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*J DENT RES* 1986 65: 939
DOI: 10.1177/00220345860650061701

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What is This?
Separation and Properties of Rabbit Buccal Mucosal Wound Hyaluronidase

C.N. BERTOLAMI1, R.H. DAY2, and D.G. ELLIS3

1Department of Oral and Maxillofacial Surgery, Massachusetts General Hospital, Fruit Street, Boston, Massachusetts 02114; 1,3Shriners Burns Institute; and 1,2Harvard School of Dental Medicine, Boston, Massachusetts

This study establishes the existence of a mammalian buccal mucosal wound hyaluronidase (hyaluronate 4-glycophosphodiesterase; EC 3.2.1.35) having properties distinct from those of the endogenous lysosomal hyaluronidase of normal (uninjured) buccal mucosa. A time-dependent change in hyaluronidase activity was measured, with the highest specific activity occurring on post-wound day 4 (7.7 ± 1.3 units/mg protein), followed by consecutive decreases until activity was no longer discernible by day 21. Mucosal wound hyaluronidase closely resembled a previously described intemumentary wound endoglycosidase in terms of a high pH optimum (5.0-6.0), distinct (but non-exclusive) substrate preference for hyaluronic acid, and ability to generate saturated depolymerization products by an endoglycosidic hydrolysis.


Introduction.

In mammalian wound repair, a family of endoglycosidic enzymes (hyaluronidases) constitutes the central catalytic system for regulating the depolymerization of hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan sulfate and for mediating characteristic changes in glycosaminoglycan (GAG) metabolism at key points in the repair process (Alexander and Donoff, 1979; Bertolami and Donoff, 1982; Fenger, 1982; Fiser-Szafarz, 1984). Aberrant endoglycosidase function may have relevance to healing derangements — such as hypertrophic scarring and keloid formation — which typically exhibit abnormalities in GAG content and distribution (Swann et al., 1985; Savage and Swann, 1985; Donoff et al., 1984). Although relatively little has been done to elucidate the characteristics of tissue hyaluronidases, the recent detection of low, baseline hyaluronidase activities in numerous normal tissues (including oral mucosa) makes clear a need for characterizing these enzymes to establish whether wound-derived hyaluronidase is identical with the enzyme found in corresponding uninjured tissues (Bollet et al., 1963; Sahu, 1981; Gold, 1982; Fenger, 1982; Bertolami and Ellis, 1985).

The hyaluronidase activity of intra-oral mucosal wounds has not been reported previously. The objective of this study was to establish whether hyaluronidase is detectable in granulation tissues of oral mucosal wounds and, if it is present, to learn whether it differs quantitatively and/or qualitatively from the hyaluronidase of normal (uninjured) mucosa.

Materials and methods.

Wounding, tissue collection, and preparation. — Thirty-nine 2.0-3.0-kg New Zealand white rabbits (Oryctolagus cuniculus) were caged separately and maintained on a Purina Laboratory Rabbit Chow HF 5326 diet (Ralston-Purina Company, St. Louis, MO) with food and water taken ad lib. Rabbits were anesthetized with intramuscular ketamine HCl (Vetalar®, Bristol Laboratories, Syracuse, NY) (35 mg/kg) and xylazine (Rompun®, Haver-Lockhart, Shawnee, KS) (5 mg/kg). Bilaterally, 1 cm × 1 cm sections of buccal mucosa were dissected from underlying muscle, and excision sites were allowed to heal for up to 21 days. Tissue samples were obtained on days 0 (normal mucosa), 3, 4, 5, 7, 9, 14, and 21. Control specimens of rabbit serum, skin, and mucosa (excised at the time of the original wounding) were used for determining baseline hyaluronidase activities of representative normal (uninjured) tissues.

Identification of mucosal wound hyaluronidase. — Hyaluronidase specific activities were determined by incubating tissue homogenates with hyaluronic acid or other GAG substrates and then measuring colorimetrically the release of oligosaccharides containing terminal reducing N-acetylhexosamine (NAH) (Orkin and Toole, 1978). Tissue samples were minced in 10 volumes of 0.05 mol/L sodium acetate, 0.05 mol/L NaCl (pH 3.5 for normal mucosa or pH 4.5 for wound tissues) (acetate buffer), and then homogenized by means of a hand-operated glass micro-tissue homogenizer at 4°C. The indicated pH values were selected on the basis of pH optimum determinations for both normal mucosal and mucosal wound granulation tissue hyaluronidases (see “Enzyme characterization,” below). Homogenate was centrifuged at 20,000 g for 20 min at 4°C. The supernatant [presumed to contain soluble endogenous substrate (Polansky et al., 1974) as well as serum and/or miscellaneous bacterial contaminants] was discarded, leaving membrane-bound hyaluronidase in the pellet. The pellet was re-suspended in 10 volumes (w/v) of 0.1% Triton X-100 detergent in 0.1 mol/L sodium formate, 0.15 mol/L NaCl (pH 3.5 for normal mucosa or pH 4.5 for wound tissues) (formate buffer), and was stirred gently for 16 hr at 4°C. Resulting homogenates were incubated with exogenous hyaluronate (Type III), chondroitin-4-sulfate, chondroitin-6-sulfate, or dermatan sulfate (Sigma Chemical Company, St. Louis, MO) at 37°C for increasing intervals up to a maximum of 24 hr. Exogenous substrate was used in concentrations of 0.04–0.8 mg/mL. Terminal NAH released by either hydrolysis or an elimination reaction was measured according to Reissig et al. (1955). Three blanks were used: distilled water, enzyme preparation boiled for five min prior to incubation with substrate, and non-boiled enzyme preparation to which exogenous substrate was not added. The specific activity of hyaluronidase was measured in “units/mg protein”; 1.0 unit was defined as the amount of enzyme releasing 1.0 μg NAH under uniform and constant incubation conditions. The standard consisted of 0.3 μmol NAH (in the form of N-acetylgalactosamine; Sigma); specificity of the colorimetric assay for NAH was established by comparing the spectra of assayed samples with that of authentic NAH over the interval 500-600 nm. Protein was measured for crude (and subsequently purified) enzyme preparations by the method of Lowry et al. (1951).

Hyaluronidase isolation. — After establishing the presence of hyaluronidase activity in buccal mucosal wound granulation tissues, we achieved partial isolation by centrifuging formate buffer homogenates at 42,000 g for 30 min and dialyzing the...
supernatant against two changes of cold phosphate buffer (0.02 mol/L, pH 6.0, ionic strength = 0.08). Anion exchange chromatography of the dialyzed supernatant was accomplished by application to a 10 mm × 200 mm column of DEAE-Sephadex (A-50, medium, 100-200-mesh exchange capacity = 3.1 mequiv/g) and elution with phosphate buffer at 4°C (Soru and Ionescu-Stoian, 1965). Fractions of 2.25 mL were collected at a flow rate of 0.25 mL/min. After phosphate buffer elution, a 0.1-0.75 mol/L NaCl step gradient elution was performed. Protein concentration was measured for each fraction, and the fractions comprising a given peak were pooled, dialyzed against cold distilled water, and lyophilized. Enzymatically active lyophilized material derived from wound granulation tissues and from normal, uninjured mucosa were then individually suspended in detergent-free formate buffer, chromatographed over a 16 mm × 400 mm column of Sephadex G-150 at 7 or 15 mL/hr, and collected into 0.6- or 1.2-mL fractions. Protein concentration and specific activity were determined for all fractions, and samples from active tubes were then electrophoresed on 5.6% sodium dodecyl sulfate (SDS)-polyacrylamide gels (Fairbanks et al., 1971).

Enzyme characterization. — Hyaluronidase preparations derived from samples of normal mucosa and mucosal wound granulation tissues were characterized by determining substrate specificity, pH optimum, and linearity of enzyme activity as a function of incubation time, depolymerization products, and molecular weight. To exclude the possibility of rate limitation due to insufficient exogenous substrate, we performed incubations in formate buffer at substrate concentrations of 0.02, 0.04, 0.05, 0.15, 0.20, 0.40, and 0.80 mg substrate/mL. To determine the percentage of enzymic activity attributable to endoglycosidic action rather than to non-specific exoglycosidases (e.g., BETA-N-acetylglucosaminidase and/or BETA-glucuronidase), we included an exoglycosidase inhibitor, saccharo-1,4-lactone monohydrate (Sigma), in representative incubation mixtures at a final concentration of 1.5 mmol/L (Levvy and Marsh, 1959). For determining pH optimum, we adjusted hyaluronidase preparations to pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or 6.0 and incubated them with exogenous substrate as described above. We calculated the enzyme’s specific activity for each pH by measuring release of terminal NAH-containing oligosaccharides and determining protein content. Boiled enzyme blanks and distilled water blanks were included. Linearity of enzyme activity as a function of incubation time was evaluated by termination of the incubations at hourly intervals up to a maximum of 24 hr and measurement of the enzyme-mediated NAH release. Comparably incubated controls consisted of enzyme samples that had been inactivated by being boiled for five min prior to incubation. Digestion products resulting from substrate degradation by hyaluronidase were examined by filter paper partition chromatography (Partridge, 1948): Aliquots of incubated preparations of substrate plus enzyme were chromatographed and examined under shortwave UV light; hexosamine and NAH-containing materials were then disclosed by means of acetylated reagent and PARA-dimethylaminobenzaldehyde (DMAB) reagent. Standards consisted of the disaccharides: 2-acetamido-2-deoxy-3-0-(BETA-D-gluc-4-enepyranosyluronic acid)-D-galactose (DETA-Di-OS), 2-acetamido-2-deoxy-3-0-(BETA-D-gluc-4-enepyranosyluronic acid)-4-0-sulfo-D-galactose (DETA-Di-4S), and 2-acetamido-2-deoxy-3-0-(BETA-D-gluc-4-enepyranosyluronic acid)-6-0-sulfo-D-galactose (DETA-Di-6S) (Miles Laboratories, Elkhart, IN).

Results.

Identification and isolation of intra-oral mucosal wound hyaluronidase. — Hyaluronidase activity was identified in formate homogenates derived from mucosal wound granulation tissues sampled over a post-wound interval of from 3 to 21 days (Fig. 1). A time-dependent change in hyaluronidase activity was measured, with the highest specific activity occurring on post-wound day 4 (7.7 ± 1.3 units/mg protein), followed by consecutive decreases until activity was no longer discernible by day 21. Although no hyaluronidase activity was detectable in uninjured skin or serum, low levels of activity (1.6 ± 0.5 units/mg protein) were found in normal mucosa.

A representative anion exchange elution profile is shown in Fig. 2. Although several proteins eluted with the NaCl step gradient, only the material that eluted with 0.02 mol/L phosphate buffer was found to be enzymatically active. This observation is consistent with earlier reports on the behavior of various mammalian hyaluronidases (Soru and Ionescu-Stoian, 1965) and suggests that the enzyme is cationic under these conditions. Gel filtration of wound-derived enzyme provided further purification (Table 1 and Fig. 2), with the most active fraction (specific activity = 222.1 units/mg protein) emerging at V O and corresponding to an apparent molecular weight of at least 250 kilodaltons (kd). In contrast, gel filtration of comparable preparations derived from normal mucosa yielded fractions with lower specific activities (most active fraction = 67.7 units/mg protein) centered over a much lower molecular weight range (approximately 51.4 kd). When active, wound-derived G-150 fractions were reduced and electrophoresed, fragments were identified corresponding to molecular weights of 62.4 kd, 107 kd, 123-150 kd, and >200 kd (Fig. 3).

We evaluated the specificity of the colorimetric assay for NAH by comparing the spectrum of enzymatically released NAH with the spectrum of authentic NAH over the 500-600 nm interval (Fig. 4). All spectra were similar and exhibited maximal absorbances at 545 nm and 580 nm. Background levels of NAH measured in samples that had been boiled prior
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MUCOSAL WOUND HYALURONIDASE

CHANGES IN SPECIFIC ACTIVITY OF TISSUE-DERIVED HYALURONIDASE AS PURITY INCREASES

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Normal Mucosa</th>
<th>Grannulation Tissue*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>S.A.†</td>
<td>Purity</td>
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<tr>
<td>Formate</td>
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<tr>
<td>Homogenate</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>0.02 mol/L Phosphate Buffer Fraction (DEAE-Sephadex)</td>
<td>13.1</td>
<td>8.2</td>
</tr>
<tr>
<td>Sephadex G-150§</td>
<td>67.7</td>
<td>42.3</td>
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</table>

**"Grannulation Tissue" refers to enzyme extracts derived from granulation tissues of four-day buccal mucosal wounds.**

†S.A.: Specific Activity; expressed as units/mg protein. One "unit" refers to the amount of enzyme that releases 1 µg terminal NAH under optimal conditions of time (8 hr), temperature (37°), and pH (3.5 for normal mucosal hyaluronidase, or 4.5 for mucosal wound granulation tissue hyaluronidase).

§The Sephadex G-150 fraction of normal mucosal preparations showing the highest specific activity (fraction 35) differed from the G-150 fraction showing the highest activity for four-day wound granulation tissue (fraction 21). This translates into different $K_v$ for the two types of preparations and, correspondingly, different molecular weights. The data presented in this Table for the G-150 run refer to the activity of the most active fraction (i.e., fraction 35 for normal mucosa, and fraction 21 for wounded mucosa).

To incubation with substrate were subtracted from the NAH values of non-boiled (i.e., active) samples. The low intrinsic absorbances of reagent blanks and of samples not containing exogenous substrate were also subtracted from the absorbances of active samples. Remaining terminal reducing NAH was considered the product of enzyme activity. Of this NAH, 60% was due to endoglycosidic action (i.e., hyaluronidase), since inclusion of saccharolactone caused a 40% decline in detectable cleavage product.

Enzyme characterization. — Wound granulation tissue hyaluronidase was found to have a pH optimum of 5.0-6.0, whereas the hyaluronidase associated with uninjured mucosa had a pH optimum of 3.5 (Fig. 5). Under conditions of optimal pH, specific activity increased in direct proportion to incubation time (first order kinetics) for enzyme derived from both tissue sources. Specific activity was also proportional to substrate (hyaluronate) concentration up to 0.15 mg hyaluronate/mL; however, beyond this level, activity declined slightly. Although unexplained, this decline has been described previously for other hyaluronidases exposed to excess substrate (Yamada et al., 1977). Wound-derived hyaluronidase exhibited a distinct substrate preference for hyaluronate; but a degree of activity against other GAG was also seen (Table 2). Previous work has shown that under the conditions of the assay, normal mucosal hyaluronidase is active only against hyaluronate (Bertolami and Ellis, 1985). Filter paper partition chromatography gave a clear representation of the disaccharide standards (DELTA-Di-OS, DELTA-Di-4S, and DELTA-Di-6S). All were disclosed by the reagent specific for NAH (i.e., DMAB), and all absorbed at UV wavelengths. In contrast, paper chromatography of depolymerization products resulting from wound-derived hyaluronidase action on hyaluronic acid was consistent with the behavior of other mammalian hyaluronidases, yielding no monosaccharide and only small amounts of disaccharide product. None of the depolymerization products absorbed at UV wavelengths. Neither standards nor cleavage products were revealed by the reagent specific for hexosamine.

Discussion.

This study establishes the presence of hyaluronidase activity in granulation tissue derived from rabbit buccal mucosal wounds; activity was highest during early repair and then decreased over the next 17 days. Tissue hyaluronidase activity may actually be higher than that measured in this study, since some hyaluronidase may be discarded in the initial, detergent-free acetate buffer supernatant. This possibility is minimized by not introducing Triton X-100 until the pellet is re-suspended; nonetheless, the risk of underestimating tissue hyaluronidase activity by discarding this supernatant seems justified in order to
eliminate endogenous substrate and exogenous contaminants (serum components and/or bacterial products).

When incubated with GAG substrates, mucosal wound enzyme generated saturated depolymerization products by an endoglycosidic hydrolysis, exhibited a pH optimum within a range typical for mammalian hyaluronidases, and exerted a distinct (though non-exclusive) substrate preference for hyaluronic acid. These characteristics justify designation of this enzyme as a "hyaluronidase". Emergence of activity in 0.02 mol/L phosphate buffer (pH 6.0) during anion exchange chromatography indicates that the enzyme is cationic (or iso-electric) under these conditions. Although the enzyme could be neutral or negatively charged at normal tissue pH, it would be positively charged in the pH range of maximal activity (5.0-6.0). The highly anionic character of the enzyme's glycosaminoglycan substrates and the cationic nature of the enzyme at its pH optimum may have relevance to enzyme-substrate interaction during glycosaminoglycan degradation.

Although intact buccal mucosa possesses hyaluronidase activity (Bertolami and Ellis, 1985), the enzyme found in mucosal wound granulation tissues appears to be a different form of hyaluronidase and displays properties more closely resembling those of a mammalian skin wound enzyme (Bertolami and Donoff, 1982). This is not surprising, since the term "hyaluronidase" refers to a large family of enzymes whose members can exist as multiple iso-enzymes (IUPAC-IUB Commission Report, 1977).

Hyaluronidases are grouped according to their mechanism of action and are designated as either hyaluronoglucosaminidases (EC 3.2.1.35), hyaluronoglucuronidases (EC 3.2.1.36), or hyaluronate lyases (EC 4.2.2.1) (Fiszer-Szafarz, 1984; IUB Commission Report, 1979). The pH optimum is the most revealing parameter for distinguishing among the various forms (Hopps and Prout, 1972; Fiszer-Szafarz, 1984). Hyaluronidases which regulate baseline metabolism of endogenous GAG usually show activity over only a narrowly acidic pH range.
Hyaluronic acid
Chondroitin-4-sulfate
Dermatan sulfate

**Vol. 65 0.02 mol/L sodium formate, 0.15 mol/L NaCl, and 0.1% Triton X-100, pH 4.5 (formate buffer).**

**Specific activity is expressed as units/mg protein. One "unit" refers to the release of 1 μg terminal NAH under optimal conditions of time (8 hr), temperature (37°), and pH (4.5).**

**Enzymic activity against sulfated glycosaminoglycans was highly variable: In four experiments, specific activity against chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan sulfate ranged from undetectable levels to the values shown above.**

(e.g., lysosomal and serum hyaluronidases, pH optimum = 3.5) (Fiszer-Szafrasz, 1984); whereas, those which function under conditions of increased GAG turnover (e.g., during developmental or reparative processes) or whose action is directed against “non-self” substrates (e.g., testicular hyaluronidase, pH optimum = 6.0) display a broad pH range of activity with optimal pH approaching neutrality (Fiszer-Szafarz, 1984; Bertolami and Donoff, 1982; Polansky et al., 1974). Both mucosal and skin wound granulation tissue hyaluronidases have high pH optima (pH 5.0-6.0), whereas the pH optimum of the hyaluronidase indigenous to uninjured mucosa is low (pH 3.5).

The substrate specificities of skin and mucosal wound hyaluronidases are similar; they are most active against hyaluronic acid, but both retain activity against chondroitin sulfate and dermatan sulfate (Table 2) (Bertolami and Donoff, 1982). Under the conditions of the assay, hyaluronidase derived from normal buccal mucosa is active only against hyaluronic acid (Bertolami and Ellis, 1985). The observation that depolymerization products possessed terminal reducing NAH groups and that the enzyme had a substrate specificity identical with that of other hyaluronoglucosaminidases suggests classification as EC 3.2.1.35 (IUB Commission Report, 1979).

The activity of hyaluronidases of injured and normal mucosal samples behave differently during gel filtration suggests that the enzymes have different molecular weights. Wound-associated enzyme eluted as a large molecule (≥ 250 kd) which could represent either a polymeric enzyme [as described for other hyaluronidases (Zaneveld et al., 1973; Yang and Srivastava, 1974, 1975)] or a complex of enzyme and endogenous substrate. Dithiothreitol (DTT) reduction of wound-derived enzyme produced fragments having a range of molecular weights (62.4- ≥ 200 kd) consistent with a large molecule (Fig. 3). The SDS-PAGE band with the lowest molecular weight (62.4 kd) approximates the size of monomeric hyaluronidase (62-70 kd) (Yamada et al., 1977; Yang and Srivastava, 1974). In comparison, gel filtration of normal mucosal preparations suggested a much smaller molecule (51.4 kd prior to reduction). On the basis of different pH optima, substrate specificities, and molecular weights, normal mucosal hyaluronidase and mucosal wound hyaluronidase are almost certainly different enzymes.

Paper chromatographic analysis revealed cleavage products to be saturated, N-acetylmuramic-containing oligosaccharides. Saturation indicates that the reaction is a simple hydrolysis (a characteristic of mammalian hyaluronidases) rather than the eliminase-type reaction of bacterial hyaluronolysases.

The role of tissue hyaluronidases has not been fully elucidated. Most are known to degrade hyaluronic acid, chondroitin-4-sulfate, and chondroitin-6-sulfate, and they are presumed to regulate metabolism of non-collagenous extracellular matrix in vivo. Such behavior could have obvious relevance to repair processes, since GAG and proteoglycans (PG) are essential for establishing and maintaining tissue integrity (Fiszer-Szafrasz, 1984). Moreover, alteration of GAG/PG structure or concentration may indirectly influence numerous cell functions, such as phagocytosis (Goggins et al., 1968; Forrester and Balazs, 1980), adhesion (Barnhart et al., 1979), motility (Hakanson et al., 1980), proliferation (Balazs and Holmgren, 1950; Fiszer-Szafrasz and Nadal, 1977), and differentiation (Fiszer-Szafrasz, 1984). Specific effects on the cellular immune response (Chevrier et al., 1982; Turley, 1982) and angiogenesis (Feinberg and Beebe, 1983) have been attributed to the hyaluronidase-hyaluronate system.

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