A Periplasmic Glutamate/Aspartate Binding Protein from *Shigella flexneri*: Gene Cloning, Over-Expression, Purification and Preliminary Crystallographic Studies of the Recombinant Protein

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Abstract Periplasmic substrate binding proteins (PSBPs) are essential components of the bacterial periplasmic transport system, which transports a wide variety of nutrients from the periplasmic space to the cytoplasm. The glutamate/aspartate binding protein S*f*Glu/AspBP is a unique PSBP consisting of 279 amino acid residues. The S*f*Glu/AspBP gene was cloned, over-expressed, and purified by immobilized metal ion affinity chromatography and size-exclusion chromatography. The recombinant protein *Sf*Glu/AspBP has been crystallized by the hanging-drop vapor-diffusion method and its X-ray diffraction data were collected at an atomic resolution of 1.15 Å. The crystals belong to the space group $P2_1$ with unit cell parameters: a=48.41 Å, b=68.18 Å, c=80.21 Å and $\beta = 98.78^{\circ}$. There are two molecules per asymmetric unit.

Keywords: Periplasmic glutamate/aspartate binding protein, *Shigella flexneri*, crystallization, crystal data.

1. INTRODUCTION

Gram-negative bacteria have a complex cell envelope consisting of an outer membrane, a thin cell wall, a periplasmic space, and a cytoplasmic membrane. Limited permeability to small solutes occurs through the outer membrane by way of water-filled channels. The cell wall is commonly regarded as an entirely permeable layer, conferring rigidity to the cell while forming a widely open network through which nutrient diffusion occurs readily. Almost every solute could not permeate though the cytoplamsic membrane unless a specialized periplasmic transport system is provided. The bacterial periplasmic transport systems have a complex composition, which transport a wide variety of substrates such as amino acids, oligopeptides, monosaccharides, oligosaccharides, vitamins, and inorganic ions [1,2]. These systems consist of a soluble periplasmic substrate-binding protein (PSBP) (the receptor) and a membrane-bound complex, which is composed of two hydrophobic integral membrane proteins and a nucleotide-binding membrane-associated protein [3, 4]. The PSBP has high affinity for the transported solutes (K_D values in the range 0.1 μM to 10 μM) and interacts with the membrane-bound complex, triggering activation of an energy-coupling mechanism and conformational changes of the liganded binding proteins that result in the release and translocation of the substrates. Periplasmic permeases are energized by ATP (or GTP) hydrolysis during the interaction, and the neucleotide-binding component is likely to be intimately involved in this step [3,5,6].

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Evidently, the structure-function relationship, and especially, the substrate-binding properties of PSBPs are essential for investigating the molecular mechanism of the action of the periplasmic transport systems. Up to date, several PSBPs have been characterized in three-dimensional structure in detail. Among them, eight are amino acid binding proteins [7-14] (Fig. **1**). As the substrates-specific binding proteins, more detailed structure information of PSBPs for binding respective unique amino acids are needed for gaining insight into the general molecular model of the periplasmic binding protein systems. Here we report the preliminary molecular biological and crystallographic investigations of a novel periplasmic amino acid binding protein from *Shigella flexneri*, termed *Sf*Glu/AspBP, as the initial step of its three-dimensional structure determination and further investigation.

The first periplasmic glutamate–aspartate binding protein, *Ec*Glu/AspBP was identified from *E. coli* K12 several decades ago [15]. It has been well characterized in both its biochemical and its genetic properties [15,16,17,18]. Its complete gene and protein sequences are now known as are its preliminary properties of its interaction with the glutamate transport system, but its 3D structure has not yet been reported. The *Sf*Glu/AspBP investigated in this paper is encoded by the *Shigella flexneri* 2a 301 gene locus sf0626 and the recombinant mature protein consists of 279 residues. A sequence-homology search revealed that *Sf*Glu/AspBP shares almost the same sequence of *Ec*Glu/AspBP with sequence identity of 99%. In a comparison of *Sf*Glu/Asp with other periplasmic amino acid binding proteins mentioned above whose structures are known, the sequence identity is lower than 24% (Fig. **1**) [19, 20]. It shows that *Sf*Glu/AspBP is a unique periplasmic amino acid binding protein. In this report, the gene cloning, expression, purification, crystallization and

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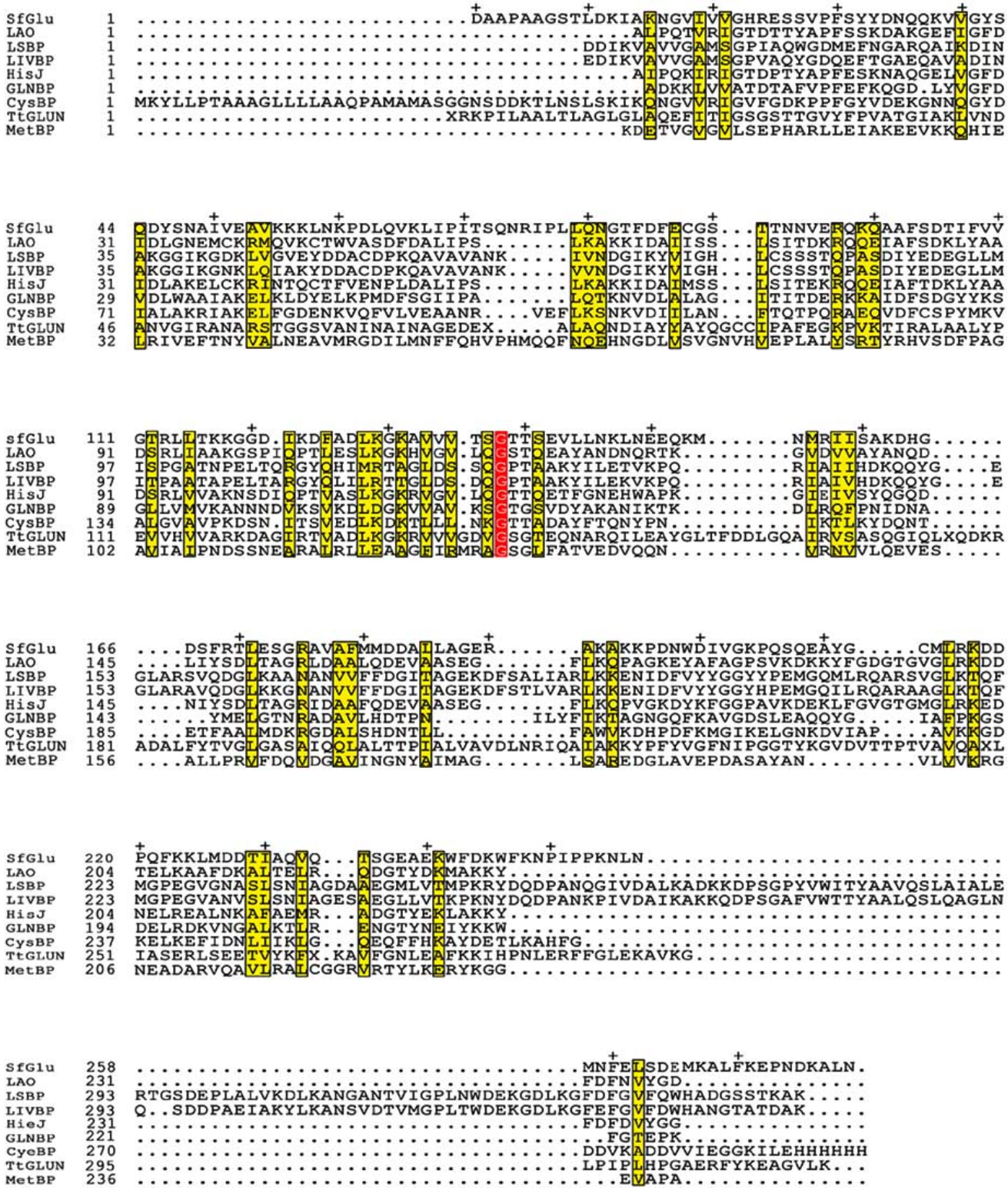

Figure 1. Sequence alignments of *Sf*Glu/AspBP with other structurally known amino acid binding proteins. Conserved residues are shaded yellow and the highly conserved residues are red. *Sf*Glu: glutamate–aspartate binding protein from *Shigella flexneri*; LAO: lysine-, arginine-, ornithine-binding protein (LAO) from *Salmonella typhimurium* [9]; LSBP: leucine-specific binding protein from *E. coli.* [8]*;* LIVBP: leucine/isoleucine/valine-binding protein from *E. coli* [7]*;* HisJ: histidine – binding protein from *Salmonella typhimurium* [10]*;* GLNBP: glutamine binding protein from *E. coli* [11]*;* CysBP : cysteine binding protein from *Campylobacter jejuni* [14]*; Tt*GLUN: putative periplasmic glutamate/glutamine-binding protein from *Thermus thermophilus* [12]*;* MetBP: methionine binding protein from *Treponema pallidum* [13]. Sequence alignment was performed with CLUSTALW [19] and the figure was prepared with ESPRIPT [20].

preliminary crystallographic analysis for *Sf*Glu/AspBP will be described. This provides a sound basis for the further 3D structure determination and structure–function relationship investigation.

2. MATERIALS AND METHODS

2.1. Cloning, Expression and Purification

The *Sf*Glu/AspBP gene was PCR – amplified from *Shigella flexneri* 2a 301 genomic DNA with the specific primers (forward 5' –ATACACTCATATGCAGACGATAACAACA CAAACAC-3'and reverse 5'- CTACTCGAGGTTCAGTGC CTTGTCATTCGGT -3') which introduce *Nde*I and *Xho*I sites at the initiator ATG and downstream of the stop codon, respectively. The *Nde*I - and *Xho*I – digested amplified fragment was ligated by T4 ligase into the *Nde*I and *Xho*I sites of the plasmid pET 22b(+) (Novagen) resulting in the recombinant plasmid *pET22b(+): Sf*Glu/AspBP, containing sequence coding for a hexahistidines tag fused to the 3' end of *Sf*Glu/AspBP gene. *E. coli*. strain TG1 competent cells were transformed with the recombinant plasmid. The recombinant plasmid was isolated and shown by PCR analysis and DNA sequencing to harbor the full *Sf*Glu/AspBP gene. *E. coli*. strain BL21 (DE3) competent cells were transformed with the recombinant plasmid and grown to OD600 of 0.6 at 310 K in 1.0 L LB broth containing 100 mg L^{-1} ampicilin. The cells were then induced with 1 mM isopropyl-β-D - thiogalatopyranoside (IPTG) and expression was continued for 4h at 310 K. Cells were harvested by centrifugation at 4000 rpm for 40 min. Subsequent purification steps were carried out 277 K. After every purification step, the purity of the sample was checked by SDS-PAGE stained with Coomassie R-250.

Cells were resuspended in 20 mL lysis buffer (50 mM NaH_2PO_4, pH8.0; 300 mM NaCl; 10 mM imidazole) containing 0.1mM phenylmethysulfonyl fluoride (PMSF), and then were disrupted by two passages through the FRENCH Pressure Cell. The lysate was clarified by centrifugation at 17000 rpm for 20 min at 277K. The supernatant was loaded directly into a Ni-NTA column (Novagen) equilibrated with the lysis buffer. After washing with the wash buffer (50 mM NaH_2PO_4, pH8.0; 300 mM NaCl; 20 mM imidazole) to remove unbound proteins, the elution buffer (50 mM Na H_2PO_4, pH 8.0; 300 mM NaCl; 250 mM imidazole) was applied. The eluate corresponding to the recombinant protein *Sf*Glu/AspBP was concentrated and then applied to a Hiload 16/60 Superdex75 prep-grade gel filtration column (Amersham Biosciences) equilibrated with 50mM Tris-HCl, pH 8.0. The peak fractions from the size-exclusion corresponding to the recombinant protein were pooled and concentrated using Amicon Ultra –15 Centrifugal Filter Unit (Millipore) prior to crystallization. The purified *Sf*Glu/AspBP protein was stored at 277 K for further crystallization experiments. And the final sample was examined by MALDI-TOF Mass Spectrometry.

2.2. Crystallization

Crystallization was performed at 293 K by the hanging drop vapour - diffusion method [21], using the Crystal Screen kits I, II and Index Crystallization Kits (Hampton Research) which exploit the sparse-matrix protocol of Jancarik & Kim [22]. The drops containing 1μl of reservoir solution and 1μl of *Sf*Glu/AspBP protein solution were equilibrated against 0.4 ml reservoir solution. After three weeks, one condition from the Index Crystallization Kits produced a large crystal with dimensions 0.9x0.7x0.5 mm (Fig. **2**).

2.3. Data Collection and Processing

The crystal was picked up from the crystallization drop using a nylon loop (Hampton Research), transferred directly into a cryostream of nitrogen gas. The data set were collected at wavelength of 0.9795 Å to a resolution of 1.15 Å at 95 K in a single pass of 180 oscillation degree, on the beamline 3W1A of the Beijing Synchrotron Radiation Facility (Beijing, China) operating at 75mA using Mar CCD 165 mm detector. The data set were processed with the program *MOSFLM* and scaled by the program *SCALA* from CCP4 [23].

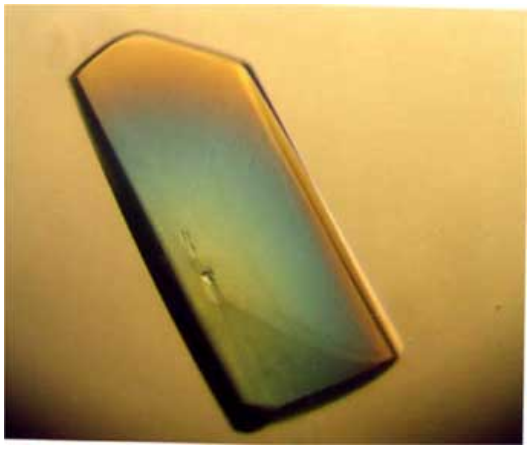

Figure 2. A single crystal of *Sf*Glu/AspBP. The size of the crystal is about 0.9x0.7x0.5 mm.

3. RESULTS

The *Sf*Glu/AspBP gene was successfully cloned into pET 22b(+) with a hexahistidines tag for efficient purification. Recombinant protein was over-expressed and purified. The yield of recombinant *Sf*Glu/AspBP after purification was 30mg/L of *E. coli* growth culture. The purity of *Sf*Glu/AspBP was estimated to be greater than 95%. The molecular weight of the recombinant protein identified by MALDI-TOF Mass Spectrometry was 32057.87 Da (Fig. **3**), which is in agreement with the estimated molecular weight of 32108.40 Da from the amino acid sequence.

Hampton Research Crystal and Index Screens were used to supply conditions for initial screening. One condition from the Index Crystallization Kits produced a large crystal. The recipe consists of 20% PEG2000 MME and 0.20 M trimethylamine N-oxide dihydrate in 0.1 M Tris-HCl pH 8.5. It is rare that just initial screening could produce such high-quality crystals.

Statistics of data collection and processing are shown in Table **1**. The crystals belonged to the monoclinic space group $P2_1$, with unit cell parameters a=48.41 Å, b=68.18 Å, c=80.21 Å, β = 98.78°. Theses data are compatible with the presence of two molecules in the asymmetric unit and a solvent content of 39.1% (Vm= 2.0 $Å^3Da^{-1}$; Vm: volume mass)

[24]. The crystals are very good diffractors of X-rays, producing data diffracting to a resolution of 1.15 Å under the experimental condition used here. It is rare that such big molecules (a total of 558 amino acid residues in one asymmetric unit) could diffract to an atomic resolution even on a rather weak X-ray radiation of synchrotron source operated at 75mA. In fact in the protein data bank (PDB), it has a very limited number for so big proteins diffracting to atomic resolution.

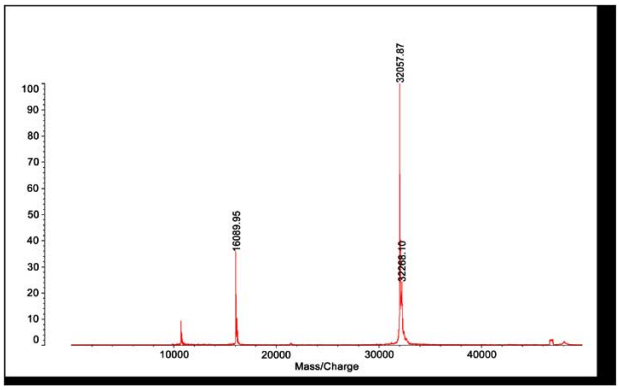

Figure 3. Mass spectrometry of *Sf*Glu/AspBP. The result showed that the molecular weight of *Sf*Glu/AspBP was 32057.87 Da.

The Se-Met derivative was prepared in order to solve this structure with multi – wavelength anomalous dispersion (MAD) method. The structure determined with the above data will provide useful information to elucidate the biological function of *Sf*Glu/AspBP and its essential role in the transport of glutamate or aspartate.

Table 1. X-Ray Diffraction Data Collection Statistics

(Values in parentheses refer to the highest resolution shell)

Spacegroup	**$P2_1$**
Unit-cell parameters	a=48.41 Å, b=68.18 Å, c=80.21 Åβ=98.78°
Resolution(Å)	41.48-1.15
Wavelength(Å)	0.9795
Total reflections	461407
Unique reflections	156629
Completeness (%)	85.9 (77.6)
Redundancy	2.9 (3.0)
Average I/σ(I)	4.8 (1.4)
Rmerge(%)	9.2 (60.6)

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