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Use of Zwitterionic Type of Detergent in Isolation of *Escherichia coli* O56 Outer Membrane Proteins Improves their Two-Dimensional Electrophoresis (2-DE)

GABRIELA BUGLA-PŁOSKOŃSKA*, BOŻENA FUTOMA-KOŁOCH, ALEKSANDRA SKWARA, WŁODZIMIERZ DOROSZKIEWICZ

Department of Microbiology, Institute of Genetics and Microbiology, University of Wrocław, Poland

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Abstract

Escherichia coli O56 were originally isolated from infected humans. Here it is reported that using the zwitterionic detergent (Zwittergent Z 3-14®) to isolate outer membrane proteins (OMPs) from *Escherichia coli* O56 is suitable for their separation by two-dimensional electrophoresis (2-DE) using pH 3-10 immobilized pH gradient IPG strips (BIO-RAD).

Key words: *Escherichia coli* O56, outer membrane proteins, two-dimensional electrophoresis

Introduction

Proteomics is a powerful tool that is used in many parts of biology. One of them is electrophoretic study of outer membrane proteins (OMPs) of Gram-negative bacteria. These structures of the bacterial cells are the keys that interact with the environment. It has been discovered that changes in OMPs expression can be a reason for the resistance of Gram-negative bacteria to the bactericidal action of serum (Bugla *et al*., 2004; Bugla-Płoskońska and Doroszkiewicz, 2006; Futoma *et al.*, 2005; Mielnik *et al*., 2001). The resistance of bacteria to serum's lytic activity as a result of the expression of many virulence factors may be essential in the development of sepsis and septic shock. Taylor and Parton (Taylor and Parton, 1976) proved that a 46 kDa OMP plays a decisive role in *Escherichia coli* resistance to serum. Studies of Kroll and co-authors (Kroll *et al*., 1983) and Taylor and Parton (Taylor and Parton, 1976) have shown that treating *E. coli* cells with sera generates changes in their composition of OMPs. Proteomics proves that OMPs are virulence factors of many diseases, for example proteins: OmpA, IbeA, IbeB, IbeC, AslA, TraJ of *E. coli* are involved in meningitidis infections (Badger and Kim, 1998).

E. coli O56 were originally isolated from infected humans from mesenterial lymph node (Orskow *et al*., 1977). It was determined (Mielnik *et al*., 2001; Bugla-Płoskońska and Doroszkiewicz, 2006), that bacteria of this serotype are sensitive to the bactericidal activity of normal cord serum (NCS) and normal bovine serum (NSB). Gamian and co-authors (Gamian *et al*., 1994) have shown that sialic acid is a component of the O-specific part of the polysaccharide chains of *E. coli* O56. Sialic acids may contribute to the pathogenicity of the microorganisms by mimicking host tissue components (Vimr and Lichtensteiger, 2002). On the other hand, sialic acid as a component of bacterial capsules activates the complement system in serum. This was shown for encapsulated *Streptococcus agalactiae*, which is the most common cause of neonatal sepsis and meningitis (Aoyagi *et al*., 2008). Recent investigations are also based on finding and testing the protective potential of OMPs against life-threating invasive bacterial infections caused *i.e. Neisseria meningitidis* serogroup B (Jessouroun *et al*., 2004) and *Pseudomonas aeruginosa* (Sorichter *et al*., 2009). Proteomics gives possibilities to find molecular candidates for vaccines. Witkowska and co-workers (Witkowska *et al*., 2006) proved that a 38 kDa OMP

* Corresponding author: G. Bugla-Płoskońska, Department of Microbiology, Institute of Genetics and Microbiology, University of Wrocław, Przybyszewskiego 63/77, 51-148 Wrocław, Poland; phone: (+48) 71-3756306; fax: (+48) 71-3252151, **e-mail: gabi@microb.uni.wroc.pl**

present in most *Enterobacteriaceae* species is a protein that generates immunological response in human organisms and is a good candidate for creating a vaccine against such species as *Escherichia coli*, *Shigella flexneri*, *Klebsiella pneumoniae* or *Proteus vulgaris.* Hamid and Jain (Hamid and Jain, 2008) showed that immunization of mice with a 49 kDa OMP gives them 100% survival after being treating with a lethal dose of *Salmonella* Typhimurium.

For any bacterial proteomic study sample preparation is a crucial step and is a critical influential factor in isoelectric focusing (IEF). The aim of this paper is to present a 2-DE procedure in conjunction with the isolation of bacterial OMPs using the zwitterionic detergent – Zwittergent Z 3-14®.

Experimantal

Materials and Methods

Bacterial cell culture. *Escherichia coli* O56 PCM 2372 from the collection of the Institute of Immunology and Experimental Therapy, Wrocław, Poland, PCM (NCTC 9056-National Collection of Type Cultures, Central Public Health Laboratory, London) were inoculated into 50 ml of Brain Heart Infusion broth (Difco) in 200 ml shake flasks and left to grow at 37°C for 18 h.

Isolation of outer membrane proteins (OMPs). The procedure of isolation of OMPs was done according to Murphy and Bartos (Murphy and Bartos, 1989) with minor modifications. Bacterial cells from an overnight culture were harvested (4000 rpm at 4°C for 15 min) and the pellet was suspended in 1.25 ml of buffer b [1 M sodium acetate (POCh), 0.001 M β-mercaptoethanol (Merck)]. Then 11.25 ml of a water solution containing 5% (w/v) Zwittergent Z 3-14® (Calbiochem) and 0.5 M $CaCl_2$ (POCh) was added. This mixture was stirred at room temperature (RT) for 1 h. To precipitate nucleic acids, 3.13 ml of 96% (v/v) cold ethanol (POCh) was added very slowly. The mixture was then centrifuged at 12 300 rpm at 4°C for 10 min. The proteins in the supernatant were precipitated by the addition of 46.75 ml of 96% (v/v) cold ethanol and centrifuged at 12 300 rpm at 4°C for 20 min. The pellet was left to dry at ambient temperature and then suspended in 2.5 ml of buffer Z [0.05% (w/v) Zwittergent Z 3-14®, 0.05 M Trizma-Base (Sigma) and 0.01 M EDTA (Sigma), pH 8.0] and stirred at RT for 1 h. The solution was kept at 4°C overnight and centrifuged at 8 700 rpm at 4°C for 10 min. OMPs were present in the soluble fraction of buffer Z after the centrifugation.

The preparations of OMPs in the soluble fraction of buffer Z were checked for the presence of succinic dehydrogenase activity, a marker for cytoplasmic membranes, using the method described by Rockwood *et al*. (1987).

Protein quantification. Protein quantification was performed with the BCA Protein Assay Kit (PIERCE®) according to Smith *et al*. (Smith *et al*., 1985) with bovine serum albumine (BSA) (Sigma) as the standard. The Pierce BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. The purple-colored reaction product (reduction Cu^{2+} to Cu^{+1} by protein in an alkaline medium) exhibits a strong absorbance at 562 nm with increasing protein concentration over a broad range: 20–20,000 µg/ml) (PIERCE Instruction).

Removal of salts, buffers and small ionic contaminants from buffer Z containing OMPs. In the next stages of preparation a 2-DE Sample Preparation for Soluble or Insoluble Proteins (PIERCE) was used according to the manufacturer's instructions. After the desalting procedure (Protein Desalting Spin Columns, PIERCE) the samples of OMPs were suspended in a buffer (PIERCE) that was compatible for 2-DE.

Two-dimensional electrophoresis (2-DE). The OMPs from *E. coli* O56 were separated on a series of 7 cm pH 3–10 immobilized pH gradient (IPG) strip. The electrophoresis separation of proteins was performed essentially as described by O'Farrell (O'Farrell, 1975). 2-DE was carried out with the PROTEAN® IEF Cell (BioRad). The main reagents for 2-DE were purchased from Bio-Rad and basically used according to the manufacturer's instructions (BioRad Instruction Manual).

Rehydratation. IPG strips (7 cm) were rehydrated prior to isoelectric focusing. The rehydratation step was performed outside the PROTEAN® IEF Cell (passive rehydratation). A total amount of 169 µg of OMPs was suspended in 125 µl of rehydratation buffer (8M urea, 0.5% CHAPS, 10mM DTT). The rehydrated strips were positioned in the focusing tray and covered with mineral oil (BioRad). The time of the rehydratation process amounted 16 hours.

Isoelectric focusing was conducted for: Step 1: 250 V, 20 min (linear); Step 2: 4 000 V, 120 min (linear); Step 3: 4 000 V, 160 min (rapid).

Total volt-hours parameter reached 14 000 V-hr. After IEF the IPG strips were removed from the focusing tray and were transferred into rehydratation/equilibration buffer for 20 min (BioRad).

SDS-PAGE. For the second dimension the IPG strips were applied onto a 9–12.5% gradient SDS-polyacrylamide gel (PAGE) using 1% (w/v) agarose in the running buffer. Gels were electrophoresed according to Laemmli (Laemmli, 1970). Tricine was used instead of glycine in the electrophoresis buffer [0.05 M Trizma Base, 0.05 M tricine, 0.1% (w/v) sodium dodecyl sulfate (SDS), pH = 8.2]. The gels were run at 5°C and at 35 V for 125 min. After that they were

stained for 24 h with coloidal Coomassie Brilliant Blue R-250 (Merck). The gel images were scanned using PDQuest 2-D Analysis Software v. 8.0.1 (BioRad). The tests were repeated three times.

Result and Discussion

Detection and identification of OMPs is of particular interest as they play important functions in signal transduction pathways, bacterial-host interactions, and other processes. However, detection of hydrophobic OMPs in 2-DE gels is associated with certain limitations (Fountoulakis, 2005). The poor solubility of hydrophobic OMPs accounts for their absence from the 2D gel map, but the addition of zwitterionic detergents can improve protein solubilization (Shaw and Riederer, 2006). The anionic nature of sodium dodecyl sulfate (SDS) detergents generally limits their effectiveness for proteomic analyses. Zwitterionic detergents have found widespread use in 2-DE (Luche *et al*., 2003; Henningsen *et al*., 2002). Zwitterionic detergents lack conductivity and electrophoretic mobility and are also suited for breaking protein-protein interactions (Srirama, 2001). Sample preparation is a very crucial step in 2-DE. Some modifications (use of other detergents, different way of sample preparation) were introduced into the 2-DE protocol suggested by O'Farrell (O'Farrell, 1975) and BioRad (Instruction Manual, 2008) which significantly impaired the resolution of proteins. In this case, the detergent Zwittergent 3-14® was used for the isolation of OMPs and not Nonidet P-40. Additionally, in the methods proposed by O'Farrell isoelectric focusing gels were made in glass tubing.

The samples of bacterial OMPs were tested for the presence of biological membranes. The preparations were assayed for the enzymatic activity of succinic dehydrogenase, a marker for the cytoplasmic membrane. The Zwittergent-extracted OMPs from *E. coli* O56 contained no detectable activity of succinic dehydrogenase which confirms that they were free of membrane contaminations.

Each sample of OMPs, appropriately prepared for 2-DE separation (*i.e.* desalting), was run in triplicate: three gels were obtained from *E. coli* O56 samples. Fig. 1 shows representative examples of the OMPs separated on a 2-DE gel, to which 169 µg of total OMPs protein (per gel) was applied. Approximately 59 (a), 62 (b), and 63 (c) protein spots were detected on Coomassie Brilliant Blue R-250 gels (Fig. 1). Fig. 2 shows the master gel which was generated on the basis of the (a), (b), and (c) gels visualisations (Fig. 1). The master gel shows 63 separated spots of OMPs of *E. coli* O56.

The obtained results show that Zwittergent Z 3-14® is suitable for the isolation of OMPs from *E. coli* O56. This paper confirms that detergents of this type may be used in the isolation of OMPs and subsequently in 2-DE with IPG strips. During 2-DE analysis we marked the main spots and were able to visualize even 63 individual protein spots. This method is a promising tool for the characterisation of bacterial virulence factors as OMPs.

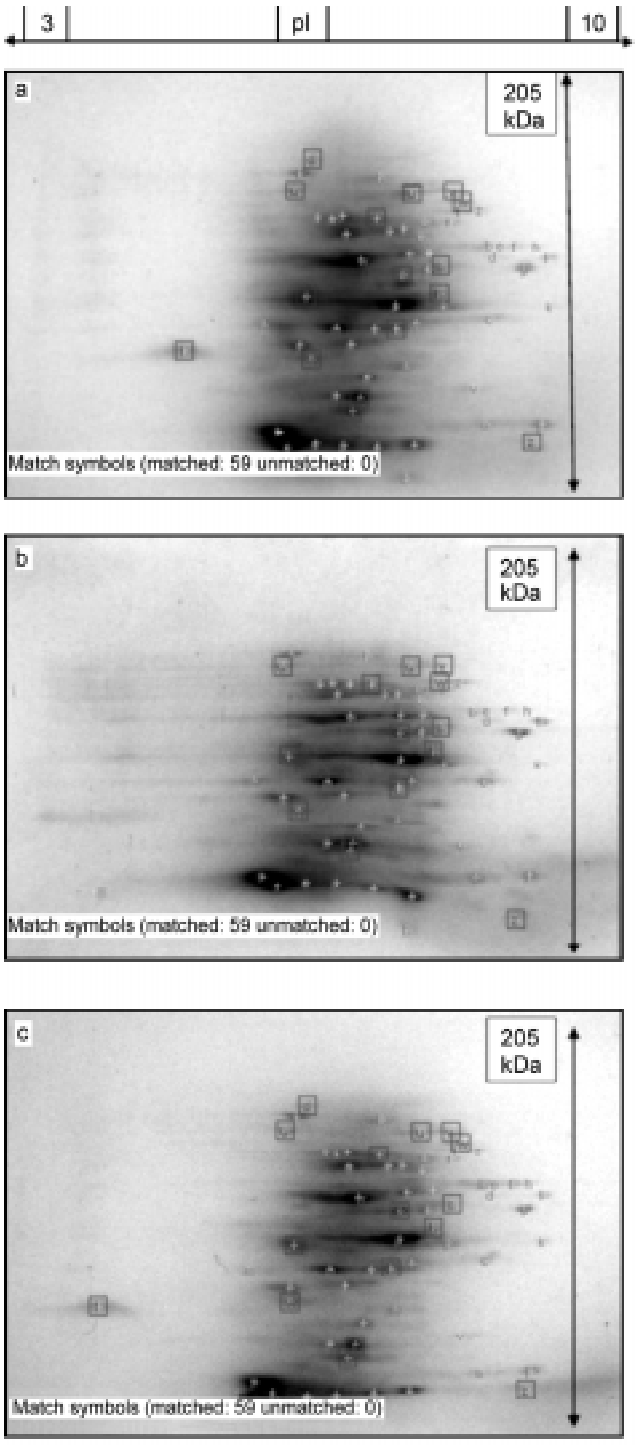

Fig. 1. 2-DE profile of OMPs of *E. coli* O56 isolated with Zwittergent Z 3-14®. OMPs were separated using pH 3–10 IPG strips (BioRad) and 9–12.5% SDS-PAGE.
Gels were stained with the Coomassie Brilliant Blue R-250 (Merck) and preliminarily analyzed with PDQuest 2-D Analysis Software v. 8.0.1 (BioRad).

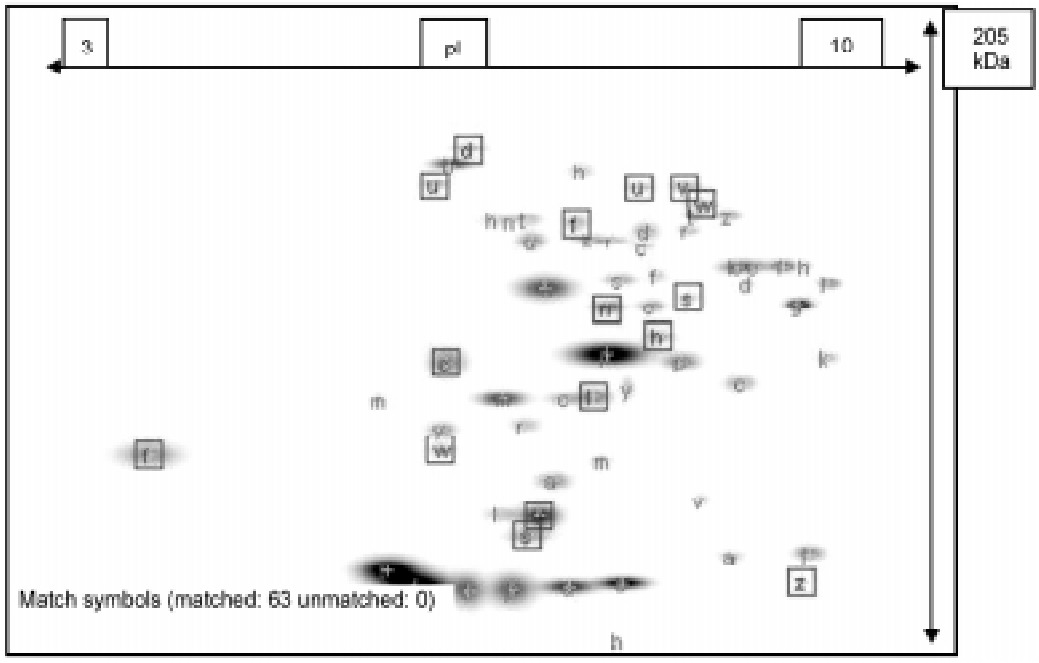

Fig. 2. Master of three 2-DE gel profiles (from Fig. 1 a, b, c) of OMPs of *E. coli* O56 isolated with Zwittergent Z 3-14® and analyzed with PDQuest 2-D Analysis Software v. 8.0.1 (BioRad).

2-DE methodology has a potential for the rapid development of specific, safe, and highly efficacious vaccines against infection caused by *E. coli* in humans and livestock. 2-DE electrophoretic methods have been successfully used for the separation of, among others, *Escherichia coli* (Molloy *et al*., 2000), *Salmonella* Typhimurium (Hamid and Jain, 2008), *Brucella abortus* (Connolly *et al*., 2006), *Edwardsiella tarda* (Kawai *et al*., 2004), *Shigella flexneri* (Peng *et al*., 2004), and *Leptospira interrogans* (Cullen *et al.*, 2002) OMPs, but none of the authors used zwitterionic detergents in the stages of OMPs preparation. They used buffer with urea (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 6M urea) (Hamid and Jain, 2008), detergent TRITON X-114 (Cullen *et al.*, 2002), sodium lauryl sarcosinate (Peng *et al*., 2004).

We conclude that Zwittergent Z 3-14® detergent is as an effective detergent for the isolation of the OMPs of *E. coli* O56 and can be adapted to 2-DE using IPG strips.

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