

Research Communication

Isolation and Characterization of a Porin-Like Outer Membrane Protein from *Xanthomonas campestris* pv. *campestris*

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Summary

Xanthomonas campestris pv. *campestris*, a plant-associated pathogenic bacterium, is the causal agent of foliar spots and blights in crucifers. The major outer membrane protein, Omp37, of 37 kDa, has been identified, purified to homogeneity, and its characterization has also been carried out. Native Omp37 behaved as a trimer, as revealed by gel filtration and SDS-PAGE. FTIR measurements revealed a high β -structure content. The pore-forming ability of the purified Omp37 was studied by the liposome swelling assay. Omp37, to our knowledge, is the first porin that has been isolated from *Xanthomonas*. This study clearly demonstrates that Omp37 is related to the family of trimeric bacterial porins.

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Keywords Outer membrane protein; porin; *Xanthomonas campestris* pv. *campestris*.

INTRODUCTION

Members of the genus *Xanthomonas* are plant-associated pathogenic bacteria distributed worldwide. They cause a variety of diseases in vegetables and fruits, resulting in considerable loss to agriculture (1). *X. campestris* pv. *campestris*, one of the most important members, is the causal agent of foliar spots and blights in crucifers. Substantial progress has been made in exploring the mechanism of its pathogenesis.

Lipopolysaccharides (LPS) and porins are major toxic components in the outer membrane of gram-negative bacteria (2, 3). Porins are integral membrane proteins that form channels in the outer membrane (4–6). They form pores that allow the influx of

small (<600 Da), hydrophilic nutrient molecules and the efflux of waste products, and exclude many antibiotics and inhibitors. Generally, porins show no particular substrate specificity except some selectivity for either cations or anions. Substrate-specific porins accelerate specific nutrient diffusion through the outer membrane (7, 8). Porins can be involved in the pathogenicity as an apoptosis inducer of epithelial cells (9). Also, porins could play an important role in the actin condensation of brain endothelial cells (10) and during adhesion in the early steps of the infection process (11).

To our knowledge, no porin from *Xanthomonas* has yet been isolated. In this study, we report the isolation and characterization of the major outer membrane protein of Mr approximately 37 kDa, named Omp37, from *X. campestris* pv. *campestris*.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Preparation of Cell Envelopes

The strain X_c-6 of *X. campestris* pv. *campestris* was obtained from the Institute of Plant Protection, CAAS (Chinese Academy of Agricultural Sciences). The cells were grown at 28 °C in a medium containing 0.3% beef extract, 0.1% yeast extract, 0.5% tryptone, 1% sucrose, and 0.1% monosodium glutamate (pH 6.8). Cell envelopes were prepared using the method of Nakae (12) with some modifications. Cells were harvested at the late exponential growth phase, washed once with distilled water, and resuspended in Tris buffer (20 mM Tris-HCl, pH 8.0). Then, they were disrupted by sonication with a CPX600 ultrasonic homogenizer (Cole Parmer). After centrifugation (10,000 × g for 10 min at 4 °C) to remove cell debris, cell envelopes were recovered by ultracentrifugation (125,000 × g for 1 h at 4 °C), and resuspended in Tris buffer by means of a Teflon homogenizer.

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Purification of Omp37. Cell envelopes were treated with buffer A (10 mM Tris-HCl, pH 7.6) containing 1% sarcosyl and 1 mM EDTA for 30 min at 30 °C to solubilize the cytoplasmic membrane but not the outer membrane (13). Outer membranes were collected by ultracentrifugation at $155,000 \times g$ for 30 min. The pellet was resuspended in buffer A to give a protein concentration of 5 mg/ml, and outer membrane proteins were solubilized with buffer A containing 1% *N,N*-dimethyldodecylamine *N*-oxide (LDAO), 100 μ g/ml lysozyme, and 0.2 M NaCl for 2 h at 30 °C. Insoluble material was removed by centrifugation at $155,000 \times g$.

Extracted outer membrane proteins were applied to an anion exchange column (Q5, BioRad) equilibrated with buffer A containing 0.1% LDAO. Proteins were eluted with a linear NaCl gradient (0 ~ 0.5 M). Fractions containing Omp37 were subjected to further purification by gel filtration chromatography on Superdex-200 (Pharmacia), and Omp37 was eluted with buffer A containing 0.8% octyl-POE and 0.1 M NaCl.

SDS-PAGE. SDS-PAGE was performed as previously described (14) using the Mini-Protean II electrophoresis apparatus (Bio-Rad). Proteins were detected by staining with Coomassie brilliant blue R-250.

Trypsin Digestion. First, 10 μ g of Omp37 was incubated with trypsin (50 μ g/ml) for 1 h at 37 °C. The reaction was stopped by addition of 0.1 mM phenylmethylsulfonyl fluoride. The sample was analyzed by SDS-PAGE. The α subunits of the heterotrimeric GTP-binding proteins ($G_o\alpha$) were treated in the same way as a control.

Mass Spectrometry MALDI-TOF (Matrix-Assisted Laser Desorption and Ionization Time of Flight). First, 1 mg of Omp37 and the matrix (a saturated solution of α -cyano-4-hydroxycinnamic acid in $\frac{2}{3}$ acetonitrile and $\frac{1}{3}$ 0.1% trifluoroacetic acid in water) were mixed at the ratio of 1:1 (v/v) on the stainless plate and dried prior to analysis. Mass spectra were obtained with a Bruker BIFLEX III. All spectra were acquired in the positive-ion mode and the acceleration voltage was set to 19,000 V. External calibration was performed with bovine cytochrome *c* (*m/z* 12361).

FTIR Spectroscopy. Omp37 (15–20 μ g) was dried on CaF₂ crystals. The spectra were recorded at a resolution of 4 cm^{-1} with an average of 20 scans in a Bruker EQUINOX55 spectrophotometer.

Liposome Swelling Assay. The liposome swelling assay was performed per procedures in Nikaido (15) with some modifications. To prepare proteoliposomes for swelling, 0.67 μ mol of asolectin was dissolved in chloroform and dried with a nitrogen stream; the lipid film was then suspended in 0.1 ml of Omp37 (3.3 μ g), and dried with a nitrogen stream again. The film containing Omp37 was resuspended in 0.2 ml of 17% (w/v) Dextran T-40 in 5 mM Tris-HCl (pH 8.0), and incubated for 1.5 h at 30 °C. Liposomes were prepared in a similar fashion without protein to use as a control. The isotonic concentration of each solute was determined by diluting liposomes with different concentrations of the solute. The concentration of the solute at which there was

no swelling or shrinking of the liposomes was considered the isotonic concentration. Proteoliposomes were mixed rapidly with different solutes at their isotonic concentrations and the change of the absorbance at 400 nm with time was monitored. Protein concentration was determined with the BCA Protein Assay Kit (Pierce).

RESULTS AND DISCUSSION

Purification of Omp37. As shown in Fig. 1, the major and significant band of the outer membrane proteins of *X. campestris* pv. *campestris* on SDS-PAGE is approximately 37 kDa, thereafter named Omp37. Because no porin from *X. campestris* pv. *campestris* has yet been isolated, we became interested in Omp37 and undertook its isolation and characterization. In the course of the extraction of outer membrane proteins with LDAO, it was noticed that Omp37 was not observed in the extractable fraction unless lysozyme or a high salt concentration was incorporated in the extraction buffer. This observation suggested that Omp37 might be a porin, because most porins are tightly associated with underlying peptidoglycan sheets through noncovalent

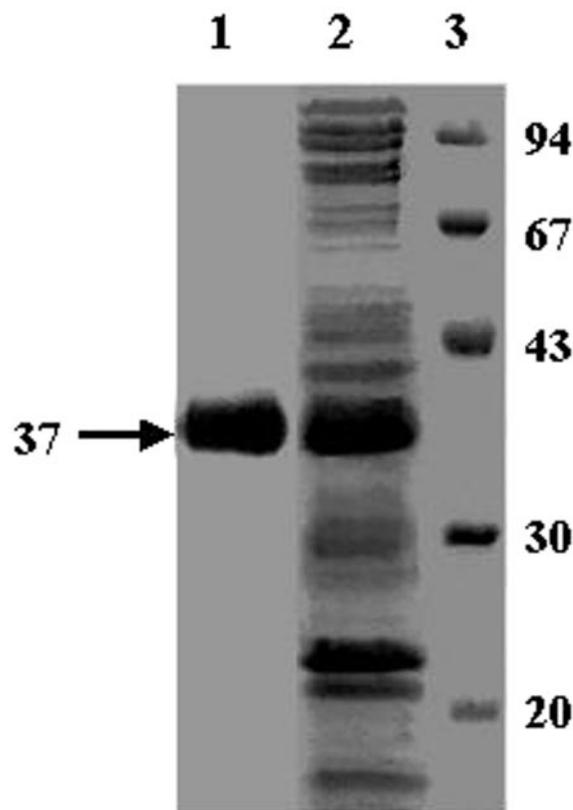


Figure 1. SDS-PAGE of purified Omp37 and outer membrane protein extractions from *X. campestris* pv. *campestris*. Lane 1, purified Omp37 in the loading buffer containing 0.4% SDS heated for 2 min at 70 °C. Lane 2, outer membrane proteins. Lane 3, molecular weight markers (molecular mass indicated in kDa).

interactions, which can be dissociated in the presence of lysozyme and/or high concentrations of salts (15).

After the extraction of outer membrane proteins, Omp37 could be purified to nearly homogeneity by means of anion exchange chromatography and gel filtration (Fig. 1). On the Superdex-200 column, Omp37 behaved as an oligomer of about 120 kDa, suggesting a trimer of native Omp37, which was also confirmed in Fig. 2 (see later). The molecular weight of Omp37 was determined by MALDI-TOF mass spectrometry, which gave (36927 ± 37) Da.

Stability of Omp37 on Heating, SDS Treatment, and Trypsin Digestion. Because porins are generally organized in trimers and are very stable in SDS below 75 °C, the temperature necessary to dissociate trimers completely (16, 17) and the sensitivity of Omp37 to the temperature or SDS was studied. As shown in Fig. 2, Omp37 behaved with different electrophoretic mobilities on SDS-PAGE under different conditions, which reflected the stability of Omp37 in vitro.

As revealed in Fig. 2A, a unique band with an apparent molecular weight of 37 kDa, corresponding to the native monomer, was obtained when the sample was heated to 70 °C. Upon heating, the trimer of native Omp37, corresponding to the band around 120 kDa, was dissociated, whereas new bands with apparent molecular weights of 75 kDa, 42 kDa, 37 kDa, and 40 kDa appeared. It has been suggested that the trimer of porins is able to dissociate into dimers and monomers on SDS-PAGE (18), so we assign the band at 75 kDa as the dimer of Omp37. An interesting finding in Fig. 2A was that most of the monomers migrated at 42 kDa at room temperature (lane 1), and completely converted into 37 kDa together with trimeric (120 kDa) and dimeric form (75 kDa) at 70 °C (lane 3). This behavior has not been observed in other porins. It has been reported that porins are tightly associated with peptidoglycan fragments and/or LPS

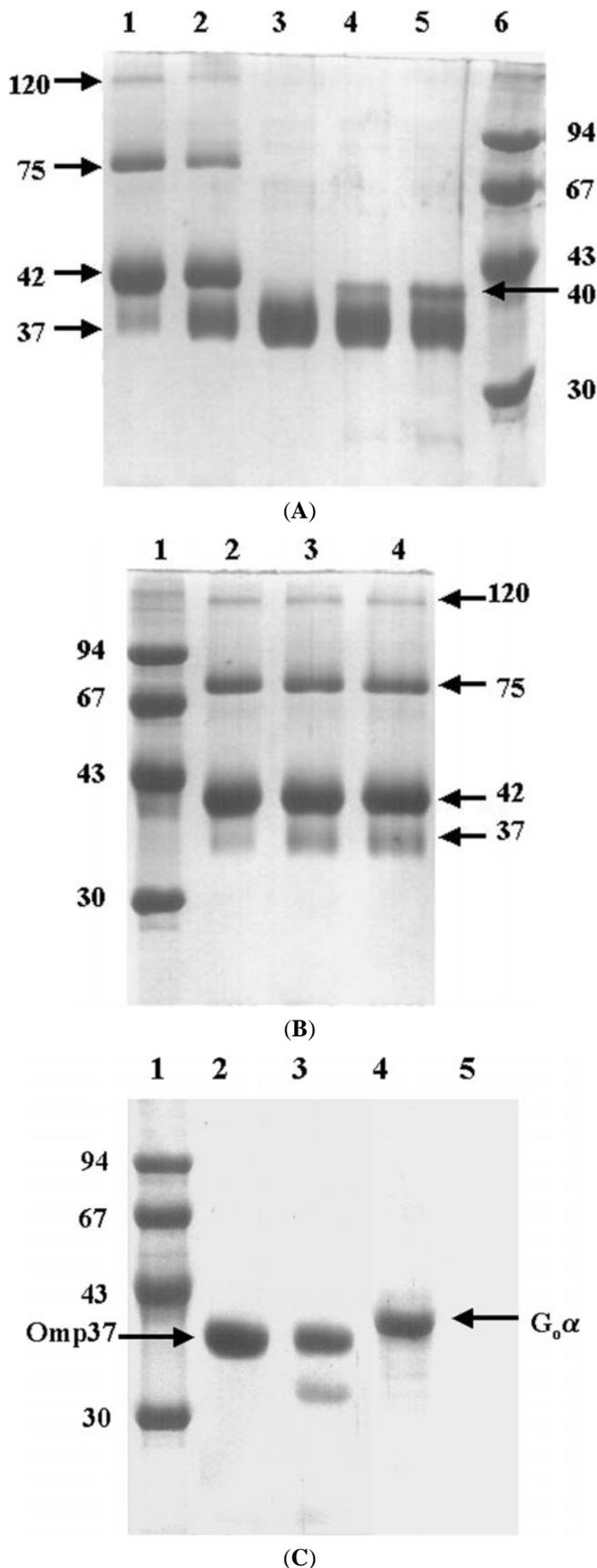


Figure 2. SDS-PAGE analysis of the stability of Omp37 after heating (A), SDS treatment (B), and trypsin digestion (C). The concentration of SDS in the gel and migration buffer was 0.1%. The experiments were performed at 4 °C. The molecular mass is indicated in kDa. (A) Heating. Aliquots of 7 μg of Omp37 in the loading buffer containing 0.4% SDS heated at different temperatures. Lane 1, 25 °C, 20 min; lane 2, 50 °C, 2 min; lane 3, 70 °C, 2 min; lane 4, 100 °C, 2 min; lane 5, 100 °C, 10 min; lane 6, molecular weight markers. (B) SDS treatment. Aliquots of 7 μg of Omp37 in the loading buffer containing different concentrations of SDS incubated for 20 min at 25 °C. Lane 1, molecular weight markers. Lane 2, 0.1% SDS. Lane 3, 1% SDS. Lane 4, 2% SDS. (C) Trypsin digestion. 10 μg of Omp37 was incubated with trypsin (50 μg/ml) for 1 h at 37 °C. The G₀α was treated in the same way to use as a control. The samples containing 3 μg of protein were subjected to SDS-PAGE analysis. Lane 1, molecular weight markers. Lane 2, Omp37, without trypsin treatment. Lane 3, Omp37, with trypsin treatment. Lane 4, G₀α, without trypsin treatment. Lane 5, G₀α, with trypsin treatment.

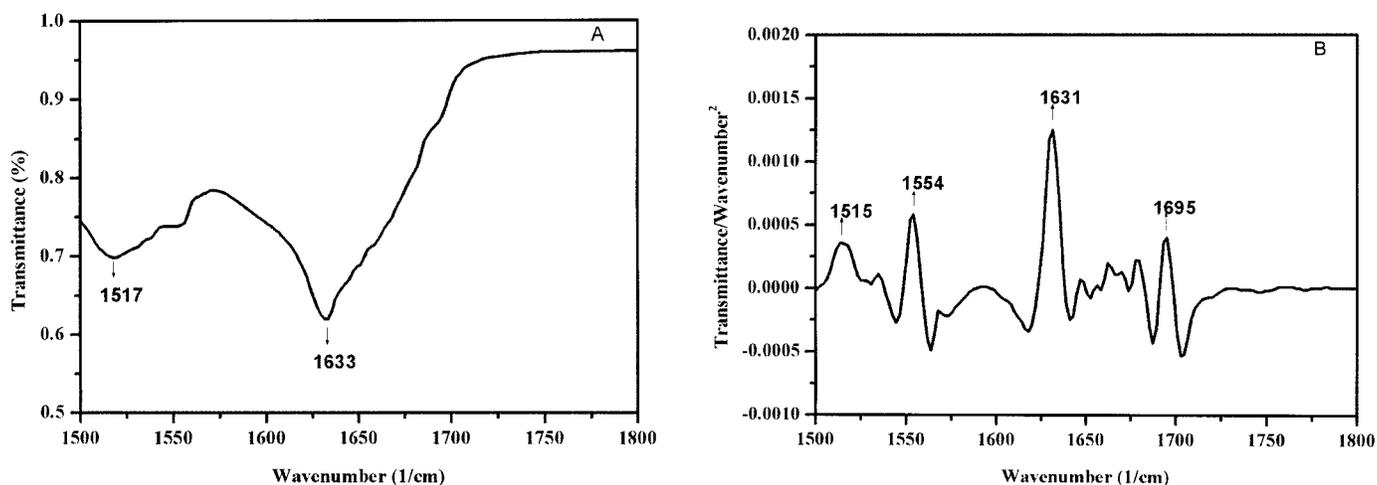


Figure 3. FTIR spectra of Omp37. (A) Transmittance spectrum. The spectrum is displayed in the amide I and amide II regions centered around 1633 and 1517 cm^{-1} . (B) Second-derivative spectrum. The main peaks at 1695 cm^{-1} and 1554 cm^{-1} correspond to the shoulders on the amide I and amide II band, respectively. The peak at 1631 cm^{-1} , in combination with the bands at 1695 cm^{-1} and 1554 cm^{-1} , indicates antiparallel β sheets in the Omp37. Measuring conditions: 20 scans, resolution: 4 cm^{-1} , temperature: 25 $^{\circ}\text{C}$.

(19–21). Accordingly, we suggest that the band at 42 kDa might be the complex of the native monomer of 37 kDa with peptidoglycan fragments and/or LPS. The band at 40 kDa emerged only above 70 $^{\circ}\text{C}$ (lane 5), indicating that it was a denatured monomer of Omp37, similar to the observation in monomers of the trimeric porin from *C. jejuni* (22). This behavior has been attributed to conformational modifications of the protein like the loss of the β -barrel structure or an incomplete folding (22).

Similar electrophoretic mobility on SDS-PAGE was also observed upon SDS treatment, except no apparent band at 40 kDa appeared, suggesting a high stability of Omp37 in SDS (Fig. 2B). This was further evidence that the band at 40 kDa in Fig. 2A was due to the heated denatured monomer of Omp37. The monomer migrated mainly at 42 kDa even in 2% SDS at room temperature, which, combined with the result in Fig. 2A, indicated that heating was necessary to dissociate peptidoglycan fragments and/or LPS from the monomer of Omp37.

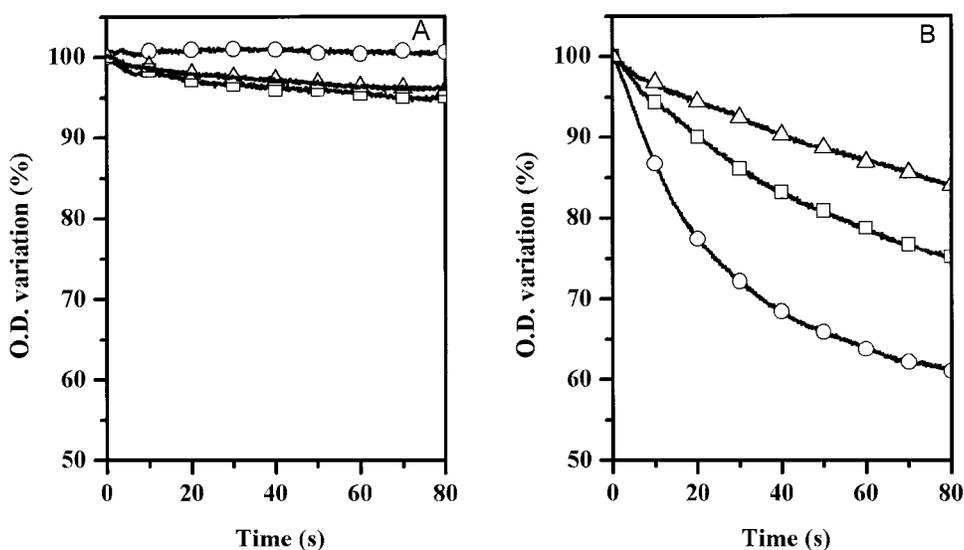


Figure 4. The change in OD (400 nm) with time in the liposome swelling assay. The preparation of proteoliposomes was described as Materials and Methods in detail. The change in OD at 400 nm with time is expressed in percent. (A) Control liposomes (without any protein included). (B) Proteoliposomes reconstituted with a lipid/protein ratio (w/w) of 150:1. (Δ), sucrose; (\square), galactose; (\circ), glycine.

The purified Omp37 was subjected to the trypsin digestion. Omp37 still remained even after the treatment with 50 $\mu\text{g/ml}$ trypsin for 1 hr at 37°C (Fig. 2C). Omp37 is similar to that of Omp32 from *Comamonas acidovorans*, which is resistant to many proteinases, e.g., LysC proteinase (23). We suggest that Omp37 is not related to OmpA of *E. coli*, which is susceptible to the proteolytic cleavage (24). Immunological study suggested that an outer membrane protein of 44 kDa was an Omp A-like protein (25).

Secondary Structure Determination of Omp37. All known porins fold into antiparallel β -barrels, instead of α -helices (26, 27). Therefore, FTIR measurements were performed to determine the secondary structure of Omp37, which revealed a high content of β -structure (Fig. 3). As seen in the second-derivative spectrum generated from Fig. 3A, the distinct peak at 1,631 cm^{-1} in the amide I band, in combination with the band at 1,695 cm^{-1} , is indicative of antiparallel β sheets. In accordance with FTIR, circular dichroism (CD) measurements also demonstrated the high content of β -structure (data not shown).

Functional Characterization. Porins have pore-forming ability, allowing the transport of molecules across lipid bilayer membranes, which can functionally be studied by the liposome swelling assay. The purified Omp37 was reconstituted into liposomes, and the swelling of proteoliposomes, reducing the turbidity, was spectroscopically monitored at 400 nm. Three solutes, glycine (75 kDa), a monosaccharide, galactose (180 kDa), and a disaccharide, sucrose (342 kDa), were chosen for the swelling assays (Fig. 4). Although no swelling of liposomes without Omp37 included was observed (Fig. 4A), proteoliposomes of Omp37 presented the swelling (Fig. 4B). This result demonstrated the pore-forming activity of Omp37. Fig. 4B also indicated that the diffusion rate of uncharged solutes through the Omp37 channels depends on the size, i.e., molecular weight, of the solute. This agrees with the observation that the smaller the solute, the higher the diffusion rate on general porins, such as the porin of OmpAb from *Acinetobacter baumannii* (28). The exclusion limit of the sieve was also estimated, and was found to be close to the size of a hydrated sugar of approximately 400 Da. This result is comparable with a porin-like protein of 45 kD from *B. fragilis* (29).

CONCLUSIONS

We conclude that the Omp37 is a porin and plays a role in the permeability of *X. campestris* pv. *campestris*. To our knowledge, Omp37 is the first porin that has been isolated from *Xanthomonas*. Further investigations such as amino acid analysis and structural studies on Omp37 are in progress.

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