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Hyaluronidase an enzyme in snake venom and its natural inhibitors: A Review

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ABSTRACT

Snakebite causes the highest rate of injury and among the population, especially in Asian countries. Snake venom is a type of zootoxins composed of a highly complex mixture of enzymes including phospholipase A_2, hyaluronidase, L-amino acid oxidase, acetylcholine esterase, phosphodiester, and low molecular weight polypeptide. The hyaluronidase or hyaluronic glucosidase venom enzyme is considered a "spreading factor" as it hydrolyzes the hyaluronic acid (generally known as hyaluronan), which plays an essential role of cell to cell adhesion in the extracellular matrix of tissue. Secondary metabolites present in a crude extract of medicinal plants are considered as capable venom neutralizing agents as they have no side effect and can neutralize a wide range of snake venom enzymes. The objective of this review is to collect, simplify and explain the role along with the importance of inhibition of hyaluronic glucosidase in snake venoms. All the accessible information on hyaluronic glucosidases was collected via electronic search (using Google Books, PubMed, SciFinder, Scopus, Google Scholar, and Web of Science) and articles of peer-reviewed journals. This review contains a brief description of the structure, occurrence, mechanism, assay procedures, and phytoconstituents reported to inhibit hyaluronidase.

Keywords: *Hyaluronidase; Zootoxin; Snake venom; Hyaluronic acid; Spreading factor*

1. INTRODUCTION

Snakebite envenoming is a neglected public health problem in several tropical and subtropical countries around the world. World Health Organization (WHO) has recognised snakebite envenoming as a Neglected Tropical Diseases (NTD) (WHO, 2017). Snakebite is estimated that worldwide, around 5 million snakebites happen yearly, with up to 2.5 million envenoming. Snake venom (zootoxin) is the complex mixture of compounds produced by poisonous animals (snakes, spiders, wasps, scorpions, and ants) is an occupational threat frequently encountered by hunters, villagers, tribal people, farmers, farm labourers, migrating population, shepherds, and army of tropical and subtropical regions (Bawaskar, 2014; Utkin, 2015). Snake venom is a biologically active components, which isble to interfere with the normal physiological process in humans. The constituents of the zootoxins include phospholipase A_2, hemorrhagic matrix metalloproteases, myotoxins and hyaluronidases which are involved in the diffusion process are generally referred to as "spreading factors".

Hyaluronidase is naturally found in several human organs and body fluids, and also in snakes, scorpions, bees, wasps, spiders, lizards, and leeches (Pinto et al., 2012). Prokaryotic and eukaryotic cells also produced hyaluronidase (Abbasian et al. 2014). It is present in the extracellular matrix (ECM) of nearly all vertebrates and functions as intercellular cement. Hyaluronidase is categories into two groups on the basis on their pH (1) acidic pH 3 and 4 (e.g. human liver and serum enzymes); and (2) neutral, pH 5 and 8 (e.g. ovine testicular enzyme) (Janardhan et al., 2014). In snake venom, hyaluronidase enzyme is known for its dynamic role as "spreading factor" as hyaluronic acid allow the spread of toxin by degrading the host (Janardhan et al., 2014; Wahby et al., 2012). This hyaluronidase enzyme plays an important role in the disruption of hyaluronic acid present in the extracellular matrix. Although, the pathophysiology of envenomation comprise both local effects (haemorrhage, edema, myonecrosis, and extracellular matrix degradation) and systemic effects (neurotoxicity, myotoxicity, cardiotoxicity), and alterations in haematological systems (Gutiérrez et al., 2017).

Classification of hyaluronidase

Firstly, the hyaluronidase activity was documented by Duran-Reynals in 1928, but the term "**hyaluronidase"** was proposed in 1940 (Bordon et al., 2015). Hyaluronidases are briefly classified into three major types (figure 1):

1. First type Enzyme Commission number 3.2.1.35 includes vertebrate enzymes (e. g. mammalian and venom hyaluronidases) that are endo-ß-N-acetyl-D-hexosaminidases which hydrolyze the ß1,4glycosidic bond between GlcNAc and GlcUA residues in hyaluronan to tetrasaccharide (GlcUA-GlcNAcGlcUA-GlcNAc) as the main product(Helting and Lindahl, 1971).

2. Second type Enzyme Commission number E.C. (3.2.1.36) is composed of hyaluronidases from annelids, such as leeches and certain crustaceans. These enzymes are endo-ß-D-glucuronidases that degrade hyaluronan to the tetrasaccharide (GlcNAc-GlcUA-GlcNAcGlcUA) by hydrolyzing the ß-1,3glycosidic bond between GlcUA and GlcNAc residues in hyaluronan(Stern and Jedrzejas, 2006).

3. Third type Enzyme Commission number E.C. (4.2.2.1, former EC 4.2.99.1) is represented by bacterial N-acetyl-D-hexosaminidases that cleave the ß,1-4glycosidic bond by a beta elimination reaction, degrading hyaluronan, chondroitin sulfate and dermatan sulfate to disaccharides with a double bond between carbons 4 and 5 (Bordon et al., 2015).

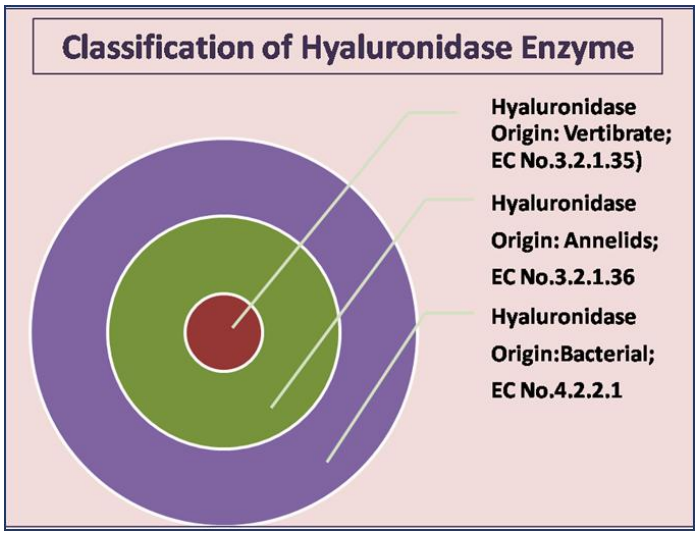

Fig. 1: Pictorial representation of a classification of hyaluronidase enzyme.

Structure and Mechanism of hyaluronidase

"Hyaluronidase" is a term given by Meyer to represent an enzyme that degrades hyaluronic acid (Meyer, 1971). The purification, characterization and systemic evaluation of hyaluronidase from Indian cobra (*Naja naja*) venom was performed. The purification was performed by gel permeation, and ion-exchange chromatography; characterization of the same was done MALDI TOF mass spectrometry. This protein has approximately 70.406 kDa molecular weight (Girish et al., 2004b). Human and snake hyaluronidase consist of five disulfide bond. Disulfide bonds (1) Cys332–Cys343, (2) Cys336–Cys371 and (3) Cys373–Cys383 are part of the epidermal growth factor-like domain, disulfide bond (4) Cys17–Cys307 and (5) Cys183–Cys196 are placed in the catalytic domain (Bordon et al., 2015). The hyaluronidase enzyme break the internal glycolsidic bonds of hyaluronic acid or mucopolysaccharides of connective tissues, resulting in a decrease in tissue viscosity, and thus, allowing the venom to penetrate into the interior of tissues (figure 2) (Bala et al., 2018).

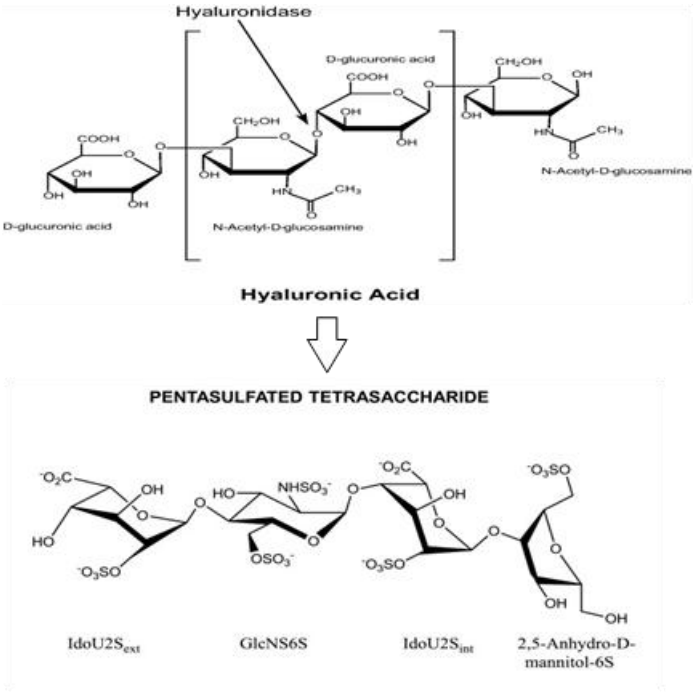

Fig. 2. Mechanism of Hyaluronidase (Hyaluronic glucosidases) (Bala et al., 2018).

Assay of Hyaluronidase

Hyaluronidase enzyme can be treated as a therapeutic target for the neutralization of snake envenomation. Several biochemical, phytochemical and microbiology related assays are performed to check the potency of enzyme and its neutralization. Hyaluronidase activity can be assayed by different methods which involve the breakdown of hyaluronic acid:

Electrophoretic Assay

The bands appearing in SDS PAGE assay are analysed and compared on the basis of difference in their molecular weight which also reveals the glycosylation patterns of the enzyme (Roldán-Padrón et al., 2019). The substrate gel assay for hyaluronidase activity was performed by incorporating hyaluronic acid at a final concentration into the 10% SDS-polyacrylamide gel matrix. SDS PAGE was shown as per the method of Lammelli. The samples and protein markers were

run on 12% gel using Tris-glycine buffer pH 8.3 and SDS. The gel is stained by using silver stain to visualize the bands (Janardhan et al., 2014).

Spectrophotometric Assay

Hyaluronidase present in crude venom was assayed turbidimetrically. The assay mixture contained hyaluronic acid and enzyme in Tris – HCl buffer. The mixture was incubated for 15 min at 37 °C, and the reaction was stopped by the addition of cetyltrimethyl ammonium bromide in NaOH. The absorbance was read at 400 nm against a blank by spectrophotometer. Inhibition study was carried out by pre-incubating venom with plant extract for 45 min (Shrikanth et al., 2014). The activity of an enzyme is evaluated by specific activity which the turbidity is reducing units/enzyme/minute.

Whole Blood Clotting Test (20 WBCT)

It is a diagnostic test for the examination of coagulopathy caused by snake envenomation (Ratnayake et al., 2017) Few millilitres of fresh venous blood is drawn and left undisturbed in the test tube for 20 min; the tube is then tilted gently to check that the blood is still liquid after 20 min, it is evidence of coagulopathy which confirms the patient is bitten by a viper (Ahmed et al., 2008).

Agar Well Diffusion Assay

In this method, 1% agar solution was mixed with hyaluronic acid, Na^+ salt, bovine serum albumin, and sodium azide. The solution was then poured in Petri plates and allowed to solidify after solidification 3-mm wells were made. To check hyaluronidase activity, overnight culture supernatants were added to the wells, followed by incubation at 37°C for overnight. The plate was then flooded with an acetic solution for 30 min. Undigested hyaluronic acid forms a precipitate under these conditions, creating hazy opacity in the agar and leaving a zone of clearance around wells containing hyaluronidase (Janardhan et al., 2014).

Hyaluronidase as a therapeutic target

The hyaluronidase exhibits absolute specificity for hyaluronan releasing tetra- and hexa-saccharides (Kemparaju and Girish, 2006). Hyaluronidase destroys the internal glycosidic bonds of few acid mucopolysaccharides of connective tissues which results in a decrease in tissue viscosity, and thus, allowing the venom to penetrate the interior of tissues. Hyaluronic glucosidases (hyaluronidases) are enzymes that hydrolyze hyaluronic acid to N-acetyl-D-glucosamine glucuronic acid in the body (Bala et al., 2018). Hyaluronic acid, a long-chain glycosaminoglycan, is a principle constituent of animal connective tissue which forms a huge hydrodynamic domain and causes expansion of the extracellular space by binding large quantities of salts, metal ions and water molecules (Kemparaju and Girish, 2006). Hyaluronidase may thus contribute to local envenomation by disrupting connective tissue and potentiating necrosis and add to systemic envenomation by accelerating venom absorption and diffusion (Pukrittayakamee et al., 1988).

Currently, antivenom immunotherapy or serotherapy is the only management available against snakebites. The side effects of antivenom immunotherapy also in consideration such as pyrogen reaction, serum sickness, renal failure, early and late anaphylactic reaction. These indications are perhaps consequences of the reaction of non-immunoglobulin proteins existing in higher amount in antivenom (Maya Devi et al., 2002). In this viewpoint, numerous efforts have been through to develop snake venom antagonists from medicinal plants. In traditional medicine, medicinal plant extract methods are passed on to generations by oral ritual and are used as antidotes against snakebites.

Neutralization of hyaluronidase through Medicinal Plants:

Numerous species of plant are termed as a folk medicine for snakebite (Singh et al., 2017). The poisonousness initiated by different proteins and enzymes present in snake venom can be inhibited through the phytoconstituents inhibitors which are naturally available in medicinal plants. Hyaluronidase inhibitors are of different chemical forms such as bioactive component, proteins, glycosaminoglycans, polysaccharides and synthetic organic compounds. Scientifically, ethanol, methanol and aqueous plant extracts are prepared out of the plant parts like root, stem barks, and leaf which works actively against the snake venom (Singh et al. 2016; Singh et al. 2019) (table 1). Timely application of these plant extracts or its sap (plant fluid) on the bitten area or drinking plant extract can neutralize snake venom (Gomes et al. 2010). Aqueous extract of *Mimosa pudica* (root) dose dependently inhibited the hyaluronidase activities of *Naja naja*, *Vipera russelii* and *Echis carinatus* venom (Girish et al., 2004a). The methanolic extract of *Leucas aspera* and its constituents were ompletely (100%) neutralized hyaluronidase activities (Gopi et al., 2014). The *Albizia lebbeck* methanolic extract of seed demonstrated significant neutralization potential against the hyaluronidases toxic enzymes of *Echis carinatus* venom (Amog et al., 2016). The leaves aqueousextract of *Rosmarinus officinalis* has shown potent inhibitors of hyaluronidase of Egyptian horned viper, Cerastes cerastes (Wahby et al., 2012). The protective efficacy of *Cassia auriculata* leaf methanol extract have significantly inhibited hyaluronidases enzymatic activities against *Echis carinatus* venom (Nanjaraj Urs et al., 2014). *Canthium parviflorum* is generally used in the traditional therapy to treat snakebite.

Partially purified methanol root extract of *C. parviflorum* has hyaluronidases enzymatic neutralization activities against *Eachis carinatus* and *Naja naja* venom (Shrikanth et al., 2016). The *Anacardium occidentale* bark extract revealed significant neutralize enzymatic (phospholipase, protease, and hyaluronidase) as well as pharmacological effects induced by *Vipera russelii* venom (Ushanandini et al., 2009). *Tamarindus indica* seed extract shown neutralization activity against the hyaluronidase, phospholipase A_2, protease, l-amino acid oxidase and 5′-nucleotidase enzyme activities of snake venom in a dose-dependent manner (Ushanandini et al., 2006). The *Morus alba* plant leaf extract completely abolished the *in vitro* proteolytic and hyaluronolytic activities of the *Vipera/Daboia russelii* venom induced local and systemic effects (Chandrashekara et al., 2009).

Table 1. Herbal plants against snake venom (Scotti et al., 2016; Singh et al., 2019; Wahby et al., 2012).

S No	Plant Extract	Common Name	Plant Parts	Percentage Inhibition
1.	*Aegle marmelos*	Bael	Leaves	94
2.	*Chamaerhodos erecta*	Nuttall's Groundrose	Aerial	80
3.	*Clitoria ternatea*	Aparajita	Leaves	94
4.	*Curculigo orchioides*	Aakundo	Tuber	97
5.	*Dryopteris cochleata*	Jatashankari	Rhizome	99
6.	*Enhydra fluctuans*	Helencha	Leaves	96
7.	*Gaultheria procumbens L.*	Eastern teaberry	Leaves	75
8.	*Lythrum salicaria L*	Purple loosestrife	Flowering herb	94.4
9.	*Mentha peprinta*	Mentha	Leaves	69
10.	*Ocimum basilicum*	Sweet Basil	Leaves and flower	78
11.	*Oenothera biennis L.*	Eveningprimrose	Aerial Part	97.9
12.	*Origanum majorana*	Sweet marjoram	Leaves	89
13.	*Payena dasyphylla*	Payena leerii	Bark	91.63
14.	*Rosmarinus officinalis*	Rosemary	Leaves	100
15.	*Sansevieria trifasciata*	Naagphan	Leaves	96

Herbal constituents active against Snake Bite:

Many bioactive plant compounds have shown their effective inhibition against the constituents of snake venom. Medicinal plants have rich bioactive constituents with potent pharmacological activity and anti-inflammatory activity. Herbal constituents such as flavonoids, alkaloids, trepanoids, glycosaminoglycans, polysaccharides, proteins, antioxidants agents, and other plants derived organic have been examined as hyaluronidase inhibitors are of great interest as they could stop or delay the diffusion of toxins after the natural envenomation. The neutralization activity of β-sitosterol and stigmasterol compounds isolated from the methanol root extract of *Pulchea indica* (flowering plant) examined for Cobra and Viper venom (Gomes et al. 2007). Aristolochic acid inhibited the *in vitro* activity of the purified hyaluronidase, and the hyaluronidase activity of whole venom in a dose-dependent manner (Girish and Kemparaju, 2006). Glycoprotein isolated from *Withania somnifera* folk medicinal plant inhibit hyaluronidase activity of Indian cobra (*Naja naja*), and Viper (*Daboia russelli*) snake venom (Gomes et al. 2010). The medicinal plants origin flavonoids having double bond between carbons 2 and 3, an unsubstituted hydroxyl groups at C-positions 5, 7 and 4′, as well as a ketone group at position 4, showed effective inhibitory activities on the snake venom hyaluronidases (Kuppusamy and Das, 1991). The medicinal plant natural products aristolochic acid and quercetin exhibited completely inhibition activity against venom hyaluronidase in dose dependently manner (Girish and Kemparaju, 2005). Polyphenols, especially tannins, have been reported to the inhibition of *Naja kaouthia* venom activities (Pithayanukul et al., 2007). The plant-derived bioactive compounds mainly tannic acid and the flavonoids luteolin, kaempferol and apigenin applied potent inhibition on the hyaluronidase enzyme of *Crotalus adamenteus* venom in mice dose-dependently (Kuppusamy and Das, 1993). Many *in vitro* and *in vivo* studies demonstrated that several flavonoids, alkaloids, terpenoids, polyphenols, saponins, sterols, glycosides, etc., from herbal medicines effectively neutralized local tissue damage induced by venom toxins/enzymes (Urs et al., 2014).

Other roles of hyaluronic glucosidase:

Hyaluronidase play a dynamic role in cancer treatment as spreading factor for cytotoxic chemotherapy, act as degrading hyaluronic acid, lowering the interstitial fluid pressure and thus intensify drug access which serves this enzyme as a potential target for the neutralization of venom (McAtee et al. 2014). Apart from this, the hyaluronidase enzyme lowers the interstitial fluid pressure (IFP) in cancerous tissues within the tumours in a non-linear concentration-dependent manner. Hyaluronidase derived from bovine testes increase the penetration and activity of the oncolytic drug adriamycin of breast cancer (Yap et al. 2011). Hence, it is used

as an additive in therapy, including not only chemotherapy but also expands to radio-immunotherapy applications.

Hyaluronidase has been used as an adjuvant for the delivery of monoclonal antibody against an osteosarcoma allied antigen. Hyaluronidase can be presented to the target tissue in a multivalent display by using nanotechnology to synthesize nano-sized water-dispersible nanostructures which are later derivatized with the enzyme. The level of hyaluronic acid surrounding tumour cells often correlates with tumour aggressiveness and overproduction of hyaluronic acid enhance anchorage-independent cell growth. Hyaluronidase on tumour cells may provide a target for antineoplastic drugs.

Conclusion

Hyaluronidase enzyme is one of the constituents of snake venom. Its concentration may induce or reduce the efficacy of snake venom to cause internal injuries and blood clots in the host body. It is termed as spreading factor as it helps to disrupt the ECM matrix by hydrolysing it. This activity of hyaluronidase enzyme has grabbed a lot of scientific attention for the neutralization of snake venom. This protein is widely explored for its structure, mechanism of action, and its role in neutralization assays. Several studies have been performed to decipher easy, simple and fast neutralization methods. It is essential to establish scientific research on the traditional use of medicinal plants and their constituents to neutralize the snake venom enzyme. Although, many ethanolic, methanolic and aqueous extracts of medicinal plants are formulated with effective neutralization activity. In future, there is need to work upon the formulation of doses with the herbal medicine for rapid and fast treatment of snakebites.

Conflict of Interest

The authors have no conflict of interest regarding the publication of this article.

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