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Crystallization and preliminary X-ray analysis of βC–S lyases from two oral streptococci

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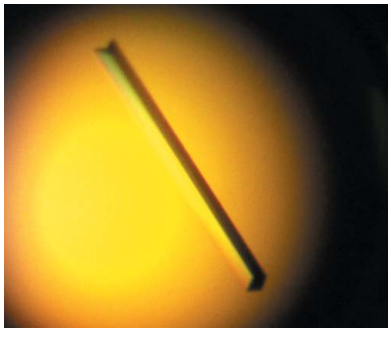


Hydrogen sulfide, which causes oral malodour, is generally produced from L-cysteine by the action of βC–S lyase from oral bacteria. The βC–S lyases from two oral bacteria, *Streptococcus anginosus* and *S. gordonii*, have been cloned, overproduced, purified and crystallized. X-ray diffraction data were collected from the two types of crystals using synchrotron radiation. The crystal of *S. anginosus* βC–S lyase belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 67.0, b = 111.1, c = 216.4 Å, and the crystal of *S. gordonii* βC–S lyase belonged to the same space group, with unit-cell parameters a = 58.0, b = 73.9. c = 187.6 Å. The structures of the βC–S lyases were solved by molecular-replacement techniques.

1. Introduction

Hydrogen sulfide (H_2S), a gas with the odour of rotten eggs, is a causative agent of oral malodour (Tonzetich, 1971). Reports have indicated that H_2S is (i) highly toxic to mammalian cells (Beauchamp *et al.*, 1984), inducing the modification and release of haemoglobin in erythrocytes (Kurzban *et al.*, 1999; Yoshida *et al.*, 2002); (ii) associated with endotoxin-induced inflammation (Li *et al.*, 2005) and apoptosis (Yang *et al.*, 2004; Yaegaki *et al.*, 2008); and (iii) one of the predominant volatile sulfur compounds in periondontal pockets (Persson *et al.*, 1990). These findings suggest that H_2S may contribute to the pathogenesis of gingivitis and periodontitis. Therefore, it is important to elucidate the mechanism of H_2S production by oral bacteria.

Generally, H_2S is produced from L-cysteine by the enzymatic action of βC–S lyase (Lcd), which is encoded by the *lcd* gene. This enzyme is a pyridoxal-5′-phosphate-dependent enzyme that catalyzes the α,β-elimination of sulfur amino acids containing αC—N and βC—S linkages, such as L-cysteine and L-cystathionine, to generate sulfur-containing molecules, pyruvate and ammonia (Guarneros & Ortega, 1970). The amino-acid sequence of Lcd is highly conserved amongst oral streptococci (Yoshida *et al.*, 2003). Interestingly, the capacity of Lcds from the anginosus group, represented by *Streptococcus anginosus*, to produce H_2S from L-cysteine was found to be considerably higher than those of Lcds from other oral streptococci, including *S. gordonii* (Yoshida *et al.*, 2008; Ito *et al.*, 2008); however, the Lcds from the abovementioned bacterial species have a similar ability to degrade L-cystathionine. However, no specific characteristics of the amino-acid sequences of these enzymes have been identified that would explain the difference in substrate specificity (Yoshida *et al.*, 2003). As an initial step towards elucidating the relationship between the structure and the properties of Lcd, we performed the expression, crystallization and preliminary X-ray crystallographic analysis of Lcds from two bacterial species.

2. Materials and methods

2.1. Cloning, expression, and purification

The recombinant Lcds of *S. anginosus* and *S. gordonii* were purified as follows. The coding sequence of *lcd* was amplified from the genomic DNA of *S. anginosus* IMU102 (Yoshida *et al.*, 2008) using the forward primer 5′-TCCGGATCCAGCAAATACAATTTTCA-

AACAG-3′ and the reverse primer 5′-TTACTCGAGTTATTTGG-GCAAGCAAC-3′. The product was then ligated into the pGEX-6P-1 vector (GE Healthcare) at the *Bam*HI and *Xho*I sites, positioning the *lcd* gene downstream of the coding sequence for glutathionine *S*-transferase (GST) and a PreScission protease-cleavage site. The plasmid for the purification of *S. gordonii* DL1 Lcd was constructed as described previously (Yoshida *et al.*, 2003). The resulting plasmids were sequenced to verify the accuracy of the PCR amplification and transformed into *Escherichia coli* BL21 cells. The production media were inoculated (1:1000) with an overnight medium and grown at 310 K until the OD_{600} reached 0.8. After the induction of protein expression with 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG), the cells were incubated at 310 K for another 2 h, harvested by centrifugation at 277 K and lysed by ultrasonication. Cell debris was sedimented by centrifugation and the portion of the GST-fusion protein that remained in the supernatant was absorbed onto a Glutathione-Sepharose 4B affinity matrix (GE Healthcare) and cleaved with PreScission protease (GE Healthcare) according to the manufacturer's protocol.

The eluate was loaded onto a prepacked anion-exchange column (HiTrap Q HP, GE Healthcare). A linear NaCl gradient elution (0.1–0.6 *M*) in 20 m*M* Tris–HCl buffer pH 8.5 was used at a flow rate of 0.5 ml min^{-1} at 277 K and 1.0 ml fractions were collected. The Lcd-containing fractions were applied onto a gel-filtration column (HiLoad 16/60 Superdex 200 pg, GE Healthcare). Phosphate-buffered saline was used as a mobile phase pumped at a flow rate of 0.7 ml min^{-1}. The purity of the samples was analyzed by SDS–PAGE.

2.2. Gel-filtration chromatography

Molecular weight was analyzed by gel-filtration chromatography with a Superdex 200 HR 10/30 column (GE Healthcare) at a flow rate of 0.25 ml min^{-1} in phosphate-buffered saline by using molecular-weight standards (Kit for Molecular Weights 12 000–200 000) obtained from Sigma. The enzyme elution was monitored at 280 nm.

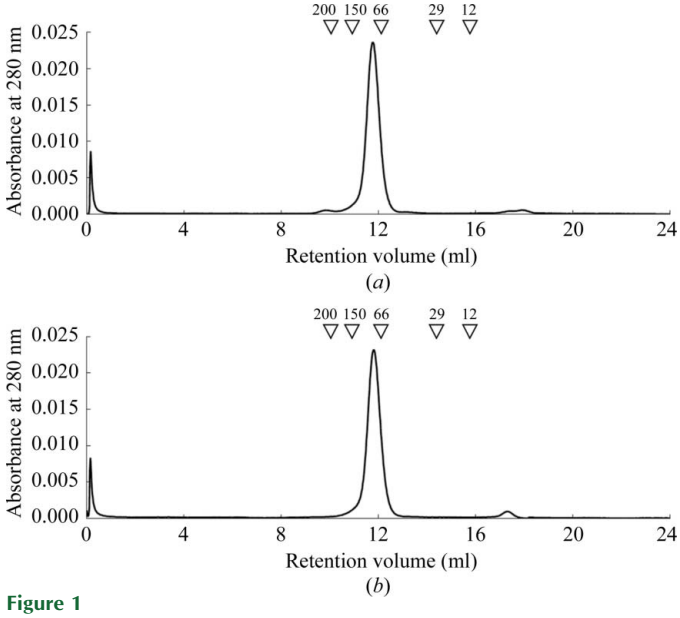


Figure 1
Gel-filtration chromatography of *S. anginosus* Lcd (*a*) and *S. gordonii* Lcd (*b*) on Superdex 200 HR10/30. The major peaks at a retention volume of 11.8 ml correspond to each Lcd protein. The positions of the molecular-weight standards (labelled in kDa) are shown above each chromatogram.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

	S. anginosus Lcd	*S. gordonii* Lcd
Experimental conditions		
Beamline	BL38B1 (SPring-8)	BL38B1 (SPring-8)
Wavelength (Å)	1.0000	1.0000
Temperature (K)	100	100
Detector	Jupiter 210 (Rigaku)	Jupiter 210 (Rigaku)
Oscillation angle (° per frame)	0.8	1.0
No. of images	450	180
Crystal parameters		
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 66.97, b = 111.14, c = 216.44	a = 58.04, b = 73.88, c = 187.64
Solvent content (%)	45	45
Monomers in ASU	4	2
Data processing		
Software	*XDS*/*XSCALE*	*XDS*/*XSCALE*
Resolution range (Å)	108.46–1.93 (1.97–1.93)	93.65–1.61 (1.64–1.61)
R_{merge}† (%)	6.1 (23.0)	4.9 (21.3)
Completeness (%)	100.0 (100.0)	99.9 (99.5)
Mean $I/\sigma(I)$	35.7 (13.8)	29.7 (6.9)
No. of unique reflections	122200 (7249)	105196 (5560)
No. of observed reflections	1792823 (107338)	726079 (24485)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation and $\langle I(hkl)\rangle$ is the mean intensity of the reflections.

2.3. Crystallization

Initial screening for crystallization was performed using the hanging-drop vapour-diffusion method. The *S. anginosus* and *S. gordonii* Lcd protein solutions were adjusted to 20.0 mg ml^{-1} (0.5 m*M*) in 20 m*M* Tris–HCl pH 7.6, 50 m*M* sodium chloride and 1.1 m*M* pyridoxal-5′-phosphate (PLP) on the basis of the extinction coefficients at 280 nm (Pace *et al.*, 1995). Drops of 0.8 µl protein solution and 0.8 µl reservoir solution were equilibrated against 0.5 ml reservoir solution at 293 K. Commercially available kits produced by Emerald BioSystems and Hampton Research were used in the screening. The initial crystallization conditions were further optimized by changing the concentrations of the precipitant, buffer and/or additive.

2.4. Data collection and processing

The crystals were mounted in cryoloops and were cryoprotected by soaking them briefly in mother liquor containing 20%(*w*/*v*) glycerol or 2-methyl-2,4-pentanediol (MPD) before flash-freezing them in a stream of nitrogen gas at 100 K. X-ray diffraction data were collected from the *S. anginosus* and *S. gordonii* Lcd crystals on beamline BL38B1 at SPring-8 using a Jupiter 210 detector (Rigaku) and were indexed, integrated and scaled using *XDS*/*XSCALE* (Kabsch, 1993). Table 1 presents the details of the data-collection conditions.

2.5. Self-rotation function

Self-rotation functions were calculated by *MOLREP* (Vagin & Teplyakov, 1997) as implemented within the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) in the resolution range 50.0–2.0 Å. The integration radii were set to 25.0 and 20.0 Å for the *S. anginosus* and *S. gordonii* Lcds, respectively.

2.6. Molecular replacement

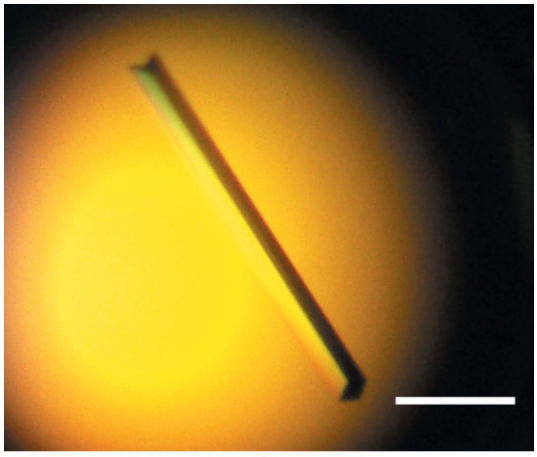
The program *MOLREP* (Vagin & Teplyakov, 1997) was used to solve the crystal structures of the two Lcds. A monomer homology model of *S. gordonii* Lcd was built on the basis of the structure of *Treponema denticola* cystalysin (PDB code 1c7n; Krupka *et al.*, 2000),

which exhibits 30% sequence identity to *S. gordonii* Lcd, using the program *MODELLER* (Sali & Blundell, 1993). The determined structure of *S. gordonii* Lcd was then used as a search model to solve the crystal structure of *S. anginosus* Lcd.

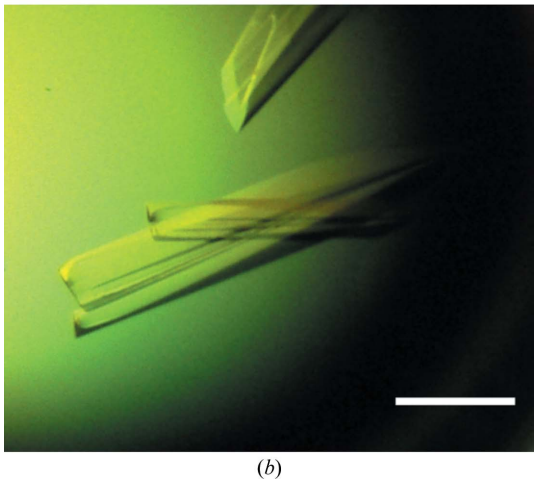
3. Results and discussion

Both *S. anginosus* and *S. gordonii* Lcds were expressed in *E. coli* and purified to apparent homogeneity. These proteins eluted as single peaks on gel-filtration chromatography, with retention volumes corresponding to molecular weights of 89.1 and 87.5 kDa for the *S. anginosus* and *S. gordonii* Lcds, respectively (Figs. 1*a* and 1*b*). These values were almost twice the theoretical molecular weights (44.8 and 45.1 kDa), suggesting that the Lcds are present as homodimers in solution.

Crystals of *S. anginosus* Lcd were grown using solution No. 32 of Crystal Screen 2 (Hampton Research) as a reservoir solution (0.1 *M* sodium chloride, 0.1 *M* HEPES pH 7.5 and 1.6 *M* ammonium sulfate). A rod-like crystal appeared within one week and reached final dimensions of 600 × 30 × 30 µm (Fig. 2*a*). The yellow colour of the crystal indicated the presence of PLP bound to the protein. The crystal diffracted to a maximum resolution of 1.93 Å and belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 67.0, b = 111.1, c = 216.4 Å. The processing statistics of the diffraction data are summarized in Table 1. A Matthews coefficient of 2.25 Å^3 Da^{-1}, corresponding to a solvent content of 45%, indicated the presence of four monomers in the asymmetric unit (Matthews, 1968). The self-rotation map showed three independent peaks representing noncrystallographic twofold axes at 22.5° intervals on the χ = 180° section (Fig. 3*a*). No peaks were found at other χ sections. The peak positions suggest that the axes are parallel to the *ab* plane and that one of the peaks is imposed by other symmetries.



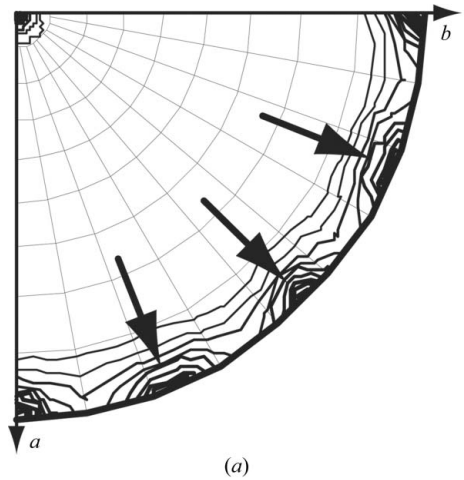
(*a*)



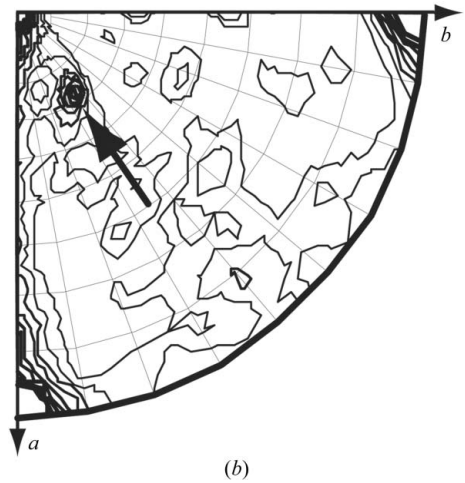
(*b*)

Figure 2
Crystals of (*a*) *S. anginosus* Lcd and (*b*) *S. gordonii* Lcd. The scale bars correspond to 100 µm.

S. gordonii Lcd was crystallized under three independent conditions, with polyethylene glycol (PEG) 3000 or PEG 3350 as the precipitant in all conditions. The crystals also had a yellow colour. A solution containing 22%(*w*/*v*) PEG 3350 and 0.2 *M* ammonium fluoride was found to be the optimum conditions and was determined on the basis of the results obtained with solution No. 3 of PEG/Ion Screen (Hampton Research). Crystals with dimensions of 600 × 150 × 50 µm grew within several days (Fig. 2*b*) using the optimized conditions. The best crystal diffracted to a resolution of 1.61 Å and belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 58.0, b = 73.9, c = 187.6 Å. Two monomers were expected to be present in the asymmetric unit based on a Matthews coefficient of 2.23 Å^3 Da^{-1}, which corresponds to a solvent content of 45% (Matthews, 1968). Table 1 summarizes the processing statistics of the collected data. A strong self-rotation peak representing a



(*a*)



(*b*)

Figure 3
Stereographic projections along the *c* axis of the χ = 180° polar section of the self-rotation function for *S. anginosus* Lcd (*a*) and *S. gordonii* Lcd (*b*). Latitude (θ angle) and longitude (φ angle) grid lines are drawn at 10° intervals. The independent noncrystallographic twofold-axis peaks are indicated by arrows. The self-rotation functions were calculated using *MOLREP* (Vagin & Teplyakov, 1997) as implemented within the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

noncrystallographic twofold axis was found on the $\chi = 180^\circ$ section of the self-rotation map (Fig. 3*b*). No peaks were seen at other χ sections. The presence of one noncrystallographic twofold axis is suggestive of dimerization of *S. gordonii* Lcd, which was examined by gel-filtration chromatography as described earlier.

Molecular replacement was used to solve the crystal structure of *S. gordonii* Lcd using a monomer homology model based on *T. denticola* cystalysin (Krupka *et al.*, 2000) as the search model. Two clear peaks in the rotation and translation functions were obtained and the solution gave readily interpretable electron densities for two monomers that closely contacted in the asymmetric unit. The orientation of the noncrystallographic twofold axis of the Lcd was consistent with that detected in the self-rotation map (Fig. 3*b*). Thus, the results of the crystallographic analysis supported those obtained from chromatography, which indicated that *S. gordonii* Lcd was a homodimeric enzyme.

The crystal structure of *S. anginosus* Lcd was also determined by the molecular-replacement technique using the preliminary refined model of *S. gordonii* Lcd as the search model. Four monomers in the asymmetric unit and three noncrystallographic twofold rotation axes were detected, as observed in the self-rotation map (Fig. 3*a*). Two dimers, each essentially identical to the close-packed dimer of *S. gordonii* Lcd in the mode of dimer formation, were identified in the asymmetric unit of the *S. anginosus* Lcd crystal. The two dimers were then related by the third noncrystallographic twofold rotation axis. Since *S. anginosus* Lcd is present as a homodimer in solution, as suggested by the results of gel-filtration chromatography, the formation of the dimer of dimers must be a crystallographic artifact. Currently, structural refinement of the *S. anginosus* and *S. gordonii* Lcds is in progress.

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