**Lcd from *Streptococcus anginosus* encodes a C-S lyase with α,β-elimination activity that degrades L-cysteine**

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**INTRODUCTION**

The genus *Streptococcus* consists of six species groups that have been recognized using 16S rRNA sequence data; these groups are designated the pyogenic, anginosus, mitis, salivarius, bovis and mutans groups (Bentley et al., 1991; Kawamura et al., 1995). There are three species in the anginosus group, *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius* (Whitworth, 1990); *S. anginosus* is the predominant member of the anginosus group associated with periapical abscesses (Fisher & Russell, 1993). It has been suggested that these microaerophilic anaerobes initiate infection and provide an environment for subsequent colonization by the strict anaerobes with which they are frequently isolated.

It has been reported that hydrogen sulfide is produced from L-cysteine by the enzymic action of L-cysteine desulfhydrase, which catalyses the α,β-elimination of L-cysteine to hydrogen sulfide, pyruvate and ammonia (Guarneros & Ortega, 1970). Since hydrogen sulfide is highly toxic for mammalian cells (Beauchamp et al., 1984) and induces the modification and release of haemoglobin in erythrocytes (Kurzban et al., 1999), enzymes participating in the production of hydrogen sulfide in the subgingival sulcus might contribute to the initiation and progression of periodontal diseases, and to abscess formation (Carlsson et al., 1993; Claesson et al., 1990; Persson et al., 1990). Based on this hypothesis,
investigations have focused on Gram-negative periodontopathogenic bacteria, as the main organisms capable of producing hydrogen sulfide (Ratcliff & Johnson, 1999), while little attention has been paid to other bacteria, even though they also have the capacity to produce hydrogen sulfide (Persson et al., 1990). Consequently, there have been few published reports on the enzymes associated with the production of hydrogen sulfide by oral bacteria other than periodontopathogenic bacteria.

Recently, we observed that the crude enzyme extract obtained from S. anginosus has a higher capacity to produce hydrogen sulfide from L-cysteine than extracts from other oral streptococci, including Streptococcus mutans, Streptococcus sobrinus, Streptococcus oralis, Streptococcus gordonii and Streptococcus salivarius. Based on this finding and clinical reports that S. anginosus is frequently isolated from several infectious sites (Fisher & Russell, 1993; Whitey et al., 1992), the high capacity of S. anginosus to produce hydrogen sulfide might play an important role in the pathogenicity of this micro-organism. In this study, we cloned the gene encoding L-cysteine desulhydrase in S. anginosus and characterized its product. In addition, the haemolytic activity of this enzyme resulting from the modification of haemoglobin by hydrogen sulfide was also determined.

METHODS

Bacterial strains and culture conditions. S. anginosus FW73 was grown in brain–heart infusion (Difco Laboratories) broth at 37 °C in a 5 % CO₂ atmosphere. Escherichia coli BL21 (F’ompT[lon] hsdS (rK mK) gal dcm iDE3), obtained from Novagen, was grown aerobically in 2×TY (tryptone/yeast extract) broth. When required, the medium was supplemented with 100 mg ampicillin l⁻¹.

DNA manipulation. Standard DNA recombination procedures, such as DNA isolation, endonuclease restriction digestion and ligation, were carried out as described by Sambrook et al. (1989). Chromosomal DNA of S. anginosus was prepared as described by Perry et al. (1983).

Purification of L-cysteine desulhydrase from S. anginosus FW73. S. anginosus FW73 was grown to an OD₅₅₀ value of about 1.0. The cells were harvested and then washed with ice-cold cell-suspension buffer (50 mM Tris–HCl, 10 mM PMSF, 10 mM EDTA, 10 μM pyridoxal 5’-phosphate, pH 7.5). A 1 ml aliquot of the cell suspension was transferred to a screw-top microcentrifuge tube containing 10 g glass beads (0.1–0.15 mm diameter; Biospec Products). The cells were vortexed with the glass beads for 45 s 10 times at 1 min intervals. After vortexing, the glass beads were allowed to settle before the supernatant was transferred to a clean tube. The supernatant was centrifuged at 18000 g for 30 min at 4 °C. To the supernatant, solid ammonium sulfate was slowly added to 70% saturation. After a 20 min incubation at 4 °C, the suspension was centrifuged at 10000 g for 10 min at 4 °C. The precipitate was then dissolved in ice-cold cell-suspension buffer.

Initial purification was performed on a 2.5 × 46 cm gel filtration column [TSKgel TOYOPEARL HW-55 (F); Tosoh], equilibrated with a buffer consisting of 50 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 0.1 mM EDTA and 10 μM pyridoxal 5’-phosphate. The column was loaded with 4 ml of the cell extract obtained as described above. The column was run at 0.25 ml min⁻¹ at 4 °C, and 2.0 ml fractions were collected. Fractions containing L-cysteine desulhydrase were identified as described below and then concentrated using a Centricon YM-30 (Amicon) with a 30000 Da cut-off. The concentrated was processed for HPLC with an amion-exchange column (TSKgel DEAE-5PW, 0.8 × 7.5 cm; Tosoh). A linear gradient (0–0.4 M) of NaCl in 50 mM Tris/HCl buffer (pH 7.0) was used at a flow rate of 1 ml min⁻¹ at 25 °C, and 2.0 ml fractions were collected. Fractions containing L-cysteine desulhydrase were subjected to HPLC with a hydrophobic column (TSKgel Phenyl-5PW, 0.75 × 7.5 cm; Tosoh). The enzyme was eluted with a linear gradient of 75–0% ammonium sulfate in 100 mM sodium phosphate buffer (pH 6.8). The column was run at 1.0 ml min⁻¹ at 25 °C, and 2.0 ml fractions were collected. Fractions containing L-cysteine desulhydrase were concentrated using a Centricon YM-30. The retentate was then subjected to HPLC with a hydroxyapatite column (Bio-Scale CHT-1; Bio-Rad) equilibrated with 10 mM sodium phosphate buffer (pH 6.8). After sample application, the column was washed with 10 ml of the same buffer at a flow rate of 1 ml min⁻¹. A linear 0–500 mM NaCl gradient in 30 ml of the same buffer was passed through the column, and fractions (2 ml) containing L-cysteine desulhydrase were collected.

Visualization of enzyme activities. The presence of L-cysteine desulhydrase in the HPLC fractions was confirmed by visualizing enzyme activities (Claesson et al., 1990). Briefly, 30 μl of each HPLC fraction were added to 130 μl of the visualizing solution, which consisted of 100 mM triethanolamine/HCl (pH 7.6), 10 μM pyridoxal 5’-phosphate, 0.5 mM bismuth trichloride, 10 mM EDTA, 1% Triton X-100 and 50 μM L-cysteine. A mixture was incubated at 37 °C to detect the black precipitate formed by the reaction of sulfide with bismuth. L-Cysteine desulhydrase activity was also detected in non-denaturing polyacrylamide gels. The samples were electrophoresed at 20 mA per gel at 4 °C for 2 h on 12.5% resolving (pH 8.8) and 3% stacking (pH 6.5) polyacrylamide gels. After electrophoresis, the gel was incubated in 10 ml of the visualizing solution at 37 °C to detect a black band at the enzyme position.

N-terminal sequencing of L-cysteine desulhydrase. The HPLC fraction containing L-cysteine desulhydrase was subjected to non-denaturing PAGE. After electrophoresis, proteins in the gel were transferred to a PVDF membrane (Millipore), as described by Towbin et al. (1979), and stained with Coomassie brilliant blue. The protein band corresponding to L-cysteine desulhydrase was excised from the membrane and destained with a solution containing 60% methanol and 10% acetic acid. The N-terminal amino acid sequence of L-cysteine desulhydrase was determined by Edman degradation at the Takara Custom Service Center in Shiga, Japan. The FASTA program was used to perform a homology search of protein databases (Lipman & Pearson, 1985).

DNA sequencing of lcd of S. anginosus. Primers that amplified lcd from S. anginosus were constructed using the S. mutans genomic DNA database (forward primer, 5’-AGGAGATG-CTGTTAATTAAC-3’; reverse primer, 5’-AGCCAAG-CAAATAAGTACCTT-3’). The 600 bp fragment amplified by PCR was cloned into the pGEM-T Easy vector (Promega). The nucleotide sequence was confirmed by the dye-deoxy chain termination technique of Sanger et al. (1977) with an ABI 310 Genetic Analyser (Perkin-Elmer). Inverse PCR was used to isolate flanking regions of the 600 bp fragment. To obtain the
Table 1. Substrate specificity of the S. anginosus l-cysteine desulfhydrase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme product</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (mM)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (μmol min&lt;sup&gt;-1&lt;/sup&gt; mg&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-Cysteine</td>
<td>+</td>
<td>Hydrogen sulfide</td>
<td>0.62</td>
</tr>
<tr>
<td>dL-Cystathionine</td>
<td>+</td>
<td>Homocysteine</td>
<td>3.27</td>
</tr>
<tr>
<td>l-Cystine</td>
<td>+</td>
<td>Hydrogen sulfide</td>
<td>1.29</td>
</tr>
<tr>
<td>S-(2-Aminoethyl)-l-cysteine</td>
<td>+</td>
<td>Cysteamine</td>
<td>2.75</td>
</tr>
<tr>
<td>3-Chloro-dL-alanine</td>
<td>+</td>
<td>ND</td>
<td>28.41</td>
</tr>
<tr>
<td>S-Methyl-l-cysteine</td>
<td>+</td>
<td>Methyl mercaptan</td>
<td>13.13</td>
</tr>
<tr>
<td>l-Methionine</td>
<td></td>
<td>ND</td>
<td>73.1</td>
</tr>
</tbody>
</table>

ND, Not done.
+*, Detected; −, not detected.

DNA sequence upstream of the 600 bp fragment, the genomic DNA of S. anginosus was digested with Mspl and a dilution of the DNA sample was self-ligated for 12 h at 16 ºC. The sample from the ligation reaction was then amplified using a forward primer (LCD-1, 5’-AAAG-GTTAAAACAGGAGA-3’) and a reverse primer (LCD-2, 5’-ATTGTCGAAAATAATGTGAA-3’), to obtain a 800 bp fragment that included 130 bp from the originally amplified 600 bp PCR product. Primers LCD-1 and LCD-2 were designed on the basis of the 600 bp sequence. To obtain the DNA sequence downstream of the original ampiclon, the genomic DNA of S. anginosus was digested with Bsal and a dilution of the DNA sample was self-ligated. The sample from the ligation reaction was then amplified using a forward primer (LCD-3, 5’-CTAACCAAGGTGTTCTCATAG-3’) and a reverse primer (LCD-4, 5’-GTTAAAAGTTATGAAGCCAC-3’), to obtain a 600 bp fragment that included 130 bp from the original PCR product. Primers LCD-3 and LCD-4 were designed on the basis of the 130 bp sequence. The resulting ampiclons were cloned into the pGEM-T Easy vector and sequenced.

Purification of recombinant l-cysteine desulfhydrase of S. anginosus. The recombinant l-cysteine desulfhydrase was purified using the expression vector pGEX-6P-1 (Amersham). To construct a pGEX-6P-1 derivative, designated pMILCD110, the l<sub>cd</sub> gene was amplified by PCR (forward primer, 5’-TCCGGATCCGCAAATACATTTCCAAAC-3’; reverse primer, 5’-GAGGTCACTTATTGGCCAAA CAATGCA-3’). These primers were designed so that BamH I and SalI restriction sites (bold) were created in the PCR product. E. coli BL21 was transformed with pMILCD110 and grown in 50 ml of 2×TY broth with ampicillin at 37 ºC until OD<sub>600</sub> value of 1.0 was attained. Expression was induced with 0.1 mM IPTG. The cells were harvested 1.5 h after induction and lysed by ultrasonication. The cell extract was obtained by centrifugation at 18 000 g for 30 min at 4 ºC. Binding to glutathione-Sepharose 4B medium (Amersham), cleavage of the fusion protein by using PreScission protease and elution of the product were performed according to the manufacturer’s instructions. The purified proteins were mixed with 50% (v/v) glycerol and stored at −20 ºC until used. The purity of the protein was analysed by SDS-PAGE.

Identification of the degradation products from several substrates. The recombinant l-cysteine desulfhydrase activity was assayed with a reaction mixture containing 50 mM potassium phosphate buffer (pH 8.0) with 10 μM pyridoxal 5’-phosphate and 4.5, 225 or 1125 μg l<sup>-1</sup> of the recombinant l-cysteine desulfhydrase with various amounts of l-cysteine or the substrates listed in Table 1. The formation of the following compounds was then used to examine the l-cysteine desulfhydrase activity: (a) sulfur compounds, such as hydrogen sulfide, methyl mercaptan, homocysteine and cysteamine; (b) pyruvate; (c) ammonia.

(a) The formation of hydrogen sulfide and methyl mercaptan was analysed by using a Shimadzu GC-14B gas chromatograph equipped with a flame photometric detector system, as described previously (Yoshimura et al., 2000; Oho et al., 2001). The reaction mixture contained 4.5 ng of the recombinant l-cysteine desulfhydrase in a final volume of 970 μl. The reaction was initiated by adding 30 μl of 33 mM l-cysteine or the substrates listed in Table 1. The reaction mixture was incubated in a sterile 15 ml polypropylene tube at 37 ºC, after the tube was sealed with a silicon plug. After a 30 min incubation, the reaction was stopped by adding 500 μl of 4.5% trichloroacetic acid. One millilitre of the vapour above the reaction mixture in the tube was collected with a gas-tight syringe and applied to a glass column packed with 25% ββ’-oxydipropionitrile on a 60–80 mesh Chromosorb W AW-DMCS-ST support system (Shimadzu) at 70 ºC. The formation of homocysteine and cysteamine as end products was determined on a reversed-phase column (TSKgel ODS-80Ts; Tosoh), using an HPLC system. The reaction mixture in a final volume of 100 μl contained 0.4 μmol of dL-cystathionine or S-(2-aminoethyl)-l-cysteine as substrate and 112.5 ng of the recombinant l-cysteine desulfhydrase. The reaction mixture was incubated for 1 h at 37 ºC and then mixed with 400 μl of 0.5 M NaHCO<sub>3</sub> (pH 8.5). The resulting supernatant was loaded onto an Amicon Microcon filter (30 kDa cut-off), and the products were separated by ultrafiltration. The ultrafiltration product was determined after derivatization with dansylchloride as described by Tapuhi et al. (1981). The dansylated products were separated at a flow rate of 1 ml min<sup>-1</sup> with a mobile phase of 70:30 (v/v) methanol/water containing 0.6% glacial acetic acid and 0.008% triethylamine.
Excitation and emission wavelengths of 350 and 530 nm, respectively, were used to detect dansylated products.

(b) Pyruvate formation was detected as described previously (Soda, 1968; Yoshimura et al., 2000) (see enzyme assay below).

(c) The formation of ammonia as an end product was determined using Ammonia-test wako (Wako Pure Chemical Industries). The reaction mixture in the final volume of 100 µl contained 20 nmol of each substrate listed in Table 1 and 22.5 ng of the recombinant L-cysteine desulfhydrase.

**Enzyme assay.** The general approach for determining the kinetic properties of L-cysteine desulfhydrase followed the method of Yoshimura et al. (2000). The kinetic parameters (Km and Vmax) were computed from the Lineweaver–Burk transformation (V−1 versus S−1) of the Michaelis–Menten equation, where V is the formation rate of pyruvate (µmol min−1 mg−1) and S is the concentration (M) of each substrate listed in Table 1. The assay was carried out with 500 µl potassium phosphate buffer (pH 7.5) containing 5 nmol of pyridoxal 5′-phosphate, 111.5 ng of the purified L-cysteine desulfhydrase and various amounts of L-cysteine or the substrates listed in Table 1. After 10 min incubation at 37 °C, the reaction was terminated by adding 250 µl of 4.5% trichloroacetic acid. The reaction mixture was centrifuged, and 250 µl of the supernatant was added to 750 µl of 0.33 M sodium acetate (pH 5.2) containing 0.017% 3-methyl-2-benzothiazolinone hydrazone. The reaction mixture was then incubated at 50 °C for 30 min (Soda, 1968). The amount of pyruvate was determined at A335. The amount of protein was determined using the Bio-Rad protein assay reagent with bovine serum albumin as a standard.

**Assay of haemoglobin release and modification.** Sheep erythrocytes (RBCs) were washed three times with PBS (0-12 M NaCl, 0-01 M Na2HPO4, 5 mM KH2PO4, pH 7.5), to remove soluble haemoglobin from them before they were used. To obtain isolated haemoglobin, the washed RBCs were centrifuged at 3000 g and then lysed with the same volume of distilled water. The reaction mixture consisted of 0-12 M NaCl, 0-01 M Na2HPO4, 5 mM KH2PO4 (pH 7.5), 10 µM pyridoxal 5′-phosphate, 25 ng purified L-cysteine desulfhydrase ml−1 and 4% (v/v) RBCs or 4% (v/v) isolated haemoglobin, with 0, 1, 3 or 6 mM L-cysteine. The reaction mixture of the positive control contained 70 µg sodium hydrosulphide ml−1 instead of both L-cysteine and the enzyme. The concentration of hydrogen sulfide produced by the degradation of sodium hydrosulphide was estimated to be 6 mM. The reaction mixture of the negative control contained 6 mM pyruvate or 6 mM L-cysteine without the enzyme. The mixture was incubated at 37 °C with slow rotation. Samples (1 ml) were withdrawn at 0, 1, 2, 3, 6, 7, 9 and 11 h, and then centrifuged at 15000 g for 3 min for spectral analysis. The supernatants of the reaction mixtures including RBCs were used to observe the changes in the amounts of haemoglobin and modified forms of haemoglobin, such as methaemoglobin, sulfaehaemoglobin, sulfmethaemoglobin and choleglobin, released from RBCs (Kurzban et al., 1999). The pellets of the mixtures including RBCs were washed with PBS and then lysed by the addition of distilled water (1 ml). These samples were then centrifuged at 15000 g for 3 min and used to show the haemoglobin modification in unbroken RBCs. The samples including isolated haemoglobin were used to demonstrate the changes in the total amounts of haemoglobin and modified haemoglobins. Spectral scans were made from 300 to 680 nm in 1 nm steps using an Ultraspex 2000 UV/Visible spectrophotometer (Amersham). The changes in absorbance at fixed wavelengths (540 and 620 nm) were recorded to measure the amounts of haemoglobin and modified forms of haemoglobin, respectively.

**Nucleotide sequence accession number.** The sequence reported here was submitted to the EMBL and GenBank databases through the DDBJ and assigned accession no. AB084812.

## RESULTS

**Purification of L-cysteine desulfhydrase from S. anginosus FW73**

Essentially all of the soluble fractions obtained after the glass-bead destruction of the S. anginosus FW73 cells showed L-cysteine desulfhydrase activity (Fig. 1B, lane 1). After ammonium sulfate precipitation, the sample was subjected to gel filtration, anion-exchange, hydrophobic and hydroxyapatite columns in that order. SDS-PAGE analysis of the purified L-cysteine desulfhydrase showed a band of approximately 44 kDa in size (Fig. 1A, lane 2). Non-denaturing PAGE also showed the L-cysteine desulfhydrase activity of the purified protein (Fig. 1B, lane 2).

**N-terminal amino acid analysis of L-cysteine desulfhydrase**

The N-terminal amino acid sequence of the purified L-cysteine desulfhydrase was MKYNFQTAPNLKS. A homology search showed that this N-terminal sequence of 15 aa residues was similar to that of the same region.
of the putative aminotransferase from *Streptococcus pneumoniae* (Hoskins et al., 2001). In addition, an ORF, whose putative product showed 56.2% identity with a putative aminotransferase from *S. pneumoniae*, was found in the *S. mutans* genomic DNA sequence database generated by the University of Oklahoma’s Advanced Center for Genome Technology (http://www.genome.ou.edu/smuts.html).

**Cloning and characterization of the gene encoding L-cysteine desulfhydrase from *S. anginosus***

Using the gene sequences from *S. pneumoniae* and *S. mutans*, PCR primers were designed to clone the gene homologue from *S. anginosus*. The amplified fragment was cloned and sequenced. However, the entire ORF was not contained in the 600 bp fragment amplified by PCR. Therefore, inverse PCR was used to isolate DNA sequences upstream and downstream of the 600 bp fragment. The amplified fragments were cloned into the pGEM-T Easy vector and sequenced. The gene was 1164 bp long, and a potential Shine–Dalgarno sequence (Shine & Dalgarno, 1974) was identified just upstream of the potential start codon. We named this gene *lcd*.

The amino acid sequence deduced from *lcd* showed 34–0 and 30–6% identity with the sequences of *Treponema denticola* cysteine desulfhydrase (Chu et al., 1995) and *E. coli* βC-S lyase (Zdych et al., 1995), respectively (Fig. 2).

**Recombinant L-cysteine desulfhydrase**

To evaluate the enzymic activity of the L-cysteine desulfhydrase from *S. anginosus*, the 1164 bp DNA ORF for L-cysteine desulfhydrase was cloned in-frame with glutathione S-transferase (GST) into the pGEX-6P-1 vector. The resulting plasmid, pMILCD110, was used to transform competent *E. coli* BL21 cells. The GST fusion protein was cleaved with PreScission protease and purified by affinity chromatography with glutathione-Sepharose 4B medium. A single protein band was observed upon SDS-PAGE analysis of the recombinant L-cysteine desulfhydrase (Fig. 1A, lane 3). The molecular mass of the denatured polypeptide, 44 kDa, agreed well with the predicted molecular mass (44–7 kDa). The non-denaturing PAGE showed that the recombinant protein had the L-cysteine desulfhydrase activity (Fig. 1B, lane 3).

**Enzymic characterization of the *lcd* gene product**

The formation of hydrogen sulfide, ammonia and pyruvate from L-cysteine by the recombinant L-cysteine desulfhydrase was confirmed. To identify substrates other than L-cysteine for the recombinant L-cysteine desulfhydrase of *S. anginosus*, several compounds were assayed. As shown in Table 1, incubation of L-cysteine with recombinant L-cysteine desulfhydrase resulted in the formation of the same end products as from L-cysteine, namely hydrogen sulfide, ammonia and...
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Fig. 3. Time-course of absorption spectra of isolated haemoglobin. After the addition of L-cysteine and L-cysteine desulphhydrase, isolated haemoglobin was incubated at 37 °C with slow rotation. The supernatants were used after a 20 dilution. O, Negative control (25 ng L-cysteine desulphhydrase ml⁻¹); △, positive control (6 mM hydrogen sulfide); ■, 6 mM L-cysteine plus 25 ng L-cysteine desulphhydrase ml⁻¹. Each graph is representative of three similar experiments.

The breakdown of substrates by the recombinant enzyme was determined by assaying the production of pyruvate, as described by Soda (1968). The $K_m$ and $V_{max}$ values of the recombinant L-cysteine desulphhydrase from S. anginosus FW73 are shown in Table 1. The values for cystathionine and 3-chloro-alanine were calculated for the mixtures of d- and l-isomers.

**Release and modification of haemoglobin**

We examined the changes in the amounts of haemoglobin and modified haemoglobins in haemoglobin samples when they were incubated with L-cysteine and the purified recombinant L-cysteine desulphhydrase. Absorption spectra of the samples including isolated haemoglobin are shown in Fig. 3. The spectrum of the negative control (no L-cysteine, no hydrogen sulfide) was unchanged during incubation. The spectrophotometric assay using the sample containing either only pyruvate or L-cysteine as a negative control exhibited a
similar pattern (data not shown). Incubation of the isolated haemoglobin with L-cysteine and the purified recombinant L-cysteine desulfhydrase resulted in spectral changes. Bands at 414 (Soret band), 540 and 576 nm dropped in intensity with the appearance of a band in the visible region at 620 nm. Isolated haemoglobin exposed to hydrogen sulfide as a positive control for 1 h underwent the same spectral changes as in incubation for 7 h with L-cysteine and the purified recombinant L-cysteine desulfhydrase. These findings indicated that haemoglobin in the reaction mixture was changed to modified types of haemoglobin (methaemoglobin, sulfhaemoglobin, sulfmethaemoglobin and choleglobin) (Kurzban et al., 1999). The changes in the absorbance readings at fixed wavelength (540 and 620 nm) are shown in Fig. 4. In the presence of both L-cysteine and L-cysteine desulfhydrase, the total amount of isolated haemoglobin decreased with time (Fig. 4a). By contrast, the total amount of modified haemoglobins increased.

More modified haemoglobins were observed as the concentration of added L-cysteine increased (Fig. 4b).

The effect of L-cysteine and the purified recombinant L-cysteine desulfhydrase on RBCs was also analysed. A spectrophotometric assay of the unbroken RBC samples was carried out. The samples were obtained by the destruction of pellets (unbroken RBCs) in the reaction mixture. The whole UV/visible spectra were similar to those of the isolated haemoglobin samples shown in Fig. 3 (data not shown). Fig. 5(a, b) show the changes in the A540 and A620 values in the pellet samples, which demonstrate the changes in the amounts of haemoglobin and modified types of haemoglobin in unbroken RBCs, respectively. The amount of haemoglobin in the unbroken RBCs decreased in a time- and concentration-dependent manner (Fig. 5a). By contrast, the amount of modified haemoglobin in the unbroken RBCs increased in a time- and concentration-dependent manner for an initial 6 h incubation. However, in the presence of 6 and 3 mM L-cysteine and L-cysteine desulfhydrase, the amounts of modified haemoglobins rapidly decreased after 6 and 9 h incubation, respectively (Fig. 5b).

**Fig. 5.** Changes in the amounts of haemoglobin (a) and modified haemoglobins (b) in unbroken RBCs. After the addition of L-cysteine and L-cysteine desulfhydrase, RBCs were incubated at 37 °C with slow rotation. After centrifugation, the pellets (unbroken RBCs) were washed with PBS, and then broken by the addition of distilled water to release haemoglobin and modified haemoglobins from unbroken RBCs. The supernatants were used after a 5 × dilution. ○, Negative control (enzyme only); △, positive control (6 mM hydrogen sulfide); □, 1 mM L-cysteine plus enzyme; ●, 3 mM L-cysteine plus enzyme; ■, 6 mM L-cysteine plus enzyme. Each graph is representative of three similar experiments.

**Fig. 6.** Changes in the amounts of haemoglobin (a) and modified haemoglobins (b) released from RBCs. After the addition of L-cysteine and L-cysteine desulfhydrase, RBCs were incubated at 37 °C with slow rotation. After centrifugation, the supernatants were used for the assay. ○, Negative control (enzyme only); △, positive control (6 mM hydrogen sulfide); □, 1 mM L-cysteine plus enzyme; ●, 3 mM L-cysteine plus enzyme; ■, 6 mM L-cysteine plus enzyme. Each graph is representative of three similar experiments.
The spectrophotometric assay of the samples including haemoglobin and modified haemoglobins released from RBCs was also performed. The 1 and 3 h incubation samples exhibited similar patterns to those shown in Fig. 3. Briefly, in the presence of hydrogen sulfide, or 6 mM l-cysteine and l-cysteine desulphhydrase, bands at 414, 540 and 576 nm were low compared with the negative control. However, after a 7 h incubation these bands were much higher than those of the negative control (data not shown). Fig. 6(a, b) show the changes in the A\textsubscript{340} and A\textsubscript{620} values in the supernatant samples, which demonstrate the changes in the amounts of haemoglobin and modified types of haemoglobin released from RBCs, respectively. Haemoglobin was not released during the first 6 h incubation in any of the samples. After the 6 h incubation, haemoglobin in the sample including 6 mM l-cysteine and l-cysteine desulphhydrase was rapidly released from the broken RBCs into the supernatant of the reaction mixture (Fig. 6a). Haemoglobin in the sample including 3 mM l-cysteine increased somewhat after an incubation of 9 h (Fig. 6a). Modified haemoglobins increased in a time- and concentration-dependent manner (Fig. 6b).

**DISCUSSION**

*S. anginosus* is frequently isolated from certain infectious sites and is thought to be associated with abscess formation (Whiley et al., 1992; Fisher & Russell, 1993). However, the mechanism by which this organism is associated with the formation of abscesses has not been elucidated. Recently, we observed that the crude enzyme extract obtained from *S. anginosus* had a much higher capacity to produce hydrogen sulfide from l-cysteine than extracts from other oral streptococci. The high capacity of *S. anginosus* to produce hydrogen sulfide might affect its role as a virulence factor, because hydrogen sulfide is reported to be highly toxic for mammalian cells (Beauchamp et al., 1984). In addition to its toxicity, hydrogen sulfide, along with methyl mercaptan and dimethyl sulfide, is considered to be the main cause of oral malodour (Tonetzich, 1977). In this study, we cloned the *lcd* gene encoding l-cysteine desulphhydrase from *S. anginosus*, and then purified the recombinant enzyme and characterized it to obtain further information concerning the virulence of this organism.

The amino acid sequence deduced from the *lcd* gene showed 34.0% identity with the *T. denticola* Hly protein (Chu et al., 1995) and 30.6% identity with the *E. coli* MalY protein (cystathionase) (Zdych et al., 1995) (Fig. 2). The Hly protein, a 46 kDa haemolysin, is capable of removing the thiol and amino groups from certain sulfur-containing compounds to produce ammonia and pyruvate (Chu et al., 1995, 1997); l-cysteine, l-cystine, S-(2-aminoethyl)-l-cysteine and cystathionine are substrates for the enzyme (Chu et al., 1997). The MalY protein is a 42-44 kDa pyridoxal-5'-phosphate-dependent enzyme with βC-S lyase activity, which forms ammonia and pyruvate from amino acids containing a βC-S linkage (Zdych et al., 1995); l-cystine, S-(2-aminoethyl)-l-cysteine, l-cystathionine and lanthionine are substrates for the enzyme (Zdych et al., 1995). The *K\textsubscript{m}* value of Hly for l-cysteine was reported to be 3.6 mM (Chu et al., 1997), which is considerably higher than that of Lcd of *S. anginosus* (0.62 mM). By contrast, the *K\textsubscript{m}* value of MalY for l-cysteine was reported to be 17 mM (Zdych et al., 1995), which is also high compared with that of Lcd (1.29 mM). The *lcd* product also showed weak homology to the metC products in *E. coli* (Belfaiza et al., 1986) and *Lactococcus lactis* (Fernández et al., 2000) (17 and 14%, respectively). MetC can degrade cystathionine and is involved in methionine biosynthesis. The homology together with the low *K\textsubscript{m}* value for cystathionine (Table 1) suggest that the *lcd* product may contribute to methionine biosynthesis in the cell.

Pyridoxal 5'-phosphate was identified as a cofactor of pyridoxal-5'-phosphate-dependent enzymes, forming a Schiff base with the ε-amino group of the active site. Pyridoxal-5'-phosphate-dependent enzymes have been classified into the α, β and γ families based on sequence alignments and the construction of protein profiles (Alexander et al., 1994). All four amino acid residues that are invariant in the comprehensive alignment of aminotransferases belonging to the α family (Mehta & Christen, 1993) are conserved in Lcd (Fig. 2). Of these four residues, the lysine residue that binds to the cofactor pyridoxal 5'-phosphate is found in Lcd at position 234 (Fig. 2), and is located between residues 209 and 256 in various members of the α family (Alexander et al., 1994). In addition, six of the eight residues that are found in most aminotransferases in the α family are conserved (Mehta et al., 1989; Mehta & Christen, 1993; Alexander et al., 1994) (Fig. 2). These findings indicate that Lcd belongs to the α family of the pyridoxal-5'-phosphate-dependent enzymes.

Chemical analysis revealed that enzymic cleavage of l-cysteine occurred in a dose-dependent manner and produced hydrogen sulfide, ammonia and pyruvate. Besides l-cysteine, sulfur amino acids containing a βC-S linkage, such as Dl-cystathionine, l-cysteine, S-(2-aminoethyl)-l-cysteine and S-methyl-l-cysteine, were cleaved at the αC-N and βC-S linkages, producing pyruvate, ammonia and other sulfur-containing molecules. In contrast, the enzyme failed to catalyse γC-S elimination reactions and also failed to cleave all of the tested amino acids that have no βC-S linkages, with the single exception of 3-chloro-DL-alanine. Interestingly, incubation of 3-chloro-DL-alanine, which is an amino acid without a βC-S linkage, with l-cysteine desulphhydrase resulted in the formation of ammonia and pyruvate. This might be ascribed to the structural identity of 3-chloro-alanine to cysteine; the difference is that the S' ion at the C-3 position is replaced with a Cl- ion.

Exposure of RBCs to both l-cysteine and l-cysteine desulphhydrase or to hydrogen sulfide resulted in the release of haemoglobin and the production of modified types of haemoglobin, while incubation of RBCs with...
any one of L-cysteine, L-cysteine desulphhydrase or pyruvate did not result in the same phenomena. Based on these findings, it is likely that the hydrogen sulfide produced from L-cysteine by the enzymatic action of L-cysteine desulphhydrase plays an important role in the release and modification of haemoglobin. It is very interesting that the amounts of modified haemoglobins in the unbroken RBCs began to decrease after an initial 6 h increase when incubated with 6 mM L-cysteine (Fig. 5b), and that the release of haemoglobin and modified haemoglobins from RBCs into the supernatant started at the same time (Fig. 6a, b). In the case of the sample including 3 mM L-cysteine, these phenomena were also observed after a 9 h incubation. These results obviously show that haemoglobin was modified in RBCs, but was not released from RBCs for at least 6 or 9 h under the experimental conditions tested. Therefore, the formation of modified haemoglobin seems to play an important role in haemolysis. These findings are consistent with a general mechanism that connects sulph-haemoglobin formation to erythrocyte lysis (Moxness et al., 1996). However, the effects of hydrogen sulfide on RBC haemoglobin might not be due solely to the presence of modified haemoglobins, because hydrogen sulfide can be cytotoxic and can lyse other types of mammalian cells.

In conclusion, we cloned the \( \text{lcd} \) gene encoding L-cysteine desulphhydrase from \( S. \ anginosus \) and then purified and characterized the gene product. This enzyme cleaved substrates at \( \text{C-N} \) and \( \text{C-S} \) linkages, producing pyruvate, ammonia and sulfur-containing molecules. In addition, the hydrogen sulfide produced by this enzyme and L-cysteine affect the release and modification of haemoglobin in RBCs. Further studies are necessary to clarify why the enzyme obtained from \( S. \ anginosus \) has such a high capacity to produce hydrogen sulfide from L-cysteine in comparison with the enzymes from other oral streptococci, and how this high capacity to produce hydrogen sulfide is associated with the virulence of the organism.

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