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Application of zwitterionic detergent to the solubilization of *Klebsiella pneumoniae* outer membrane proteins for two-dimensional gel electrophoresis

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ABSTRACT

Klebsiella pneumoniae is a frequent cause of nosocomial respiratory, urinary and gastrointestinal tract infections and septicemia with the multidrug-resistant *K. pneumoniae* being a major public health concern. Outer membrane proteins (OMPs) are important virulence factors responsible for the appropriate adaptation to the host environment. They constitute of the antigens being the first in contact with infected organism. However, *K. pneumoniae* strains are heavily capsulated and it is important to establish the OMPs isolation procedure prior to proteomics extensive studies. In this study we used Zwittergent Z 3-14® as a detergent to isolate the OMPs from *K. pneumoniae* cells and resolve them using two-dimensional electrophoresis (2-DE). As a result we identified 134 protein spots. The OMPs identified in this study are possible candidates for the development of a protein-based vaccine against *K. pneumoniae* infections.

1. Introduction

Klebsiella pneumoniae is an opportunistic pathogen which causes many nosocomial infections including the most common urinary tract infections, pneumonia and septicemia especially in immunocompromised patients. Recent investigations connect *K. pneumoniae* infections with inflammatory bowel diseases or liver abscesses. Also, they rank them second to *Escherichia coli* cause of the bacteremia due to biliary infection (Ebringer et al., 2007; ECDC Surveillance report, 2007; Yang et al., 2009; Lee et al., 2011; Ortega et al., 2012). *K. pneumoniae* is (among ten) the most frequently detected pathogen in infections related to hospitalization (Hidron et al., 2008). Despite the use of appropriate antibiotic therapy, the morbidity and mortality due to *K. pneumoniae* remain extremely high, with mortality rates up to 60% (Coovadia et al., 1992; Sahly and Podschun, 1997; Yadav et al., 2005). Moreover, multiresistant strains are frequently isolated, emphasizing the need to find new ways to prevent and treat *K. pneumoniae* infections (Lytsy et al., 2008; Chagas et al., 2011). Unfortunately, no vaccine capable of preventing infection by *K. pneumoniae* has been developed yet.

Among the different cell structures, surface components are being discussed as candidates for vaccine: siderophores, exotoxins, lipopolysaccharides (LPS) and capsular polysaccharides (CPS) (Podschun and Ullmann 1998; Yadav et al. 2005). However, the LPS released from the bacterial cells may cause renal scaring, even after one episode of bacterial infection (Cryz et al., 1984). The best characterized virulence factor of *K. pneumoniae* is CPS, which protects bacteria from complement attack and antimicrobial peptide-mediated killing (Campos et al., 2004; Moranta et al., 2010). Also, bacterial CPS prevents phagocytosis by host cells (Athamna et al., 1991). Most of the studies have been focused on the role of LPS and CPS in pathogenicity of *K. pneumoniae*. LPS has nine different O-type antigens, but its possible application for vaccination is hampered by high toxicity during immunization. CPS, in turn, has proved to be highly immunogenic and non-toxic. However, it has a large variety of K-type antigens (77 different) and hence requiring the application of multivalent vaccines. A vaccine with 24 different K-types has been reported, but there is no information of any further development (Cryz et al., 1986; Yadav et al. 2005). Due to the disadvantages of these antigens as vaccine components, research has been directed toward more conservative proteins, such as extracellular toxins, outer membrane proteins (OMPs) or fimbriae proteins (Klipstein et al., 1983; Goetsch et al., 2001; Lavender et al., 2005; Struve et al., 2009). Of these, FepA, OmpA OmpK17, OmpK36 and colicin have been selected as potential vaccine candidates (Lai et al., 2001; Struve et al., 2003; Kurupati et al., 2006). Positive results have been reported for a DNA vaccine based on outer membrane proteins in a study conducted on a mouse model (Kurupati et al., 2011).

Data supporting the hypothesis on surface-related proteins being involved in bacterial pathogenesis, for example in adhesion, invasion and protection against host immune response, has been recently

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accumulated. Proteins attached to the outer membrane are important for membrane integrity and provide transport of molecules from the environment (Lin et al., 2002). Also OMPs are receptors for bacteriophages and play a key role in signal transduction, intracellular transport, and energy transformation processes ensuring proper bacterial cell functioning (Navarre and Schneewind, 1999; Cabanes et al., 2002; Fluegge et al., 2004; Maione et al., 2005). Therefore, identifying novel OMPs may aid research on new strategies of preventing colonization and infection with *K. pneumoniae*.

OMPs are hard to purify and solubilize for general analysis due to their hydrophobic nature. They are often associated with other substances such as membrane lipids. In this investigation we used Zwittergent Z 3-14® as an effective detergent to isolate the OMPs of *K. pneumoniae* and resolve them using two-dimensional electrophoresis (2-DE). The Zwittergent 3-14® has already been used for isolation of OMPs from a number of bacterial species (Kokeguchi et al., 1991; Tagawa et al., 1993; Kokeguchi et al., 1994; Pal et al., 1997; Halling and Koster, 2001; Gatto et al., 2002; Siritapetawee et al., 2004; Augustyniak et al., 2010; Bugla-Płoskońska et al., 2009; Bugla-Płoskońska et al., 2011; Futoma-Kołoch et al., 2009; Zhang et al., 2011), but its application for OMPs of *K. pneumoniae* has not been tried yet. Rationale of these studies on *Klebsiella* was thick capsule produced by these strains and the need to establish the 2-DE conditions prior to proteomics extensive studies.

2. Material and methods

2.1. Bacterial strains and growth conditions

K. pneumoniae strain PCM 2713 from the Polish Collection of Microorganisms (PCM) at Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wroclaw, Poland was selected for this study. Culture was grown for 18 h at 37 °C, under shaking (200 rpm) in 50 ml of Brain Heart Infusion Broth (Difco).

2.2. Isolation of outer membrane proteins

Outer membrane proteins were isolated using Murphy and Bartos' method (1989) with minor modifications (Bugla-Płoskońska et al., 2009; Futoma-Kołoch et al., 2009; Bugla-Płoskońska et al., 2011). Overnight cultures were centrifuged (4000 rpm, 4 °C, 20 min) and bacteria were resuspended in 1.25 ml of 1 M sodium acetate (POCH) with 1 mM β-mercaptoethanol (Merck). Subsequently, 11.25 ml of 5% (w/v) Zwittergent Z 3-14® (Calbiochem) and 0.5 M $CaCl_2$ (POCh) in water were added and stirred for 1 h at room temperature (RT). To precipitate nucleic acids, 3.13 ml of 96% (v/v) cold ethanol (POCH) was added very slowly and centrifuged (12,300 rpm, 4 °C, 10 min). Proteins were precipitated from supernatant after centrifugation (12,300 rpm, 4 °C, 10 min) by addition of 46.75 ml of 96% (v/v) cold ethanol. Pellet was dried at RT, then suspended in 2.5 ml of 50 mM Trizma-Base (Sigma-Aldrich) buffer, pH 8.0 with 0.05% (w/v) Zwittergent Z 3-14® and 10 mM EDTA (Sigma-Aldrich), stirred for 1 h at RT and subsequently kept at 4 °C overnight. Insoluble material was removed from OMPs solution by centrifugation (8700 rpm, 4 °C, 10 min).

Protein content was quantified by BCA Protein Assay according to Smith et al. (1985) with bovine serum albumin (BSA) (Thermo Scientific) as the standard.

Prior to 2-DE, OMPs preparations were pretreated with ReadyPrep™ 2-D Cleanup Kit (Bio-Rad) according to the manufacturer's instructions to reduce interference with substances contaminating samples for isoelectric focusing (IEF) and 2-DE.

To assure that soluble fractions of buffer Z are not contaminated with cytosolic proteins, the enzymatic activity of succinic dehydrogenase (marker of cytoplasmic proteins) was checked in previous preparations of OMPs from *E. coli* and *Salmonella* spp. (Bugla-Płoskońska et al., 2009; Futoma-Kołoch et al., 2009; Bugla-Płoskońska et al., 2011) using Rockwood et al.'s method (1987) and the result was negative.

2.3. Two-dimensional electrophoresis

The OMPs from *K. pneumoniae* were separated with 3–10 immobilized gradient IPG strips and 4–7 pH immobilized gradient IPG strips (7 cm) (Bio-Rad). 2-DE was carried out with the Mini-PROTEAN® Tetra Cell System (Bio-Rad). The main reagents for 2-DE were purchased from Bio-Rad and used according to the manufacturer's instructions (Bio-Rad). Prior to the first dimension, precast IPG strips were rehydrated with 120 μl rehydration buffer (Bio-Rad), 15 μg of proteins and 3 μl of proteome marker for 2-DE (Serva) (16 h, RT). The rehydrated strips were positioned in the focusing tray and covered with 2 ml of mineral oil (Bio-Rad) to prevent evaporation. Isoelectric focusing (IFE) was conducted by stepwise increase of the voltage as follows: 250 V, 20 min, 4000 V, 120 min (linear) and 4000 V (rapid) until the total volt-hours reached 14 kVh. After IEF separation, the strips were equilibrated in 6 M urea, 0.375 M Tris, 2% SDS, and pH 8.8, reduced with 2% (w/v) DTT and alkylated with 0.135 M iodoacetamide. IPG strips were then loaded onto the top of a 9–12.5% gradient polyacrylamide gel (10 × 8 cm, 1.0 mm thick) using 0.5% agarose (Bio-Rad) in the running buffer. Molecular mass standards — Precision Plus Protein™ Standards (Bio-Rad) were applied at the basic end of the IPG strips.

Modified Laemmli (1970) system with tricine (Calbiochem) instead of glycine in the electrophoresis buffer (50 mM tricine, 0.1% (w/v) sodium dodecyl sulfate, pH 8.2) was used as previously described (Bugla-Płoskońska et al., 2009). Electrophoresis was performed at 4 °C with constant power (1 W) until the dye front reached the bottom. Following separation in the second dimension, the gels were stained using Gromova and Celis' method (2006).

Spot patterns were visualized under white light and photographed using a GelDoc XR camera system (Bio-Rad). Spots of proteins were analyzed by PDQuest software (Bio-Rad). To create master gel three repetitions of 2-DE procedures were performed. Master or reference gel is a virtual representative profile for match-set among the sample gels. The master gel summarizes all identified spots and serves as the basic image. Molecular masses of analyzed proteins were calculated by comparing with that of molecular mass markers for 2-DE and the pI values were calculated according to the linearity of IPG strips using the software.

3. Results and discussion

In this investigation we used Zwittergent Z 3-14® as an effective detergent to isolate the OMPs of *K. pneumoniae* and resolve them using two-dimensional electrophoresis. OMPs from *K. pneumoniae* were separated by 2-DE in two pH ranges: 3–10 and 4–7 (Figs. 1A and 2A). More accurate separation was achieved when pH 4–7 IPG strips were used. Three replicates of 2-DE gels were stained for this strain and showed similar results. After 2-DE, silver stained gels revealed about 85 spots within the 3–10 range and 134 spots within the 4–7 pH range (Figs. 1B and 2B). Most of the proteins had molecular mass between 25 and 50 kDa. The pI and mass of spots are shown in Table 1.

In order to obtain the highest concentration and the best quality of proteins that will promote the likelihood of identifying as many proteins in 2-DE as possible, another method was employed, including isolations using Triton X-100. However, this method yielded poor quality of separation (Figs. 3A, B and 4A, B).

Isolation by Zwittergent Z 3-14® produced substantially higher concentration and better quality of protein spots and was therefore selected for use in this study to obtain the soluble and insoluble bacterial outer membrane proteins for analysis.

Zwittergent Z 3-14® was successfully applied for highly efficient isolation of OMPs from *Neisseria gonorrhoeae* (Blake and Gotschlich, 1984),