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Research Article
Extraction of Antigenic Membrane Proteins from *Salmonella* Using Detergent and Phase Partition Method

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Abstract

Background and Objective: *Salmonella* porins have been shown to be good immunogens and these proteins carry an important value as they can be utilized as target proteins for diagnostics. In general, purification of porins from outer membrane complexes of bacteria is a tedious task due to the presence of enormous amounts of lipopolysaccharides and flagellar proteins associated with the outer membrane complex along with the porin proteins. The objective of study was to employ a modified method using a combination of detergent and phase separation to get a good efficacy of the classical procedure. **Materials and Methods:** The *Salmonella enterica* strain was obtained from MTCC, IMTECH-Chandigarh, India. The bacterial cultures were grown in standard nutrient broth and harvested for extraction of outer membrane proteins with Zwittergent™ 3-14 and phase separation using Triton X-114 (TX-114). The extracted protein was analyzed and confirmed using ESI-QTOF mass spectrometry. **Results:** Varying percentage of sodium chloride and glycerol were tried out and all the phase separated profiles gave the same result with varying protein concentrations of the outer membrane porin (Omps) and Lipopolysaccharide (LPS) in the upper and lower phase. A good protein profile in terms of absence of LPS and protein enrichment was obtained when 5% sodium chloride and 5% glycerol was used. The proteins identified by mass spectrometric analysis matched predominately to *Salmonella* species. These proteins gave unique peptide matches for outer membrane proteins (OMPs) aided by NCBI database. **Conclusion:** The modified detergent extraction protocol along with phase separation was effective in extracting the membrane proteins from *Salmonella* sp.

Key words: Outer membrane protein, membrane protein extraction, Triton X-114, phase separation, *Salmonella* porins

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Salmonella is an important Gram negative enterobacteriaceae pathogen causing non-typhoidal and typhoidal enteric fever[1]. *Salmonella enterica*, a member of the *Salmonella* species is responsible for severe gastroenteritis and also infects a broad range of hosts causing varying severity of the pathogenicity[2,3]. Studies have shown that outer membrane proteins (OMPs) of bacterial systems as a possible candidate for vaccine development, suggesting their strong nature as immunogenic components[4,5]. One such major OMPs are the porin proteins (Omp A, Omp C, Omp D) which can be used as a candidate for immunological studies since their involvement in eliciting an immune response has been demonstrated and verified[6]. Purification of porins from outer membrane complexes of bacteria is a tedious task due to the presence of enormous amounts of lipopolysaccharides (LPS) and flagellar proteins which are also associated with the outer membrane complex along with the porin proteins. There is already an established method of detergent-based extraction of outer membrane porin protein from *Salmonella typhimurium*[7]. But such detergent mediated extraction sometimes may not be feasible for all common known serovars of the bacterium. They often yield lipopolysaccharides otherwise known as endotoxins and often pose a challenge in immunological studies because of their toxic nature. LPSs are usually removed with the aid of a good chromatography technique but the presence of ionic detergents sometimes hinders further processing of the extracted sample and also the extraction profile of the sample varies from serovar to serovar. Hence, in this study, a modified approach was carried out using a Zwittergent 3-14 for solubilization of membrane proteins followed by endotoxin removal using Triton X-114.

Zwittergent 3-14 is a zwitterionic detergent used for the solubilization and extraction of membrane proteins from Gram negative bacteria[8]. Triton X-114 is a nonionic detergent homogenous at 0°C but separates in to an aqueous phase and detergent phase above 25°C. Mixed micelles are formed by membrane proteins with non-ionic detergents and hence proteins can be separated using it. Unlike ionic detergents, non-ionic and zwitterionic detergents do not possess a net charge, lack conductivity and electrophoretic mobility. Also, they do not alter the structure of the protein and hence suited for breaking protein-protein interaction[9]. This technique has been used to separate out integral membrane proteins from other organisms such as mouse[10] and it is known that Triton X-114 remove endotoxins from protein preparations[11]. Moreover, many methods have been reported wherein the protein has been recombinantly expressed in *Escherichia coli* with a histidine tag to aid in purification techniques there on. Expressing one Enterobacteriaceae Omp in other closely related bacteria of the same family, where in both the bacteria sharing the same Omp will pose a major hurdle in extraction as the bacterial Omps are similar in profile and molecular weight. Expressing the protein with a tag involves reduced protein stability. In this method, possible isolation of outer membrane porin proteins using classical techniques were used. Since bacterial cells have a complex LPS layer when compared to animal cells. The direct usage of Triton X-114 didn't seem feasible and hence the mixed detergent approach was considered for this study.

MATERIALS AND METHODS

Bacterial culture: The *Salmonella enterica* strain was obtained from MTCC, IMTECH-Chandigarh, India. The bacteria were grown in nutrient broth obtained from HIMEDIA, India. The bacteria were cultured in a total volume of 8 L for 36 h at 37°C with a 200 rpm. After 36 h, the cultures were centrifuged at 6,300 rpm for 30 min at 26°C (Eppendorf 5810R, Germany) to collect the cell pellet. The final pellet was washed with saline and the total biomass obtained was around 10 g. The pellet was resuspended in sonication buffer (50 mM Tris-HCl, pH 7.6) and subjected to sonication with an ultrasonicator (Misonix S-4000, USA) at 100 Hz, thrice. The cell sonicate was taken and centrifuged at 6,300 rpm for 30 min at 26°C to remove any cell debris. The supernatant was carefully transferred to a clean tube and subsequently centrifuged at 173,037 rpm for 1 h at 26°C using a Beckman Coulter Optima™ L-90K Ultracentrifuge (USA). The supernatant was collected and the pellet was processed for extraction.

Extraction of OMPs and phase separation using Triton X-114: Most of the chemicals used for preparation of buffers were obtained from Sigma (India). Zwittergent™ 3-14 and Triton X-114 were purchased from Calbiochem, USA and Acros Organics™, USA respectively. About 2 mL of extraction solution (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM EDTA, 0.4% Zwittergent™ 3-14) was added to the pellet and incubated for 4-6 h at 37°C and was centrifuged at 10,000 rpm for 10 min at 26°C. This extract was further subjected to phase partitioning using 5% Triton X-114 freshly prepared with a gradient of sodium chloride and glycerol (phasing agent). The extraction solution and phasing agent (1:10,000) were mixed and incubated on ice for 15 min. This was followed by centrifugation of the sample at 3214 rpm for 20 min at 26°C. After centrifugation, two distinct phases were formed and the

upper phase was carefully isolated and ice-cold acetone was added (1:25). This was incubated on ice for 6 h at 4°C to precipitate the proteins. After incubation, the samples were centrifuged at 3214 rpm for 30 min at 4°C. The pellet was then resuspended in 0.5 mL of phosphate buffer saline (50 mM sodium phosphate monobasic+50 mM sodium phosphate dibasic+150 mM sodium chloride, pH 7.6).

ESI-QTOF mass spectrometric identification of OMPs: The extracted and phase partitioned samples were subjected to SDS-PAGE (10%) under reduced conditions. The gels were stained with Coomassie Brilliant Blue. Chemicals used for SDS-PAGE were bought from Sigma (India). The bands were excised and subjected to in-gel tryptic digestion according to the established method[12]. The tryptic digested products were analysed by LC-MS/MS with 1290 Infinity LC and 6540 UHD accurate mass Q-ToF mass spectrometry with Agilent Jet Stream ESI source systems (Agilent Technologies, USA). Tryptic digested sample was injected and resolved on Zorbax Eclipse Plus C18 column (2.1×150 mm, 1.8 µ particle size). The mobile phase solvents were water with 0.1% formic acid and ACN with 0.1% formic acid and flow rate was 0.25 mL min^{-1}. The following gradient was used: 0-3 min 5% B, 3-23 min 60% B, 23-25 min 90% B and 25-31 min 5% B.

The Q-ToF MS was operated in positive ionization and in auto MS/MS mode with a medium isolation width. Following were the source parameters: Gas temperature 300°C, gas flow 8 L min^{-1}, nebulizer 35 psi, sheath gas temperature 350°C, Vcap-3500 V and fragmentor 175 V. The MS/MS acquisition was in range of 100-1700 with maximum for 8 abundant ions and charge state preference was set to 2 or more charges for fragmentation. The data acquisition was done with in-built Agilent Mass Hunter Work station B.04.00.

The spectra data was analyzed with Agilent's Spectrum Mill MS Proteomics Workbench B.04.01.141. Spectra of each band was extracted and interpreted with following parameters: Precursor MH+ range 300-4000 Da, sequence tag length was >1, precursor scan +/- 1.4 m/z and S/N 25. NCBI database was used for search, limited to the microorganism and with trypsin digest mode and number of missed cleavage was set to 2. The precursor and product mass tolerance was set to +/- 20 ppm and +/-50 ppm, respectively. The MS/MS search result was auto validated for peptide and protein modes and the protein was identified.

Statistical analysis: The experiments were done in triplicate and the data were given as average values. The statistical analysis was analyzed using Microsoft Excel.

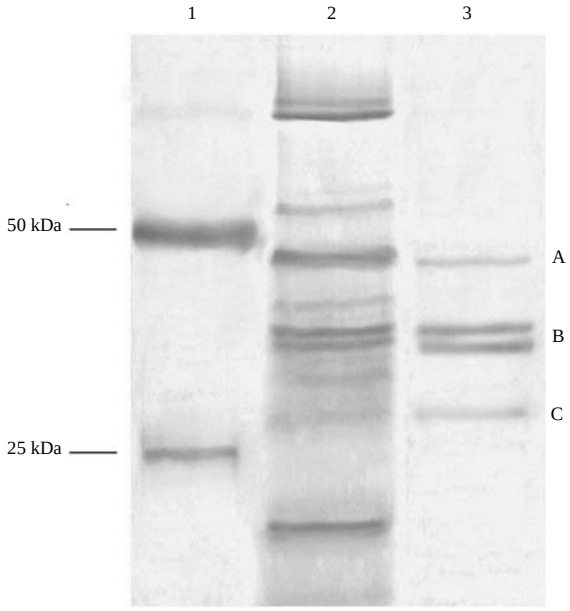

Fig. 1: Zwittergent extraction and phase partitioning of *Salmonella enterica* outer membrane proteins
Lane 1: In-house reduced IgG, Lane 2: Zwittergent 3-14 solubilized *Salmonella enterica* extract, Lane 3: Phase partitioned extract of lane 2

RESULTS

Extraction and phase separation of *Salmonella* porin proteins: The bacterial membrane protein that was extracted with Zwittergent™ 3-14 was having a lot of contaminants (Fig. 1, Lane 2). The extraction solution obtained after incubation of the processed cell pellet for 4-6 h at 37°C (Fig. 1).

In the present study, Triton X-114 was used for phase separation. It was observed that, the optimized membrane protein extraction method was effective as it had low levels of LPS in the finally phase-separated extract. The phase separated extraction solutions using Triton X-114, which were then precipitated with cold acetone is presented in Fig. 1, lane 3. Also, it was observed that other proteins associated with the extraction appeared in the surfactant rich phase.

The extract was subjected to phase partitioning using 5% Triton X-114 freshly prepared with a gradient of sodium chloride and glycerol (phasing agent). It was observed that all the phase separated profiles were almost same with varying protein concentrations of the Omps and LPS in the upper and lower phase. In Fig. 2, the lane 4 represents the phase separated extraction using 5% glycerol and 5% sodium chloride where a good profile in terms of lack of LPS and protein enrichment was observed. Lanes 3 (6% glycerol and

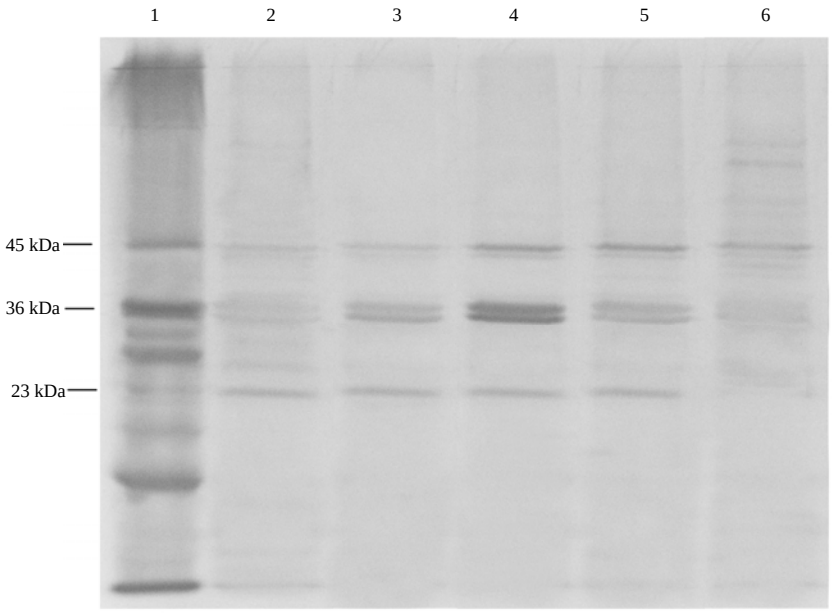

Fig. 2: SDS-PAGE (12%) showing LPS and porin profile

Lanes, 1: Zwittergent 3-14 solubilization, 2: (10% glycerol), 3: (6% glycerol+4% sodium chloride), 4: (5% glycerol+5% sodium chloride), 5: (4% glycerol+6% sodium chloride), 6: (10% sodium chloride)

4% sodium chloride) and 5 (4% glycerol and 6% sodium chloride) carry considerably low protein levels in their upper phase even though much LPS wasn't seen in the lanes.

Mass spectrometric identification of OMPs: The three bands denoted as A, B and C (Fig. 1) were excised from the gel and subjected to in-gel digestion. The protein, its molecular mass and species to which it belong was identified from MS/MS spectral data using Agilent Spectrum Mill MS Proteomics Workbench. The list of the proteins identified along with their accession number is presented in Table 1. The proteins were identified which matched predominately to *Salmonella* species.

DISCUSSION

In this study, a modified detergent extraction protocol for extraction *Salmonella* membrane proteins was followed with a zwitterionic detergent, Zwittergent 3-14. The bacterial membrane protein that was extracted with Zwittergent was having a lot of contaminants (Fig. 1, Lane 2). Unlike the previously well established detergent based protocol for isolating porins[7] which included working with a mutant variant of a *Salmonella* serovar, the strain which was taken in this study was wild type strain and was very much prone to variations and adaptations even though the conditions for growth applied were constant (this yet again stands true to the proof that *Salmonella enterica* is the major species of *Salmonella* from which other serovars rise). So establishing a working membrane protein extraction method which yielded a constant reproducible result was an absolute necessity. Working with the already well established methods seems to bottleneck the approach for porin extraction since the strains used differ from laboratory to laboratory. When working with different strains, especially wild type strains the integrity of any extraction method or technique employed poses a serious problem irrespective of the genus or species of the bacteria taken for the study yielding heterogeneous mixtures[13-16].

In the present study, the extraction method was modified by incorporating a phase separation step using Triton X-114. It was observed that, the optimized membrane protein extraction method was effective as it had lower level of LPS in the finally phase partitioned extract, since LPS usually gives a ladder like formation in both Coomassie brilliant blue and silver staining[17]. The phase separation using TX-114 facilitates the separation of proteins based on their varying hydrophobicity. The addition of glycerol to the detergent solution helps in giving a better efficiency when working with phase separation[18]. Sodium chloride mildly affects the micelle formation. Though the varying percentage

Table 1: List of the proteins identified along with their accession number

Bands	Spectra	Distinct peptides	Distinct summed MS/MS search score	AA (%) coverage	Total protein spectral intensity	Protein MW (Da)	Species	Database accession number	Protein name
Band A	34	23	414.88	66.5	3.89E+07	37658.5	*Salmonella enterica* subsp. enterica serovar Typhimurium	342359729	Omp A
Band B	44	20	423.44	79.3	2.90E+07	39271.7	*Salmonella enterica* subsp enterica serovar Typhimurium	1.53E+08	Omp C
Band C	14	10	160.14	26.2	3.15E+06	37658.5	*Salmonella enterica* subsp enterica serovar Typhimurium	3.42E+08	Omp A

of sodium chloride and glycerol were tried out, all the phase separated profiles showed the same results with varying protein concentrations of the Omps and LPS in the upper and lower phase.

The three protein bands denoted as A, B and C (Fig. 1, lane 3) were subjected to mass spectrometric identification. The proteins that were identified matched predominately to *Salmonella* species. As listed in Table 1, the B and A showed around 66.5% sequence to *Salmonella enterica* subspecies enterica serovar Typhimurium outer membrane protein A. The highest amino acid coverage B showed 74.5% sequence coverage to *Salmonella enterica* subspecies enterica serovar Typhimurium outer membrane protein B. Where as the highest amino acid B and C showed was only 26.2% sequence coverage to *Salmonella enterica* subspecies enterica serovar Typhimurium outer membrane protein A.

Though some methods with optimization yield different porin proteins from other *Salmonella* serovars[19], this study method has shown to enrich Omp A and C among the various porins present on the cell surface of the *Salmonella enterica*. Though not a new method, phase partitioning using Triton X-114 appears to study well when working with a non-engineered or a mutant variant of bacteria. The technique should be reproducible but the results may vary with the detergent used, species, buffer conditions, chemical grade and temperature. The LPS-free extraction method gives us an advantage to explore further the chance of membrane proteins to act as biomarkers as LPS are known to interfere with any immunological study with associated proteins of the bacterium.

CONCLUSION

In this study, the *Salmonella enterica* strain obtained from MTCC, IMTECH-Chandigarh, India was used for extraction of outer membrane proteins with Zwittergent™ 3-14 and then phase separation with Triton X-114. The protein profile obtained using 5% sodium chloride and 5% glycerol was found to be good. The mass spectrometric analysis of the proteins matched predominately to *Salmonella* species and gave unique peptide matches for OMPs aided by NCBI database. It was observed that the modified detergent extraction protocol along with phase separation was effective in extracting the membrane proteins from *Salmonella* species.

SIGNIFICANCE STATEMENTS

Extraction method employs a modified detergent solubilization using a mild zwitterionic detergent followed

by immediate phase partitioning of the solubilized detergent extract to separate out important membrane proteins from a concoction of membrane proteins and endotoxins. This modified extraction method yields a homogenous porin protein extract that can be used for further analysis and for various applications.

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