Purification and characterization of a major 40 kDa outer membrane protein of *Acinetobacter baumannii*

Kondapalli Jyothisri, V. Deepak, Moganty R. Rajeswari*

Department of Biochemistry, All India Institute of Medical Sciences, New Delhi 110029, India

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Abstract ***Acinetobacter baumannii*, an opportunistic pathogen, is well known to cause a wide spectrum of nosocomial infections particularly in intensive care units. The major outer membrane (OM) protein, OmpAb, of 40 kDa from *A. baumannii* has been identified and purified to homogeneity from cultures grown at 30°C and 100 mM NaCl. The synthesis of OM proteins of *A. baumannii* is thermoregulated and osmoregulated. The pore forming ability of the purified OmpAb and the diffusion of uncharged solutes in proteoliposomes has been demonstrated by following the liposomal swelling assay. The trimeric OmpAb is characterized as a porin with a pore size of 1.3 nm and is found to be similar to the OmpF of *Escherichia coli* and can possibly be classified as a general diffusion pore. It appears that OmpAb plays an important role in the diffusion properties of the outer membrane of *A. baumannii*.**

Key words: Outer membrane protein; Porin; OmpAb; *Acinetobacter baumannii*

1. Introduction

Recently, numerous outbreaks of nosocomial infections caused by *Acinetobacter baumannii* have been reported and these are of great concern because of widespread and increasing resistance to antibiotics [1]. Infections with *Acinetobacter* are common in patients in the intensive care units of our hospital. *A. baumannii*, a Gram-negative bacterium and an opportunistic pathogen, is resistant to a wide range of antibiotics such as β-lactams and aminoglycosides [1,2]; imipenem resistance, however, is relatively rare [3,4]. One of the important factors contributing to this drug resistance is perhaps the impermeability of certain classes of antibiotics across the outer membrane (OM) or some structural change in the proteins of the OM. It is well known that the OM acts as a molecular sieve for diffusion of hydrophilic molecules including drugs, nutrients and other essential substances for supporting growth and protects against hazardous agents such as host lysozyme. The association of porins in some Gram-negative bacteria such as *Escherichia coli* [6–9], *Salmonella typhimurium* [10], *Pseudomonas aeruginosa* [11] and *Proteus mirabilis* [12] has been studied in detail. The best characterized porin of *E. coli* is OmpF, whose X-ray crystallography structure has been determined [13], and there are no reports on the purification of OM proteins and characterization studies of porins of *A. baumannii*. To understand the role of porins in the drug resistant and multidrug resistant strains of *A. baumannii*, the first step would be to know the structure and function of porins in the wild type. And there are no reports on the purification of OM proteins in *A. baumannii*. It is for this reason that we have isolated and purified the major 40 kDa protein from *A. baumannii* (ATCC) and reconstituted it in artificial membranes to study the pore forming ability.

2. Materials and methods

2.1. Chemicals

All the routine chemicals used in the experiments were of analytical grade from standard sources. Egg phosphatidylcholine was purchased from Sigma, molecular weight standards from Bio-Rad, Sephacryl S-300 and dextran T-40 were from Pharmacia Biotech. The purity of the lipid was checked by thin layer chromatography.

2.2. Bacterial strain

A. baumannii ATCC 19606 strain was used in the present study.

2.3. Absorbance measurements

All the OD measurements were made on a Cary-Varian 1 E UV-Vis spectrophotometer with an accuracy of OD ±0.001.

2.4. Preparation of cell and OM proteins

Cells were grown in LB broth with constant shaking at different temperatures (30°C and 37°C) and salt (100 mM, 300 mM sodium chloride) conditions. Log cultures (OD 0.6) of 500 ml grown under each growth condition were harvested, sonicated and centrifuged for 30 min at 100 000 × *g*. The pellet containing the cell envelope was treated with 1% Sarkosyl for 30 min at 25°C to solubilize the inner membrane and centrifuged for 30 min at 100 000 × *g*. The pellet containing the OM fraction was treated with 2% SDS to obtain SDS soluble and SDS insoluble fractions and the latter was treated with lysozyme to remove the associated peptidoglycan. The concentration of total OM protein was measured by the method of Bradford [14] using BSA as standard and analyzed by SDS-polyacrylamide gel electrophoresis.

2.5. Purification of OmpAb

The OmpAb was purified from 5 l culture grown at 30°C with 100 mM NaCl. The total OM proteins were separated by gel filtration using a Sephacryl S-300 column [15]. Total OM protein of 35 mg of the SDS soluble fraction (or 8 mg of the SDS insoluble fraction) was loaded on a column after equilibrating with 0.1% SDS, 0.2 M $LiCl_2$, 50 mM Tris-HCl pH 7.5, 0.1 mM EDTA and 3 mM NaN_3 and eluted at a flow rate of 1 ml/2 min. The protein content in the eluate was monitored by measuring OD at 280 nm. The column was precalibrated with a mixture of standard proteins. The main protein was collected, dialyzed and stored at −20°C.

2.6. SDS-PAGE

Protein samples were analyzed using the Mini-Protean II electrophoresis apparatus of Bio-Rad on a 5–20% polyacrylamide gradient gel and proteins were detected by staining with Coomassie brilliant blue [16].

2.7. SDS-PAGE: non-denaturing conditions

To examine whether the monomers are held together by any covalent linkages (S-S bonds), the purified OmpAb after gel filtration was analyzed by SDS-PAGE without β-mercaptoethanol in the sample buffer to exclude the possibility of -S-S- linkages between monomers

*Corresponding author. Fax: (91) (11) 6863631.
E-mail: mrraji@medinst.ernet.in and mrraji@hotmail.com

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of the trimer. Samples were not boiled in solubilizing buffer and the run was carried out at 4°C.

2.8. Liposomal swelling assay

Vesicles were reconstituted from phospholipids and proteins and the liposomal swelling assay was carried out by the method used for *E. coli* [5,17,18]. Proteoliposomes were prepared by dissolving 2.5 μM of egg phosphatidylcholine (EPC) and 0.2 μM of diacetyl phosphate in chloroform and dried under a stream of nitrogen atmosphere followed by dissolving and drying in benzene and ether. To prepare proteoliposomes for swelling, the lipid film was finally suspended in 3 ml of 17% (w/v) dextran T-40 in 5 mM Tris-HCl, pH 7.5 and 25 μg of OmpAb SDS soluble protein was added and sonicated. 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 solutes. The concentration of solutes 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 absorbance at 450 nm was monitored; the minimum time for the recording of the first reading was 10 s due to experimental limitations.

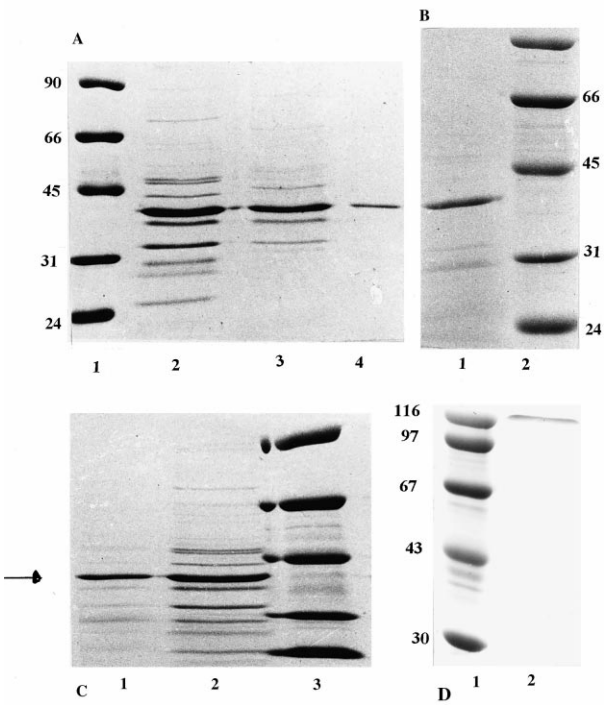

Fig. 1. SDS-PAGE profile of outer membrane proteins from *A. baumannii* extracted from cells grown under different growth conditions. The samples containing 8 μg total OM protein or 1.8 μg of purified OmpAb were heated at 100°C for 5 min in the sample buffer (6.25 mM Tris-HCl pH 6.8, 10% glycerol, 1% β-mercaptoethanol, 1% SDS) and were separated on a 5–20% gradient gel and proteins detected by Coomassie blue staining. A: OM proteins at 30°C and 100 mM NaCl. Lane 1, molecular weight marker; lane 2, total OM protein at 30°C and 100 mM NaCl; SDS soluble proteins expressed at 30°C and 100 mM NaCl; lane 3, SDS soluble proteins expressed at 37°C and 100 mM NaCl; lane 4, purified OmpAb after gel exclusion with Sephacryl S-300 column. B: lane: 1, SDS insoluble fraction of OM proteins expressed at 30°C, 100 mM NaCl; lane 2, molecular weight marker as in A. C: Lane: 1, OM proteins expressed at 30°C and 300 mM NaCl; lane 2, OM proteins expressed at 30°C and 100 mM NaCl; lane 3, molecular weight marker as in A. D: SDS-PAGE under non-denaturing conditions (4°C). Lane 1, numbers at the margin indicate the molecular weight sizes of the marker in kDa, 206 (not shown in the photograph), 116, 96, 67, 43 and 30; lane 2, SDS-PAGE of trimeric OmpAb in the absence of β-mercaptoethanol in the sample buffer. Numbers in the margin indicate molecular mass in kDa. The arrow indicates the position of the 40 kDa protein, OmpAb.

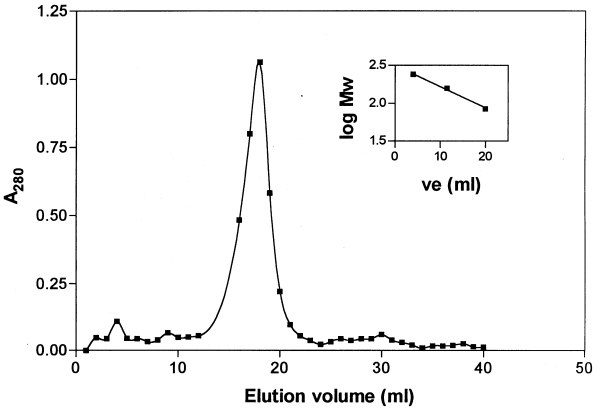

Fig. 2. Elution profile of the outer membrane proteins of the SDS soluble fraction (35 mg) by the gel filtration. The total OM of the SDS soluble fraction of 35 mg protein in Tris-HCl buffer was loaded on a 1.25×42 cm Sephacryl S-300 column after equilibration with 0.1% SDS, 0.2 M $LiCl_2$, 50 mM Tris-HCl pH 7.5, 0.1 mM EDTA and 3 mM NaN_3 and eluted with the same buffer at a flow rate of 1 ml/2 min. Peak fraction 17 contained the protein OmpAb. The column was precalibrated with a mixture of proteins, 4 mg alkaline phosphatase (86 kDa), 2.5 mg of human immunoglobulin G (160 kDa) and 63 mg of catalase (244 kDa). Inset: dependence of molecular weight (Mw) on elution volume.

3. Results and discussion

The major and significant band of the OM proteins of *A. baumannii* on SDS-PAGE is a 40 kDa porin (Fig. 1A, lanes 2–4). The protein monomeric form with 40 kDa is hereafter named OmpAb. Besides the OmpAb, *A. baumannii* contains other major proteins having molecular masses of 33, 36, and 44 kDa accompanied by traces of other proteins, notably at 22, 28, and 30 kDa, and a doublet around 50 kDa as seen on SDS-PAGE (Fig. 1A, lane 2). The single band of protein with 33–36 kDa as reported by Clark [4] is actually well resolved on our SDS-PAGE as 33 and 36 kDa. Although the protein profile is similar under different growth conditions, the osmolarity and temperature affected the expression of the total OM proteins. The expression of all OM proteins decreased by twofold when the temperature was raised from 30°C to 37°C while maintaining the salt concentrations at 100 mM (Fig. 1A, lane 2 (30°C), lane 3 (37°C)). The expression was also decreased by 50% when the salt concentration was increased from 100 mM to 300 mM NaCl at 30°C (Fig. 1C, lane 2 (100 mM) and lane 1 (300 mM)). It is known that in *E. coli* the expression of the OM protein is influenced by phosphate starvation, salt, temperature, etc. [18,19]. Although the physiological role of the thermo- and osmoregulation of porin production is not clear, it appears that the bacterium recognizes the non-parasitic state and depending on medium composition and growth temperature expresses different porins (OmpF, OmpC, etc.) [6].

Surprisingly, the total OM proteins from the SDS insoluble fraction consisted of proteins with 40 kDa and lower and no detectable concentration of proteins above 40 kDa was observed (Fig. 1B, lane 1); this may be because the peptidoglycan is digested by lysozyme releasing proteins with smaller molecular weight. Nakae et al. reported that in *E. coli* the

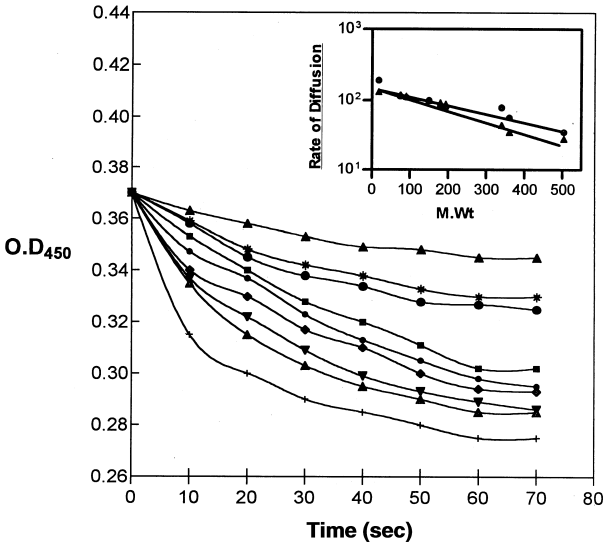

Fig. 3. The change in OD (450 nm) with time in the liposome swelling assay. The liposomes were reconstituted with 2.5 μM of EPC and 25 μg of OmpAb. On addition of various solutes: water (+); glycine (▲); alanine (▼); arabinose (♦); glucose (•); methyl mannosine (■); sucrose (●); lactose (*); and raffinose (▲) the changes with time were recorded with liposomes. Inset: Relative rate of diffusion (with respect to arabinose) in liposomes as a function of molecular weight; OmpAb from the SDS soluble fraction (●) and the SDS insoluble fraction (▲).

SDS insoluble fraction of OM proteins is more potent than the SDS soluble fraction in conferring sucrose permeability on the reconstituted vesicle membranes [6]. So we also purified the 40 kDa protein from the SDS insoluble fraction, however, in our present study we did not see any difference in activity (see Section 3.1).

The total OM proteins after dialysis were loaded on the Sephacryl S-300 column and elution volume vs. OD_{280} is shown in Fig. 2. However, after purification the yield of the major fraction OmpAb was about 4 mg for the SDS soluble and 1 mg for the SDS insoluble fraction. The OmpAb accounts for approximately 30–40% of the total OM proteins of *A. baumannii*. The molecular weight of the main protein peak was estimated to be about 115 kDa (inset, Fig. 2). In order to make sure that the 115 kDa protein is a multimer of the major 40 kDa protein, we analyzed the 115 kDa protein on SDS-PAGE. Peak fraction 17 was seen as a single band at 40 kDa on SDS-PAGE analysis (Fig. 1A, lane 4). The peak fraction was loaded on SDS-PAGE under native conditions to confirm the existence of the trimeric form of the OmpAb in non-denaturing conditions; the gel showed a single band around 120 kDa (Fig. 1D, lane 2, lane 1 molecular weight marker). This proves the observation that the native OmpAb exists as a trimeric form.

3.1. Functional characterization

Liposome swelling was followed by measuring the change in OD at 450 nm as a function of time and the graphs were extrapolated to zero time (Fig. 3). The OD at which all the curves met was taken as the zero time reading using solutes: glycine (75 Da), alanine (90 Da), arabinose (150 Da), glucose (180 Da), methyl mannosine (194 Da), sucrose (342 Da), lactose (360 Da) and raffinose (504 Da). The diffusion rates of the solutes shown in Fig. 3 are normalized with respect to arabinose (taken as 100). The rate of diffusion of solutes from the membrane by OmpAb as a function of molecular weight of the solute is shown in Fig. 3 (inset); the slopes of the graphs are practically the same when OmpAb from the SDS soluble or insoluble fraction was reconstituted in the membrane. This observation suggests that the pore forming ability of OmpAb from both the SDS soluble and insoluble fraction is almost the same (Fig. 3, inset). On the basis of swelling experiments it was found that the rate of diffusion of uncharged solutes through the porin channels depends on the size, i.e. molecular weight, of the solute (Fig. 3, inset). Using the Renkin [18] equation we estimated the approximate diameter of the pores to be 1.3 nm, which is close to that of *E. coli* OmpF of 1.2 nm [18]. The exclusion limit of the sieve is found to be close to the size of a hydrated sugar of approximately 700 Da [6].

The specific activity of the pore was determined according to the definition 'the change in optical density × 1000 per minute per microgram of protein' [19]. The specific activity for arabinose diffusion into proteoliposomes for OmpAb was estimated to be 760. This value is comparable with earlier results for *E. coli* of 380 [18], *P. aeruginosa* H103 of 97 [20] and *Vibrio cholerae* of 950 [21]. In mycobacteria (Gram-positive bacteria) a larger pore size (2 nm) with a very low specific activity of 27 [19] has been reported. The diameter can define the exclusion limit, which is the bottleneck of the channel. The specific activity in fact tells us about the speed or velocity of solute mobility, which in turn depends on the length, size and number of pores. So, while discussing the solute permeability of the membrane, one should take into account both the factors, i.e. specificity and pore size.

We therefore conclude that the OmpAb is a porin and plays an important role in the permeability of *A. baumannii*. Further investigations such as amino acid analysis and structural studies on OmpAb are in progress. As there are no reports on the porins of *A. baumannii*, these initial studies on the ATCC strain could help in understanding the structure-function relationships as in the case of *E. coli* OmpF [22].

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