J. Microbiol. Biotechnol. (2004), **14**(6), 1343–1349

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Molecular Cloning and Characterization of the Gene for Outer Membrane Protein H in a *Pasteurella multocida* (D:4) Isolate from Pigs with Atrophic Rhinitis Symptoms in Korea

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Received: February 13, 2004
Accepted: April 27, 2004

Abstract A native strain of *Pasteurella multocida* was isolated from pigs suffering from severe atrophic rhinitis at domestic farms in Gyeonggi Province, Korea, and was identified as capsular serogroup "D" and somatic serotype "4" by disc diffusion decapsulation and gel diffusion precipitation tests, respectively. The *P. multocida* (D:4) induced atrophic rhinitis in healthy pigs by the secondary infection. The gene for outer membrane protein H (*ompH*) of *P. multocida* (D:4) was cloned in *Escherichia coli* DH5α by PCR. The open reading frame of the *ompH* was composed of 1,023 bp, possibly encoding a protein with 341 amino acid residues containing a signal peptide of 20 amino acids at N-terminus, and the gene product with molecular mass of ca. 38 kDa was identified by SDS-PAGE. Hydropathy profiles indicated that there are two variable domains in the OmpH. To express the *ompH* in *E. coli*, the gene was manipulated in various ways. Expression of the truncated as well as full-length forms of the recombinant OmpH was fatal to the host *E. coli* BL21 (DE3). However, the truncated OmpH fused with GST was consecutively expressed in *E. coli* DH5α. A large quantity of the fused polypeptide was purified through GST-affinity chromatography.

Key words: *Pasteurella multocida* (D:4), pig atrophic rhinitis, bacterial outer-membrane proteins, outer-membrane protein H (OmpH)

The Gram-negative facultative anaerobic bacterium, *Pasteurella multocida*, is one of the most notorious animal pathogens causing widespread infections; snuffles in rabbits, pneumonia and hemorrhagic septicemia in cattles, atrophic rhinitis in pigs, foul cholera in chickens, and common secondary invasions in various animals [11, 14, 19]. On occasion, people suffer from the infections mainly through animal bites, therefore, it is considered as a zoonosis. In the Western world, hemorrhagic septicemia is not common and the other infections are controlled with vaccines and antibiotics [1, 2, 18]. In less developed countries, however, pasteurellosis is poorly controlled, thus inflicting severe economic losses on domestic animal industries. Currently available vaccines, either inactivated or live, have their intrinsic defects [1, 18]: Inactivated vaccines can induce only serotype specific immunity, while attenuated live vaccines are able to provide limited heterologous protection, however, often to induce the diseases themselves [1, 2]. Therefore, more studies are needed to understand mechanism(s) of immunity to pasteurellosis.

Pathogenic strains of *P. multocida* have been classified into five serogroups (A, B, D, E, and F) based on capsular antigens, or into 16 serotypes based on antigens of somatic lipopolysaccharide (LPS) [3, 21, 22]. The LPS appears to be the major antigen involved in the serotype classification scheme, but its role in immunity is unclear [1, 20]. In fact, outer-membrane proteins (OMPs) of *P. multocida* have been recognized as immunodominant antigens, and they are thought to be responsible for cross-protective immunity, since LPS alone induces only partial protection against pasteurellosis in mice [1]. Vaccination of mice [19], chickens [25], and rabbits [16] with *P. multocida* OMPs stimulates significant protection against challenge with live bacteria [8, 16, 19, 25].

Porins located in outer membrane are attractive vaccine candidates for induction of homologous and heterologous immunity against infections of Gram-negative bacteria [18]. They are transmembrane polypeptides presiding

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in the outer membrane of Gram-negative bacteria and function as a molecular channel/gate to allow the diffusion of small hydrophilic molecules through the outer membrane, or as receptors for phages and bacteriocins [17, 18]. Several regions of various porins are well conserved among bacterial species as well as families. They share a basic structure containing a high proportion of antiparallel beta-chains that take on a barrel conformation [4]. Some beta-barrels are associated with the outer membrane as a homotrimer or remain as monomers. So, they have a high degree of homology in their primary amino acid sequences and secondary structures [12, 18]. These characteristics make porins attractive vaccine candidates.

Porin H is the major OMP in the envelope of *P. multocida* [17]. This protein, OmpH, has been purified and characterized as a porin in various serotypes and serogroups, because it has a high level of structural and functional similarity with the superfamily of porins in Gram-negative bacteria [4, 17]. It has homotrimeric conformation and stability in sodium dodecyl sulfate (SDS) at room temperature. The molecular masses of dissociated monomers vary between 34 and 42 kDa, depending on their serotypes of the bacteria and electrophoretic system used for analysis [18]. A number of OmpHs were purified, and their N-terminal amino acid sequences have been determined [4]. These N-terminal sequences are almost identical to those of OMP 179 and OMP 153 from strain P-1059, which are in the high-molecular-mass range [24].

In this study, we isolated a native pathogenic strain of *P. multocida* from pigs suffering from atrophic rhinitis at a farm located in Gyeonggi Province, Korea. It was identified as serogroup D and serotype 4. The outer membrane protein H gene (ompH) of *P. multocida* (D:4) isolate was cloned in *E. coli*. The activity and structure of the gene product were composed with those of porins. It is hoped that our results will be useful in protecting pigs from atrophic rhinitis in husbandry industries.

Molecular Cloning of *OmpH* from *P. multosida* (D:4) by PCR

Since the first event by L. Pasteur of immunization against infection with Gram-negative facultative bacterium, like *Pasteurella multocida* (the organism bearing his name), it has been known that many of the bacterial outer-membrane proteins (OMPs) are strong antigens [3, 11]. In particular, OmpH, one of the major OMPs in *P. multocida*, is considered as a good candidate antigen related to the immunization. Several other OmpHs have been identified in various strains of *P. multocida* and used in protective experiments against pathogens of severe pasteurellosis [1, 4, 5, 11]. In the last few years, domestic pigs suffered from severe atrophic rhinitis in Gyeonggi Province, Korea. During that period, we identifed the pathogen as *P. multocida*, and it was shown to be close to serogroup "D" and serotype "4" by disc diffusion decapsulation [3, 20, 21] and gel diffusion precipitation tests [22], respectively. This identification is correct, since swine atropic rhinitis has been known to be caused by serogroup D of *P. multocida* [14]. Soon after the pathogenicity of the isolate was confirmed by performing secondary infection of the disease to normal healthy pigs, we decided to study this pathogen at the molecular level. Thus, the isolate was grown overnight on Bacto™ brain-heart infusion (BHI) (Difco Laboratories, Detroit, MI, U.S.A.) agar at 37°C in a 5% CO_2 environment [5]. For broth culture, overnight seed cultures were used to inoculate BHI broth, and grown to a mid-logarithmic phase at 37°C with vigorous shaking. Genomic DNA isolated from the pathogen [9, 15] was used as template for PCR-based *ompH* cloning. Nucleotide sequence of the PCR primers was determined with the aid of BLAST sequence database (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD, U.S.A.). The forward oligomer (OMPH-F) was from the 32nd to the 55th nt (5'-GTGATACTATGAA-AAAGACAATCG-3'), and the reverse oligomer (OMPH-R) was from the 1,193rd to the 1,216th nt (5'-GATCCA-TTCCTTGCAACATATTGA-3') in the nucleotide sequence (Fig. 2). The transcription initiation codon (ATG) was

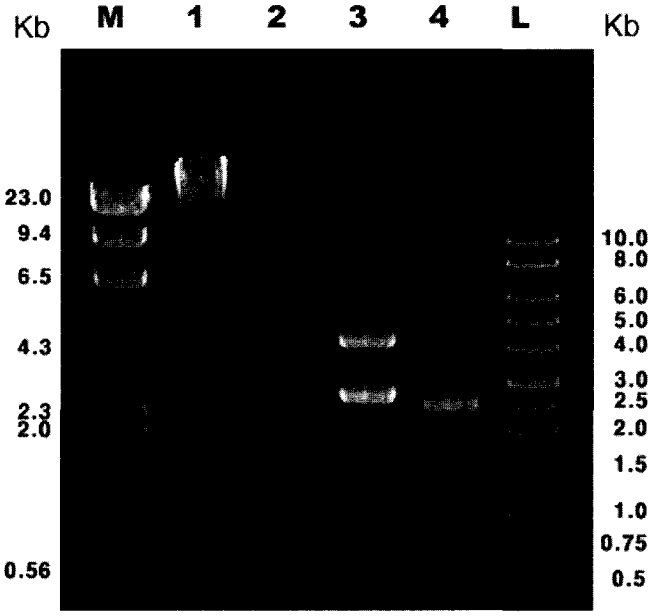

Fig. 1. Electrophoresis patterns of PCR products amplified with *ompH*-specific primers, cloning vector (pGEM-T/OmpH), and expression vector (pRSET B/OMPH-ORF-F).
The expected sizes of the PCR products and the pGEM-T/OmpH plasmid are 1 kb (lane 2) and 4 kb (lane 3), respectively. Lane M, lambda/*Hin*dIII DNA size marker; lane 1, Genomic DNA purified from *P. multocida* isolated from domestic pigs suffering from severe atrophic rhinitis in Gyeonggi Province, Korea; lane 2, the amplified fragment of *ompH* by PCR with the OMPH-F and OMPH-R primers; lane 3, PCR cloning vector, pGEM-T/OmpH; lane 4, expression vector, pRSET B/OmpH-ORF-F; L, 1 kb DNA ladder.

Fig. 2. Nucleotide and deduced amino acid sequences of the *ompH* in *P. multocida* D:4 (GenBank accession number AY603962).
Twenty amino acid residues at the N-terminus shown in italics are the putative signal peptide for membrane targeting. ORF of the *ompH* is 1,026 nts and encodes 341 amino acids. Translation start and termination codons are underlined.

(TAA) appeared from the 1,063rd to the 1,065th nt (Fig. 2). Some results of the gene manipulation steps are presented in Fig. 1. The genomic DNA was isolated from pathogenic *P. multocida* (D:4) (Fig. 1, lane 1), and PCR was performed with OMPH-F and OMPH-R primers. Briefly, the PCR mixture consisted of 100 ng of genomic DNA, 30 pmol of *ompH*-specific forward (OMPH-F) and reverse (OMPH-R) primers, 0.2 mM each of four deoxyribonucleotide triphosphates (dNTPs), 1.0 mM $MgCl_2$, and 2 U of *Taq* DNA polymerase (Strategene Co., La Jolla, CA, U.S.A.) in 50 l of appropriate reaction buffer. The amplification reaction included 1 cycle at 94°C for 5 min; 35 cycles at 94°C for 1 min; 53°C for 1 min; and 72°C for 1 min; and finally 1 cycle at 72°C for 5 min. The reactions were carried out on a Primus 96 plus PCR system (MWG Biotech, Ebersberg, Germany). The amplified DNA fragment (1,185 bp; Fig. 1, lane 2) was ligated onto the linearized vector pGEM-T (3,000 bp; Promega Co., Madison, WI, U.S.A.) to generate a circular recombinant plasmid (4,185 bp), named pGEM-T/OmpH (Fig. 1, lane 3). It was then transformed into *E. coli* DH5α for the subsequent cloning and sequencing. A new clone, named EOmpH, has been established among Amp^R resistant transformants, from which pGEM-T/OmpH was further purified and sequenced by the method of Sanger *et al.* with minor modifications [9, 10, 15].

DNA Sequence Analysis of Native OmpH in *P. multocida* D:4

PCR products and subcloned PCR inserts in plasmids were sequenced, using Applied Biosystems model 377 Genetic analyzer (Foster City, CA, U.S.A.). Sequence analysis was conducted with DNASTAR software (DNASTAR Inc., Madison, WI, U.S.A.) and Hitachi DNASIS software (Hitachi Software Engineering Co., San Bruno, CA, U.S.A.) [15]. Its nucleotide sequence and deduced amino acid (AA) residues are presented in Fig. 2. The coding region, or open reading frame (ORF), of the *ompH* is 1,026 bp long.

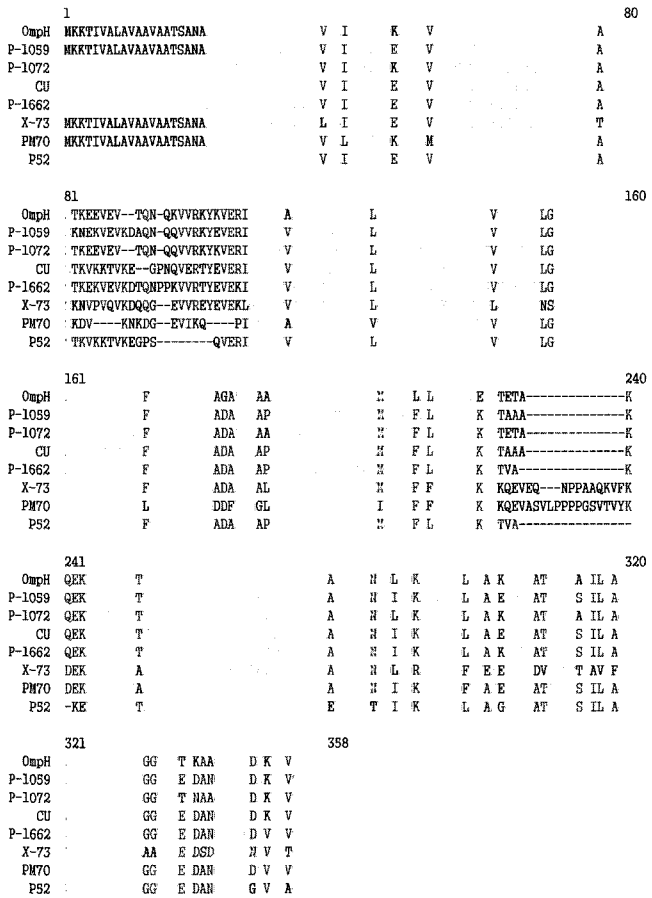

Fig. 3. Multiple sequence alignment of deduced amino acids of various OmpH isolated from various types of *P. multocida*.
OmpH is a clone in this study. Sequence information was obtained from databases of SwissPort and GenBank. Conserved amino acid sequences (shaded boxes) are predicted as antiparallel β-strands, and the other regions containing gaps (short dashes) and sequence variations are assumed to be external loops or periplasmic turns.

The predicted primary polypeptide is composed of 341 amino acids containing signal peptide of 20 amino acids. The molecular mass was estimated to be about 38 kDa by SDS-PAGE. The signal peptide has the common characteristics associated with such motifs, including a stretch of hydrophobic amino acids and an Ala-X-Ala cleavage site (Figs. 2 and 3). Nucleotide homology searches of the newly cloned *ompH* were performed at the National Center for Biotechnology Information with the BLAST network service, and its deduced amino acid sequence (OmpH) was found to be serotype D:4 (94% identity) (Fig. 3). Multiple sequence alignment of OmpH amino acid sequences of different serotypes revealed high sequence homology (over 70% overall identity), with variations of amino acid composition and sequence length in some regions. Several regions of various porins are well conserved among bacterial species as well as families: They share a basic structure containing a high proportion of antiparallel β-strands that take on a barrel conformation [4]. Some β-barrels are associated with the outer membrane as a homotrimer or remain as monomers. Therefore, they have a high degree of homology in their primary amino acid sequences and secondary structures [12, 18]. These characteristics make porins attractive vaccine candidates.

Hydropathy Analysis of OmpH

Secondary structure analyses were performed using multiple sequence alignment and the following supportive analyses: self optimized prediction method (SOPM), prediction of coiled coil regions (Coils), Transmembrane region and orientation prediction (TMpred), and Classic and membrane prediction (SOSUI) at the BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/). Hydropathy analysis of OmpH showed a typical hydrophobic stretch at the N-terminus corresponding to the putative signal peptide and hydrophilic regions, which coincided to prophesied surface domains and cytosolic domains generated by the predicted secondary structure of OmpH (Fig. 4). The last phenylalanine residue at the C-terminus constituted a sequence characteristic

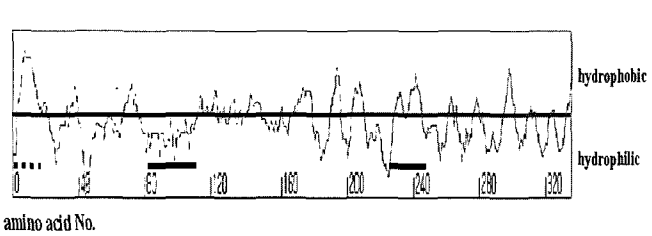

Fig. 4. Hydropathy profile of OmpH generated by Classic and membrane prediction (SOSUI) at BCM Search Launcher.
The putative signal peptide is indicated by dotted line, and the solid bars indicate the two variable domains found in OmpH. The SOSUI system (version 1.0, /Mar 10, 1996) is a useful tool for secondary structure prediction of membrane proteins from a protein sequence. The basic idea of prediction in this system is based on the physicochemical properties of amino acid sequences such as hydrophobicity and charges.

of bacterial OMPs which is important for their correct assembly into the membrane (Figs. 2 and 4). Hydrophilic domains within the OmpH corresponded to predicted surface-exposed domains in the secondary structure that are highly variable in their amino acid sequence, compared with similar OMPs. Furthermore, residues at 83-107 and 228-249 constituted variable regions which corresponded to hydrophilic domains of OmpH (Figs. 3 and 6). Therefore, these variable domains seem to be unique epitopes. Overall, the structure of OmpH among several types of the *P. multocida* also supports these presumptions. The structures of OMPs are conserved, however, small variable regions are present in the proteins of each serotype. This indicates that the variable regions may work as epitopes that play an important role in immune response.

Expression of Several Recombinant OmpHs in *E. coli* BL21 (DE3) and *E. coli* DH5α

It has been known that bacterial porin genes are often difficult to clone in *E. coli*, because exogenous porin peptides tend to be lethal to the cell: The non-self porins may cause osmotic destabilization in the cell, displacement of *E. coli*'s intrinsic porin(s), or a change in the structural integrity of the outer membrane of *E. coli*. Therefore, for successful expression of this *ompH*, several gene manipulation strategies were made in the present study (Fig. 5). The full ORF of *ompH* [1,026 bp; from the initiation (ATG) to the termination (TAA) codon; *ompH-ORF-F*] coding the entire primary amino acid sequence was amplified by PCR using template DNA of pGEM-T/OmpH with two primers of ORF-F (forward; 5'-GGGAT-TGG*GGATCC*TATGAAAAAGA-3') and ORF-R1 (reverse; 5'-CTAACA*AAGCTT*AGAAGTGTACGCG-3'). The forward oligomer contains *Bam*HI (*GGATCC*) recognition site, while the reverse oligomer has a *Hind*III (*AAGCTT*) recognition site, respectively. The amplified DNA fragments were restricted by the two enzymes, and then ligated to the vector, pRSET

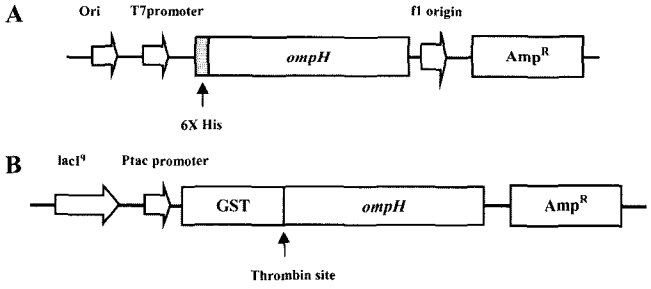

Fig. 5. Construction of the expression vectors for the *ompH* expression.
A. Full-length *ompH* (*ompH-ORF-F*) and truncated *ompH* (*ompH-ORF-T*) were inserted into pRSET B expression vector, resulting in pRSET B/OmpH-ORF-F and pRSET B/OmpH-ORF-T, respectively. B. Another truncated *ompH* (*ompH-ORF-T*) was inserted into pGEX-4T-1 vector, resulting in pGEX-4T-1/OmpH-ORF-Tα.

Fig. 6. Functional tests for the recombinant *ompH* gene expressions. In the transformation test with pRSET B/OmpH-ORF-F, a number of colonies were grown in *E. coli* DH5α, while no colonies in *E. coli* BL21 (DE3).

B cleaved by *Bam*HI/*Hin*dIII, to obtain the recombinant plasmid, pRSET B/OmpH-ORF-F. It was then transformed into two hosts; *E. coli* DH5α and *E. coli* BL21 (DE3) [13, 15]. We observed transformants in the former, but not in the latter (Fig. 6). Transformation was repeatedly unsuccessful in the competent cells of *E. coli* BL21 (DE3), even though the cells were not induced with IPTG. This was quite unexpected, since the cloned *ompH-ORF-F* is positioned after the T7 promoter, so that the expression of the inserted gene occurs only after the host is induced by IPTG. The lethality of the host, *E. coli* BL21 (DE3), or failure of the transformation led us to hypothesize that a small amount of OmpH (the primary protein) was leaked (or expressed) by the inserted *ompH-ORF-F*, without the addition of IPTG. Our results confirmed the facts that the recombinant plasmid, pRSET B/OmpH-ORF-F, was indeed carrying a bacterial porin gene, and that even a small amount of porin expressed by pRSET B/OmpH-ORF-F was lethal to *E. coli* BL21 (DE3) [5, 18].

It has been known that the signal peptide of the OmpH is responsible for the targeting of recombinant OmpH and the lethality of the host, *E. coli* BL21 (DE3). If the signal peptide was removed from the primary structure, targeting of the OmpH to the outer membrane would be inhibited, making the OmpH harmless in the host. In other words, if there is no signal peptide, the truncated OmpH will remain in the cytoplasm, thus causing no disturbance to the outer membrane of *E. coli.*

In order to express the gene for truncated ORF (the ORF excluding the signal peptide of 20 amino acids), the expression vector (pRSET B/OmpH-ORF-T) was constructed based on pRSET B. The expression of pRSET B/OmpH-ORF-T was observed in *E. coli* BL21 (DE3) as fused polypeptide, whose molecular mass was determined to be *ca.* 39 kDa on polyacrylamide gel (12.5%) containing 1% SDS (data not shown). It was an estimated molecular weight, since the fusion protein was composed of 352 amino acids (truncated ORF of 321 amino acids plus 31 amino acids tagged on pRSET B). We also observed the following impacts of the truncated OmpH on the lethality of *E. coli* BL21 (DE3): Even though the signal peptide was removed to block the delivery of the expressed OmpH to outer membrane in the periplasmic space of the host, only a few colonies were grown on the plate, *E. coli* BL21 (DE3). The degree of the gene expression also was not enough to be extracted in a large quantity. However, we could not find good rationales explaining the instability of the plasmid transformed to BL21 (DE3), and low efficiency of the plasmid expression. Thus, we employed another expression vector, pGEX-4T-1, tagged with the gene for glutathione S-transferase (GST; 26 kDa). This vector system was expressed in *E. coli* DH5α without host lethality.

To clone the truncated ORF into the pGEX-4T-1, another sets of primers with the following sequences were designed. A forward oligomer, named ORF-T2, was 27-mer having a *Bam*HI site (5'-ACTTCAGCA*GGATCC*GCAACAGTTTAC-3'), while a reverse oligomer, named ORF-R2, was 27-mer having a *Xho*I site (5'-TTCTAA*CTCGAG*TTAGAAGTGT-ACGCG-3'). The truncated gene (966 bp for 321 amino acids, *ca.* 35 kDa) amplified with the pair of primers was cleaved with *Bam*HI/*Xho*I and then ligated to the vector, pGEX-4T-1, digested with *Bam*HI/*Xho*I. The recombinant plasmid, named pGEX-4T-1/OmpH-ORF-Tα, was transformed into *E. coli* DH5α as the cloning and expression vector (Fig. 5). A new clone, named EOmpH-ORF-Tα, was established among Amp^R transformants. The truncated OmpH (*ca.* 35 kDa) fused with GST (26 kDa) was observed in the total protein extract of EOmpH-ORF-Tα by SDS-PAGE (Fig. 7, lane 1). The fusion protein (*ca.* 61 kDa) was purified from the total protein extract by GST affinity chromatography (Fig. 7, lane 3). A large quantity of the mature form of recombinant OmpH was obtained after cleavage of the fusion protein with thrombin to hydrolyze GST (Fig. 7, lane 4).

Since the first attempt by Louis Pasteur to immunize against infection with the Gram-negative facultative bacterium, like *P. multocida*, it has been well known that many of the bacterial OMPs are strong antigens. Specifically, OmpH, one of the major OMPs in *P. multocida*, is considered as a good candidate antigen related to the immunity. Several other OmpHs have been identified in various strains of *P. multocida* and used in protective experiments against agents of severe pasteurellosis [1, 5, 8, 11, 16, 18, 19, 25]. It has been suggested that certain serological types are the etiologic agents of severe pasteurellosis, and that a cross-protective immune response may be achieved by immunization with a mixture of serotype-specific antigens that are recognized by convalescent sera [23, 24] Recently, characterization and diversity of pathogenic *P. multocida* have been

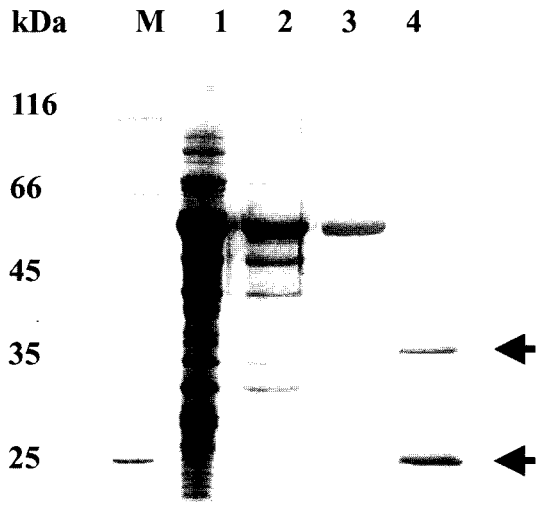

Fig. 7. Purification profile of recombinant OmpH protein.
Lane M, protein molecular weight standard; lane 1, cell lysate of the *E. coli* host, EOmpH-ORF-Tα, harboring pGEX-4T-1/OmpH-ORF-Tα; lane 2, crude extracts from the cell lysate; lane 3, purified recombinant OmpH fused with GST (*ca.* 61 kDa); lane 4, thrombin digestion of the purified recombinant OmpH. The M_r of the thrombin-digested recombinant OmpH and the thrombin-digested GST were *ca.* 35 kDa and *ca.* 26 kDa, respectively.

investigated on the basis of capsular type and OMP type by PCR analysis [6, 7].

In conclusion, we cloned a native gene (*ompH*) coding a major outer-membrane protein in *P. multocida* (D:4) which was isolated from a swine suffering from atrophic rhinitis in Korea. Also, we were successful to produce a large amount of recombinant OmpH. Hopefully, these results will contribute in regulating swine atrophic rhinitis in addition to other infections caused by *P. multocida*.

Acknowledgment

This paper was supported by Samsung Research Fund, Sungkyunkwan University, 2001.

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