研究快报

Protein Preparation, Crystallization and Preliminary X-ray Crystallographic Analysis of Smu.260 From *Streptococcus mutans* —— a Cariogenic Dental Pathogen*

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Abstract *Smu.260* encodes a putative protein of 200 residues in *Streptococcus mutans*, a primary pathogen for human dental caries. *Smu.260* was cloned into expression vector pET28a and expressed in good amount from the *E.coli* strain BL21 (DE3). Smu.260 protein was purified to homogeneity in a two-step procedure of Ni^{2+} chelating and size exclusion chromatography. The purified protein exists in two forms, a dimer form about 46 ku with yellow color and a tetramer form without apparent color. Crystals were obtained from the dimer protein by hanging-drop vapor-diffusion method. The crystals diffracted to about 2.3 Å resolution and belong to orthorhombic space group $P2_12_12_1$ with cell dimensions of a = 89.88 Å, b = 90.91 Å, c = 105.17 Å. The asymmetric unit is expected to contain two dimers with solvent content of 53%.

Key words *Streptococcus mutans*, dental caries, Smu.260, protein crystallography

Streptococcus mutans is the principal etiological agent in human dental caries [1] and also a primary pathogen of infective endocarditis [2]. Studies on cariogenesis mechanism and prevention vaccine of caries so far were only focused on virulence factors including the cell-surface fibrillar proteins, which mediate adherence to the tooth surface [3]; the glucosyltransferase enzymes, which synthesize adhesive glucans and allow microbial accumulation on teeth[4]; as well as acidogenicity and acid tolerance of the bacteria, which makes the cell survive in acidic conditions while it promotes the formation of dental caries[5]. The genome of *Streptococcus mutans* UA159, a serotype c strain, has been sequenced recently and found to contain 1 963 ORFs (open reading frames), 63% of which have been assigned putative functions[6]. In order to study the cariogenesis mechanism and to discover new drug or vaccine targets at the genomic level, we have initiated a structural genomics project on *S.mutans*. We have chosen firstly the 37% function unknown genes in the *S.mutans* genome as our targets. The ORF of *Smu.260* is one of such targets.

Smu.260 is located at 25 170~25 175 bp of *S.mutans* genome. The 600 bp sequence encodes a hypothetical protein, with a molecular mass of 22 ku and calculated isoelectric point (p*I*) of 4.8. The gene product contains a conserved domain belonging to nitroreductase protein family (Pfam 00881)[7], which is involved in the reduction of nitrogen-containing compounds. Proteins of this family utilize FMN as a cofactor and are often found to be homodimers, possible characteristics of this protein family include oxygen-insensitive NAD (P)H nitroreductase (FMN-dependent nitroreductase or dihydropteridine reductase, EC 1.5.1.34) and NADH dehydrogenase (EC 1.6.99.3). Sequence search showed that most proteins with high sequence identity to Smu.260 are from bacteria (Figure 1), and the annotations for the homologous proteins are either hypothetical ones or only with putative function. Smu.260 has no structural homolog in PDB, the highest hit found there is only 12% in sequence identity.

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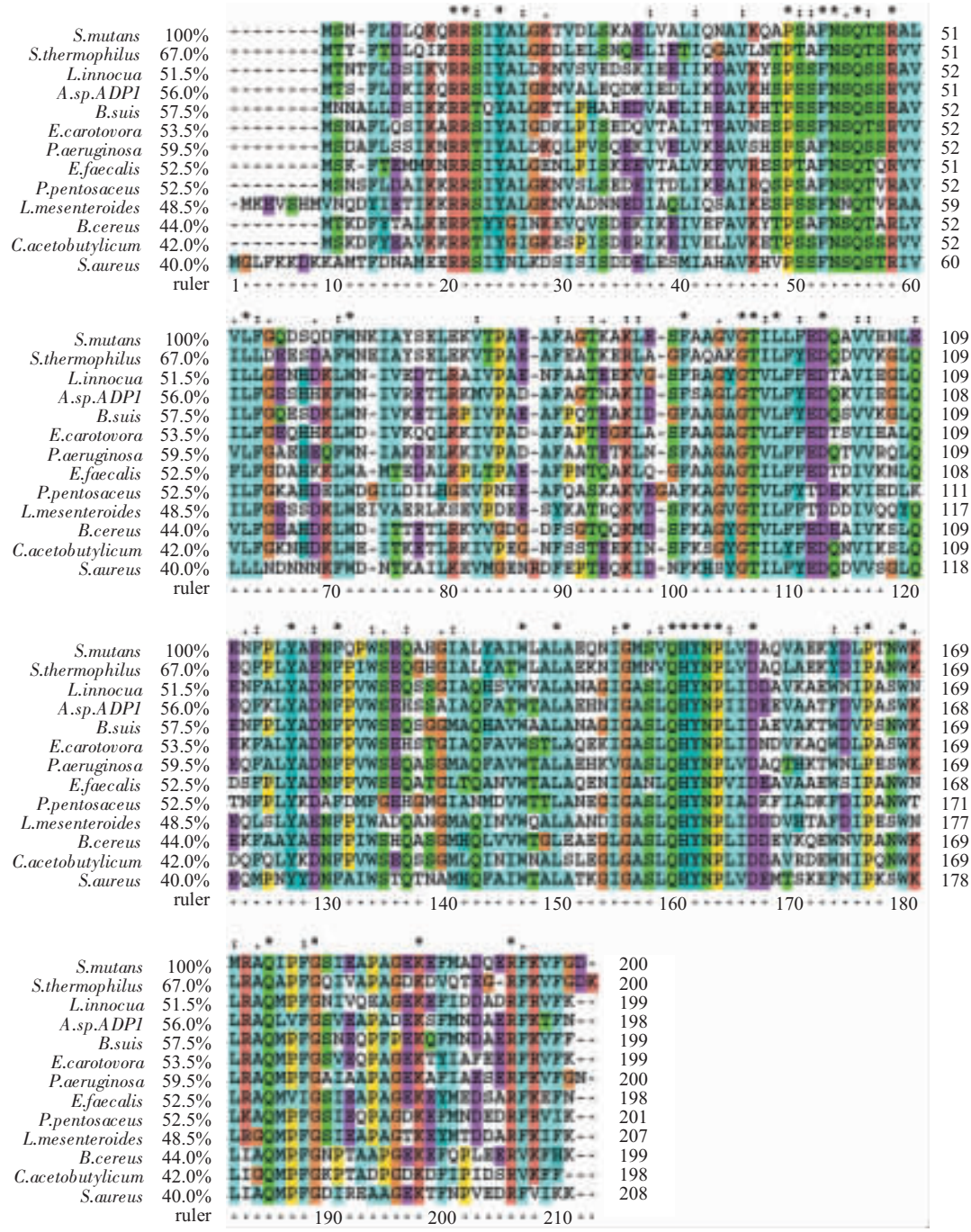

Fig.1 Multiple sequence alignment of some Smu.260 homologous

The alignment is performed using the program Clustal X [8]; the percentage identities with *S.mutans* Smu.260 are indicated after the organism names; the asterisks indicate the strict conserved residues.

1 Materials and methods

1.1 Cloning, expression and purification

To construct expression plasmid, *Smu.260* gene was PCR amplified from chromosomal DNA of *Streptococcus mutans* ATCC25175, using primers SMU.260-F, 5′ gcggatccatgtcaaatttttagatttaca 3′, and SMU.260-R, 5′ atagtttagcggccgcctagtcaccaaatactttga 3′, which contains *Bam*H Ⅰ and *Not* Ⅰ digestion sequence respectively. After digested by *Bam*H Ⅰ and *Not* Ⅰ at room temperature overnight, the PCR product was ligated into the expression vector pET28a (Novagen) which has been digested with the same enzymes.

Transformed cells (*Escherichia coli* strain BL21 (DE3)) were cultured in 20 ml LB (Luria-Bertan)

medium supplemented with 50 mg/L kanamycin at 310 K overnight and then inoculated to 1 L LB medium. Expression of the recombinant protein was induced by 0.5 mmol/L IPTG (isopropyl-β-d-thiogalactopyranoside) when the culture reached an A_{600} of 0.6~0.8. The cultivation continued at 310 K for 4 h before harvesting cells by centrifugation at 5 000 r/min, for 10 min. The pellet was re-suspended with Buffer A (20 mmol/L Tris-HCl, 500 mmol/L NaCl, pH 7.5) and lysed by sonication on ice. The lysate was centrifugated at 13 000 r/min, 277 K for 30×2 min to get rid of the debris prior a two-step purification procedure utilizing Ni^{2+} chelating column and S75 size exclusion column.

1.2 Crystallization and X-ray data collection

Protein purified from gel filtration (in 200 mmol/L NaCl) was directly concentrated with Millipore centrifugal filter device. Crystallization experiments were performed at room temperature using hanging-drop vapour-diffusion method, equal volumes (1 μl) of protein solution and reservoir solution were mixed in one drop and the droplets were equilibrated against 500 μl of the reservoir solution. Conditions from Crystal Screen Ⅰ, Crystal Screen Ⅱ and Index Kit (Hampton Research) were applied as initial screening conditions. The diffraction data from a cryogenic crystal were collected at 100 K using an in-house X-ray instrument, Bruker SMART 6000 CCD detector mounted on a Bruker Nonius FR591 rotating-anode generator with Cu K_α radiation. A_φ scan was performed with 0.2°φ oscillation per frame and 2θ angle was set to −5°. A total of 800 frames were collected, the exposing time per frame was 120 s. Data were processed using the Bruker on-line software PROTEUM suite.

2 Results and discussion

Smu.260 was successfully cloned into pET28a expression vector, which has been verified by sequencing. The protein was expressed in soluble form in BL21(DE3) cells. For the recombinant protein with 6-his tag on the N-terminal, it has been purified by 5 ml Hitrap Ni^{2+} chelating chromatography first which produced a kind of yellowish protein. The expressed Smu.260 protein had the right molecular mass as shown by SDS-PAGE. Further purification with 120 ml S75 gel-filtration column gave two peaks (Figure 2), SDS-PAGE results indicated that they were both 22 ku protein of Smu.260. According to the gel-filtration peak location, however, it could be deduced that the two peaks were for tetramer and dimer form of the protein, respectively, and the dimer form (second peak) was yellow (Figure 2). After concentrated by Millipore centricon tube, protein concentration in tetramer and dimer form reached 22 and 17 g/L, respectively.

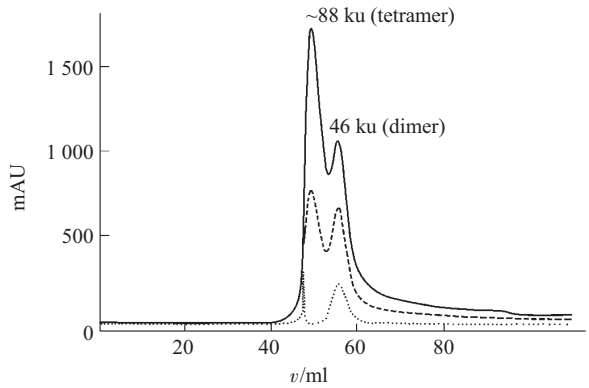

Fig.2 Hiload S75 chromatography graph of Smu.260

——: sm23 S75 041110:10_UV1_280 nm; ----: sm23 S75 041110:10_UV2_254 nm; ·····: sm23 S75 041110:10_UV3_450 nm.

Whole wavelength optical density scan of the dimer protein gives peaks at 220 nm, 270 nm, 370 nm and 450 nm, which are the characteristic absorbances of FMN (data not shown). It agrees well with the prediction from sequence analysis that Smu.260 belongs to a member of nitroreductase protein family that utilizes FMN as a cofactor.

Both forms of the protein were applied for crystallization screenings. The diamond shaped crystals of dimer Smu.260 were obtained under condition of 30% (*w*/*v*) Polyethylene Glycol 8000, 0.2 mol/L sodium acetate tri-hydrate, 0.1 mol/L sodium cacodylate, pH 6.5 (Figure 3).

Fig.3 Crystals of dimer form Smu.260

For the diffraction data collection on the dimer crystal, a total of 161 456 measured reflections were merged to 36 671 unique reflections with an R_{sym} (on intensity) of 7.9%. The merged data set is 94.1% complete to 2.3 Å resolution. The crystal belongs to space group $P2_12_12_1$, with unit-cell parameters of a = 89.88 Å, b = 90.91 Å, c = 105.17 Å. Assuming there are 4 molecules per asymmetric unit, the value of the crystal packing parameter V_m is 2.67 $Å^3 \cdot u^{-1}$ based on molecular mass, corresponding to a solvent content of 53%, which falls well within the range commonly observed for protein crystals[9]. Data statistics for the X-ray data collection and processing are summarized in Table 1. Structure determination using in-house SAD (single-wavelength anomalous diffraction) method is underway.

Table 1 X-ray data collection and processing statistics

Resolution/Å	50-2.3 (2.4-2.3)[1)]
Completeness /%	94.1 (82)
R_{sym} /%[2)]	7.9 (22.4)
Averge $I/\sigma(I)$	6.5 (1.7)
Spacegroup	$P2_12_12_1$
Cell parameters/Å	a=89.88 b=90.91 c=105.17
No. of observed reflections	161 456
No. of unique reflections	36 671
Mol/Asym	4
V_m/$Å^3 \cdot u^{-1}$	2.67
Solvent content/%	53

[1)]Data in parentheses correspond to the highest resolution shell; [2)]$R_{sym} = \sum |I_{obs} - I_{avg}| / \sum I_{obs}$ where the summation is over all reflections.

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龋齿致病菌变形链球菌蛋白 Smu.260 的制备、结晶及初级晶体学分析 *

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摘要 变形链球菌 (*Streptococcus mutans*) 是最主要的龋齿致病菌，其基因 *Smu.260* 编码一个约 23 ku (200 个氨基酸) 的蛋白质. *Smu.260* 的 DNA 片段被克隆到表达载体 pET28a 后在大肠杆菌 BL21(DE3)菌株中表达得到很好的产量. 产物 Smu.260 蛋白通过 Ni^{2+} 亲和柱和分子筛两步法纯化，并发现纯化后的蛋白以两种形式存在，二聚体 (约 46 ku) 和四聚体，前者呈亮黄色，后者无色。采用悬滴气象扩散法得到了二聚体形式的晶体. 晶体的 X 射线衍射分辨率达到 2.3 Å，晶体属正交空间群 $P2_12_12_1$，晶格参数为 a=89.88 Å, b=90.91 Å, c=105.17 Å. 晶胞不对称单元内估计含有一个二聚体，溶剂含量为 53%.

关键词 变形链球菌，龋齿，Smu.260，蛋白质晶体学

学科分类号 Q51，O72

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