Extraction and purification of two outer membrane proteins (porins) from *Klebsiella pneumoniae* local isolate.

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Abstract

Background: The porins are present in large amounts in the outer membrane of gram negative bacteria and form water-filled channels that permit the diffusion of small hydrophilic solutes across the outer membrane. Porins are generally divided into two classes: nonspecific porins (e.g., OmpC and OmpF), which permit the general diffusion of small polar molecules (600 Da), and specific porins (e.g., LamB), which facilitate the diffusion of specific substrates.

Objective: To purify and characterize outer membrane proteins (porins) from a local isolate of *Klebsiella pneumoniae*.

Materials and methods: An identified local isolate of *Klebsiella pneumoniae* was used as a primary source for the isolation and purification of porins. Outer membrane protein (porins) was purified and characterized and the contaminating lipopolysaccharides (LPS) were detected by thiobarbituric acid assay.

Results: The final preparation contained porins in a concentration of 3.2 mg/ml. The results of electrophoretic separation revealed that porins appeared as two distinct bands with molecular weights of porins were estimated to be 35 and 36 kDa, respectively.

Conclusions: Porins were expressed by the local isolate of *Klebsiella pneumoniae* with molecular weights highly similar to that of porins preparations produced by other gram negative bacteria and *Klebsiella pneumoniae* expressed two types of porins under standard laboratory conditions.

Keywords: Porins, Thiobarbituric acid, Gel filtration chromatography, Ketodeoxyoctinate.

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Introduction

Approximately, 50% of the dry mass of the outer membrane of gram-negative bacteria consists of proteins, and more than 20 immunochemically distinct proteins (termed outer membrane proteins [OMPs]) have been identified in *E. coli*. Apart from their structural role, OMPs have also been shown to have other functions, particularly with regard to transport, and have been classified as permeases and porins. Furthermore, several OMPs have been shown to be potent inducers of cytokine synthesis [(1)].

Porins are OMPs which form trimers that span the outer membrane and contain a central pore with a diameter of about 1 nm. These porins (e.g., OmpC and OmpF of *E. coli*) are permeable to molecules with molecular masses lower than approximately 600 Da. Porins play a crucial role in the interactions between the environment and bacteria, in addition, or probably as a consequence, they are present in large amounts in the outer membrane of gram-negative bacteria [(2)].

Materials and methods

Porins were extracted according to the method described by Nurminen [(3)]. Briefly, the bacterial cells were harvested by centrifugation at 4000 rpm for 30 minutes. One gm of bacterial cells was washed twice with 0.01M tris buffer (pH 7.8) and suspended in10 ml of 0.01 M tris

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buffer containing 0.01 M EDTA and 1.3 mg lysozyme, then 0.4 ml solution of 1 M $MgCl_2$ containing 50 mg DNas & RNase each was added. One gm (wet weight) prepared above was extracted twice (separated by centrifugation at 3000xg) with 2% TX-100 buffer containing 0.01M $MgCl_2$.

Half mg trypsin / ml suspension was added and incubated overnight at 37 °C. One hundred ml of the digested mixture was centrifuged at 20000xg, the supernatant was collected, and the pellet was digested once more with trypsin. The supernatant of both digestions was ultrafiltered using the amikon apparatus. The retained material was washed with 1L of D.W. by further ultra filtration. The precipitate was suspended into 100 ml of D.W. and centrifuged at 20000xg for 20 minutes. The sediment was finally suspended in 10 ml of D.W.

For further purifying porins, the final preparation was subjected to gel filtration chromatography using Sephacryl S-200 gel.

Preparation and packing of the gel

Sephacryl S-200 gel was prepared according to the instructions of the manufacturing company. It was suspended for 2 hrs in 250 ml of 0.01 M EDTA buffer (pH 7.5) containing 0.2% TX-100 and then it was degassed by using vacuum pump. Gel was poured with care (to avoid bubbles) onto a column with dimensions of 1.5x88 cm. Finally the column was equilibrated over night with the same buffer.

Method of Gel filtration chromatography

Five ml of porins solution was loaded onto the column, and fraction of 5 ml each were eluted after settling the flow rate to about 30 ml/hrs. Absorbance at 280 nm was measured for all of the fractions.

Concentration

The porins peaks, were collected as 80 ml of elution buffer and concentrated by sucrose to a final volume of 10 ml for each peak.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of the porins and the apparent masses of their variants were estimated by SDS-PAGE. SDS-PAGE was done according to the method of Laemmli described by Garfin, [4]. The protein concentration in the final preparation of porins was measured by the absolute method:

$$\frac{\text{O.D. at 235nm - O.D. at 280 nm}}{2.51} = \text{protein concentration (mg/ml)}$$

as mentioned by Whitaker and Granum, [5].

Thiobarbituric acid assay for the estimation of lipopolysaccharide (LPS) concentration (Ketodeoxyoctinate).

Standard curve of LPS: Several known concentrations of LPS were plotted versus their relevant absorbance at 550 nm, and a standard curve was created. By the aid of the standard curve, it was possible to measure LPS concentrations in the final porins preparation.

Thiobarbituric acid assay was performed according to the method described by Hanson and Philip, [6] and to alleviate the cytotoxic effects of contaminating LPS, polymyxin B was added to the final porins solution in a dose of 5 g/ml and the mixture was incubated for one hour at 20°C.

Results

The results revealed that porins were eluted as two peaks (Figure 1); the fractions enriched in protein, identified by absorbance at 280 nm, were pooled and extensively concentrated by sucrose and checked

for protein heterogeneity by SDS-PAGE. Figure 2 illustrates the electrophoretic experiment of the present study which shows clearly that porins are represented by two bands. The first is at molecular weight 36 kDa and the other at molecular weight 35 kDa. The two bands are related to peak no. 1 and peak no. 2 shown in figure 1, respectively. The final preparation resulted after purification steps contained porins in a concentration of 3.2 mg/ml as estimated by the absolute method and it was shown that porins solution had contaminating LPS in a concentration of 117 µg / ml (Figure 3).

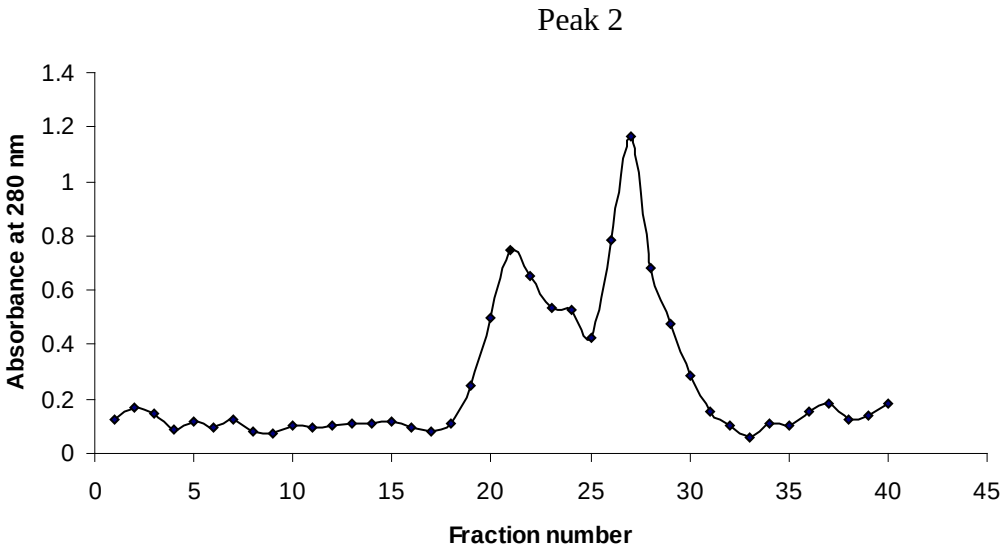

Figure 1: Purification of *Klebsiella pneumoniae* porins with Sephacryl S-200. The dimensions of the column was 1.5x88 cm, flow rate was adjusted to 30 minutes/hrs. 0.2% TX-100 (pH 7.8) containing 0.01 M EDTA was used as elution buffer and fraction of 5 ml each were eluted.

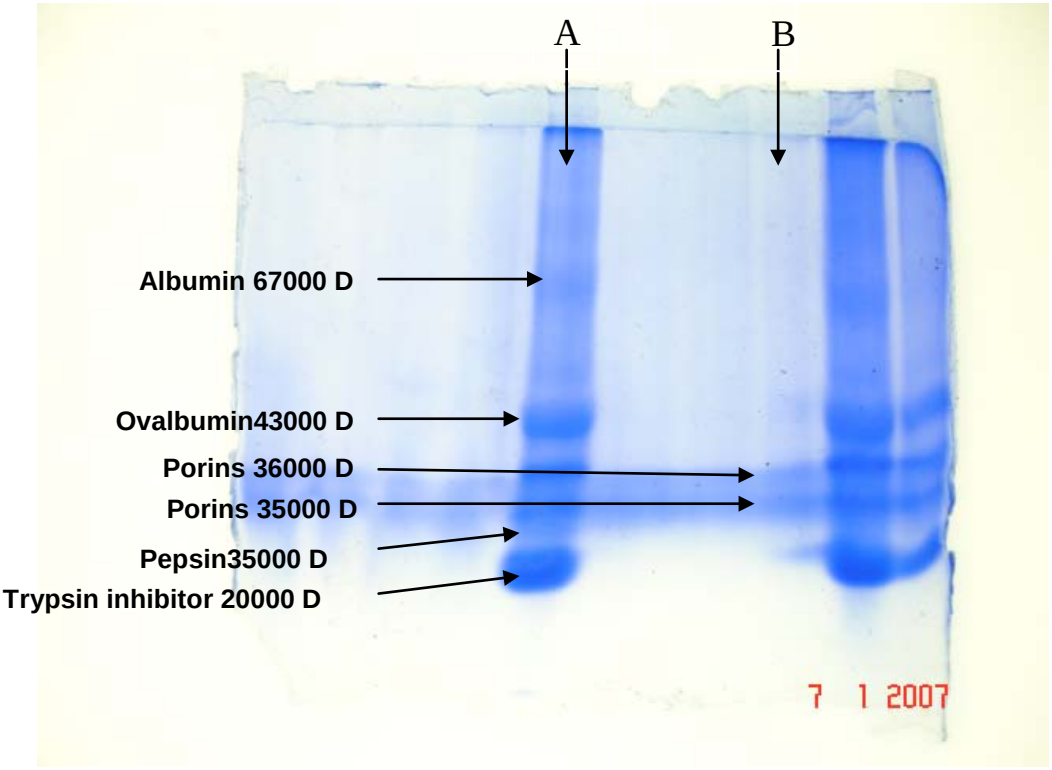

Figure 2: SDS polyacrylamide gel electrophoresis of porins.
A. Standard proteins.
B. Porins sample.

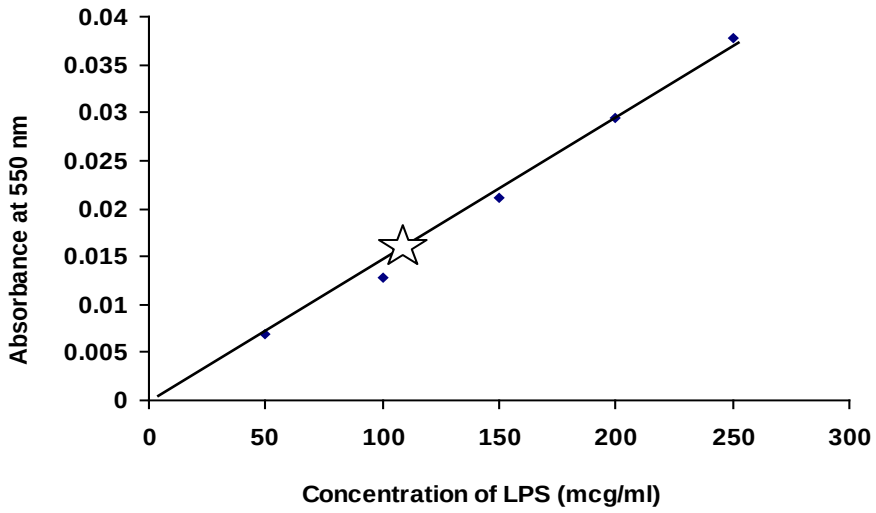

Figure 3: Standard curve of Lipopolysaccharides (LPS)

Discussion

Porins have been purified from a number of gram negative bacteria. In all so far examined cases; the apparent molecular weights of the proteins are in the range of 30 to 45 kDa, while some of the porins can be purified as oligomers in SDS. They are usually acidic proteins and in many cases show association with peptidoglycan. In addition, outer membrane contains multiple species of porins. The separation of one porin from the other is difficult, for example, one can suppress the production of one or more porins through manipulation of culture conditions [(7)].

Among the outer membrane proteins found in gram-negative bacteria are the abundant porins which form diffusion channels for small molecules such as metabolizable sugars [(8)]

It is well documented that disruption of cells will increase membrane protein yield. Thus, enzyme digestion was used to disrupt the bacterial cells, and the use of lysozyme – EDTA greatly enhances membrane destruction [(9)]. Furthermore, the use of DNase and RNase would result in degradation of nucleic acids and increasing the purity of proteins [(10)].

The protease resistance capability of the porins could be utilized for their isolation. For example, trypsine solubilizes practically all the proteins of the TX- treated envelopes degrading at the same time most of them with the exception of porins [(3)].

The results of this study are expectedly consistent with the results of Galdiero & Co-workers, (1994) [(11)] and Meghji &Co-workers, (1997) [(8)] who mentioned that the purified porins from *Salmonella typhimurium* showed the two expected bands with molecular masses of 34 and 36 kDa and the purified porin from *Pseudomonas aeruginosa* showed two bands with a molecular mass of 36 to 38 kDa, respectively. The apparent similarity of the results might indicate phyllogenetic relationship since outer membrane proteins are conserved, with minor differences, in all members of the gram negative bacteria.

Porins possess a high proportion of β-sheet structure, which traverses the membrane in a tightly packed β - barrel organization. This makes them relatively resistant to denaturation by SDS or other detergents at low temperature but not at higher temperatures [(12)]. Therefore, porins display different motilities when they were separated at low or high temperatures [(13)].

Porins usually have a strong association with LPS and it is difficult to obtain the proteins completely free of LPS contamination [(14)].

It is stated that, by binding to the lipid A of LPS, polymyxin B completely inhibits the strong cytopathic effect of this lipid whereas binding to the porins leaves the biological activity of the protein unmodified [(15)].

It is stated that a concentration of 0.5-1.0 mg/ml of LPS is required for *in vitro* cytotoxic effect [(16)]. The results of the current study revealed close proximity to that of Luo & Co-workers, (1997) [(17)] when they estimated LPS concentration of 0.418 µg of Ketodeoxyoctinate (KDO) / mg of protein. While in another study, it is mentioned that the content of LPS in porins preparations was in the order of 1 pg/mg of porin [(7)]. Furthermore, in another experiment to extract and purify porins from *Pseudomonas aeruginosa*, much lower value for LPS was recorded in the final preparation (about 20 µg/ ml) which was neutralized by incubation with polymyxin B as mentioned above [(15)].

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