Salivary gland hyaluronidase in various species of phlebotomine sand flies (Diptera: psychodidae)

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Abstract

Hyaluronidase activity was detected and partially characterized in salivary gland extracts of females of six sand fly species. In *Phlebotomus papatasi* and *Lutzomyia longipalpis* the enzyme was active over a broad pH range; the pH optimum was 5.0. Besides high cleaving activity towards hyaluronic acid, it hydrolyzed chondroitin sulfates A and C. Hyaluronidases of various sand fly species differed in structure and sensitivity to reducing conditions. In the subgenera *Phlebotomus* (*P. papatasi* and *P. duboscqi*) and *Adlerius* (*P. halepensis*) the predominant active form of the enzyme was monomeric with the same apparent molecular weight under nonreducing and reducing conditions (around 65 kDa for *P. papatasi* and *P. duboscqi* and 110 kDa for *P. halepensis*). In *P. sergenti* the enzyme occurred as a putative homodimer but remained active under reducing conditions when separated into 60 kDa subunits. In *L. longipalpis* and *P. perniciosus* the activity was detectable under non-reducing conditions only. In *P. duboscqi*, low enzyme activity was found also in males. Salivary gland hyaluronidases of sand flies share characteristics with endo-N-acetylhexosaminidases of mammalian sperm cells and corresponding venom enzymes of Hymenoptera. Hypothetically, they facilitate blood meal acquisition but also may modulate immune reactions of the host and promote pathogen transmission.

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1. Introduction

Sand flies (Diptera, Phlebotominae), similarly to other blood-sucking arthropods, possess an array of salivary compounds that modulate hemostasis and immunity of the host in order to facilitate blood meal acquisition without inducing an immediate hypersensitivity response (reviewed by Champagne and Valenzuela, 1996 and Wikel et al., 1996). Sand fly salivary compounds also have a key role in transmission of parasitic protozoa of the genus *Leishmania*, serious human pathogens. The immunosuppressive effect of saliva decreases the infective dose of the parasite and has an enhancing effect on *Leishmania* infection in the vertebrate host (Titus and Ribeiro, 1988; Theodos et al., 1991). Studies carried out on two important vectors, *L. longipalpis* and *P. papatasi*, revealed that saliva inhibits activation of T-cells (Titus, 1998) and some macrophage functions, including the oxidative burst (Gillespie et al., 2000) and NO production (Hall and Titus, 1995; Katz et al., 2000). In natural conditions this allows the establishment of the infection from a low number of highly infective metacyclic forms ejected from the sand fly proboscis (Warburg and Schlein, 1986).

One of molecules which may participate in both, a blood meal acquisition and enhancement of *Leishmania* infection, is a hyaluronidase, an enzyme recently detected in saliva of *L. longipalpis* and *P. papatasi* (Charlab et al., 1999; Ribeiro et al., 2000). Hyluronidases cleave hyaluronic acid (HA) which is a high molecular weight glycosaminoglycan and a major component of the extracellular matrix in vertebrates. In addition, of most of them also cleave other components of extracellular matrix, particularly chondroitin sulfates. There are, however, at least three types of hyaluronidases hydrolyzing HA via different mechanisms. Testicular hyaluronidases of mammals and venom hyaluronidases of Hymenoptera are endo-N-ace-
tyl-hexosaminidases degrading high molecular weight substrate to tetrasaccharides. Hyaluronidases from leeches and nematodes belong to endo-glucuronidases that specifically hydrolyse hyaluronic acid (with tetrasaccharides as the final product), while bacterial hyaluronidases act via β-elimination, yielding disaccharides as the main end products (reviewed by Kreil (1995)).

In venomous snakes and arthropods like spiders, scorpions and hymenopteran insects, hyaluronidases are frequently found in toxins. They are called “spreading factors” as they facilitate the spreading of toxic compounds by degradation of the extracellular matrix (see review by Kreil (1995)). In bloodsucking arthropods other than sand flies the hyaluronidase activity was detected in the tick Amblyomma hebraeum (Neitz et al., 1978) and the black fly Simulium vittatum (Ribeiro et al., 2000). The activity is thought to play an important role in blood-meal acquisition by increasing the permeability of host tissue for other pharmacological compounds present in saliva; by depolymerizing hyaluronic acid it may help diffusion of antihaemostatic agents into the vicinity of the feeding lesion or help to enlarge the size of the feeding hematoma.

In this study, we present some physico-chemical properties of sand fly hyaluronidases and compare their activities among six species of the genera Lutzomyia and Phlebotomus.

2. Materials and methods

Seven colonies of six sand fly species were used: Phlebotomus papatasi Scopoli, P. duboscqi Neveu-Lemaire, P. sergenti Parrot, P. perniciosus Newstead, P. halepensis Theodor and Lutzomyia longipalpis Lutz and Nieva. In P. papatasi, two colonies of different in geographical origin (Turkey and Cyprus) were compared. The flies used to establish the colonies were collected in Sanliurfa, Turkey (P. papatasi and P. sergenti) or were provided by Dr. M. Maroli (P. perniciosus), Prof. R. Ward (L. longipalpis) and Prof. R. Killick-Kendrick (other colonies). Colonies were maintained at 26 °C and 14/10 LD photoperiod, adults were given a 50% sucrose solution on cotton wool.

Salivary glands were dissected out in buffer A (20 mM Tris, 150 mM NaCl, pH 7.8) and stored in batches (20 glands in 20 µl of TRIS buffer) at −70 °C. Previous studies (Volf et al., 2000) revealed age-dependent differences in protein composition of the saliva. Therefore, females aged 5–6 days were used for comparison of hyaluronidase activities. Males of P. duboscqi used for zymography were at least 3 days old. Salivary gland extract (SGE) was obtained by disruption of glands by three freeze-thaw cycles in liquid nitrogen and centrifugation at 17,000 g (5 min, 2 °C). Protein concentration in SGEs was determined by the BCA method according to instructions of the producer (Sigma) using bovine serum albumin (BSA) in buffer A as a standard. Three SGE samples (each containing ten pairs of salivary glands from 10 individuals) from each colony were used for assessment of average protein content in an “optimal gland” (i.e. average protein content in an individual divided by two). Maximum care was devoted to dissect both glands from each individual undisrupted as the size of the salivary glands in one pair are not equal in some species.

Heat stability and substrate specificity of the enzyme activity of L. longipalpis, P. papatasi (Turkish origin) and P. duboscqi were detected by the dot method on 10% polyacrylamide gels with copolymerized hyaluronic acid (HA, potassium salt, from human umbilical cord, Irvine, CA) and chondroitin sulfate (A, B or C, Sigma), respectively, all at 0.002% final concentration. Sand fly SGEs (1 gland in 1 µl) were dotted on a gel and sheep testicular hyaluronidase, (Sigma, cat.# H6254) 1 µg in 1 µl, was used as a control. For heat stability assessment, SGEs were preincubated in water bath (25, 60, 80 and 100 °C) for 30 min. All gels were prepared using 0.1M acetate, pH 5 containing 0.1 M NaCl and 0.05% Tween-20. Incubation was carried out for 24 h at 37 °C in a moist chamber. The gels were then washed in water, soaked in 50% formamide for 30 min and stained in Stains-all (Sigma) solution (50 µg/ml in 50% formamide) for 1 day in the dark. After a rinse in distilled water the gels were scanned and photographed.

To quantify hyaluronidase activity in SGEs, a sensitive assay on microtitration plates coupled with biotinylated HA (bHA) was adapted. Preparation of microtiter plates for hyaluronidase assays was performed according to the method of Frost and Stern (1997), with minor modifications. Briefly, HA (1mg/ml) was dissolved in 0.1 M MES, pH 5.0, overnight. Then, N-hydroxysuccinimide (final concentration 0.1 mg/ml), biotin hydrazide (1 mM) and aqueous solution of 1-ethyl-3-3-diethylaminoethyl carbodiimide (30 µM) were added and the solution was left stirred overnight at 4 °C. The reaction was terminated by addition of 4 M guanidine-HCl and the product (bHA) was dialysed against tap and then distilled water.

For covalent coupling of bHA the Covalink®NH microtitrator plates (NUNC, Placerville, NJ) were used. Fifty µl of bHA (0.2 mg/ml) containing 0.1 mg/ml N-hydroxysuccinimide was mixed in wells with equal volume of 0.64 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and coupled overnight at 4 °C. The plates were then washed with 0.1 M phosphate buffer, pH 7.2, containing 2 M NaCl and 50 mM MgSO4 (buffer B), postcoated for 1 h with 2% BSA in PBS, pH 7.2 and equilibrated with appropriate assay buffer. SGE samples were serially double diluted in assay buffer (100 µl/well) and incubated for 30 min at 37 °C. The assays were performed three times in octaplicates for each sample.
and in triplicates for each control. The reaction was terminated by the addition of 6 M guanidine (200 µl/well) followed by several washes with buffer B containing 0.05% Tween 20 (B-Tw) and equilibrated with PBS containing 0.1% Tween 20 (PBS-Tw). Then, 100 µl/well avidin-peroxidase (2.5 µg/ml in PBS-Tw) was added for 30 min at room temperature. After extensive washing with B-Tw the wells were developed by adding 100 µl of substrate solution (1 mg/ml o-phenylenediamine and 0.022% H₂O₂ in citrate/phosphate buffer, pH 5.3) and scanned using a 492 nm filter using a Fluoroskan II plate reader (Labsystems, Finland). Wells without bHA, enzyme or avidin-peroxidase served as controls. Bovine testicular hyaluronidase (Sigma, cat.#H3631) in formate buffer pH 4.0 was used as a standard for the calibration of enzyme activity against relative turbidity reducing units (rTRU). A standard curve ranged from 0.1 to 7.81×10⁻⁴ rTRU/well.

The optimum pH for the activity of P. papatasi and L. longipalpis enzymes was explored at the range of 4.0–9.0. Four different buffers were used: 0.1 M formate, pH 4.0; 0.1 M acetate, pH 5.0; 0.1 M bis-TRIS, pH 6.0 and 7.0; 0.1 M Tris, pH 8.0 and 9.0; all buffers contained 0.1 M NaCl. Controls were performed for each pH separately. For comparison of enzyme activities among various sand fly species the optimal pH 5 was chosen (0.1 M acetate buffer).

Electrophoresis (SDS PAGE) was carried out on 10% slab gels (0.75 mm thick) using Mini-Protean II apparatus (Biorad) and constant voltage 150 V. For silver staining, 2 µg of total SGE protein was loaded per lane in either nonreducing or reducing (2-mercaptoethanol) sample buffer. Control staining with SYPRO Orange (Bio-Rad) was performed according to manufacturer’s manual. Substrate gels were copolymerized with 0.002% HA. As the hyaluronidase activities and band patterns varied among particular sand fly species, different loads were used per lane in order to obtain bands of equal intensity (four gands in P. sergenti and P. duboscqi, two gands in L. longipalpis, one gland in P. papatasi and P. halepensis, 0.5 gland in P. perniciosus). In case of P. duboscqi males, ten gands were used. Following the electrophoresis, gels were rinsed 2×20 min in 0.1 M TRIS, pH 7.8, 20 min in 0.1 M MES, pH 5.0 (both with 1% Triton X-100) and incubated in MES (without detergent) for 90 min at 37 °C. After rinsing in water the gels were stained with Stains-all as described above. Hyaluronidase activity was visible as a pink band on a dark blue background.

The Mw of L. longipalpis hyaluronidase detected in our experiments was higher than the predicted Mw of the polypeptide chain (Charlab et al., 1999). In order to see whether this could be caused by posttranslational modifications, the published aminoacid sequence (NCBI Entrez-Protein entry AAD32195) was used to perform a PROSITE scan for sites of potential N-glycosylation.

3. Results

Protein content in SGEs differed between species. The highest protein level was observed in P. duboscqi - 0.78 µg per “optimal gland” (SD 0.071), followed by P. papatasi from Cyprus colony - 0.51 µg/gland (SD 0.053), P. halepensis - 0.41 µg/gland (SD 0.055), P. papatasi from Turkish colony - 0.33 µg/gland (SD 0.049) and P. sergenti - 0.23 µg/gland (SD 0.017). The lowest protein content was found in L. longipalpis - 0.18 µg/gland (SD 0.038).

The dot method on gels with copolymerized glycosaminoglycans was used to study the substrate specificity and heat stability of P. papatasi, P. duboscqi and L. longipalpis hyaluronidases. In all three species, HA is the preferred substrate for the enzyme. The two types of chondroitin sulfate, A and C, showed moderate hydrolysis (Fig. 1A), whereas no detectable hydrolysis occurred of chondroitin sulfate B. As shown in Fig. 1B, P. papatasi and L. longipalpis hyaluronidases were heat stable. Preincubation at 80 °C for 30 min weakened but not abolished the enzyme activity. When boiled for 30 min at 100 °C, the activity was not measurable in the plate assay after 30 min incubation but was still detectable using overnight incubation of gels. The sheep testicular hyaluronidase, used as a control, produced similar result (Fig. 1B).

Assay on microtitration plates coupled with bHA was used for assessment of the pH profile of hyaluronidase activity in P. papatasi and L. longipalpis (Fig. 2). In both species, the activities occurred over a broad pH range with an optimum at pH 5. Substantial activity was retained at pH 4 and 6; under more acidic and alkaline conditions the activity declined markedly but was still clearly detectable up to pH 8.

Hyaluronidase activity in SGEs of females of five species was compared using quantitative assay on microtitration plates. Average activities related to individual
Fig. 2. pH dependence of hyaluronidase activity of *Phlebotomus papatasi* (PAP) and *Lutzomyia longipalpis* (LON). Values represent means of three measurements.

Indviduals and to total protein content in SGEs are summarised in Fig. 3. Activity levels significantly differed between species; in *L. longipalpis* the activity was distinctly lower than in the members of the genus *Phlebotomus*. The highest activities were found in *P. papatasi* and *P. halepensis*. In *P. duboscqi*, the hyaluronidase activity per individual is in the middle of the range but the specific activity (rTRU/µg of total protein) is low.

SDS PAGE revealed that SGE protein profiles exhibit species-specific patterns. Hyaluronidases are minor protein bands hardly detectable on the gel after silver staining (Fig. 4). Similarly, SYPRO Orange staining did not detect any bands in the presumptive area (data not shown). However, enzyme activity was clearly detectable by zymographic analysis on gels with incorporated HA under both nonreducing and reducing conditions (Fig. 5A–5C).

Activities of the enzymes in nonreduced samples (Fig. 5B) were high and zymography revealed major diffuse bands. Putative enzyme oligomers were seen in some species, namely *P. papatasi* and *L. longipalpis*; the proportion of the enzyme forms varied between colonies of the same species as shown in *P. papatasi* originating from Turkey and Cyprus, respectively. The molecular weight of the enzyme differed between members of the genera *Phlebotomus* and *Lutzomyia* but also between various subgenera. In *L. longipalpis* the major band was present around 60 kDa. The estimated molecular weights of the enzymes in *P. duboscqi* and two colonies of *P. papatasi* (subgenus *Phlebotomus*) were around 65 kDa, in *P. (Larroussius) perniciosus* 100 kDa and in *P. (Paraphlebotomus) sergenti* and *P. (Adlerius) halepensis* 110 kDa. Hyaluronidase activity in SGE of *P. duboscqi* males had molecular weight identical with that of females (Fig. 5A).

Under reducing conditions, SDS PAGE revealed sharper enzyme bands allowing more precise assignment of corresponding molecular weights (Fig. 5C). In members of the subgenus *Phlebotomus* (*P. duboscqi* and two colonies of *P. papatasi*) and in *P. (Adlerius) halepensis*, hyaluronidase activity was observed within the same Mw range as in non-reduced conditions (around 65 and

Fig. 3. Comparison of hyaluronidase activity in salivary gland extracts of five sand fly species using the microtitration plate method. Values represent means of three octuplicate measurements; bars show mean +/- 0.95 confidence intervals. Wells were loaded with 0.5 “optimal” gland of females, 5–6 days old. LON= *Lutzomyia longipalpis*, PAT= *Phlebotomus papatasi* (Turkish colony), DUS= *P. duboscqi*, SER= *P. sergenti*, HAL= *P. halepensis*. Activity is expressed in relative turbidity reducing units (rTRU) per either µg of total protein or individual female using bovine testicular hyaluronidase as a standard.

Fig. 4. Silver-stained SDS PAGE gels (10%). Salivary gland extracts from *P. duboscqi* males (dM) (10 glands per lane) and females (2 µg per lane) of six species were run under nonreducing conditions. LON= *Lutzomyia longipalpis*, PAC= *Phlebotomus papatasi* (Cyprus colony), DUS= *P. duboscqi*, SER= *P. sergenti*, PER= *P. perniciosus*, HAL= *P. halepensis*. 
Moreover, all females were strictly of the same age. To obtain both glands from each individual undisrupted. Therefore a special effort was paid in our experiments usually the larger one, which is easier to dissect out. May be affected by collecting only one of the two glands, of individual glands in a pair is not equal and the results published previously (Ribeiro et al., 1986; Volf et al., 2000). This could be caused by different colonies used, conditions of their maintenance and by sensitivity of methods for protein concentration measurement. In addition, in members of the subgenus Phlebotomus (P. papatasi and P. duboscqi) the volume of individual glands in a pair is not equal and the results may be affected by collecting only one of the two glands, usually the larger one, which is easier to dissect out. Therefore a special effort was paid in our experiments to obtain both glands from each individual undisrupted. Moreover, all females were strictly of the same age.

Hyaluronidases of P. papatasi and L. longipalpis showed an optimum at acidic pH 5.0 but the activity was observed up to pH 8.0. This broad pH range explains the results of Charlab et al. (1999) and Ribeiro et al. (2000), who demonstrated enzyme activity in sand flies at neutral pH. A broad pH optimum among hyaluronidases is not an exception; some, e.g. lysosomal enzymes, show activity in a narrow pH range whereas others, e.g. testicular hyaluronidases, operate within a broad pH range. In the black fly Simulium vittatum, the salivary gland hyaluronidase had an optimum around pH 6.0 (Ribeiro et al., 2000). As shown by Charlab et al. (1999) in a cDNA clone from L. longipalpis, there is also high structural similarity of sand fly salivary hyaluronidases with corresponding enzymes of Hymenoptera, namely Polistes wasps.

Considerable differences have been found in the levels of hyaluronidase activity in different sand fly species. In L. longipalpis, the activity was low either when related to an individual or to microgram of total SGE protein. L. longipalpis, in contrast to Phlebotomus species, possesses a peptide maxadilan, the most effective known vasodilator (Lerner et al., 1991). Despite maxadilan and hyaluronidase have distinct and specific pharmacological roles, they both participate in bloodfeeding. Therefore, we can speculate that the low levels of the enzyme could be partially substituted by the presence of vasodilator.

In P. duboscqi the specific activity (units/µg of total protein) is low, too, but the protein content per individual is the highest among the species tested; therefore the lower enzyme activity might be compensated by the larger amount of saliva injected into host skin. Amounts of hyaluronidase (in % of total protein) in SGEs have not been measured in this study but its specific activity seems to be incredibly high; no distinct protein bands, corresponding to major diffuse bands of lysis in zymographs, could be found on silver stained gels. Sensitivity of the silver staining used was as low as 2 ng per band (Giulian et al., 1983); comparing to the load (2 µg per lane) we can conclude that the presumable hyaluronidase content in a SGE is less than 0.1%, i.e., the activity per µg of pure hyaluronidase may be, hypothetically, 1000 times or more higher.

Our experiments imply relatively high thermostability of the enzymes. However, these data should be proved using the purified molecule; other proteins present in SGEs might protect the enzyme against loss of molecular configuration or help with its recovery. To determine whether the enzyme activity was specific for cleaving HA, we also tested other glycosaminoglycan components of extracellular matrix, chondroitin sulfate A, B

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**Fig. 5.** SDS PAGE zymography under nonreducing and reducing conditions on 10% gels with incorporated 0.002% hyaluronan. (A) Salivary gland extract from males of P. duboscqi (nonreducing conditions). (B) Salivary gland extracts from females of seven sand fly colonies (nonreducing conditions). (C) Same female extracts as in (B), but under reducing conditions. Abbreviations same as in Figs. 3 and 4.
and C. Although the commercial preparations of chondroitin sulfates are not 100% pure, due to difficulty in separating the different types of these mucopolysaccharides, moderate cleaving activity was demonstrated for chondroitin sulfates A and C only. The chondroitinase activity further suggests that sand fly salivary enzymes belong to the class of endo-N-acetyl-hexosaminidases that includes mammalian and hymenopteran hyaluronidases, but not the class of endoglucuronidases of leeches and nematodes (see review by Kreil (1995).

Comparison of hyaluronidase activity in electrophoretic substrate gels under nonreducing conditions disclosed that oligomeric forms of the enzyme may occur in *L. longipalpis* and *P. papatasii*. Oligomeric series have been found, e.g., in ram sperm hyaluronidase; the specific activity of the multiple enzyme forms was not affected by reductive conversion into monomers (Harrison, 1988). On the other hand, in macaque sperm hyaluronidase PH-20, reduction resulted in a complete loss of activity (Li et al., 2002). In sand flies, reducing conditions affected stability of the enzymes; in *P. perniciosus* and *L. longipalpis*, 2-mercaptoethanol inhibited the hyaluronidase activity. This implies that reduction-sensitive residues are either important for the function of the active site of the enzyme or steric relations in the molecule (oligomeric structure or intramolecular disulfide bonds) have been disrupted.

The enzymes of other species remained active under reducing conditions. In members of subgenera *Phlebotomus* (P. *papatasii* a *P. duboscqi*) and *Adlerius* (*P. halepensis*) reduction did not result in differences in the apparent molecular weight, suggesting that the enzymes consist of a single polypeptide chain rather than possessing subunit structure. In *P. (Paraphlebotomus) sergenti* reducing conditions decreased the molecular weight of the activity to about 55%. We suppose that hyaluronidase in this species prevails as a dimer which remained active even after separation of subunits.

In *L. longipalpis* hyaluronidase the cDNA clone described by Charlab et al. (1999) encodes a product of predicted Mr 44,232. This is more than 70% of the molecular weight we observed by zymography. Perhaps the polypeptide chain undergoes posttranslational modifications—hyaluronidases are generally glycosylated and in some cases their activity even regulated by enzymatic (de)glycosylation (e.g., Gmachl and Kreil (1993); Deng et al. (1999); Li et al. (2002). For example, in the honeybee venom hyaluronidase, which contains three N-glycosylation sites, the saccharide content is 7.5% (Kemeny et al., 1984). A PROSITE search for functional motifs in the aminoacid sequence of *L. longipalpis* hyaluronidase showed 16 sites of putative N-glycosylation, which would markedly increase the Mr of the protein.

Protein content of salivary glands and the SDS PAGE profiles of SGEs highly differ between sand fly males and females (Volf et al., 2000). This finding was not surprising as only females regularly take blood and, therefore, must produce various compounds with anti-hemostatic and immunomodulatory properties. Hyaluronidase activity, however, was present also in males which feed mainly on plant juices rich in saccharides. Hyaluronidases in saliva of arthropod vectors may possess several functions. In addition to their crucial role in bloodfeeding they may also affect pathogen transmission. Hyaluronan oligosaccharide fragments generated by hyaluronidase can downregulate production of INFγ (Romano et al., 1983), and modulate chemokine and NOS gene expression in macrophages (McKee et al., 1997). Moreover, a target for the fragments could be CD44, the hyaluronan receptor present on different cell types including monocytes/macrophages and T-lymphocytes which plays important roles in inflammatory reactions (for review see Lesley et al. (1997). Therefore we hypothesize that sand fly hyaluronidases are important in *Leishmania* transmission and establishment within the vertebrate host, and may be included in the salivary enzymes responsible for the infection-enhancing effect of sand fly saliva observed with various *Leishmania*-sandfly models (for review see Gillespie et al. (2000).

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