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Expression，Purification and Crystallization of Heat Shock Factor Binding Protein 1*

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Abstract Heat shock response rises when the organisms in the environmental stimulate. During heat shock, a lot of proteins which is so called heat shock protein that do not express in normal state largely express. These proteins′ expression is regulated by a family of transcription factor. These proteins were called heat shock transcriptional factors (HSFs). Among these HSFs, HSF1 is the most important protein. And HSFs are also regulated by other proteins, such as heat shock factor binding protein1 (HSBP1). HSBP1 interact with the trimer formation of HSF1 to convert the HSF1 from its active trimer state to inert monomer state. In order to do the further functional investigation, the gene of HSBP1 was cloned by PCR using the virus cDNAs as templates and expressed in *Escherichia coli* BL21 (DE3). Furthermore, the expressed HSBP1 protein was purified and crystallized. The distinct crystal form was obtained by the hanging-drop vapor-diffusion process to carry on screening and the optimization to the crystallization condition. The crystals belong to R3 space group with the cell parameter $a = b = 35.2$Å, $c = 233.3$Å.

Key words heat shock factor binding protein 1 (HSBP1), purification, crystallization
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Heat shock response (HSR) is one kind of response that living cells react to external stresses such as elevated temperatures, chemical toxicants, and pathogen infections. In that process, a small set of genes, the heatshock protein genes (hsp), is activated[1]. Heat shock proteins (HSPs) encoded by the hsp genes include mainly molecular chaperones, proteases, and other proteins essential for protection and recovery from cellular damage resulting from misfolded proteins [2]. HSR is mainly regulated by heat shock transcription factors (HSFs) [2]. HSF1 is one type of HSF and upregulates hsp gene expression by functioning both as a transcription factor and a polyadenylation stimulatory factor. Posttranslational modification, the oligomeric status of HSF, its DNA-binding ability, transcriptional competence, nuclear and subnuclear localization, as well as its interactions with regulatory cofactors or other transcription factors all appear to be fine tuned[3]. In the different formations, the monomeric form HSF1 (HSF1-M) is inactive, and the trimeric form HSF1 is active (HSF1-T). To understand the regulation of HSF1 activity, intensive studies have been focused on identifying proteins that potentially interact with HSF1[4–11]. Heat shock factor binding protein 1 (HSBP1) was identified in a yeast-two-hybridization (Y2H) analysis and subsequently characterized as the HSF1 binding protein[4–10].

HSBP1 is highly conserved across species and is ubiquitously expressed and localized in the nucleus. The molecular mass of HSBP family proteins are usually less than 10 ku. As a member of HSBP family, human HSBP1 consists of 76 amino-acid residues, which is predicted to have a coiled-coil structure and is found to self-assemble into homo-oligomers in solution. HSBP1 could reduces the DNA-binding ability of HSF1. Furthermore, overexpression of an

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HSBP1 homolog results in inhibition of HSR and lowers the survival rate after a heat shock stress[4]. On the basis of these observations, HSBP1 is considered as a negative regulator of HSR. Its functions under physiological condition may include preventing incidental activation of HSF1. However, the detailed structural information on HSBP1 oligomerization remains elusive now. To understand the structural basis of HSR regulation by HSBP1, we carried out structural studies on this protein. Here we report gene clone, protein expression, purification, crystallization and preliminary crystallographic study of HSBP1 protein.

1 Materials and methods

1.1 Gene cloning

The forward primers 5′ **CCG GAA TTC** ATG GCC GAG ACT GAC CC CAA 3′ and reverse 5′ **ACG CGT CGA** CTC AAC TCT TTT GCG TGG CAG 3′ were used to amplify the HSBP1 gene from cDNA library. The primers included *Eco*R Ⅰ and *Sal* Ⅰ restriction sites (shown in bold and underline). The HSBP1 gene was amplified by polymerase chain reaction (PCR) cloned into the pET-28a vector (GE healthcare). The sequence of the insert was verified by sequencing. The recombinant plasmid was transformed into *Escherichia coli* strain BL21 (DE3).

1.2 Expression and purification

The protein was expressed at high levels in *E. coli* BL21 (DE3) cells as a fusion protein with a His tag at the N-terminal. Bacteria were grown in LB medium containing 100 mg/L Ampicillin to A_{600} = 0.6～0.7, then induced with 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) and treatment at 16 ℃ overnight. Cells were harvested by centrifugation, resuspended in lysis buffer (20 mmol/L Tris-HCl pH 8.0, 500 mmol/L NaCl, 5% glycerol, 5 mmol/L β-ME) and then homogenized by sonication. The cell lysate was centrifuged at 15 000 r/min for 30 min at 4 ℃ to remove the cell debris. The supernatant was collected and the fusion protein was loaded onto a 20 ml Ni-NTA column equilibrated in lysis buffer. After washing away the unbound protein with three column volumes of wash buffer (20 mmol/L Tris-HCl pH 8.0, containing 500 mmol/L NaCl, 5% glycerol, 5 mmol/L β-ME, 20 mmol/L imidazole), tightly bound proteins, mainly target HSBP1, were then eluted with 50 ml elution buffer (20 mmol/L Tris-HCl pH 8.0, containing 500 mmol/L NaCl, 5% glycerol, 5 mmol/L β-ME, 250 mmol/L imidazole). In order to avoid possible interference of some strong negative charged molecules, Resource Q column anion exchange chromatography was conducted according to the predicted p*I* 4.17 of HSBP1 using ExPASy primary structure analysis tools (available at http://www.expasy.org). The sample eluted from Ni-NTA affinity chromatography column was centrifuged at 4 500 r/min for 30 min and diluted to the salt concentration of approximate 10 mmol/L using, 25 mmol/L HEPES, pH 7.5, and applied to a Resource Q column (GE Healthcare) running in 25 mmol/L HEPES, pH 7.5 and developed with a 0～1 000 mmol/L NaCl gradient and target HSBP1 protein were eluted by 25 mmol/L HEPES, pH 7.5, 200 mmol/L NaCl. The purity of HSBP1 protein was examined by 15% SDS-PAGE at each purification step and was estimated to be greater than 95% finally.

1.3 Crystallization

For crystallization purposes, the protein with a His tag fused at the N-terminus was concentrated using a 5K ultrafiltration membrane (Filtron) in a buffer containing 25 mmol/L HEPES pH 7.5, 100 mmol/L NaCl to 20 g/L by ultrafiltration (Millipore Amicon, USA) and subjected to Crystal Ⅰ, Ⅱ, Index Screen, and PEG/ion Screen Kit (Hampton Research, USA) as initial screening kits. Crystallization experiments were performed at 16 ℃ using the hanging-drop vapour-diffusion method. 1 μl of protein solution were mixed with 1 μl of reservoir solution and equilibrated against 0.2 ml of the reservoir solution. Crystals with good shape were obtained within 14 d.

1.4 Data collection and processing

The native crystal of HSBP1 was soaked in a saturated sodium citrate solution as the cryo-protectant and flashfrozen in 100 K cold nitrogen stream for data collection. It diffracted to 2.2Å resolution in-house on a Rigaku Micro-007 rotating copper-anode X-ray generator with a RAIXS IV++ image-plate detector. All intensity data were indexed, integrated and scaled with the HKL2000 package. The details of data collection was summarized in Table 1.

2 Results and discussion

2.1 Gene clone

The positive clones were screened and identified by agarose gel electrophoresis, endonuclease digestion and PCR (Figure 1). Sequencing was completed by Shanghai Bioasia Inc (Shanghai) and DNA sequencing

verified the correct insertion of coding fragment of HSBP1 with the length of 228 bp.

Table 1　Data-collection and processing statistics of HSBP1

Paramters	Native HSBP1
Wavelength/Å	1.541 8
Resolution range/Å	50～2.2
Completeness/%	91.1(77.1)
Total reflections	14 547
Unique reflections	5 019
Redundancy	2.9 (1.9)
R_{merge}/%	5.4 (37.1)
$I/\sigma(I)$	18.0 (3.6)
Space group	$R3$
Unit-cell parameters	a=35.197Å, b=35.197Å, c=233.905Å

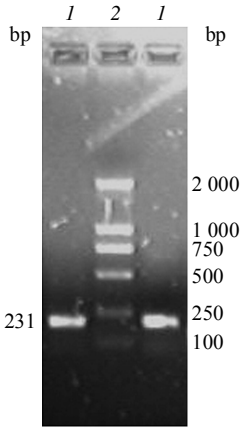

Fig. 1　Agarose gel electrophoresis of two-digested recombinant plasmid

1: HSBP1; *2*: Marker.

2.2　Expression and purification

HSBP1 protein fused with an N-terminal His-tag was solubly expressed in *E. coli* with a yield of about 10 mg/L LB culture. The fusion protein was determined by SDS-PAGE and mass spectrum with a molecular mass of 8.5 ku and found to be identical as expected previously. Anion exchange chromatography was applied after affinity chromatography to avoid the interference of some strong negative charged molecules (chromatography profile data not shown). The purified HSBP1 protein was calculated to be > 95% pure based on SDS-PAGE analysis (Figure 2). Since HSBP1 protein shows nearly 14 ku in SDS-PAGE, we also performed mass spectrum to confirm its molecular mass to be 8.5 ku exclude residues in vector (data not shown here).

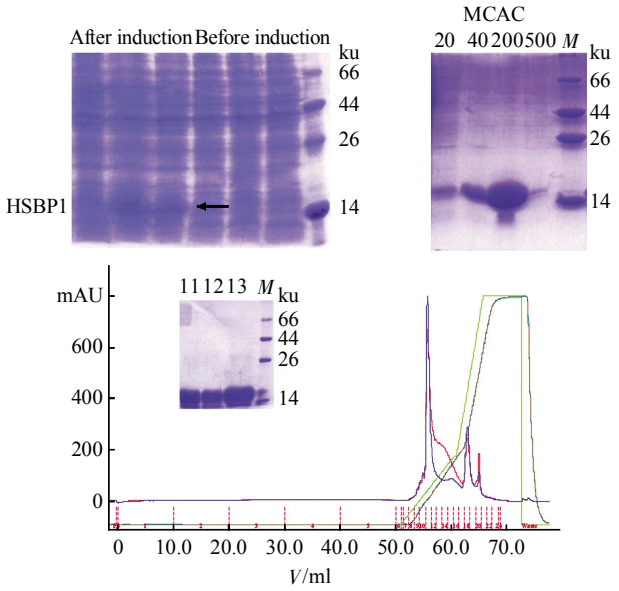

Fig. 2　The expression and preliminary purification of HSBP1

M: Marker; 11, 12, 13: Elution volume.

2.3　Crystallization

The hanging drop vapor diffusion method was applied for crystallization. Initial crystals were obtained using 1.5 mol/L Ammonium Sulfate, 20 mmol/L HEPES (pH 7.5) and 10% glycerin at 16℃. Rod-like crystals grew with a few days. In order to differentiate protein crystals from salt crystals, X-ray crystallographic analysis was applied. Additive Screen kit of Hampton was used to improve the diffraction quality. Gradient dehydration was also used to improve diffraction by adding 25 mmol/L HEPES, pH 7.5, 15% ～30% glycerin to the well buffer respectively. Finally, optimization of the condition to 1.7 mol/L ammonium sulfate, 20 mmol/L HEPES (pH 7.5) and 15% glycerin greatly improved the quality and the size of the crystal (Figure 3).

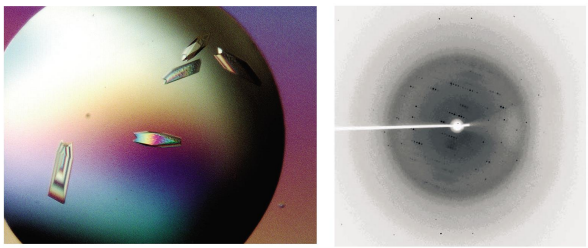

Fig. 3　Single crystal and typical diffraction pattern of HSBP1

The condition for crystallization is 1.7 mol/L ammonium sulfate, 20 mmol/L HEPES (pH 7.5) and 15% glycerin at 16℃.

Initial molecular replacement was performed using CNS1.2 and PHASER, but no obvious and correct solution was found according to rotation and translation functions. This was caused by the low similarity between the searching model and HSBP1. Heavy atom and seleno-methionine derivations have been produced to get the correct phases. Structure determination is underway.

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热休克因子结合蛋白(HSBP1)的表达、纯化、结晶与初步晶体学研究 *

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摘要 热休克反应(heat shock response，HSR)是细胞在缺氧、病毒感染等应激因素刺激下，为适应微环境的改变而发生的一种自身保护性反应，以暂时性下调细胞正常代谢和选择性上调热休克蛋白(heat shock protein，HSP)的表达为特点．HSR 是通过热休克转录因子(heat shock transcription factor，HSF)与相应的启动子结合，启动转录过程，进而促使 HSP 的表达来实现，其中 HSF1 是最有代表性、研究最多的一种 HSF．HSF1 在无活性的单体及有活性的三聚体之间的转变和平衡是转录调控的关键．热休克因子结合蛋白(heat shock factor binding protein，HSBP1)是一个含有两个伸展的疏水重复区，与 HSF1 的三聚体区域相互作用，以其伴侣形式实现对 HSF1 的 DNA 结合活性有负调节作用，通过抑制 HSF1 与 DNA 结合活性，从而抑制 HSF1 的转录活性．为了深入研究 HSBP1 行使功能的结构基础，对 HSBP1 蛋白成功地进行了克隆表达和结晶，该晶体属于 R3 空间群，其晶胞参数为 $a = b = 35.2$Å, $c = 233.3$Å.

关键词 热休克因子结合蛋白(HSBP1)，纯化，结晶

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