Crystallization of the immunodominant outer membrane protein OmpC; the first protein crystals from *Salmonella typhi*, a human pathogen

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Abstract OmpC, a surface antigen of *Salmonella typhi* was crystallized after several attempts, using PEG 3350. Well shaped hexagonal crystals were grown from vapor diffusion method using octyl glucoside and $C_{12}E_9$ as detergents. Crystals are sensitive to X-ray and diffract weakly up to 7 Å. Porin isoforms, due to the bound lipopolysaccharides, could be the cause for poor diffraction. Crystal quality depends largely on the purification method, and in case of LPS contamination, the genetic background of the bacteria. Crystallization and initial data collection suggest optimum conditions and the method of choice for OmpC crystallization.

Key words: OmpC; Surface antigen; Crystallization; X-ray diffraction; *Salmonella typhi*

1. Introduction

Porins form water filled channels on the outer membrane of Gram-negative bacteria and allow small hydrophilic solutes to pass through the pore [1]. They are present as homotrimers in vivo and shown to be phage receptors and B-cell mitogens [2,3]. In the porin superfamily, there are general and specific porins and their expression is modulated by the living environment of the bacteria [4]. Porins show a trimeric structural organization with a similar β-barrel fold, irrespective of low sequence homology between porins of *Enterobacteria* and other bacterial origin. Porins that are general diffusion channels, have each β-barrel made of 16 antiparallel β-strands in the membrane spanning regions whereas those of the specific porins such as LamB and ScrY have 18 strands [5]. However, porins differ in their pore characteristics and more importantly in their surface exposed loop regions which are the main focus in immunological and structure-function related studies. Expression of major outer membrane proteins, OmpF and OmpC, of *Enterobacteria* is under the two-component regulatory system in vivo and influenced by the osmolarity conditions. *Salmonella typhi* OmpC was found to be expressed both in low and high osmolarity conditions [6] unlike *Escherichia coli* OmpC which is expressed more during high osmolarity.

Outer membrane porins are potential surface antigens and are immunologically important in terms of diagnosis and vaccine design [7]. *Salmonella* porins induce both humoral and cell mediated immunity [8,9] in animal models. *S. typhi* OmpC has been demonstrated to have potential to display rota viral epitopes on the cell surface [10]. Possible expression of OmpC, all through the infection period, has been emphasized [11]. A high level of antiporin antibodies have been detected from the sera of patients with typhoid fever [12,13]. Though the role of OmpC in infection and pathogenesis is not understood yet, it is a possible target antigen in diagnostics and a candidate antigen for multivalent vaccine design [10]. We have earlier predicted the possible sequential epitopes for OmpC [14] based on the sequence alignment and the structures of *E. coli* porins. The prediction was improved based on the homology model built for OmpC (PDB code: 1IIV). The model helped in analyzing the structural features of surface exposed antigenic loops in comparison with other *Enterobacterial* porin structures. At present, we are interested in determining the crystal structure of OmpC to probe the unique structural features of these antigenic regions which are surface exposed.

In spite of the improvement in the methods for membrane protein crystallization [15,16], conditions to get stable and well diffracting crystals are still unpredictable [17]. It is dependent on many factors like choice of the major and additive detergents, precipitant, and especially on the protein purity. In the case of OmpC, from the galE mutant of *S. typhi* vaccine strain Ty21a, removal of non-covalently attached lipopolysaccharides (LPS) is a major hurdle in the sample preparation for crystallization. The chemical heterogeneity of the LPS results in isoforms of OmpC purified from *S. typhi* Ty21a (unpublished). Here, we report on the crystallization of OmpC using PEG/NaCl system which yielded reproducible crystals for X-ray crystallographic study. This is the first report on the crystals of major protein antigen from *S. typhi*, an obligatory human pathogen which causes typhoid. The crystals are well shaped. However, they diffract only to low resolution (up to 7 Å). The reasons for poor diffraction and problems in getting stable and good quality crystals of *S. typhi* OmpC are analyzed and their implications for porin crystallization are discussed.

2. Materials and methods

2.1. Purification and buffer exchange

Porin was extracted from the avirulent, vaccine strain *S. typhi* Ty21a [18], using a modified salt extraction method. This method was developed to extract only the major outer membrane protein (OmpC) in large quantities from the crude membrane preparation. In order to remove the free LPS, extracted porin was passed through the gel filtration column, packed with Sephacryl S-200 HR matrix. Extraction and column buffer had 50 mM Tris-HCl (pH 7.7), 5 mM EDTA, 0.4 M NaCl, 3 mM NaN_3 and 0.05% β-mercaptoethanol. Buffer exchange and additional LPS removal were simultaneously achieved, prior to crystallization, using Amicon Stirred cell and ultrafiltration devices with 10 kDa and 50 kDa cutoff membranes. Porin in

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Abbreviations: $C_{12}E_9$, polyoxyethelene-9-lauryl ether; SDS, sodium dodecyl sulphate; PEG, polyethelene glycol; OG, octyl glucoside

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extraction buffer was exchanged initially with a buffer containing 50 mM sodium phosphate pH 6.5, 0.1% $C_{12}E_9$ (Sigma), 100 mM NaCl and 3 mM NaN_3 and later on with crystallization buffer containing 0.8% β-octyl glucoside and 0.05% $C_{12}E_9$ (Sigma).

2.2. Crystallization

Crystallization was carried out using microbatch, macrobatch under oil and sitting/hanging drop vapor diffusion methods. PEG/NaCl system [19] was used in all methods with varying molecular weights of PEG (Mol. wt. 400, 1450, 3350, 6000, 8000) as precipitant. In case of microbatch method, 2 μl each of protein sample (10.5 and 14.5 mg/ml) and precipitant solution were mixed and layered down, under 10 μl of paraffin oil, that had been filtered through 0.2 μm filter, in the wells of Terazaki plates. For macrobatch, 20 μl of protein precipitant mixture and 50 μl of oil was used in 96 well U-bottom plate and sitting drop stages (locally made) in 24 well plates. For vapor diffusion experiments, either 5 or 10 μl each of protein (14.5, 10.5, 15 and 23.3 mg/ml) and precipitant solutions were mixed in equal proportions, before setting up the drops in 24 well plates with 1 ml of reservoir. Unless mentioned otherwise, all the setups were initially maintained at 22–25°C, until the crystals started appearing, and then later shifted to 22–30°C. It took a minimum of 4 weeks for crystals to appear in most cases depending on the method. In the case of the microbatch method, crystals appeared within 10 days also. Crystals were confirmed to be of protein using the Izit Crystal dye (Hampton research, USA) before further analysis using X-rays.

2.3. Data collection

Crystals were mounted in thin-walled glass capillaries and checked for X-ray diffraction. Initial data collection was done using a Rigaku rotating anode X-ray source operated at 40 kV, 58 mÅ and an MAR Image plate (MAR Research Inc). The diffraction data were processed using the Mar XDS software [20].

3. Results

3.1. Microbatch under oil

Initial screening resulted in reproducible microcrystals of < 50 μm^3 size under the conditions with 8–21.5% of PEG 3350, in a period from 2 weeks to a few months. Increase in the NaCl concentration in the drop promoted faster crystal growth and also the phase separation occurred earlier. The boundary between the oil and the drop disappeared before crystals started growing. Increase in the drop size, from 2 to 4 μl in 10 μl oil, did not produce noticeable changes in crystal formation and size. The same conditions at room temperature did not yield crystals and only resulted in precipitation. The macrobatch method yielded reproducible and slightly bigger crystals, though the crystal size did not improve much. Porin samples used in these methods were passed through only 10 kDa cutoff membrane. The entire drops were filled with clusters of microcrystals due to large number of nucleation sites. Phase separation, typified by oil droplet appearance, occurred after the crystal formation. The crystals got covered and in a couple of days the entire well looked dirty. Slight temperature fluctuations do not seem to affect the crystal formation. Using crude porin, containing free and bound LPS, the microbatch method did not produce crystals when SDS was used as the only detergent. Conditions using various concentrations of PEG 400, 6000 and 8000 did not yield crystals.

3.2. Vapor diffusion

The crystallization attempts were then made to prevent excessive nucleation and phase separation. In vapor diffusion experiments crystals formed after a month, in 13–15% PEG 3350 and in the presence of detergents β-OG (0.8%) and $C_{12}E_9$ (0.05%). Though 13% PEG induced slow crystal growth, single, well ordered crystals were formed (Fig. 1). Increase in PEG concentration beyond this resulted in disordered crystals. Crystals with hexagonal and rod morphology appeared in the same well. Increased amount of NaCl in the drop and reservoir favored faster crystal growth. The change in protein concentration from 14.5 mg/ml to 23.3 mg/ml gave rise to long, thick hexagonal towers of $0.4 \times 0.4 \times 0.2$ mm in size. 15% PEG 1450 also yielded hexagonal crystals with well defined faces which took a few months to grow. Beyond this concentration, PEG 1450 induced faster growth of needles. Long hexagonal towers were seen at the protein concentration of 23 mg/ml.

3.3. X-ray diffraction

None of the crystals were stable on X-rays for a long time.

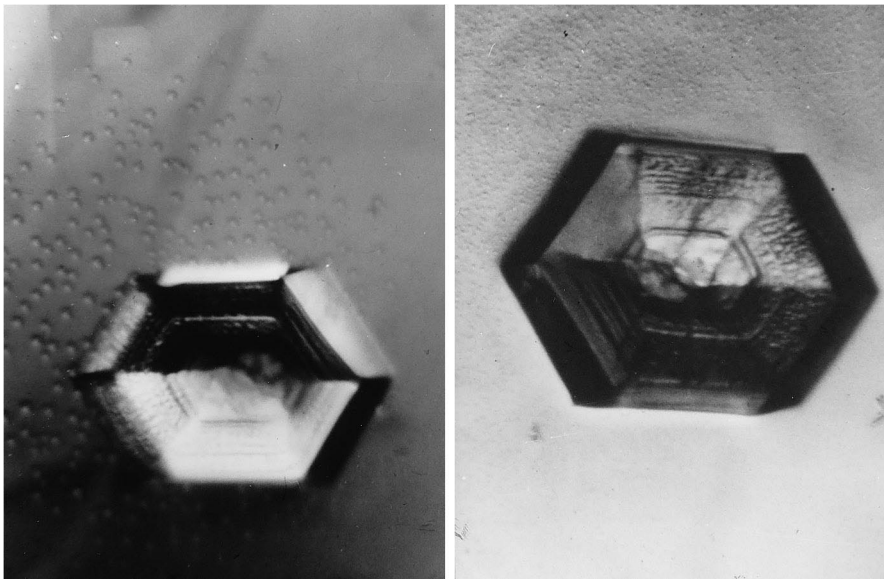

Fig. 1. Crystal grown under sitting drop method. One μl of Izit crystal dye was delivered in the drop and allowed to diffuse through for about 3–4 h. Crystal shown on the left side is before adding dye and the same crystal, after adding the dye, is shown on the right. The crystal turned blue indicating that it is a protein crystal. This was subsequently confirmed by the X-ray diffraction.

Soaking the crystals in stabilization buffer, containing increased amount of precipitant, did not improve the stability much. Initial data collected were only partial due to weak diffraction of crystals and short life times upon X-ray exposure. Data processing was very difficult due to too few and weak reflections in each of the frames. Crystal to crystal variation caused problem in indexing. The data can be indexed in the trigonal system with typical cell dimensions $a = b = 121.77$ Å, $c = 197.85$ Å, $\alpha = \beta = 90°$ $\gamma = 120°$. The dimensions are rather close to that of ScrY [21], suggesting three trimers in the unit cell. Data completion is 33% from 30 to 7Å with the $R_{merge}(I) = 14.8\%$ (for 1858 reflections).

4. Discussion

Integral membrane protein structures contribute only 1.2% of around 7600 protein structures available in the PDB database. This is mainly due to problems in getting quality crystals and partly due to non-availability of well defined expression systems for many integral membrane proteins when compared with soluble proteins of cellular localization. Though major outer membrane proteins, which are expressed constitutively in large amounts, may not require such expression systems for large scale preparation, their structure-function studies are limited due to problems in getting pure protein, LPS association and choice of the detergent, etc.

Crystals from both micro- and macrobatch under oil could not be stabilized in any solution. Harvesting in any other buffer resulted in immediate disappearance of crystals. Crystals from macrobatch were mounted directly from the well. The oily layer which covered the crystal surface could not be removed due to direct mounting. Single crystals could not be grown under both micro- and macrobatch methods. It is likely that in this case, as the sample was passed through only 10 kDa cutoff membrane, there were many nucleation sites in the drops which may be due to micellar aggregation as reported earlier [22].

The sitting drop method with PEG 3350 as a precipitant was promising in terms of single as well as bigger crystals, after initial trials. PEG 6000 and 8000 induced rapid precipitation even at lower concentrations. It is necessary to improve the quality of the crystals. Our analysis indicates that the problem could be due to bound LPS of different chemotypes. Purified porin still had bound LPS which was released on boiling with sample buffer. This could be visualized in SDS-PAGE gels stained with silver nitrate which also detects LPS. Purified *S. typhi* OmpC shows anomalous mobility in the gel unlike *E. coli* OmpC which migrates as a single band, whereas the recombinant *S. typhi* OmpC, expressed in *E. coli*, migrates as a single band (unpublished). This may be due to more amounts of aberrant LPS attached to *S. typhi* OmpC extracted from the LPS mutant Ty21a. Though the bound LPS was removed by repeated buffer exchange using ultrafiltration devices, complete removal of LPS was not possible. It has been shown, using monoclonal antibodies, that the *E. coli* porin monomer had still bound LPS as noticed in the Western blot after separation in SDS-PAGE [23]. Buffer exchange using 50 kDa Mol. wt. cutoff ultrafiltration devices, prior to setting up for crystallization, gave rise to single and larger crystals at 22–25°C. However similar conditions at room temperature (30–35°C) did not yield crystals, but induced precipitation.

A recent report on the crystallization attempts with the osmoporin *E. coli* OmpC [24] also discusses the problems due to contaminating LPS. Though *E. coli* OmpC could be crystallized under many conditions with different buffers, pH and detergents, the crystal quality seems to be depending on the purity, in terms of the bound LPS. Extensive buffer exchange and additional ion-exchange chromatography are likely to help in effective removal of LPS to a large extent thereby improving crystal quality, which is crucial for the three-dimensional structure determination of *S. typhi* OmpC. Other possibilities such as porin-antibody (Fab) complexes and use of recombinant *S. typhi* OmpC are also being explored to improve the crystal quality.

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