An extracellular enzyme with hyaluronidase and chondroitinase activities from some oral anaerobic spirochaetes

D. Scott,† I. R. Siboo,† E. C. S. Chan and R. Siboo

INTRODUCTION

Extracellular products of oral bacteria have been implicated in the initiation and progression of periodontal disease (Loesche, 1993; Stevens & Hammond, 1988). Bacteria isolated from saliva (Mahler & Lissanti, 1952), gingival sulci (Hershon, 1974; Rosan & Williams, 1964; Schultz-Haudt & Scherp, 1955) and periodontal pockets (Tam et al., 1982) produce hyaluronidase (Hase). The severity of gingivitis in young male adults correlated with increased Hase activity in their saliva (Rovelstad et al., 1958). Injection of Hase into interdental papillae of monkeys produced gingivitis (Aisenberg et al., 1951) and administration of Hase into gingival sulci of healthy human adults resulted in a loss of cementing ground substance, tract formation and invasion of the corium by bacteria (Schultz-Haudt et al., 1953). It was consequently postulated that disruption of the crevicular epithelium might be the first step in the pathogenesis of periodontal disease (Schultz-Haudt & Scherp, 1955). Most putative periodontopathogens do not produce Hase (Grenier & Michaud, 1993) but the production of Hase and the role that this enzyme may play in periodontal disease have been largely ignored in recent years.

Glycosaminoglycans (GAGs), polymers of a hexosamine and a uronic acid disaccharide (Iozzo, 1985), are constituents of the extracellular matrix of connective tissues (Last et al., 1988). Hyaluronic acid (HA) (p-glucuronic acid and N-acetylglucosamine repeating units) is commonly detected in the gingival crevicular fluid (GCF) and is the only major GAG found in GCF collected from patients with chronic gingivitis (Last et al., 1985). Chondroitin 4-sulphate (C4S) is found in the GCF during advanced periodontal disease (Last et al., 1985, 1988; Smith et al., 1995). Variation in the GAG content of the GCF has, therefore, been proposed as a marker of degenerative changes in the periodontium due to disease or to orthodontic treatment (Last et al., 1985, 1988; Smith et al., 1995; Waddington & Embery, 1991). In periodontal tissues, the major GAGs are HA, chondroitin sulphate (CS) and dermatan sulphate, with C4S present in significantly higher amounts than chondroitin 6-sulphate (C6S) in alveolar bone, cementum, periodontal ligaments and gingiva (Last et al., 1985, 1988; Okazaki et al., 1993; Smith et al., 1995; Waddington & Embery, 1991). HA, the non-sulphated GAG (Iozzo, 1985), is found in high amounts in the gingiva (Last et al., 1988). CS is a polymer of p-glucuronic acid and N-acetylgalactosamine repeating units. GAGs are thought to be involved in maintaining
the structural integrity of connective tissue, in water retention, and in cell migration (Iozzo, 1985). Sulphated GAGs are protein-linked in connective tissues, forming proteoglycans (Iozzo, 1985; Isaacs, 1994) which tend to be rich in aspartate, glycine and glutamate (Waddington & Embry, 1991). Proteoglycan can be associated with HA via link proteins (Iozzo, 1985). Streptococcal Hase, bovine Hase and flavobacter chondroitinase (Case) (Dell'Orbo et al., 1995; Quacci et al., 1992) have been shown to cause structural alterations to collagen fibrils. Hase-, Case- and collagenase-producing bacteria have also been associated with infections of root canals (Hashioka et al., 1994).

The nomenclature in use to describe GAG-degrading enzymes is in need of clarification. There are many names used to describe GAG-degrading enzymes, such as hyaluronate lyase, Hase and chondroitin sulphatase. Care should be taken before assigning a descriptive name to the enzyme under investigation. Chrontroitin sulphatase should only be used where sulphate moieties are, in fact, removed from the core GAG. Hase is a general term which should be refined to better describe enzymic activity; for example, a Hase whose specificity is restricted to the cleavage of β-glucuronate-(1 → 3)-N-acetylglucosamine glycosidic bonds in HA should be referred to as a hyaluronoglucosaminidase, whereas an enzyme that is able to cleave the β-N-acetyl-hexosamine-(1 → 4) glycosidic bonds in both HA and in CS is a hyaluronoglucosaminidase (HGase). A review of the nomenclature of GAGs and their relationship to GAG-degrading enzymes has recently been published (Scott, 1993).

There has also been some confusion as to the specificity of complex polysaccharide-degrading enzymes in the literature. This has been partly due to the impurity of bovine submaxillary mucin, the substrate commonly used to monitor neuraminidase activity (Pritchard & Lin, 1993). Characterization of the degradation products of a group B streptococcal enzyme thought to be a neuraminidase due to its ability to hydrolyse this substrate (Hayano & Tanaka, 1969; Milligan et al., 1980; Brown & Straus, 1987) revealed that a hyalobiuronic acid derivative was liberated from HA containing bovine submaxillary mucin preparations and that the enzyme is, in fact, a Hase (Pritchard & Lin, 1993). Affinity columns containing N-аци обезьяной (p-aminophenyl)oxamic acid-agarose have been employed to purify bacterial neuraminidase but are now known also to bind Hase (Pritchard & Lin, 1993). Interference by proteases in the BSA precipitation method for the assay of Hase may give rise to false-positive results (Grenier & Michaud, 1993). In addition, Hase may not migrate through HA-containing gels (Hotze et al., 1993) due to binding of the enzyme to its substrate. This may result in a significant overestimation of the molecular mass.

We conjectured that oral anaerobic spirochaetes may produce Hase as they are found in high numbers in the most apical aspect of periodontal pockets (Omar et al., 1990) and are seen intercellularly in gingival tissue sections of patients with periodontitis (Loesche, 1988).

We have identified the secreted HA- and CS-degrading enzyme of Treponema denticola ATCC 35405 as an HGase (EC 3.2.1.35). This enzyme may function as a virulence factor by breaking down the extracellular matrix of the gingiva, thus contributing to the progression of periodontal disease.

**METHODS**

**Reagents.** Purified bovine Hase, HA, C4S, CS, Stains-all, Alcan blue, rabbit anti-Apis mellifera antibodies, gold-labelled protein G, Clostridium perfringens neuraminidase, Penicillium dextranase and routine chemicals were purchased from Sigma. Gold sodium thiomalate was obtained from Aldrich. Nitrocellulose membranes, molecular mass markers, protein assay kit, Affi-Gel 701 and gold-labelled rabbit anti-human IgG were supplied by Bio-Rad Laboratories. It should be noted that the commercial substrates, HA, C4S and CSs are impure. HA can contain 2–3% CS, and traces of heparin (Sigma), or as much as 20% CS (United States Biochemical). In addition, C4S contains CS and C6S contains C4S.

**Bacteria and culture conditions.** Oral spirochaetes Treponema denticola ATCC 35405, Treponema vincentii ATCC 35580 and Treponema socranski ATCC 35536 were grown anaerobically in new oral spirochaete (NOS) broth as described previously by Cheng & Chan (1983). Streptococcus agalactiae was obtained from the Department of Microbiology and Immunology Culture Collection for Teaching, McGill University, Canada, and was grown in Robertson's Cooked Meat medium, at 37°C, under anaerobic conditions. The spirochaetes were harvested at their late-exponential growth phases (T. denticola, 7 d; T. vincentii and T. socran-ski, 12 d) and S. agalactiae after 24 h, by centrifugation (4000 g, 15 min, 4°C). Preliminary experiments had established that maximal Hase activity was present in the growth medium at these times. The cell-free culture supernatants were collected and their protein content was estimated with the Bio-Rad protein assay kit. Samples of the supernatants were used immediately or frozen and used within 48 h for assay for Hase and Case activity.

**Assays for Hase and Case activities.** The enzymic activity of each culture supernatant was determined with the substrates HA, C4S and C6S essentially by the method of Hotze et al. (1993). Triplicate assays on each batch of supernatants were performed in 1.5 ml disposable plastic cuvettes. Each cuvette received 100 µl HA in 0.1 M sodium acetate buffer, pH 6.0, to give a final concentration of 40 µg ml⁻¹ and 10 µl of the T. denticola supernatant. The cuvettes were sealed, shaken and incubated for 3 h at 37°C. Thereafter, 890 µl of a stock solution of Stains-all (50 µg ml⁻¹ in 50% formamide/0.06% acetic acid solution) was added, the contents of the cuvettes were mixed and the A₆₄₀ was measured immediately. The assay is based on the shift to a longer wavelength when Stains-all binds to HA or CS. The T. vincentii and T. socranski culture supernatants were assayed for Hase activity in an identical manner. The Case activity of the T. denticola supernatant was determined as described above with C4S and C6S as substrates and the Case activity of T. socranski and T. vincentii supernatants was assayed with CS. The amount of substrate cleaved was determined by subtraction (i.e., absorbance of the substrate at A₆₄₀ – absorbance of the substrate at tₖₐ₀ – cleaved substrate). The concentration of the substrates was determined from standard curves prepared with HA, C4S or C6S in acetate buffer, and Stains-all. The enzyme activity is expressed as µg HA or CS cleaved h⁻¹ (ml supernatant)⁻¹. Control assays were carried out with bovine Hase, un inoculated NOS supernatant and acetate buffer with each of the substrates. Preliminary assays were carried out at a pH range of 4.0–9.0 and the enzyme was

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found to hydrolyse HA, C6S and C4S optimally at pH 6.0. The experiment was repeated with \textit{T. denticola} and \textit{S. agalactiae} culture supernatants (0–40 μl), with a 3 h digestion period. Enzyme kinetics were not investigated due to the impurity of the commercial substrates HA, C4S and C6S.

**Inhibition assays.** The specificity of the Hase and Case activities was determined initially with gold sodium thiomalate, a known inhibitor of Hases (Hotez et al., 1993). \textit{T. denticola}, \textit{T. vincentii} and \textit{T. socranskii} supernatants (40 μl) were each pipetted into separate microcentrifuge tubes, 40 μl gold sodium thiomalate (1 mg ml⁻¹ in acetate buffer) was added to each tube and they were shaken continuously for 20 min at 37 °C. Each mixture was then assayed for Hase and Case activities with HA, C4S and C6S by the Stains-all method. \textit{T. denticola}, \textit{T. vincentii} and \textit{T. socranskii} supernatants incubated separately with acetate buffer only served as controls for enzyme degradation over the incubation period. The \textit{T. denticola}, \textit{T. vincentii} and \textit{T. socranskii} supernatants, with and without gold sodium thiomalate, were assayed at the same time.

The effect of several ions (Cd²⁺, Hg²⁺, Cu²⁺ and Mn²⁺) and of EDEA, SDS, p-chloromercurobenzoe Natroxy-1-phenylalanine chloromethyl ketone (TPCK), soybean trypsin inhibitor, PMSF, DTT, glutathione and t.cysteine HCl on the Hase activity of the \textit{T. denticola} supernatant was determined using the Stains-all assay, with appropriate controls, over a 3 h period as described above; 20 μl \textit{T. denticola} supernatant was used. The concentration of each inhibitor/activator is given in Table 2. The Hase activity of the \textit{T. denticola} culture supernatant alone served as the 100% control.

As a further means of examining the specificity of the Hase activity, 0–40 μl \textit{T. denticola} culture supernatant was used and the degradation of HA was, again, measured by the Stains-all assay after a 3 h incubation. Under identical conditions, the effect of 20 μl gold sodium thiomalate (1 mg ml⁻¹ in acetate buffer) and 20 μl of a 1/100 and a 1/500 dilution of commercial rabbit anti-\textit{A. meliifera} (honey-bee) venom antibodies in acetate buffer at each concentration of \textit{T. denticola} supernatant was monitored. The effect of gold sodium thiomalate and of rabbit anti-\textit{A. meliifera}-venom antibody on increasing concentrations of \textit{S. agalactiae} supernatant (0–20 μg ml⁻¹) was also examined under identical conditions.

The effect of dextranase (3 U) and neuraminidase (1 U) on the HA substrate was monitored by the Stains-all assay after a 3 h incubation at 37 °C.

**Conjugation of HA and C6S to Affi-Gel 701.** Affi-Gel 701 (5 ml) was sedimented by centrifugation (4000 g, 10 min). The resin was washed twice with HCl-acidified water, pH 4.7, by centrifugation, resuspended in 2.0 ml acidified water and 2.0 ml HA [1 mg (ml acidified water)⁻¹] was added to the resin. A total of 4 mg carbodiimide was added, in small aliquots, to the mixture and the tube was vortexed after the addition of each aliquot. The mixture was shaken continuously for 1 h at 37 °C. An additional 4 mg carbodiimide was added to the tube and the pH of the reaction mixture was maintained at 4.7 for 4 h. Conjugation was allowed to proceed with continuous shaking of the tube for 18 h at 4 °C. The HA-conjugated Affi-Gel 701 (HA-Gel) was packed by centrifugation, and the supernatant was collected and assayed for unbound HA. The HA bound to the Affi-Gel 701 (HA-Gel) was washed twice with acetate buffer (5 min per wash) by inversion of the tube and centrifugation, and resuspended to a total volume of 5.0 ml in acetate buffer. C6S was conjugated to Affi-Gel 701 (C6S-Gel) and assayed for unbound C6S as described for HA. \textit{T. denticola} supernatant (500 μl) was pipetted into each of two microcentrifuge tubes. HA–Gel (500 μl) was added to one of the two tubes, C6S–Gel (500 μl) was added to the other tube and they were shaken continuously for 2 h at 4 °C. The tubes were centrifuged and the supernatants (unbound proteins) were collected. The gels were washed with acetate buffer to remove proteins trapped in the gels. Each of the two washed gels was resuspended in 1.0 ml of a 0.5 M NaCl/0.25 M glycine, pH 3.2, buffer, mixed continuously for 15 min by inversion and centrifuged (4000 g, 10 min). The eluted protein(s) was collected and dialysed against acetate buffer. The unbound and the eluted protein(s) were assayed for both Hase and Case activities by the Stains-all method (described above). \textit{T. denticola} supernatant was incubated with acetate buffer instead of HA– or C6S–Gels, and served as a control for the dilution effect and stability of the Hase and Case in the \textit{T. denticola} supernatant during the adsorption/incubation period. Bovine Hase served as a positive control to ascertain the efficacy of the HA– and C6S–Gels in the adsorption of Hase and Case activities.

**Electrophoresis and electroblotting.** \textit{T. denticola}, \textit{T. vincentii} and \textit{T. socranskii} culture supernatants, supernatants treated with gold sodium thiomalate (as described above) as well as the unbound and eluted protein(s) after adsorption of the supernatants with HA– or C6S–Gels were subjected to SDS-PAGE under non-reducing conditions and translotted onto nitrocellulose membranes as described previously (Chan et al., 1991). The membranes were washed twice (5 min per wash) in acetate buffer and then immersed in a solution of either HA or C6S (20 μg ml⁻¹ in acetate buffer) for 15 min at room temperature. Unbound HA and C6S were drained from the membranes, which were dried for 30 min at 37 °C. Thereafter, the membranes were soaked in acetate buffer for 2–3 min and drained of excess buffer. The saturated membranes were incubated for 24 h at 37 °C in a closed container and then immersed in a solution of Alcian blue (20 μg ml⁻¹ in acetate buffer, pH 4.7) for 1 h at room temperature. Thereafter the membranes were washed extensively with acetate buffer, pH 4.7, and examined for unstained degradative bands representing Hase and Case activity. Alcian blue binds to the membrane-bound HA and C6S but it does not bind to the degradative products of HA and C6S. The molecular mass of the enzyme(s) responsible for HA and CS degradation was thus established.

Preliminary experiments with the Stains-all assay showed that neither Hase nor Case was inactivated by the concentrations of SDS and methanol in the buffers used in the SDS-PAGE and translort blot techniques. Miura et al. (1995) have previously reported that SDS did not inhibit the activity of several GAG-degrading enzymes. \textit{T. denticola} culture supernatant, and the HA–Gel– and C6S–Gel-purified \textit{T. denticola} Hase, were subjected to electrophoresis through a 10% SDS-PAGE system at 200 V, as described previously (Chan et al., 1991), and double-stained with Coomassie blue and silver to assess purity.

To determine if the spirochaete Hase could be recognized by anti-bee-venom antibodies, which could then be used in electron microscope studies, Western blotting was also performed after electrophoresis of purified \textit{T. denticola} Hase, bovine Hase and \textit{A. meliifera} venom through a 10% SDS-PAGE system. The immunoblot was developed using rabbit anti-\textit{A. meliifera}-venom antibodies, alkaline-phosphatase-conjugated goat anti-rabbit IgG, \textit{p}-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

**Electron microscopy.** \textit{T. denticola} was sedimented from NOS culture medium by centrifugation (4000 g, 10 min) and the cells were resuspended in acetate buffer, washed by inversion of the tube and sedimented by centrifugation. The cells were resuspended in acetate buffer and adjusted to 10⁵ cells ml⁻¹. Two aliquots of the cells (40 μl per aliquot) were placed onto two
separate areas of a plastic Petri dish. To one aliquot was added 40 µl HA (1 mg ml⁻¹ in acetate buffer) and to the second aliquot was added 40 µl acetate buffer. Each aliquot was stirred and incubated for 15 min at 37 °C. Then, 40 µl gold sodium thiomalate (1 mg ml⁻¹ in acetate buffer) was added to each of the aliquots, which were stirred and incubated for 30 min at 37 °C. Nickel Formvar grids (mesh size 300) were submerged separately into each sample preparation for 1 min; the grids were then washed twice with acetate buffer, blotted dry on bibulous paper, and the cells were fixed with 2% (v/v) glutaraldehyde.

Gold-labelled rabbit anti-human IgG was incubated with an equal volume of a 1/100 dilution of anti-A. meliitiera-venom antibody in acetate buffer was added and the cells were incubated for 1 h at 37 °C. The cells were then washed twice with acetate buffer, then incubated with a 1/10 dilution of gold-labelled protein G in acetate buffer for 1 h at 37 °C. The gold-labelled protein G was first centrifuged at 14000 g for 5 min to remove any gold aggregates. The protein G preparation contained 2.7 × 10¹⁵ gold particles ml⁻¹, as purchased, with a mean diameter of 9.6 nm. The cells were washed twice with acetate buffer. Nickel Formvar grids were submerged for 1 min, rinsed with acetate buffer, submerged into 0.2% phosphotungstate for 1 min, re-rinsed and blotted, then fixed with 2% glutaraldehyde. Cells were also prepared for examination as above after first being incubated with 1 mg HA ml⁻¹ in acetate buffer and as above using gold-labelled protein G without the prior incubation with anti-venom antibodies, as negative controls. The prepared grids were examined with a Philips 410 electron microscope.

RESULTS

Hase and Case activities in culture supernatants

The enzyme(s) secreted by T. denticola, T. vincentii and T. socranszki, and active in the culture supernatants, each hydrolysed HA and C6S (Table 1). T. denticola produces about three- and fivefold more of the HA-hydrolysing enzyme than T. socranszki and T. vincentii, respectively. The T. denticola enzyme, however, hydrolysed C6S and C4S less efficiently than HA. The T. vincentii and T. socranszki enzyme(s) hydrolysed HA and C6S at a much lower rate than the T. denticola enzyme. The hydrolysis of C4S by the T. vincentii and T. socranszki enzyme was not tested. Freezing of the supernatants did not appear to affect enzyme activity. Gold sodium thiomalate, an inhibitor of Hase (Hotez et al., 1993), completely inhibited the hydrolysis of HA and C6S by the enzyme produced by T. denticola, T. vincentii and T. socranszki (Table 1). The apparent higher rate of hydrolysis of HA and C6S by the culture supernatants of T. denticola, in comparison with the supernatants of T. socranszki and T. vincentii, may be due to the amount of enzyme produced and secreted by each species. Although the supernatants were harvested at the time of maximal enzyme production, it is recognized that the apparent higher rate of hydrolysis may also reflect differences in growth yields of the three oral spirochaete species.

Affinity chromatography

The T. denticola eluate from the HA–Gel (affinity purified) hydrolysed 650 µg HA h⁻¹ ml⁻¹ and 208.3 µg C6S h⁻¹ ml⁻¹. Likewise, the T. denticola eluate from the C6S–Gel also hydrolysed 633.3 µg HA h⁻¹ ml⁻¹ and 233.3 µg C6S h⁻¹ ml⁻¹.

Effect of inhibitors

The effects of polysaccharide-degrading enzyme inhibitors, ions, thiol reagents and protease inhibitors on the Hase activity of the T. denticola supernatant is given in Table 2. There was no observed effect on T. denticola supernatant Hase activity by thiol reagents, protease inhibitors, SDS, p-chloromercuribenzoate, EDTA or by Mn²⁺, Cd²⁺ or Hg²⁺. The protease inhibitors were used to confirm that HA hydrolysis was not due to proteolytic degradation of proteoglycans in commercial preparations of hyaluronate. The Hase activity was inhibited by

<table>
<thead>
<tr>
<th>Culture supernatant of:</th>
<th>Hydrolysis [µg substrate hydrolysed h⁻¹ (ml supernatant)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HA</td>
</tr>
<tr>
<td>T. denticola</td>
<td>3120 ± 255 (5)</td>
</tr>
<tr>
<td>T. denticola + GST</td>
<td>0</td>
</tr>
<tr>
<td>T. socranszki</td>
<td>1110 ± 18 (2)</td>
</tr>
<tr>
<td>T. socranszki + GST</td>
<td>0</td>
</tr>
<tr>
<td>T. vincentii</td>
<td>595 ± 40 (2)</td>
</tr>
<tr>
<td>T. vincentii + GST</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1. Hydrolysis of HA, C4S and C6S by the culture supernatants of T. denticola, T. socranszki and T. vincentii, and inhibition of Hase and Case activities by gold sodium thiomalate (GST)
Table 2. Effect of various ions and inhibitor molecules on the hydrolysis of HA by the culture supernatant of *T. denticola* and of neuraminidase and dextranase treatment of the HA substrate

Results show the percentage hydrolysis of HA by 20 μl *T. denticola* supernatant with sodium acetate buffer, pH 6.0, as described in the text, in the presence of various ions and inhibitor molecules. The amount of HA hydrolysed in 3 h by 20 μl *T. denticola* supernatant only served as the 100 % control. All results are the mean of triplicate assays.

<table>
<thead>
<tr>
<th>Culture supernatant with:</th>
<th>HA hydrolysis (%)</th>
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</thead>
<tbody>
<tr>
<td>No additive</td>
<td>100</td>
</tr>
<tr>
<td>HgCl₂ (2 mM HgCl₂)</td>
<td>93</td>
</tr>
<tr>
<td>CdCl₂ (2 mM CdCl₂)</td>
<td>95</td>
</tr>
<tr>
<td>CuCl₂ (2 mM CuCl₂)</td>
<td>82</td>
</tr>
<tr>
<td>MnCl₂ (2 mM MnCl₂)</td>
<td>98</td>
</tr>
<tr>
<td>SDS (0.1 %)</td>
<td>93</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate (2 mM)</td>
<td>96</td>
</tr>
<tr>
<td>EDTA (1 mM)</td>
<td>103</td>
</tr>
<tr>
<td>TPCK (2 mM)</td>
<td>99</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor (10 μg ml⁻¹)</td>
<td>101</td>
</tr>
<tr>
<td>DTT (5 mM)</td>
<td>98</td>
</tr>
<tr>
<td>L-Cysteine·HCl (5 mM)</td>
<td>99</td>
</tr>
<tr>
<td>Glutathione (5 mM)</td>
<td>97</td>
</tr>
<tr>
<td>PMSF (0·1 mM)</td>
<td>90</td>
</tr>
<tr>
<td>pH 4·0</td>
<td>59</td>
</tr>
<tr>
<td>pH 8·5</td>
<td>12</td>
</tr>
<tr>
<td>Neuraminidase*</td>
<td>3</td>
</tr>
<tr>
<td>Dextranase†</td>
<td>0</td>
</tr>
</tbody>
</table>

* Three hour digestion of HA by 1 U *C. perfringens* neuraminidase in acetate buffer, pH 6·0, as described in the text.
† Three hour digestion of HA by 3 U *Penicillium* dextranase in acetate buffer, pH 6·0, as described in the text.

approximately 20 % by 2 mM CuCl₂. Neuraminidase and dextranase exhibited negligible ability to degrade HA, as determined by the Stains-all assay. In preliminary studies, the optimum pH of the *T. denticola* Hase was determined to be 6·0, unlike the bovine Hase which hydrolyses HA over a broad range (pH 4·5–6·0).

The inhibitory effect of gold sodium thiomalate and of rabbit anti-*A. mellifera*-venom antibodies on the ability of *T. denticola* and *S. agalactiae* culture supernatants to degrade HA over a 3 h period is presented in Fig. 1.

**SDS-PAGE, transblots and hydrolysis of HA and G6S**

The *T. denticola, T. vincentii* and *T. soranskii* secreted enzyme(s) separated by SDS-PAGE and transblotted onto membranes hydrolysed HA or G6S which were subsequently adsorbed onto the membranes. The hydrolysis of HA and G6S results in degradative products that are not stained by Alcian blue. Each enzyme(s) secreted by *T. denticola, T. vincentii* and *T. soranskii* hydrolysed both HA and G6S (Fig. 2a, c) and produced discrete single unstained (clear) bands against a blue background. From the location of the bands, the molecular mass of the enzyme was estimated as 39 kDa. The *T. denticola* culture supernatant treated with gold sodium thiomalate did not hydrolyse either HA or G6S, i.e. unstained bands were not evident (Fig. 2b, d). The inhibition of the *T. denticola* enzyme indicates that gold sodium thiomalate binds firmly to the extracellular enzyme as neither SDS-PAGE nor transblotting reversed the effect of gold sodium thiomalate. The unbound protein which passed through the HA-Gel or the G6S-Gel affinity columns did not hydrolyse either HA or G6S (Fig. 2b, d).

The eluates from the HA-Gel and the G6S-Gel each hydrolysed both HA and G6S. Each biologically active eluate contained a single molecular species with a molecular mass of 59 kDa, as detected in SDS-PAGE, gels doubly stained with Coomassie blue and silver (Fig. 3). The affinity-purified enzyme and the enzyme in the supernatants have comparable molecular masses (compare Figs 2 and 3). Taken together, these data suggest that Hase and Case activities may be attributed to a single protein in the *T. denticola* culture supernatant with dual activity towards both GAGs. HA and CS share a common hexuronyl–hexosaminyl glycosidic linkage (Iozzo, 1985), and this linkage would appear to be the best candidate target for the enzymic action. Further studies involving

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**Fig. 1.** Inhibition of the Hase activity of the culture supernatants of *T. denticola* and *S. agalactiae*. The effect of gold sodium thiomalate and anti-*A. mellifera*-venom antibodies on the amount of HA degraded by the culture supernatants of *T. denticola* (a) and *S. agalactiae* (b). (a) ○, Culture supernatant alone; ●, supernatant incubated with a 1/100 dilution of anti-venom antibody; □, supernatant with a 1/500 dilution of anti-venom antibody; ■, supernatant incubated with 1 mg gold sodium thiomalate ml⁻¹. (b) ○, Culture supernatant alone; ●, supernatant incubated with a 1/100 dilution of anti-venom antibody. The error bars represent the standard error of the mean. All results are the mean of triplicate assays.
Fig. 2. Analysis of the enzymic activity of the *T. denticola*, *T. socranskii* and *T. vincentii* culture supernatants by SDS-PAGE, transblot and degradation of HA or C6S adsorbed onto the transblot membranes. (a) Culture supernatant of *T. denticola* (lane 1), *T. socranskii* (lane 2), *T. vincentii* (lane 3); bovine Hase (lane 4); HA is adsorbed onto the membrane. (c) The supernatants in lanes 1-4 are the same as in (a) but C6S is adsorbed onto the membrane. (b) Culture supernatant of *T. denticola* (lane 1), after adsorption with HA-Gel (lane 3) and C6S-Gel (lane 5), after incubation with gold sodium thiomalate (lane 6), and incubation with buffer without HA-Gel (lane 2) and without C6S-Gel (lane 4); HA is adsorbed onto the membrane. (d) Culture supernatant of *T. denticola* (lane 2), after adsorption with HA-Gel (lane 4) and C6S-Gel (lane 6), after incubation with gold sodium thiomalate (lane 1), and incubation with buffer without HA-Gel (lane 3) and without C6S-Gel (lane 5); lane M, molecular mass markers (110, 84, 47, 33, 24 and 16 kDa, from top to bottom); C6S is adsorbed onto the membrane. See Methods for details.

Fig. 3. SDS-PAGE of *T. denticola* culture supernatant and the protein(s) of *T. denticola* supernatant bound to HA-Gel or C6S-Gel and eluted. Lanes: 2, *T. denticola* culture supernatant; 1, 3, eluate of the protein from the C6S-Gel and the HA-Gel, respectively. Molecular mass markers are indicated.

Fig. 4. Western blot detection of antigens cross-reacting with anti-*A. mellifera* antibodies. Lanes: 1, HA-column affinity-purified Hase from *T. denticola*; 2, bovine Hase; 3, *A. mellifera* venom; M, molecular mass markers (112, 84, 53.5, 27.9 and 21 kDa, from top to bottom).
Hyaluronoglucosaminidase of some oral spirochaetes

**Fig. 5.** Electron micrograph of anti-A. melifera-venom antibody and gold-labelled protein G binding along the periphery of T. denticola. (a) T. denticola incubated with anti-venom antibody and gold-labelled protein G. (b) T. denticola pre-incubated with HA prior to the addition of anti-venom antibody and gold-labelled protein G. Gold-labelled protein G alone did not bind to T. denticola (data not shown). Bar, 0.1 μm.

**Fig. 6.** Electron micrographs of gold sodium thiomalate binding along the periphery of T. denticola. (a) T. denticola incubated with gold sodium thiomalate. (b) T. denticola incubated with a mixture of HA and gold sodium thiomalate. The arrow points to one of many sites to which gold sodium thiomalate binds. Bar, 0.1 μm.

infrared spectroscopy and HPLC are required, however, in order to accurately determine the mechanism of degradation. Affinity purification of T. socranskii and T. vincentii supernatants was not carried out.

On Western blotting, the affinity-purified enzyme from the HA-Gel beads produced a single line of 59 kDa as seen in lane 1 of Fig. 4. The honey-bee venom, in lane 3, gave two major bands of 48 kDa and 11 kDa.

**Electron microscopy**

Anti-A. melifera antibodies bound on, or near to, the surface of T. denticola, as seen in Fig. 5. The protein G labelled with 10 nm gold particles did not bind to the T. denticola cells without the prior addition of the anti-venom antibodies. Prior incubation of the T. denticola cells with 1 mg HA ml⁻¹ completely blocked the binding of anti-A. melifera-venom antibodies, as determined by electron microscopy. Gold sodium thiomalate binds to the extracellularly located enzyme of T. denticola, T. vincentii and T. socranskii and HA also blocks the binding of gold sodium thiomalate. Gold-labelled rabbit anti-human IgG does not bind to the cells. The cluster density of the gold sodium thiomalate molecules is more abundant nearer to than farther away from the outer membrane of the spirochaetes (Fig. 6). The enzyme appears to be trapped around the periphery of cell in the exopolysaccharide layer or slime layer (Johnson, 1977), as well as around clumps of polysaccharide that are shed from the surface of the cells, for some time before ending up in the supernatant.

**DISCUSSION**

Low levels of Hase and Case activities have been previously reported to be produced by nine small-sized unclassified oral spirochaetes (Fiehn, 1987). The insensitivity of the turbidimetric plate assay (Fiehn, 1987) could account for the reported low Hase and Case activities of these spirochaetes or this may be due to a false-positive reaction that results from the degradation of BSA by protease secreted by the spirochaetes (Grenier & Michaud, 1993). Others have reported that T. vincentii and
T. socianskii do not produce Hase (Fitzgerald & Gannon, 1983). These investigators incubated the viable spirochete cells in 500 µg HA ml⁻¹ for a maximum of 40 h and measured the relative viscosity of the mixture for Hase activity (Fitzgerald & Gannon, 1983). With such a short incubation period, these two oral spirochaetes might not have produced sufficient extracellular Hase to effect changes in the viscometric flow of 500 µg HA. For example, Hase and Case activities were detected after 2 weeks incubation by the turbidimetric plate assay (Fiehn, 1987) and in our investigation Hase activity was detected in the supernatants after 7 d (T. denticola) and 12 d (T. socianskii and T. vincentii) of growth. Moreover, both the plate and viscometric assays are not as sensitive as the Stains-all spectrophotometric assay, which has been reported to detect Hase activity in 60 ng protein from *Ancylostoma braziliense* (Hotez et al., 1993). However, we found that the lower limit of Hase activity was detected in 150 ng culture supernatant protein. The extracellular enzyme produced by *T. denticola*, *T. vincentii* and *T. socianskii* readily hydrolysed HA and CS. The affinity-purified 59 kDa enzyme of *T. denticola* hydrolysed both HA and CS and a specific inhibitor of Hase, gold sodium thiomalate (Hotez et al., 1993), inhibited the extracellular enzyme hydrolysis of both HA and CS. There are only two major proteins present in honey-bee venom, a Hase and a phospholipase A (Gmachl et al., 1993), and anti-*A. mellifera*-venom antibodies also inhibited the Hase activity of *T. denticola*. A single band of 59 kDa was observed on electrophoresis and transblotting of the affinity-purified *T. denticola* Hase. That a component(s) of the anti-venom antibodies exhibits specificity to the Hase of *T. denticola* is also suggested by the ability of HA to block the binding of the antibodies to *T. denticola* cells, as observed by electron microscopy and the inhibition of Hase activity in the Stains-all assay.

The inhibition assays confirm that enzymic activity is due to Hase. The *T. denticola* Hase is not activated by reducing agents or ions, since DTT, glutathione and EDTA did not inhibit the activity. The lack of a requirement for activating factors has also been reported for the Hase of *C. perfringens*. Information on inhibitors and activators of polysaccharide-degrading enzymes was obtained from commercial suppliers and from Colowick & Kaplan (1972).

We selected a wide range of potential inhibitors and activators as there has been some confusion in the literature over the identity and specificities of polysaccharide-degrading enzymes. It has recently been reported that *Porphyromonas gingivalis* does not produce a Hase (Grenier & Michaud, 1993), contrary to previous reports (Seddon & Shah, 1989; Steffen & Henges, 1981). The degradation of BSA, used to form a precipitate under acidic conditions with HA, by bacterial proteases might have led to false-positive results (Grenier & Michaud, 1993). It has also been shown that group B streptococci produce a Hase (Pritchard & Lin, 1993), an enzyme previously considered to be a neuraminidase (Hayano & Tanaka, 1969; Milligan et al., 1980; Brown & Straus, 1987). The confusion surrounding the specificity of this enzyme associated with neonatal meningitis and sepsis caused by group B streptococci (Milligan et al., 1978) was probably due to the impurity of the substrate employed, that is bovine submaxillary mucin which contains HA (Pritchard & Lin, 1993). *S. agalactiae*, one of the group B streptococci, is a known Hase producer (Ożegowski et al., 1994) and was therefore selected as a positive control of bacterial origin, to complement the use of purified bovine Hase in our Stains-all assays.

The Hase of *Ancylostoma braziliense* larvae can migrate through substrate-containing polyacrylamide gels on electrophoresis (Hotez et al., 1993). In initial experiments we found that the Hase of the oral anaerobic spirochaetes under study would not migrate through HA-containing polyacrylamide gels by SDS-PAGE at 200 V. All lytic activity was observed only at the top of the gel. This can be explained by retardation of the enzyme mobility due to interaction of the Hase with the substrate incorporated into the gel. For this reason, the technique was adapted to adsorb HA or CS onto the nitrocellulose membrane after the separation of the supernatant proteins by SDS-PAGE and transblotting. The migration of the enzyme was then observed by the localized degradation of HA and CS. This gives a more accurate estimation of the molecular mass of the enzyme. The use of this technique by other investigators should permit the visualization of Hase whilst giving rise to more accurate estimations of molecular mass.

The term Hase has been used generically for hyaluronate lyases (EC 4.2.2.1), hyaluronoglycanidase (EC 3.2.1.36) and HGase (EC 3.2.1.35). The latter hydrolyses both HA and CS while the two other enzymes specifically hydrolyse HA. Therefore, *T. denticola*, *T. vincentii* and *T. socianskii* produce a Hase that can more appropriately be termed HGase.

The HGase produced by *T. denticola*, *T. vincentii* and *T. socianskii* might function as a virulence factor. Spirochaetes have been isolated from the gingival sulci (Gornitzky et al., 1991) and periodontal pockets (Gornitzky et al., 1991; Omar et al., 1990). They move in highly viscous environments (Klitörinos et al., 1993) and are located histologically in the intercellular matrix of diseased gingival tissue (Loesche, 1988). The HGase could disrupt the integrity of the gingival epithelium and could continue to degrade the extracellular matrix as the spirochaetes move through the gingival matrix. The oral spirochaetes may create avenues through which other bacteria and their products penetrate into the deeper tissue.

**ACKNOWLEDGEMENTS**

This project was supported by a grant (MA-10509) from the Medical Research Council of Canada. D. Scott was supported by a Science and Engineering Research Council, UK/NATO grant. The electron microscope facilities were kindly provided by Kathy Hewitt, Department of Biology, McGill University, Montreal, Canada. Some of the results reported here were presented at the 94th General Meeting of the American Society for Microbiology, Las Vegas, NV, USA, 23–27 May, 1994.
REFERENCES


Received 12 January 1996; revised 28 May 1996; accepted 29 May 1996.