

## Isolation of Outer Membrane Proteins (OMP) from *Salmonella* Cells Using Zwitterionic Detergent and Their Separation by Two-Dimensional Electrophoresis (2-DE)

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Received 26 May 2009, revised 25 September 2009, accepted 30 September 2009

### Abstract

We report that using the zwitterionic detergent Zwittergent Z 3-14<sup>®</sup> to isolate outer membrane proteins (OMPs) from *Salmonella* O48 is suitable for their separation by two-dimensional electrophoresis (2-DE) in a capillary tube system. Sample preparation is a very crucial step for any bacterial proteomic study. Some modifications were introduced to the 2-DE protocol suggested by O'Farrell and BioRad, which significantly impaired the resolution of proteins. 2-DE analysis of OMPs may be helpful in the interpretation of the variable susceptibility of *Salmonella* O48 rods to the bactericidal activity of serum.

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**Key words:** *Salmonella*, outer membrane proteins, two-dimensional electrophoresis

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*Salmonella* O48 strains are Gram-negative bacteria that are clinically important microorganisms causing acute enteritis in children and in immunodeficient adults. The outer membrane of Gram-negative bacteria is characterized mainly by the presence of lipopolysaccharides (LPS) and outer membrane proteins (OMPs) (Raetz and Whitfield, 2002). The LPS of *Salmonella* serotype O48 has been shown to contain long-chain sialic acid-containing O-specific polysaccharides (Gamian *et al.*, 2000). Sialic acids may contribute to the pathogenicity of the microorganisms by mimicking host tissue components (Vimr and Lichtensteiger, 2002). OMPs are also important virulence factors and have a significant role in pathobiology and bacterial adaptation to environmental conditions. These proteins are of special interest to us as they can modify the susceptibility of bacteria to, for example, the bactericidal activity of normal human serum (NHS) (Alberti *et al.*, 1993). The resistance of bacteria to the lytic activity of serum may be essential in the development of sepsis and septic shock. It is well documented that the cytolytic activity of serum depends on the presence of the complement system (Lutz *et al.*, 2007). Some OMPs undoubtedly amplify the sensitivity of bacteria, such as *Neisseria meningi-*

*tidis* (Zollinger *et al.*, 1987), *Aeromonas hydrophila* (Merino *et al.*, 1998), and *Klebsiella pneumoniae* (Alberi *et al.*, 1993), to the bactericidal activity of serum. Other OMPs enhance the resistance of bacteria to the cytolytic activity of complement, for example OmpA (*Escherichia coli*) (Weiser and Gotschlich, 1991), DsrA (*Haemophilus ducreyi*) (White *et al.*, 2005), and UspA2 (*Moraxella catarrhalis*) (Attia *et al.*, 2005). Our long-term investigations have shown that LPS with sialic acid does not secure *Salmonella* serotype O48 against an immunological response (Bugla-Płoskońska and Doroszkiewicz, 2006; Mielnik *et al.*, 2001). Therefore, we focused on OMPs as factors which can modulate the susceptibility of the rods to serum. 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 like non-ionic detergents do not possess a net charge, they lack conductivity and electrophoretic mobility, and do not bind to ion-exchange resins. Like ionic detergents, they are also suited for breaking protein-protein interactions (Srirama, 2001). Sample preparation is a very crucial step in 2-DE. The aim of this paper is to

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present a 2-DE procedure in conjunction with the isolation of *Salmonella* OMPs using zwitterionic detergent – Zwittergent Z 3-14<sup>®</sup>. The procedure of isolation of OMPs was done according to Murphy and Bartos (1989) with minor modifications. A method involving extraction of OMPs with the Zwittergent Z 3-14<sup>®</sup> was adapted for *Salmonella* O48 strains from methods developed for isolating OMPs of *Branhamella catarrhalis* (presently *Moraxella catarrhalis*) (Murphy and Bartos, 1989) and *Haemophilus influenzae* (Murphy and Bartos, 1988). The preparations of OMPs were assayed for the presence of succinic dehydrogenase, a marker for cytoplasmic membrane (Rockwood *et al.*, 1987). The Zwittergent-extracted OMPs of two *Salmonella* strains as well as OMPs of *Moraxella catarrhalis* (Murphy and Bartos, 1989) contained no detectable succinic dehydrogenase activity, which indicates that Zwittergent-extracted OMPs preparations were free of contamination by cytoplasmic membrane. The procedure of isolation of OMPs was modified at two stages of preparation: bacteria were multiplied in a liquid medium BHI (Brain Heart Infusion, Difco) left to grow overnight at 37°C for 18 h. The next modification concerning the fractions of OMPs suspended in a buffer Z which were kept at 4°C overnight (before centrifugation at 8700 rpm at 4°C for 10 min).

Bacteria (*Salmonella enterica* subsp. *arizonae* PCM 2543, and *S. enterica* subsp. *enterica* sv. Dahlem PCM 2512 from the Polish Collection of Microorganisms (Institute of Immunology and Experimental Therapy, Wrocław, Poland, PCM) were inoculated into 50 ml of BHI in 200-ml shake flasks and left to grow at 37°C for 18 h. Bacterial cells from the overnight culture were harvested (4000 rpm at 4°C for 15 min) and the pellet was suspended in 1.25 ml of buffer  $\beta$  [1 M sodium acetate (POCh), 0.001 M  $\beta$ -mercaptoethanol (Merck)]. In turn, 11.25 ml of a water solution containing 5% (w/v) Zwittergent Z 3-14<sup>®</sup> (Calbiochem) and 0.5 M CaCl<sub>2</sub> (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 12300 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 12300 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<sup>®</sup>, 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 8700 rpm at 4°C for 10 min. OMPs were present in the soluble fraction of buffer Z after centrifugation. Protein quantification was performed with the BCA Protein Assay according to Smith *et al.* (1985) with bovine serum albumin

(BSA) (Sigma) as the standard. Solutions containing 100 mg of protein were diluted with 72% (w/v) of trichloroacetic acid (TCA) (Chempur) to reach 6% (w/v) of TCA and incubated at RT for 10 min. The samples were then centrifuged at 8700 rpm for 10 min at 15°C. The supernatant was removed and the pellets were washed with 0.5 ml of 90% cold acetone (POCh). The samples were kept on ice for 15 min and harvested at 8700 rpm for 15 min at 15°C. The supernatant was removed and the remaining pellet was left to dry. In the next stages of preparation a ProteoExtract<sup>™</sup> Protein Precipitation Kit (Calbiochem) was used according to the manufacturer's instructions. 2-DE was carried out with the Mini-PROTEAN<sup>®</sup> 3 System (BioRad). The main reagents for 2-DE were purchased from Bio-Rad and used practically according to the manufacturer's instructions (BioRad). It was necessary to reduce the molar concentration of urea and replace Bio-Lyte 5/7 ampholyte with ddH<sub>2</sub>O in three reagents: the first-dimension sample buffer, the first-dimension sample overlay buffer, and the first-dimension gel monomer solution. Tricine (Calbiochem) was also used instead of glycine in the electrophoresis buffer (second dimension). Furthermore, the time and the voltage of the isoelectrofocusing (IEF) run were established empirically. The protein pellets obtained after precipitation were diluted in 20 ml of sample buffer [6.0 M urea, 2% (v/v) Triton X-100, 5% (v/v)  $\beta$ -mercaptoethanol, 1.6% (v/v) Bio-Lyte 3/10 ampholyte]. After 10 min of incubation at RT the samples were collected (7800 rpm 2 min, 15°C) and 20  $\mu$ l of the supernatant was applied to the top of a tube gel containing ampholytes [6.0 M urea, 4% (w/v) acrylamide, 2% (v/v) Triton X-100, 1.6% (v/v) Bio-Lyte 3/10 ampholyte, 0.01% (w/v) ammonium persulfate, 0.1% (v/v) TEMED]. The tube gels were pre-electrophoresed by running at 200 V for 10 min, 300 V for 15 min, and 400 V for 15 min (electrode preparation, Apex PS 900S TX). After pre-electrophoresis, the upper and lower chamber buffers were replaced with fresh solution. The samples were loaded on the top surface of the gels and overlaid with 20  $\mu$ l of sample overlay buffer [3.0 M urea, 0.8% (v/v) Bio-Lyte 3/10 ampholyte, 0.25 ml 0.05% (w/v) bromophenol blue stock solution]. OMPs (100  $\mu$ g) were separated by IEF on pH 3.0–10.0 at 300 V for 16 h, 400 V for 2 h, and 800 V for 1 h (Apex PS 900S TX). Three tube gels per sample were run. After the IEF run was complete, the gels were removed from the tubes along with the running buffer [0.05 M Trizma Base, 0.05 M tricine, 0.1% (w/v) sodium dodecyl sulfate (SDS)] using a 2-ml syringe. The tube gels were extruded onto pieces of Parafilm laboratory film. Unused gels were immediately stored at –70°C without prior equilibration. The tube gels which were extracted, in the next stages of the protocol were equilibrated in SDS

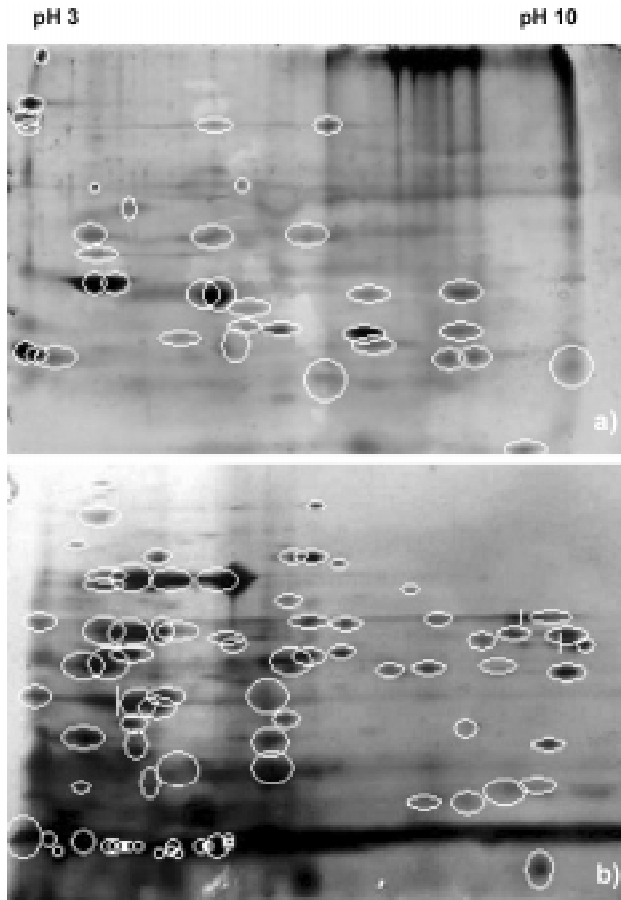


Fig. 1. 2-DE profile of OMPs isolated with Zwittergent Z 3-14<sup>®</sup>: *S. arizonae* PCM 2543 (a), *S. Dahlem* PCM 2512 (b).

OMPs were focused with capillary tubes in the pH range of 3–10 and run on 9–12.5% PAGE. Equivalent amounts (100 mg) of proteins were separated on 2-DE. Gels were stained with a Silver Stain Plus<sup>™</sup> kit (BioRad) and preliminarily analyzed with PDQuest 2-D Analysis Software v. 8.0.1. (BioRad).

buffer [0.0625 M Tris-HCl, 2.3% (w/v) SDS, 5% (v/v)  $\beta$ -mercaptoethanol, 10% (v/v) glycerole, 2.5 ml 0.05% (w/v) bromophenol blue] for 20 min, rinsed in running buffer, and 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 (1970) for 2 h (20 mA/gel) and stained with a Silver Stain Plus<sup>™</sup> kit (BioRad). The gel images were scanned using PDQuest 2-D Analysis Software v. 8.0.1. (BioRad). Four gels were obtained from two cultures of *Salmonella*: two from the OMPs of the *S. enterica* subsp. *arizonae* PCM 2543 samples, and two from *S. enterica* subsp. *enterica* sv. Dahlem PCM 2512. Each sample was run in triplicate. Tested *Salmonella* strains which demonstrate sensitivity to the bactericidal activity of human and animal serum (Futoma *et al.*, 2005; Bugla *et al.*, 2004) were grown in BHI medium to the logarithmic phase. OMPs were separated according to isoelectric points by 2-DE in a capillary tube system (pH 3–10) (Hamid and Jain, 2008) and then in 9–12.5% gradient SDS polyacrylamide

gel (Fig. 1). In this paper we present electroforegrams of OMPs derived from strains: *S. enterica* subsp. *arizonae* PCM 2543 (Fig. 1-a) and *S. enterica* subsp. *enterica* sv. Dahlem PCM 2512 (Fig. 1-b). The results show that Zwittergent Z 3-14<sup>®</sup> is suitable for OMPs isolation from these rods. This paper also confirms that detergents of this type may be used in 2-DE capillary tube systems as well. It was reported by Bugla-Płoskońska *et al.* (2009) that the use of the Zwittergent Z 3–14 to isolate OMP from *Escherichia coli* O56 is suitable for their separation by 2-DE using pH 3–10 immobilized pH gradient IPG strips.

During 2-DE analysis we marked the main spots and were able to visualize more than 84 individual protein spots (*S. enterica* subsp. *enterica* sv. Dahlem PCM 2512, Fig. 1-b). Figure 1-a (from *S. enterica* subsp. *arizonae* PCM 2543) contains a total of 35 spots. The major proteins were found in the acidic regions of the gels. In this study we described an experimental approach for the resolution of OMPs of *Salmonella* serotype O48 strains. 2-DE is a very useful method for finding correlation between the presence of some OMPs in the outer membranes of *Salmonella* and the susceptibility of the strains to the bactericidal activity of NHS. Among the spots visualized on the gels are probably proteins which modulate the sensitivity of the bacteria to the cytolytic activity of serum. As reported by several workers, OMPs can stimulate the innate response (Zollinger *et al.*, 1987; Merino *et al.*, 1998; Alberti *et al.*, 1993). The next investigations will comprise the immunogenicity studies to analyse this phenomenon. The power of 2-DE was demonstrated by Hamid and Jain (2008), who confirmed that OMPs provide promising targets for the development of a candidate vaccine against typhoid. 2-DE methodology has a potential for the rapid development of specific, safe, and highly efficacious vaccines against salmonellosis in humans and livestock. In this study we used Zwittergent Z 3-14<sup>®</sup> as an effective detergent to isolate the OMPs of *Salmonella* O48 and adapted the 2-DE technique in a capillary tube system for their resolution.

#### Acknowledgments

We would like to thank Prof. Andrzej Gamian for providing the *Salmonella* strains. We thank Dorota Kotowska and Aleksandra Skwara for their contribution to this study.

This work was supported by grants from the University of Wrocław (2780/W/IGiM/07 and 2515/W/IGiM) assigned to Dr Gabriela Bugla-Płoskońska.

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