker; Lane 2, 3, pET-OmpK and pET-OmpW with IPTG induction; Lane 4, 5, pET-OmpK and pET-OmpW without IPTG induction; Lane 6, pET-30a(+) with IPTG induction (**b**) Lane 1, protein molecular marker; Lane 2 and 3, pET-OmpK and pET-OmpW with IPTG induction; Lane 4, pET-30a (+) with IPTG induction

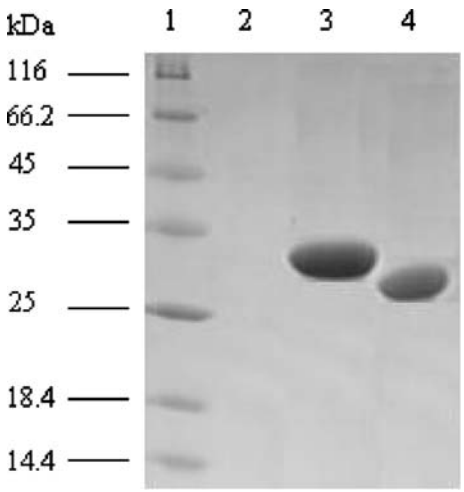


**Fig. 3** SDS-PAGE of the purified OmpK and OmpW proteins. Lane 1, standard protein molecular marker (molecular mass are indicated in kDa); Lane 2, the washed fraction (50 mM imidazole); Lane 3 and 4, the eluted OmpK and OmpW fractions

Generally, valuable vaccines and diagnostic reagents against their microorganisms primarily induce antibodies against surface structures. Outer membrane proteins have been thought of as useful antigens for development of both diagnostic reagents and vaccine candidates. In *V. cholera*, OmpW has been demonstrated to be strong immunogenic, which have been considered as vaccine development (Nandi et al. 2005). OmpW and ctxA as target genes in *V. cholera*, were used for clinical diagnostication and rapid screening of both toxigenic and nontoxigenic strains by one-step multiplex PCR assay (Nandi et al. 2000; Aeckersberg et al. 2001).

In this study, two major membrane protein genes (*OmpK* and *OmpW*) were firstly cloned from *V. alginolyticus*, and obtained over-expression in pET-30a(+) expression system. Since nickel reagent may capture recombinant protein fused with six-His tag, the recombinant proteins were purified with Ni-NTA resins. The result of Western blot analysis indicated that OmpK and OmpW proteins were immunoreactive, which could provide a base for further study on the diagnostication and evaluation if they could be used as effective vaccine candidates against infection by *V. alginolyticus* and other *Vibrio* species.

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