Characteristics of a protease of *Streptococcus sanguis* G9B which degrades the major salivary adhesin

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1. SUMMARY

An endogenous enzyme present in cell surface extracts of *Streptococcus sanguis* strain G9B degraded the major salivary adhesin of the organism. The enzyme showed optimal activity between 50 and 65°C and was inactivated at higher temperatures. The activity at these unusually high temperatures seemed to be a consequence of release from the cell surface since intact whole G9B cells showed greater activity at 37°C. The enzyme was not found in culture supernatants of G9B cells. The pH range for the enzyme was between 5 and 9. It was inhibited by iodoacetic acid, Hg²⁺, Cu²⁺, EDTA, SDS, and PMSF, but not by TLCK, TPCK, soybean trypsin inhibitor, cysteine, dithiothreitol, leupeptin, Ca²⁺, Mg²⁺ or saliva. The enzyme did not show any activity against human or rabbit IgG or human IgA. Enzyme activity was also found in *S. sanguis* strains Adh⁻ (a spontaneously occurring non-adherent mutant of G9B), and M-5.

2. INTRODUCTION

*Streptococcus sanguis* adheres strongly to the salivary pellicle which coats tooth surfaces. The adhesins which mediate this adherence to the salivary molecules have not been fully characterized, however attention has focused on the fibrillar appendages [1,2] and surface proteins [3,4] which may or may not be associated with the fibrils. Most studies of these adhesins have relied on indirect methods to demonstrate activity [1–3]. Such indirect methods include inhibition of adherence of whole cells by specific antibodies and absorption of antibody inhibitory activity by defined molecules. Although direct blocking of whole bacteria by isolated adhesins has been reported, this activity has either been poorly reproducible [4] or entailed streptococcal components cloned in *E. coli* [5]. Speculation on the reasons for the difficulty in obtaining biologically active adhesins has included conformational changes in the molecules upon release from the cell wall and partial

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degradation of the proteins by endogenous enzymes.

A protein adhesin from *S. sanguis* strain G9B, comprising 80, 62 and 52 kDa polypeptides (designated the 80 kDa complex), has been identified in our laboratory [3,6]. The 80 kDa complex is the substrate for a proteolytic enzyme which is also present in cell wall extracts of G9B [3]. This enzyme has complicated purification of the adhesin from cell wall extracts and may be responsible for the inability to demonstrate direct blocking activity of crude adhesin preparations. In this report we describe some of the properties of the *S. sanguis* G9B proteolytic enzyme with activity against the 80 kDa adhesin complex.

### 3. MATERIALS AND METHODS

#### 3.1. Bacteria and culture conditions

*S. sanguis* G9B and M-5 were originally isolated from dental plaque. Strain Adh is a spontaneous mutant of G9B which adheres poorly to salivary pellicle [7]. All are Biotype I [8]; G9B and Adh are serotype 1, whereas M-5 is serotype 2 [9]. Cells were grown overnight at 37°C in Trypticase peptone broth (BBL Microbiology Systems, Cockeysville, MD) supplemented with 5 g/l yeast extract (BBL) and 0.5% glucose as a carbon source. Culture supernatants of G9B were concentrated in an Amicon Ultra-filtration unit (PM5000, Amicon Corp., Lexington, MA.), dialyzed and lyophilized.

#### 3.2. Adhesin and enzyme preparation

Streptococcal surface molecules were extracted by shaking in a 2 mM barbital buffer pH 8.6, dialyzed and lyophilized, as reported previously [6]. The barbital extract of G9B contains the 80 kDa adhesin complex and the proteolytic enzyme for which it is a substrate [3].

#### 3.3. Immunoblotting

10% SDS-PAGE gels were run with the buffer system of Laemmli [10] and electrophoretically blotted onto nitrocellulose [11]. The blots were probed with antibody as described previously [6].

#### 3.4. Antisera

Antibodies were produced in rabbits to formalinized whole G9B cells as reported previously [9]. Anti-80 kDa complex antibodies were produced by excising the 80 kDa band from SDS-PAGE gels, mixing with adjuvant and injecting into rabbits [6].

#### 3.5. Protease assay

Preliminary observations indicated that the G9B barbital extract contained an enzyme which degraded the 80 kDa antigen complex but had no effect on any of the other antigens in the extract [3]. Activity of this enzyme was greater at 56°C compared to 37°C and was abolished at temperatures above 80°C [3]. Therefore, to obtain enzyme free of detectable substrate, G9B barbital extract (1 μg protein/μl in 20 mM Tris-HCl, pH 7.0) was incubated at 56°C for 20 min. To prepare substrate without enzyme, G9B barbital extract (1 μg protein/μl in 20 mM Tris-HCl, pH 7.0) was heated to 80°C for 20 min. Substrate preparations subsequently incubated at 56°C for 30 min retained the 80 kDa antigen complex, indicating that the enzyme was inactivated. Enzyme and substrate preparations were stored at −20°C. Protease activity was determined by incubating the enzyme preparation (10 μg protein) with the substrate preparation (10 μg protein) at 56°C for 20 min in 20 mM Tris, pH 7. The mixture was then examined by immunoblotting with anti-80 kDa antibodies to test for the presence of the 80 kDa antigen complex (the substrate). Controls of enzyme preparation only (56°C, 30 min) and substrate preparation only (80°C, 20 min) were included in each experiment.

#### 3.6. Determination of optimum temperature

The optimum temperature for enzyme activity was determined by performing the protease assay at 22, 30, 37, 45, 50, 55, 65, 70, 75, 80, 90 and 100°C.

#### 3.7. Determination of optimum pH

Enzyme and substrate were prepared as described above except they were resuspended in 10 μl of one of the following buffers: 0.5 M glycine, pH 2–4; 0.5 M acetate, pH 4–6; 0.5 M phosphate,
pH 6–8; 0.5 M Tris-HCl, pH 7–9; or 0.5 M bicarbonate, pH 9–11. The protease assay was then carried out as described above. Separate experiments of substrate only were included to control for possible instability of the 80 kDa complex at any of the pH values.

3.8. Effects of protease inhibitors and metal ions

The enzyme preparation was preincubated for 30 min at 56 °C with a 10 mM solution of the following protease inhibitors: phenylmethylsulfonyl fluoride (PMSF), N-α-p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK), EDTA, and iodoacetic acid. Leupeptin was used at 10 μM; soybean trypsin inhibitor was 100 μg/ml. Dithiothreitol, L-cysteine, and SDS were assayed at 20 mM and the effects of CaCl₂, MgCl₂, ZnCl₂, CuCl₂, and HgCl₂ were determined at 10 mM. The protease assay was performed as described above. Separate experiments with substrate only in the presence of inhibitors and metal ions were performed to control for any possible direct effects of these compounds on the 80 kDa complex.

3.9. Effects of saliva

An equal volume of pooled clarified whole saliva was added to the enzyme/substrate mixture prior to incubation at 56 °C for 20 min.

3.10. Distribution of the enzyme

G9B culture supernatant (10 μg protein) in 20 mM Tris-HCl, pH 7.0, was added to an equal volume (10 μl) of substrate preparation (10 μg protein) and incubated at 56 °C and 37 °C for 30 min. G9B whole cells were washed and resuspended in 20 mM Tris-HCl pH 7.0. 10 μl containing 1 × 10⁵ cells were added to 10 μl substrate preparation (10 μg protein) and incubated at 56 °C and 37 °C for 30 min. The cells were then removed by centrifugation (×3) at 10,000 × g. In both cases enzyme activity was determined by assaying for the 80 kDa antigen in immunoblots.

3.11. Activity of enzyme against immunoglobulins

Human colostral IgA and IgG, and rabbit IgG (Sigma Chemical Co., St. Louis, MO) were suspended to 50 μg/μl in 20 mM Tris-HCl pH 7.0 and an equal volume (10 μl) enzyme preparation (500 μg protein) added. The mixtures were incubated for 30 min at 56 °C and 37 °C. Proteolytic activity was determined by assaying for lower molecular weight degradation products in immunoblots probed with peroxidase conjugated anti-IgA and anti-IgG antibodies (Sigma). Proteolytic activity was also investigated by immunoelectrophoresis (IEP) as described by Frandsen et al. [12].

4. RESULTS

4.1. Optimum temperature

Fig. 1 shows the immunoblots of the substrate preparation after incubation with the enzyme preparation at various temperatures. The enzyme preparation was optimally active between 50 and 65 °C. Below 50 °C the activity of the enzyme could only be demonstrated after incubation with the substrate for several hours (not shown). The enzyme did not show any activity above 65 °C.
even after prolonged incubation and subsequent reduction of the temperature to 56°C. Thus, the enzyme appears to be denatured above 65°C.

4.2. Optimum pH
The enzyme was active between pH 5 and 9; no activity was observed at pH 2, 3, 4, 11 and 12, however some activity was seen at pH 10.

4.3. Inhibition studies
The thiol inhibitor iodoacetic acid inhibited enzyme activity, as did Hg^{2+} and Cu^{2+}, indicating the enzyme may be a thiol protease. Activity was also inhibited by EDTA, suggesting a requirement for a metal cofactor. Of the serine protease inhibitors tested, PMSF, but not TLCK, TPCK, leupeptin or soybean trypsin inhibitor, resulted in inactivation. The enzyme was sensitive to denaturation by SDS. Cysteine, dithiothreitol, Ca^{2+}, Mg^{2+} and Zn^{2+} did not inhibit enzyme activity.

4.4. Effect of saliva
A 1 in 2 dilution of whole saliva had no effect on the activity of the enzyme.

4.5. Distribution of the enzyme
Barbital extracts of Adh^- and M-5 demonstrated activity against the G9B 80 kDa adhesin complex. Titering the enzyme preparation indicated similar enzyme levels in G9B, Adh^- and M-5 extracts. Enzyme activity at 56°C was not detected on G9B whole cells but when the cells and substrate were incubated at 37°C, the 80 kDa complex was degraded. This suggests that the enzyme is present on the cell surface, however, it exhibits different kinetics when free then when bound to the cell. No enzyme activity was found in the culture supernatant of G9B.

4.6. Activity against immunoglobulins
No effects on either human or rabbit IgG or human IgA were detected at 56°C or 37°C.

5. DISCUSSION
Studies of the S. sanguis adhesins have been hampered by the difficulty in obtaining biologically active preparations. We have previously identified an 80 kDa antigen complex in S. sanguis strain G9B which appears to be responsible for specific attachment to the salivary pellicle on tooth surfaces [3,6]. Although the 80 kDa protease may alter the salivary binding domains necessary for adhesion competition or blocking activity, it does not, at least initially, affect the antigenic determinants required for antibody inhibitory activity [3,6]. Since enzyme activity was also present in an extract of strain M-5, which is a different serotype to G9B, such enzymes may be widespread amongst S. sanguis strains and must be considered in the purification of S. sanguis adhesins. The soluble enzyme showed optimal activity between 50 and 65°C; however the optimal activity on whole cells was at 37°C. Although it is not entirely certain that both activities are due to the same enzyme, the observation suggests that the kinetics of the soluble enzyme differs from those of the enzyme on the cell surface. Variations in temperature dependence and heat stability between enzymes immobilized and free in solution are well documented (13-15) and are believed to be associated with either conformational changes in the enzyme, environmental differences, partitioning of the substrate between the solution and solid phase, or diffusion effects [13].

The enzyme was inhibited by iodoacetic acid, Hg^{2+} and Cu^{2+}, but not by the reducing agents dithiothreitol or cysteine, characteristics of a thiol protease. The enzyme was also inactivated by PMSF, but not by other serine protease inhibitors, and by EDTA, which suggests a requirement for divalent cations. Such characteristics are unusual but not unprecedented. Clostridium histolyticum produces a thiol protease which requires metal ions for action and is sensitive to TLCK but not to other serine protease inhibitors [16]. A low molecular weight protease isolated from Bacteroides gingivalis can be inhibited by both serine protease and thiol protease inhibitors [17]. The IgA1 degrading enzymes from many Bacteroides species are thiol proteases with a requirement for metal cofactors [18].

IgA proteases have been found in S. sanguis [19], however the G9B enzyme described here did not degrade IgA nor did it attack IgG. Therefore
it should not interfere with antibody absorption experiments performed with enzyme containing streptococcal extracts.

The enzyme is active in the presence of saliva and on the cell surface where its behavior appears to be governed by more physiological conditions. Thus, the enzyme may be active in vivo, albeit its function is as yet unclear. The presence of similar levels of the enzyme in the non-adherent mutant of G9B, Adh−, would tend to preclude a direct involvement in adherence to salivary pellicle. Further studies are in progress to purify this interesting enzyme in order to investigate more fully its characteristics and function.

REFERENCES