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A Low Molecular Weight Isoform of Hyaluronidase: Purification from Indian Cobra (*Naja naja*) Venom and Partial Characterization

K. S. Girish and K. Kemparaju*

Department of Biochemistry, University of Mysore, Mysore 570 006, India; E-mail: kemparaj@rediffmail.com

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Abstract—A low molecular weight isoform of hyaluronidase (NNH2) has been isolated from Indian cobra (*Naja naja*) venom by successive chromatography on Sephadex G-75 and CM-Sephadex C-25 columns. The apparent molecular weight determined by SDS-PAGE is 52 kD, and the p*I* value is 9.7. NNH2 is an endoglycosidase and exhibits *in vitro* absolute specificity for hyaluronan; it also hydrolyzed hyaluronan in human skin sections. NNH2 is nontoxic, but it indirectly potentiates the hemorrhagic activity of hemorrhagic complex-I. Curcumin, indomethacin, and tannic acid inhibited dose dependently the degradation of hyaluronan by NNH2.

Key words: hyaluronidase, hyaluronan, spreading factor, *Naja naja* venom

Envenomation due to snakebite constitutes a medical hazard in most regions of the world [1, 2]. The pathology includes both local and systemic effects. The later involve direct effects on the central nervous system and hemostasis by target-specific toxins, which can results in death of victims [3-7]. Local effects, like hemorrhage, edema, myonecrosis, dermonecrosis, and involvement of tendon and cartilage can lead to severe morbidity [8, 9]. Zinc dependent hemorrhagic metalloproteinases (the "metzincin" group of enzymes), myotoxins (enzymatic/non-enzymatic), and hyaluronidases are responsible for local tissue damage [8, 10-14]. The enzymes of metzincin group are predominant in *Viperid* venoms, while elapid venoms are rich in myotoxic phospholipase A_2 and cardiotoxins; in contrast, hyaluronidase is present in all snake venoms [15-18]. Hyaluronidase hydrolyzes hyaluronan and chondroitin sulfates A and C in the extracellular matrix (ECM). This results in structural collapse in the integrity of ECM, promoting easy diffusion of systemic toxins into the circulation for distribution to their target sites from the site of the bite. The enzyme isolated from *Heloderma horridum* increased the hemorrhagic area in mice that were injected with a hemorrhagic toxin from *Trimeresurus flavoviridis* venom [19]. Hence, hyaluronidase is commonly known as a spreading factor. Although hyaluronidase potentiates the toxicity of venoms by decreasing the diffusion time of systemic toxins [14], the enzyme has been greatly ignored in snake venoms. Hyaluronidase is nontoxic by itself, and this could be the reason for the lack of interest. In view of its role both in the local and systemic effects of envenomation, much attention has now been focused to target the enzyme for better management of snakebite [18]. Recently Girish et al. [14] reported the isolation and characterization of a high molecular weight isoform of hyaluronidase (NNH1) from Indian cobra (*Naja naja*) venom. Here we present the isolation and partial characterization of a low molecular weight isoform (NNH2) from the same venom.

MATERIALS AND METHODS

A pooled and dried venom sample from five or six adult *Naja naja* snakes was procured from Hindustan Park, Kolkata, India. The quantitative estimation of hyaluronidase activity was by ELISA-like assay using biotinylated hyaluronic acid binding protein (HABP) (Calbiochem, USA). Each well of a microtiter plate was coated with 30 μg human umbilical cord hyaluronan. The hyaluronan–HABP complex was detected with streptavidin–biotin–peroxidase complex coupled to the tetramethyl benzidine/hydrogen peroxide system (Bangalore Genei Private Limited, India). The absorbance was read

* To whom correspondence should be addressed.

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at 450 nm as described by the manufacturer. Maximum absorbance was obtained by incubating the hyaluronan-coated wells in the absence of any hyaluronidase [20]. The inhibition of NNH2 activity by inhibitors was expressed as $1 - (A_{max} - A_{sam})/(A_{max} - A_{min})$, where A_{max} is the absorbance of wells not exposed to hyaluronidase, A_{min} is the absorbance of wells exposed to NNH2 plus an equal volume of buffer, and A_{sam} is the absorbance of wells exposed to NNH2 plus an equal volume of sample with inhibitor.

RESULTS AND DISCUSSION

NNH2 has been purified to homogeneity (26-fold) using successive chromatography on a Sephadex G-75 and CM-Sephadex C-25 columns [14]. NNH2 was co-eluted with NNH1 from a Sephadex G-75 column and then separated on a CM-Sephadex C-25 column. NNH2 eluted as a single peak with the retention time of 48.1 min in reversed-phase HPLC on a Vydac C8 column (Fig. 1). The column was eluted using a linear gradient of acetonitrile concentration (from 0.1% solution of trifluoroacetic acid in water to 0.1% solution of trifluoroacetic acid in acetonitrile) for 60 min with the flow rate of 1 ml/min and the eluate absorbance was monitored at 280 nm.

NNH2 moved as a sharp band in SDS-PAGE (12.5%) [21] to the same extent under reduced and non-reduced conditions (Fig. 2a). The apparent molecular weight was found to be 54 kD by gel permeation on a Sephadex G-100 column (data not shown) and 52 kD by SDS-PAGE (with or without β-mercaptoethanol) suggesting single polypeptide nature. The molecular weight determined for NNH2 is in contrast to 70.4 kD reported for NNH1 [14]. Thus, NNH2 is a low molecular weight isoform that exists in the same venom. The molecular weight determined agrees well with the range 33 to 110 kD reported for venom hyaluronidases [14].

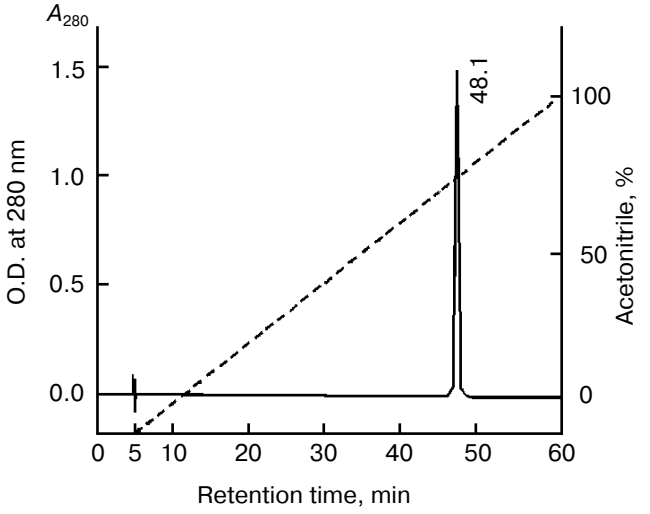

Fig. 1. Reversed-phase HPLC elution profile of NNH2.

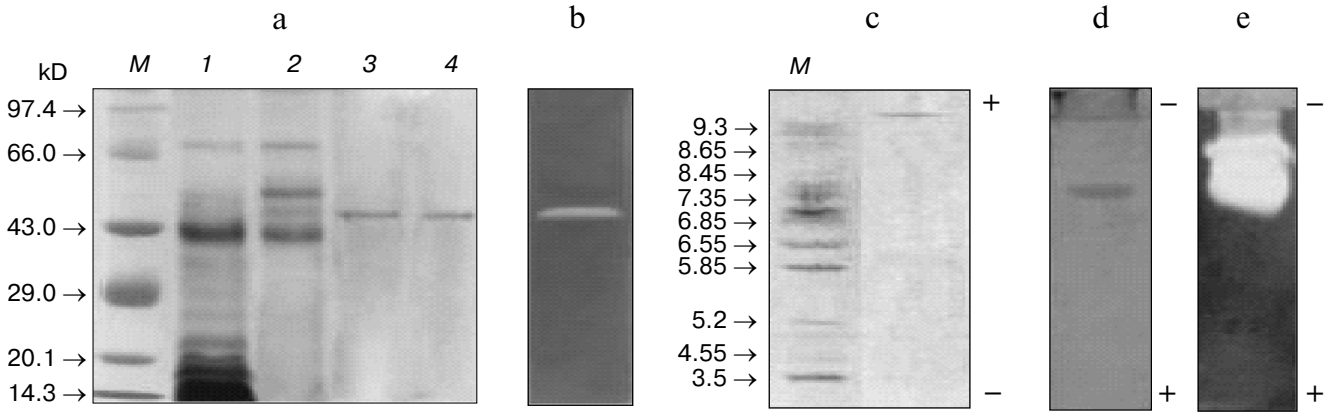

Fig. 2. Purification of NNH2 as shown in SDS-PAGE, isoelectrofocusing, native PAGE, and zymogram assay. a) SDS-PAGE (12.5%) of 75 μg *Naja naja* venom (*1*), 50 μg Sephadex G-75 fraction (*2*), 15 μg NNH2 under non-reducing (*3*) and reducing (*4*) conditions. M, molecular weight markers in kD (from top to bottom): phosphorylase *b* (97.4 kD), bovine serum albumin (66 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), soybean trypsin inhibitor (20.1 kD), and lysozyme (14.3 kD). b) Pattern of enzyme activity (zymogram) of NNH2 (3 μg) resolved in SDS-PAGE containing hyaluronan under non-reducing condition. c) Isoelectrofocusing of NNH2 (5 μg). M, isoelectric point markers (from top to bottom): trypsinogen (9.30), lentil lectin basic band (8.65), lentil lectin middle band (8.45), lentil lectin acidic band (8.15), myoglobin basic band (7.35), myoglobin acidic band (6.85), human carbonic anhydrase B (6.55), bovine carbonic anhydrase B (5.85), β-lactoglobulin A (5.20), soybean trypsin (4.55), and amyloglucosidase (3.50). d) Native polyacrylamide gel (10%) electrophoresis of 15 μg NNH2 at pH 4.3 using β-alanine-acetic acid buffer with malachite green as a tracking dye at a constant current of 100 V for 4 h. After electrophoresis, the gel was stained with 0.1% Coomassie brilliant blue R-250. e) Pattern of enzyme activity (zymogram) of NNH2 (10 μg) in PAGE containing hyaluronan.

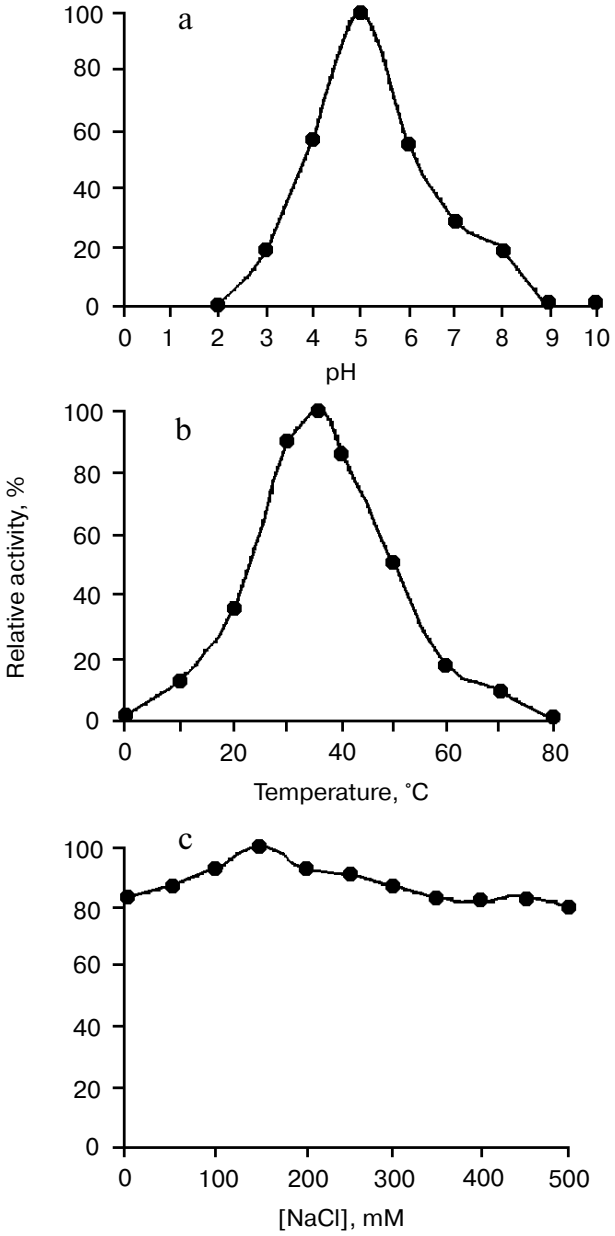

Fig. 3. Effect of pH (a), temperature (b), and NaCl (c) on the activity of NNH2. Percent enzyme activity was calculated by considering enzyme activity in 0.1 M sodium acetate buffer, pH 5.0, containing 0.15 M NaCl at 37°C as 100%.

Furthermore, NNH2 revealed a single translucent band in SDS-PAGE (12.5%) hyaluronan zymogram assay [22, 23] with a mobility similar to that observed by us in SDS-PAGE (Fig. 2b). NNH2 was slightly more basic than NNH1 and revealed a sharp band with the calculated isoelectric point of 9.7 (Fig. 2c). NNH2 moved as a sharp band in native PAGE at pH 4.3 (Fig. 2d), and similar mobility was observed in zymogram assay (Fig. 2e). NNH2 showed fluorescence emission maximum at 345 nm when excited at 280 nm, while NNH1 had two fluorescence emission maxima, i.e. 310-320 and 340-350 nm. NNH2 was maximally active at pH 5 (Fig. 3a) and at 37°C (Fig. 3b). In the presence of 150 mM NaCl, about 20% enhanced activity was observed; however, concentrations lesser and greater than 150 mM NaCl did not alter the basal activity (Fig. 3c). NNH2 hydrolyzed hyaluronan with a specific activity of 1.35 units/min per mg protein. It did not hydrolyze chondroitin, chondroitin sulfates A, B, C, and D, heparin, and different molecular forms of chitosans (high, medium, and low molecular weight forms) (Table 1). This suggested absolute specificity for hyaluronan similar to that of NNH1. Furthermore, NNH2 did not hydrolyze chromogenic substrates such as *p*-nitrophenyl-β-D-glucuronide and *p*-nitrophenyl-β-D-glucosaminide and thus it can be assigned to endoglycosidases. Therefore, NNH2 appears to belong to the endo-β-N-acetylhexosaminidase class of hyaluronidases, similar to NNH1 [14] and bovine testicular hyaluronidase [24]. However, the later, in addition to hyaluronan, hydrolyzed several other glycosaminogylcans such as chondroitin, chondroitin sulfates A, C, and D, and different molecular forms of chitosans but was similarly inactive towards chondroitin sulfate B and heparin (data not shown).

Table 1. Substrate specificity of *Naja naja* venom (NNH2) and bovine testicular hyaluronidases

Substrate	Specific activity, units/min per mg protein	
	Naja naja venom hyaluronidase (NNH2)	bovine testicular hyaluronidase
Hyaluronan*	1.35	0.55
Chondroitin**	0.0	0.00021
Chondroitin sulfate A***	0.0	301
Chondroitin sulfate C***	0.0	243
Chondroitin sulfate D***	0.0	160
Chitosan (high molecular weight form)**	0.0	0.00073
Chitosan (medium molecular weight form)**	0.0	0.00024
Chitosan (low molecular weight form)**	0.0	0.00011

* One unit of activity is the amount of enzyme required to cause decrease in absorbance by 0.06 at 450 nm during 10 h.

** One unit of activity is the amount of enzyme required to release 1 μmol of N-acetylglucosamine per 1 min.

*** One unit of activity is the amount of enzyme required to hydrolyze 50% of the substrate in 1 min.

Table 2. Hemorrhagic activity of hemorrhagic complex-I in the presence and in the absence of NNH2

Treatment	Hemorrhagic spot, mm^2
Saline	0.0
NNH2 (25 μg)	0.0
Hemorrhagic complex-I (2 μg)	10 ± 1
Hemorrhagic complex-I (2 μg) + NNH2 (4 μg)	16 ± 2
Hemorrhagic complex-I (2 μg) + NNH2 (6 μg)	20 ± 1.5

Table 3. Inhibition of NNH2 by bioactive compounds*

Inhibitor	Inhibition, %			IC_{50}, μM
	50 μM	100 μM	200 μM	
Curcumin	43 ± 2	80 ± 5	96 ± 4	57
Tannic acid	26 ± 4	55 ± 3	90 ± 6	86
Indomethacin	35 ± 2	72 ± 4	89 ± 6	70

Note: Tannic acid was dissolved in dimethyl sulfoxide. Curcumin and indomethacin were dissolved in ethanol. NNH2 (0.75 μg) was pre-incubated with varied concentrations of inhibitors for 15 min at 37°C. Assay was initiated by adding pre-incubated NNH2 and inhibitor sample. Values are mean ± SEM of five experiments.

Pharmacological studies of NNH2 revealed that it is nontoxic to mice at doses up to 14 mg/kg body weight (intraperitoneally). The mice were sacrificed after 24 h, and the postmortem examinations revealed neither bleeding in the peritoneal cavity nor any visible damage to vital organs such as heart, liver, lungs, kidneys, and brain. NNH2 was not myotoxic as there was no change in the levels of cytoplasmic markers (CPK and LDH enzymes) in the serum. NNH2 was not cytotoxic as it did not alter the viability of EAT cells even at a dose of 100 μg/10^6 cells in 2 ml reaction mixture. At a tested dose of 25 μg, NNH2 was devoid of hemorrhagic, edema, and both direct and indirect hemolytic activities and furthermore it did not alter the clotting time of re-calcified human plasma. However, the spreading property of NNH2 was demonstrated using the *Vipera russelli* venom hemorrhagic complex-I [25] according to the method of Kondo et al. [26]. The magnitude as well as the intensity of hemorrhagic spots induced by hemorrhagic complex-I increased with increasing concentrations of NNH2. Less intense hemorrhagic spots were observed when injected with hemorrhagic complex-I alone. In contrast, NNH2 alone did not induce any hemorrhage even at a concentration of 25 μg (Table 2).

NNH2 hydrolyzed hyaluronan in human skin tissue sections (5 μm thick). Tissue hyaluronan was localized by the modified histochemical procedure [27, 28] using biotinylated hyaluronic acid binding protein in a system of streptavidin–peroxidase with benzidine tetrahydrochloride chromogenic substrate. Intense brown color appeared in the extracellular spaces of epidermis and dermis in control sections, confirming the localization of hyaluronan (Fig. 4a). In contrast, when stained after treatment with NNH2, the sections were either translucent with no intense brown color or displayed faint and discontinuous staining (Fig. 4b). The disappearance of the brown color in the NNH2 treated sections suggested hyaluronan degradation. Thus, it is highly likely that NNH2 degrades hyaluronan in the ECM of victims at the site of the bite during natural envenomation and facilitates easy diffusion of systemic toxins.

In an attempt to screen certain flavonoids, antioxidants, and antiinflammatory drugs for inhibition of

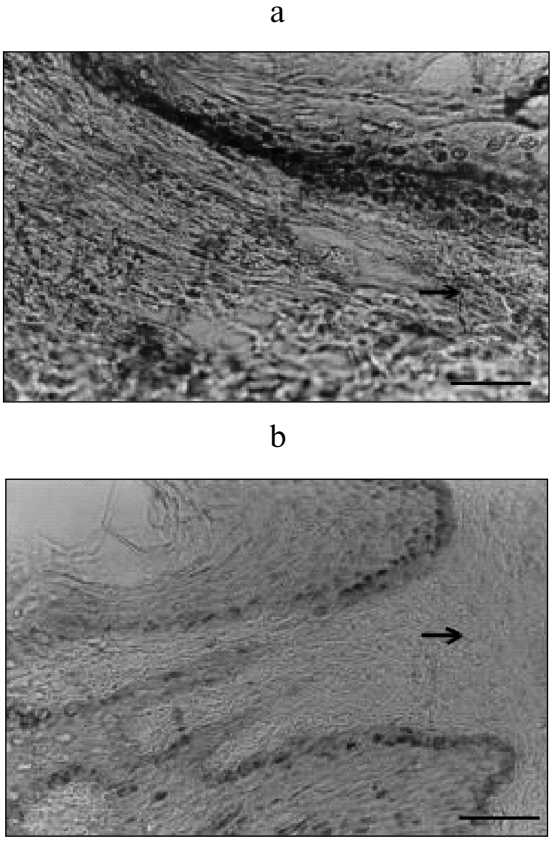

Fig. 4. Human skin sections stained for hyaluronan with biotinylated HABP before (a) and after (b) preincubation with NNH2. Arrows indicate the localization of hyaluronan as dark patches in (a) and their disappearance in (b). Scale bar: 50 μm.

NNH2, it was found that tannic acid (a flavonoid), curcumin (an antioxidant), and indomethacin (an antiinflammatory drug) all inhibited the activity of NNH2. The inhibition of hyaluronan degradation was found to be dose dependent (Table 3). All three inhibitors inhibited NNH2 to almost a similar extent with varied IC_{50} values. Furthermore, all three inhibitors inhibited NNH2 degradation of hyaluronan in human skin sections as the sections revealed intense brown staining of intact hyaluronan (data not shown). As hyaluronan is an essential component of ECM and found to act as a connecting material to bridge adjacent cells in tissues, its degradation undoubtedly alters the structural integrity of ECM during natural envenomation.

It is thus likely that NNH2, in addition to increasing the rate of diffusion of toxins, contributes to local tissue damage. *Naja naja* venom hyaluronidase and its role in spreading of toxins have been established recently [14]. Inhibition of hyaluronidase resulted in reduced local tissue damage and prolonged the survival time of mice injected with *Naja kaouthia* and *Calloselasma rhodostoma* venoms [18]. Thus, hyaluronidase needs prompt consideration and much more investigation before beginning clinical trials. One such aspect is assigning the precise functions for hyaluronan degradation products that are generated due to envenomation. The neutralizing antibodies for jararafibrase-1, a hemorrhagic metalloproteinase from *Bothrops jararaca* venom, inhibited the venom induced systemic coagulopathy [13]. Inhibitors of metalloproteinases inhibited the local hemorrhage and dermonecrosis of *Bothrops asper* bite [29, 30]. Antivenoms, despite success in neutralizing the systemic toxins, have minimal protective effect on local tissue damage [31, 32]. Thus, inhibitors of hyaluronidase and other locally acting enzymes/toxins, in addition to minimizing the local tissue damage, transiently limit the distribution of toxins to the site of the bite. This will retard the rapid burst in the concentration of systemic toxins to reach the lethal level. Hence, new therapeutic strategies that involve direct application of a cocktail of inhibitors of locally acting agents in the bitten region, at least as a first aid, should minimize the local tissue necrosis and increase survival rate of victims.

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