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Articles

Biological Characterization of the Omp1-like Protein from *Actinobacillus actinomycetemcomitans*

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Actinobacillus actinomycetemcomitans is a gram-negative, nonmotile coccobacillus bacterium that is associated with several human diseases, including endocarditis, meningitis, osteomyelitis, subcutaneous abscesses and periodontal diseases. A full-length Omp1-like protein gene from *A. actinomycetemcomitans* was cloned into a pQE30 vector and over-expressed in *Escherichia coli* BL21(DE3) cells. The protein revealed sequence homologies to Seventeen kilodalton proteins (Skp) from *Pasteurella multocida* and *E. coli* that have been characterized as periplasmic chaperones. This soluble Omp1-like protein was successfully purified to homogeneity for further folding and functional studies. The purity, identity, and conformation of the protein were determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis, matrix-assisted laser desorption ionization mass spectrometry, circular dichroism, fluorescence spectroscopic, and differential scanning calorimetric studies. We showed that the protein formed an oligomer larger than a tetramer. We found, further, that it is comprised of mostly α-helices and boasts high thermal stability.

Key Words: Outer membrane protein 1, *A. actinomycetemcomitans*, Stability, Oligomer

Introduction

Gram-negative bacteria are encased by inner and outer membranes, and these membranes are separated by a periplasmic space. The inner membrane is a symmetrical phospholipid bilayer; the outer membrane is of asymmetric composition. For pathogenic bacteria, many specific proteins required for virulence is located in the outer membrane.[1] The membrane also forms a barrier that protects the cell against external agents, including antibiotics, detergents, heavy atoms and other damaging substances.[2]

Actinobacillus actinomycetemcomitans is a gram-negative, nonmotile coccobacillus that is associated with several human diseases, including endocarditis, meningitis, osteomyelitis, subcutaneous abscesses and periodontal diseases. The pathogenic mechanism for *A. actinomycetemcomitans* purportedly impairs the host immune mechanism by bacterial virulence factors that might contribute to disease processes.[3] In particular, the pathogenesis of periodontitis might be explained by immune stimulation by periodontopathic bacteria, which induce large scale T cell activation, cytokine production and polyclonal B cell activation.[4]

The N-terminal amino acid sequence of outer membrane protein Omp26 (202 aa) from Nontypeable *Haemophilus influenzae* has a revealed homology to a *Yersinia enterocolitica* OmpH, as well as homologies to Skp (17 kDa), OmpH, and Hip-1 proteins in *Pasteurella multocida, Yersinia pseudotuberculosis, Escherichia coli* and *Salmonella enterica*.[5] Seventeen kilodalton protein (Skp) has been characterized as a molecular chaperone that interacts with unfolded proteins as they emerge in the periplasm from the Sec translocation machinery. Skp is required for efficient release of translocated proteins from the plasma membrane. Recent studies have shown that Skp and lipopolysaccharide (LPS) are required for efficient folding and insertion into lipid bilayers of OmpA, a model β barrel OMP of *E. coli*.[6,7]

In the present study, a 22 kDa full-length Omp1-like protein (191 amino acids) exhibited approximately 71% amino acid sequence similarities with the Skp from *P. multocida* and 31% with that from *E. coli*. The folding and function of the 22 kDa novel Omp1-like protein have still remained undefined. The recombinant Omp1-like protein gene from *A. actinomycetemcomitans* was cloned and expressed in *E. coli*. It was purified to generate a homogeneous protein for further structural studies. In order to identify the stability and oligomerization of the protein, various biochemical and biophysical techniques including CD, fluorescence spectrum and DSC were used.

Materials and Methods

Cloning and expression. A full-length Omp1-like protein gene from *A. actinomycetemcomitans* was cloned into the N-terminal his-tagged fusion protein vector pQE-30 (QIAGEN). The recombinant Omp1-like protein fusion was transformed into the expression host *Escherichia coli* BL21(DE3) (Stratagene). A single colony was inoculated into 5 mL of LB (Luria Bertani) medium containing 50 μg/mL ampicillin and grown overnight at 37 °C. Bacteria were cultivated using a flask containing 1 L of LB medium at 37 °C until the OD_{600} reached 0.5 -

0.7. Isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM to induce the expression of the recombinant Omp1-like protein (191 amino acids). After 4 - 6 h, the cells were harvested by centrifugation at 4,500 rpm for 25 min at 4 °C, and supernatant was discarded. Cell pellets were either immediately used or stored frozen at −70 °C until needed. The cells were resuspended in buffer A (50 mM Tris-HCl [pH 7.5] and 200 mM NaCl), and disrupted by sonication on ice. After sonication, remaining whole cells and debris were removed by centrifugation at 13,500 rpm for 45 min.

Purification of Omp1-like protein. The clear supernatant was loaded onto a Ni-NTA (Amersham-Pharmacia Biotech) column and pre-equilibrated with buffer A. The column was washed with buffer A containing 20 mM imidazole at a flow rate of 1.5 mL/min and elution of the bound protein was achieved using varying amounts of imidazole from 50 to 200 mM. Protein elution was monitored at 280 nm and the resulting fractions were analyzed by electrophoresis on 12% SDS-PAGE gel. The eluted protein solution from the previous step was applied to a column of Q Sepharose fast flow (Amersham-Pharmacia Biotech) for ion exchange chromatography and washed extensively with buffer A. Elution was performed using a gradient of NaCl solutions in 50 mM Tris-HCl buffer. Then gel-filtration was performed on an HPLC using a Superdex 75 column (Amersham-Pharmacia Biotech) equilibrated in buffer A. The Omp1-like protein was separated and the fractions were collected and concentrated to a final volume of 1ml by centrifuging at 2,000 rpm using ultrafiltration devices. All the purification steps were analyzed and visualized by SDS-PAGE using 12% polyacrylamide gel and Coomassie brilliant blue staining.

Protein concentration analysis. The Omp1-like protein concentration was measured using the Bradford method with a Bio-Rad protein assay kit with Bovine Gamma Globulin (BGG) as a standard. Measurements were determined by BioPhotometer using a UVette of 10 mm optical path length.

MALDI-TOF mass analysis. For in-gel digestion, 10 μL of trypsin solution (2 ng/L in 25 mM of ammonium bicarbonate [pH 8.0] was added for digestion overnight at 37 °C. Peptides were extracted with 50% CAN/1% TFA (trifluoroacetic acid) and dried in a vacuum desiccator overnight, followed by reconstitution with 3μL of CHCA (α-Cyano-4-hydroxy-cinnamic acid) matrix solution (8 mg of CHCA in 1 mL of 50% CAN/1% TFA). One microliter of sample was loaded onto a 96 × 2 MALDI plate. The peptide mass was acquired with the Voyager DE-PRO (Applied Biosystems, Framingham, MA) in reflector mode under 20,000 V of accelerating voltage, 76% grid voltage and 0.002% guide-wire voltage. The Cal Mix 2 of the MALDI-TOF MS calibration kit (Applied Biosystems, Framingham, MA) was used for external calibration, and autolysis fragments of trypsin were used for internal calibration. Peptide matching and protein identification were carried out with the Mascot peptide mass fingerprint.

Circular dichroism (CD) and temperature controller measurements. The circular dichroism spectra of the folded Omp1-like protein were carried out with a spectropolarimeter (JASCO J-815) using a 0.01 cm path length cell at 10 nm interval at room temperature. For denaturant-induced unfolding, 3 M urea was added to Omp1-like protein in buffer A. The CD spectrum for the purified Omp1-like protein was recorded in the 190 - 250 nm range and scanning speed was 200 nm/min at room temperature. Each spectrum was the average of five scans. Temperature controller spectra were taken at a protein concentration of 1% (w/v) at room temperature (using cell length 1 cm). Scanning speed was the same as above, and used up to 3-fold accumulation. Thermal denaturation experiments showed that the recombinant Omp1-like protein his-tag can be reversibly unfolded. Thermal unfolding was performed using the same set-up by heating the samples with a constant temperature gradient from 20 °C to 75 °C. Calculation for the contents of the protein's secondary structure elements was performed using the CDNN program.[8]

Fluorescence spectroscopy. Fluorescence emission spectra were acquired by an Edinburgh (UK) FLS920 TCSPC (Time Correlated Single Photon Counting Spectrometer). The counts were recorded for the 300 - 450 nm wavelength range and a 295 nm excitation. For measurements of the unfolded Omp1-like protein, 3 M urea was added to the Omp1-like protein and it was incubated for 1 hr on ice. All spectra of the unfolded and folded Omp1-like protein were recorded at room temperature and at concentrations of 0.3 mg/mL.

Differential scanning calorimetry (DSC). Differential scanning calorimetric (DSC) measurements were carried out on a DSC 204 F1 (NETZSCH DSC, USA). An 8 mg protein was placed in an aluminum pan and immediately sealed. It was heated to 0 °C - 90 °C, and the heat flow was 1 °C/min. An empty pan was used as a reference. The sensitivity was 0.1 μw and the sensor time constant was 0.6. During the protein analysis using DSC, the reference had the same state (liquid), and it was placed in a reference crucible made of aluminum. The onset temperature (T_m), peak transition or denaturation temperature (T_d), enthalpy of denaturation (ΔH) and cooperativity, represented by the width at half-peak height ($\Delta T_{1/2}$), were computed from the thermograms by Universal Analysis Version 3.0.3. All samples were examined at least 2 times by repeating the heating-cooling cycles. In all cases, the sealed pans containing protein samples and references were equilibrated.

Formaldehyde cross-linking of the Omp1-like protein. To determine if the Omp1-like protein forms monomer or oligomer, full-size Omp1-like protein was tested for cross-linking using 1.5% formaldehyde. Purified Omp1-like protein was incubated with 1.5% formaldehyde from 15 min to 60 min. Protein analysis was by 10% SDS-PAGE using a 225 kDa protein marker.

Results and Discussion

The full-length Omp1-like protein from *A. actinomycetemcomitans* was verified by DNA sequencing. It was then expressed in an *E. coli* BL21(DE3) strain. The pQE-30 vector was used in the expression of a His fusion Omp1-like protein construct. Overexpression of the Omp1-like protein was observed mainly in the supernatant of the large-scale culture. The expressed protein was identified at the expected molecular weight of 25 kDa for the His-tagged Omp1-like protein. The Omp1-like protein was purified using a Ni-NTA column with an affinity to the fusion partner, the His-tag. Subsequently, the fractions, including the Omp1-like protein, were loaded onto a Q-Sepharose fast flow anion-exchange column and eluted with a gradient of NaCl

Table 1. Yield volumes and concentrations of Omp1-like proteins at each prep steps

Ni-column	
Volume (mL)	50
Concentration (mg/mL)	0.5
Purity (%)	80
Q-Sepharose	
Volume	50
Concentration	0.4
Purity (%)	90
Superdex 75	
Volume	60
Concentration	0.3
Purity (%)	95

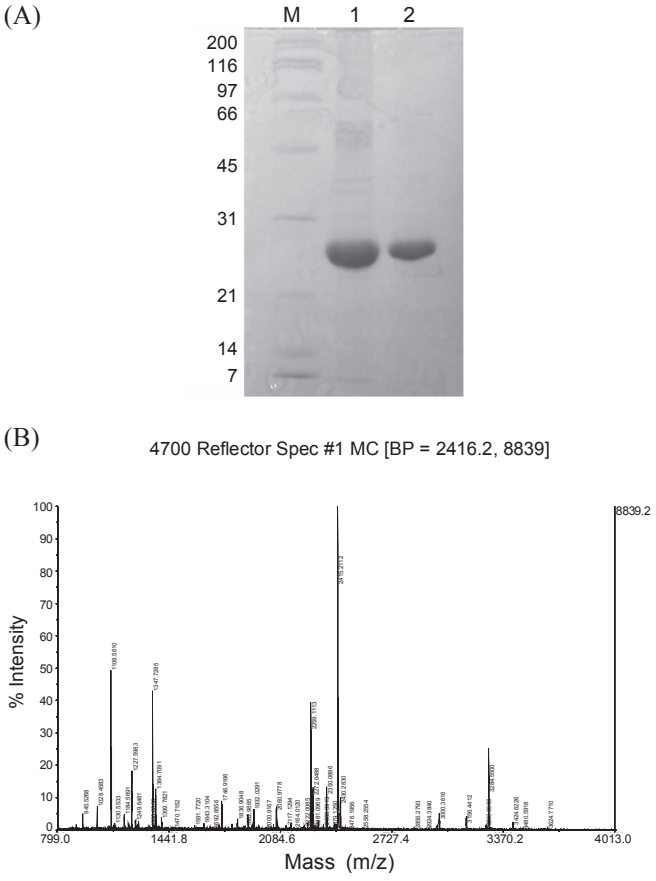

Figure 1. SDS-PAGE of the soluble Omp1-like protein and MALDI-TOF mass spectrum. (A) The concentration of the Omp1-like protein was about 34 mg/mL. Lane 1, protein molecular weight; lane 2, 7 μL of the protein; lane 3, 3 μL of the protein. (B) MALDI-TOF peptide spectrum of the protein from *A. actinomycetemcomitans*.

solution. The fractions that included the Omp1-like protein came out in the unbound fractions. The protein was purified using gel-filtration chromatography. Multi-step purifications of the protein under non-denaturing conditions were performed, as shown in Table 1. The soluble Omp1-like protein was successfully purified to homogeneity and concentrated to 34 mg/mL (Fig. 1A).

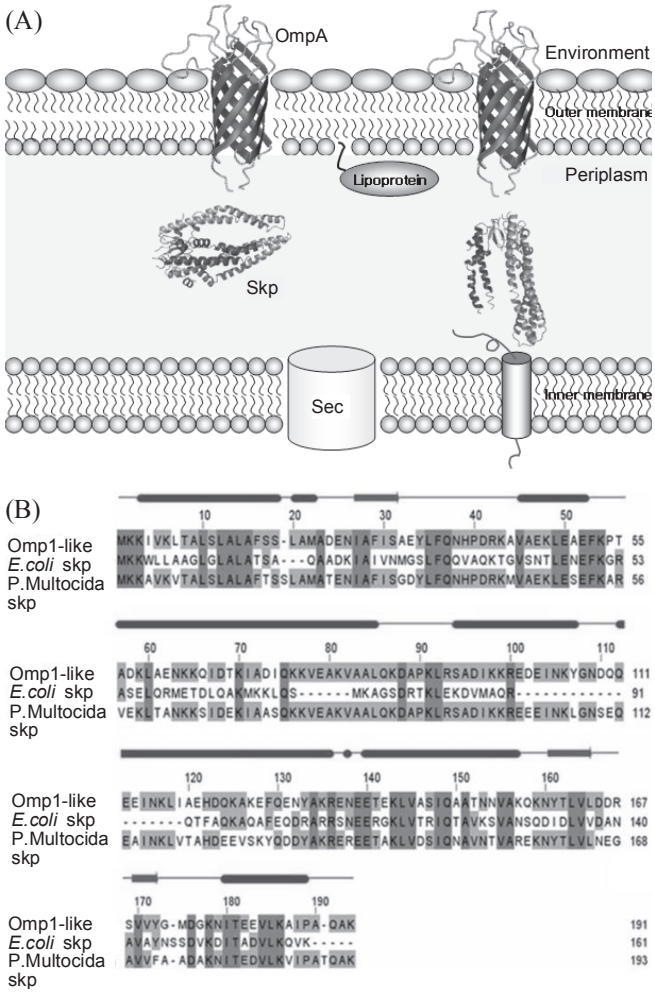

Figure 2. Architecture and secondary structure of the Omp1-like protein. (A) A schematic diagram for the locations of Skp in the periplasm. The Omp1-like protein was identified as a sequence homologous to Skp from *E. coli*. It can be located at the periplasm. (B) The amino acid sequences of the Omp1-like protein were compared with those of Skp from *E. coli* and *P. multocida*. The secondary structure of the Omp1-like protein was predicted. Alpha-helices were shown as ellipses, and β-sheets were shown as arrows. Loops were shown by lines.

MALDI-TOF MS studies revealed the approximate molecular mass of the recombinant protein, which mass was in accordance with the theoretical mass prediction for the Omp1-like protein. The monoisotopic masses obtained for the individual peptides were unrestricted. Mass fingerprinting analysis of the protein was performed according to the peptide mass tolerance of 50 ppm. The maximum appeared at *m/z* 2416.2 (Fig. 1B). The sequences for the digested peptides were matched with protein sequences in the database using the MASCOT program (http: // www. matrixscience.com). The protein was identified as the novel Omp1-like protein (Accession No. AF321231, pI value 6.78, and MW 21.478 kDa).

The Omp1-like protein was identified as a sequence homologous to Skp from *E. coli*. It can be located at the periplasm: a schematic diagram of its location of Omp1-like protein is provided in the Fig. 2A. The secondary structure of the Omp1-like protein was successfully predicted (Fig. 2B). Its amino acid se-

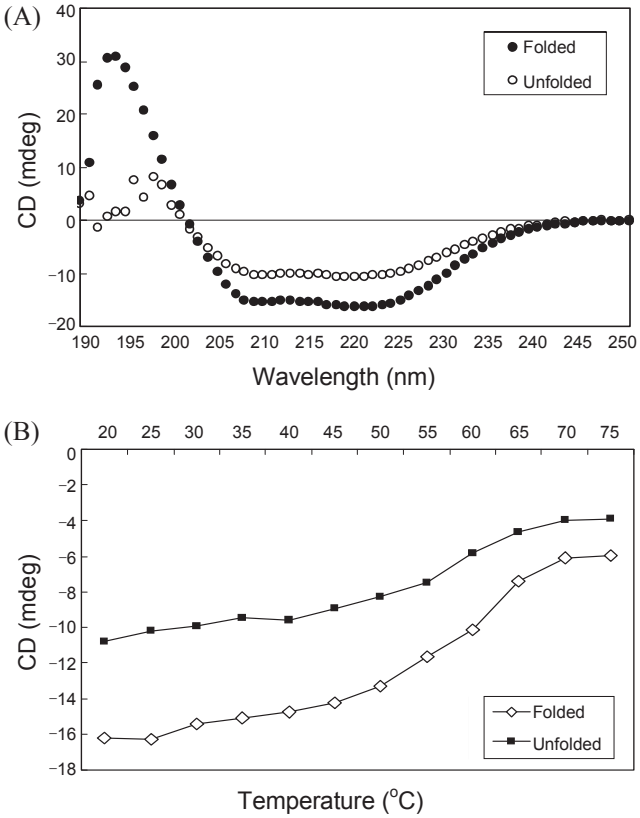

Figure 3. CD analyses of the folded and unfolded Omp1-like proteins. (A) Secondary structure of the folded Omp1-like protein at room temperature. For the unfolded structural study of the Omp1-like protein, 3 M urea was treated to the protein. Spectra were recorded at 190 - 250 nm using a 0.01 cm path length cell, and scanning speed of 50 nm/min. (B) Graphs of the folded and unfolded Omp1-like protein were verified at temperatures from 20 °C to 75 °C at 210 nm.

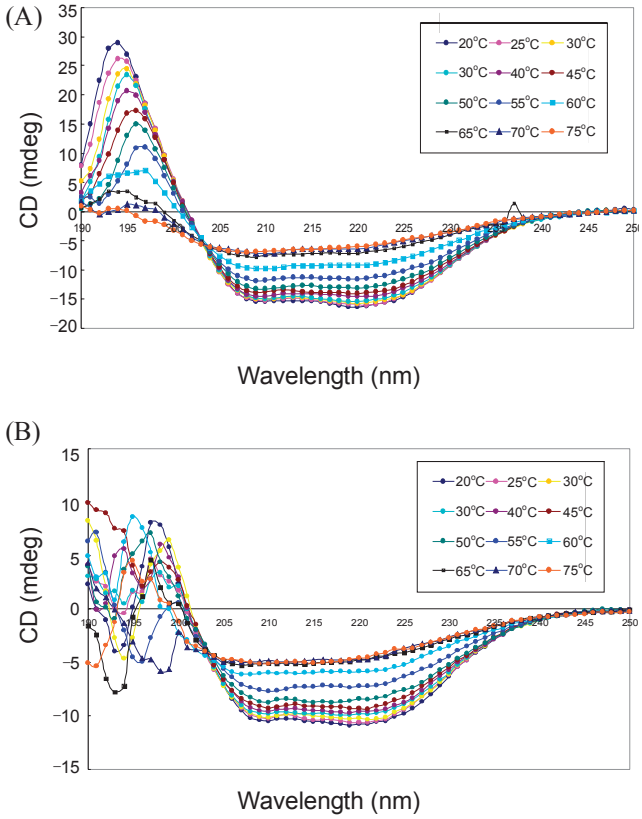

Figure 4. Temperature controller spectra of the folded and unfolded Omp1-like proteins. (A) CD spectra of the Omp1-like protein at different temperatures (20 °C - 75 °C). (B) CD spectra of the protein treated with 3 M urea. Protein solutions (1% (w/v) in buffer A) were introduced and spectra were recorded using a constant temperature gradient of 20 °C - 75 °C from 190 to 250 nm (band width 1 nm, scan speed 200 nm/min, using up to 3-fold accumulations) and corrected for solution blanks.

quences were compared with those of Skp from *E. coli* and *P. multocida*, and the secondary structures were revealed.

Proteins in the native state are highly ordered in one main conformation, whereas those of the denatured state are disordered in many different conformations. To observe the folding properties, the folded and unfolded Omp1-like proteins were determined by CD spectroscopy. The CD spectrum of the folded protein exhibited two negative maximums at about 208 nm and 222 nm. A positive maximum below 195 nm suggested that the protein adopts a biologically active natured state (Fig. 3A). The spectrum of the unfolded Omp1-like protein showed structural changes in the 190 - 250 nm interval. The positive peak was destroyed, and the magnitude for the negative peak was less than for the folded conformation. This indicated that the conformational changes of the unfolded Omp1-like protein occurred in both the α-helices and β-sheets structures. The folded Omp1-like protein showed a predominant α-helices structure, but for the unfolded protein the proportions of the α-helices and β-sheets structures were lower than for the folded protein. Deconvolution of the spectra using the CDNN program indicated the following secondary structure contents: for the folded Omp1-like protein, 89.7% α-helices, 5.0% β-sheets, 4.6% turns and 0.7% non-ordered forms: while for the unfolded Omp1-like protein, 71.4% α-helices, 4.3% β-sheets, 13.4% turns and 10.9% non-ordered forms. The CD spectra of the folded and unfolded Omp1-like proteins at varying temperatures from 20 to 75 °C at 210 nm are shown in Figure 3B. As can be seen, there is a gap between the dual peaks at 210 nm, characteristically for α-helices.

In order to identify the stability of the protein, various biochemical and biophysical techniques including CD, fluorescence spectrum and DSC were used.[9-12] Additional thermal denaturation experiments for the folded and unfolded Omp1-like proteins were carried out using CD spectroscopy (Fig. 4A and B). From the results, we found that the folded Omp1-like protein was reversibly unfolded. An interesting point is that at low temperatures, the spectrum showed bands characteristic of α-helices. Heating caused progressive unfolding of the protein, and the α-helical and β-sheets bands were lost, characteristic bands of random or disordered structures appearing. With the gradual increase of the Tm curve, the general characteristics of disordered or loosely folded proteins were shown.

The fluorescence emission spectra for the purified folded and unfolded Omp1-like proteins were recorded for the 300 - 450 nm wavelength range and a 295 nm excitation wavelength (Fig. 5A). The folded Omp1-like protein and the unfolded Omp1-like protein showed similar behaviors, with emission

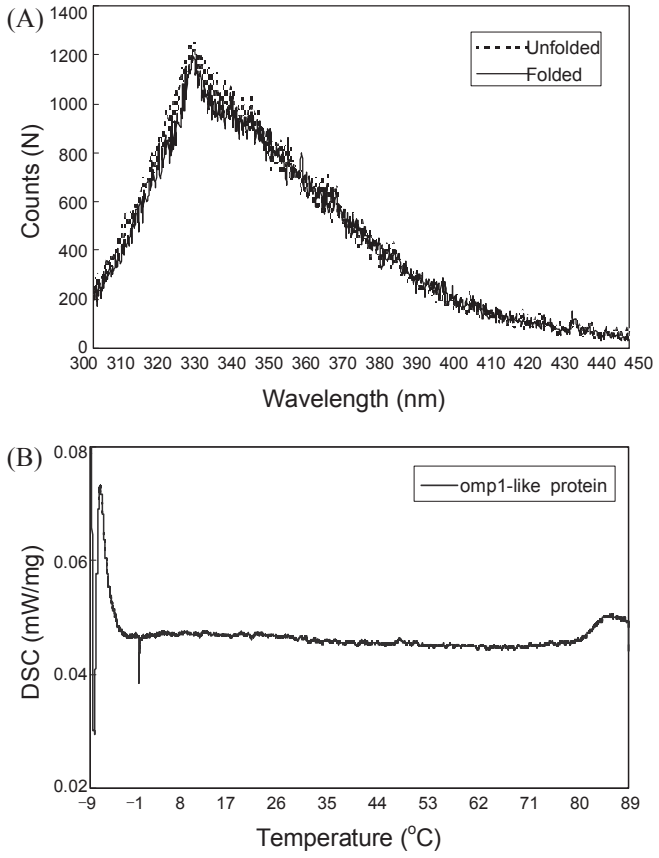

Figure 5. Fluorescence and DSC analyses of the Omp1-like protein. (A) Fluorescence analysis of the folded and unfolded Omp1-like proteins. The fluorescence spectra were recorded with 295 nm excitation wavelength using TCSPC (Time-Correlated Single Photon Counting) Spectrometer. (B) DSC analysis of Omp1-like protein. Temperatures were set from −10 °C to 90 °C. The heat flow was 1 °C/min. The concentration of the Omp1-like protein was 1 mg/mL. The melting temperature (Tm) and width were −0.9 °C and 0.2 °C.

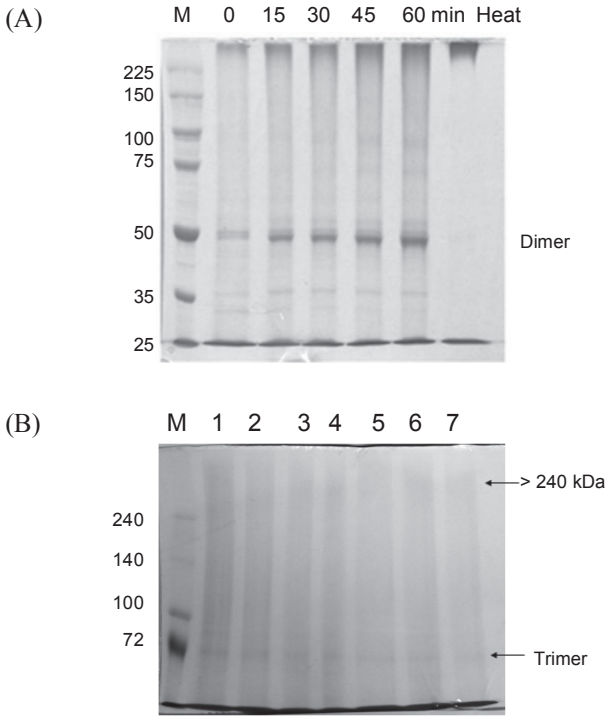

Figure 6. Formaldehyde cross-linking and native gels of the Omp1-like protein. (A) Omp1-like protein cross-linking by 1.5% formaldehyde. The Omp1-like protein was prepared under different conditions either not cross-linked (lane 2) or cross-linked with 1.5% formaldehyde in buffer A (lanes 3-7). The proteins were incubated at room temperature for various times (15, 30, 45 or 60 min). Only one cross-linked protein was boiled at 72 °C for 10 min (lane 7). The proteins were analyzed by electrophoresis 10% SDS-PAGE gel. (B) The Omp1-like proteins treated at various pH levels. The Omp1-like protein trimers were presented in the 6% native gel. Some bands were also observed greater than 240 kDa. The proteins were treated with 1 M Tris-HCl for 1 hr at various pH levels (pH 4.6, 5.6, 6.5, 7.5, 8.5, 9.5, and 10.5), but not boiled.

peaks observed at 328 nm. The fluorescence intensities (counts) were about 1214 N for the folded protein, and about 1174 N for the unfolded one.

DSC was used to analyze the effects of heating the protein on protein stability (Fig. 5B).[13-14] DSC scans were performed for conditions under which the protein was irreversibly unfolded. The temperature for the minimal heat adsorption by the protein was −0.9 °C. The height of the heat adsorption was 0.008217 mW/mg ($\Delta H_m = -0.08976$ J/g). The protein did not show thermal transitions or denaturation from 0 °C up to 80 °C, and a small peak was found near 87 °C.

The highly expressed Omp1-like protein in *E. coli* was analyzed to oligomer. The protein was treated with a cross-linking reagent, 1.5% formaldehyde, in buffer A. In this study, the protein band was observed near 51 kDa as a dimer. As the incubation time increased, the intensity of the dimer of the Omp1-like protein was enhanced. After the protein was boiled at 72 °C, it was denatured and the dimer disappeared in the gel (Fig. 6A). In the 6% native gel, the bands were located at the trimer between 75 and 100 kDa. At the various pH levels, the trimer bands did not changed their intensity. Also, an additional, greater-than 225 kDa band was observed, suggesting that the Omp1-like protein formed an oligomerization larger than a tetramer (Fig. 6B).

The secondary structure of the Omp1-like protein, a type of OMP, is similar to that of Skp from *P. multocida*. The amino acid sequences of the Omp1-like protein and of the Skp from *P. multocida* were 71% matched; those of the Omp1-like protein and of the Skp from *E. coli*, meanwhile, showed a 31% sequence homology. As previously mentioned, Skp is a homotrimeric periplasmic chaperone for newly synthesized OMPs.[15-18] Three hairpin-shaped α-helical extensions extend ~60 Å from the trimerization domain, which is composed of 3 intersubunit β-sheets that wind around a central axis. From observations of its secondary structure, the amino acid sequences of the Omp1-like protein from *A. actinomycetemcomitans* were not well matched with those of the Skp from *E. coli*, but both sets were composed mostly of α-helices. Using the SWISS MODEL REPOSITORY program, an automated protein model of the Omp1-like protein was compared with the outer membrane chaperone Skp (OmpH) family from *E. coli*.[19] The model residues of the experimental Omp1-like protein, based on template 1sg2A of the Skp 3D structure, ranged from 26 aa to 186 aa.

In the present study, to identify the stability and oligomeri-

zation of novel Omp1-like protein, various biochemical and biophysical techniques including CD, fluorescence spectrum and DSC were used. We will make an attempt at a further structural and functional study of the Omp1-like protein for the purposes of a comparison with the outer membrane Skp family from *E. coli* and *P. multocida*.

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