Egyptian Journal of Chemistry
http://ejchem.journals.ekb.eg/

Purification and Biochemical Characterization of Hyaluronidase from Egyptian *Echis pyramidum pyramidum* Venom

Walaa H. Salama *

Molecular Biology Department, *National Research Centre, Cairo12622, Egypt*

In Loving Memory of Late Professor Doctor ""Mohamed Refaat Hussein Mahran""

Abstract

A novel hyaluronidase was purified to apparent homogeneity from Egyptian *Echis pyramidum pyramidum* venom. The enzyme was purified 7.6-fold by Sephacryl S-200 size exclusion followed by CM-Sepharose cation-exchange chromatography with a specific activity of 610 U/mg over the activity of whole venom (80 U/mg). The molecular mass of the purified enzyme was approximately 48 kDa in its native and reduced forms. The maximum activity was estimated at pH 5.5 and 40 ºC. The purified hyaluronidase has an absolute activity towards hyaluronic acid with K_m and V_{max} values of 20µg/ml and 40µg/min, respectively. The Na^+ ion enhanced the hyaluronidase activity, whereas Fe^{+2}, Ni^{+2}, Cu^{+2}, Co^{+2}, Zn^{+2}, Mn^{+2}, o-phenanthroline (5 mM) and heparin (10 units) inhibited the enzyme activity by 100%. Antisera against the venoms of *Echis pyramidum*, *Echis coloratus*, Cerastes *cerastes*, *Cerastes vipera* and *Naja haje* greatly neutralized *E. pyramidum* hyaluronidase to a varied extent. In contrast, antisera against *Naja nigricollis* negatively neutralized the hyaluronidase activity. The study concluded that the purified enzyme shared similar immunogenic properties with venoms of vipers rather than elapids. It also demonstrated the importance of characterization of enzymes from venoms of different species to explore the venomics and antivenomics of Egyptian snake species.

Keywords: Snake venom; Enzymes; Inhibition; Hyaluronic acid; Neutralization; Antigenic; Epitope

1. Introduction

Bites by venomous snakes are considered neglected health issues that cause severe complications and mortality. Envenomation by vipers is predominantly characterized by local tissue destruction, edema, hemorrhage, and systemic circulatory disturbances leading to death [1]. Antivenom administration is the only therapy for neutralizing venom toxicity [2, 3].

Snake venom is a liquid mixture of enzymes and polypeptides, along with other lipids, carbohydrates, biogenic amines, and inorganic metal ions that act as activators and stabilizing agents of venom components [4]. Metalloproteases, serine proteases, collagenases, and hyaluronidases are the most predominant enzymes presented in vipers rather than elapids [5]. Proteases and collagenases catalytically degrade basement membrane proteins, and factors involved in the blood clotting cascade [6, 7] result in extravasodilation, coagulapathy, and circular shock [1]. Hyaluronidases are present in snakes, along with bees, spiders, scorpions, and caterpillars [8].

Snake venom hyaluroniadases (EC 3.2.1.35) are a group of hydrolytic enzymes that catalyze hyaluronan degradation, a high molecular-mass polysaccharide and a component of extracellular matrix and connective tissue. In addition, they are key mediators in not only increasing toxins influx but also potentiating the activities of venom enzymes, resulting in local tissue damage and inflammation [8].

In Egypt, the Echis and Cerastes genera are venomous vipers that inhabit mainly deserts. *Echis pyramidum pyramidum* (Egyptian saw-scaled viper) belongs to the *Viperidae* family, is a highly medically important viper, and inhabits northern Egypt, particularly El-Faiyum and Giza deserts. Annually, more than 60% of snakebite deaths in Africa are caused by Echis vipers [9].

Today, obtaining the venom proteins in their pure and active forms is important for both developing drugs based on snake venom components and enhancing the potency of antivenoms. Therefore, understanding the chemical structure and functional characteristics of venom enzymes is of great interest for biomedical purposes [8]. Previously, several

*Corresponding author e-mail: walaahsalama82@gmail.com
Receive Date: 23 December 2023, Revise Date: 15 January 2024, Accept Date: 24 January 2024
DOI: 10.21608/ejchem.2024.257468.9028

enzymes were purified and characterized from the venoms of Egyptian snakes to investigate the biological activities of these proteins.

In the current study, our aim is to purify and biochemically characterize the hyaluronidase enzyme from the venom of the Egyptian Echis pyramidum viper, as well as to investigate its neutralizing ability with the venoms of several Egyptian elapids and vipers, in order to discover new targets for medical purposes and better antidotes for snakebite management.

2. Experimental

2.1. Venom

Lyophilized Echis pyramidum (Epy) venom was obtained from the laboratory animal unit of Medical Research Center, The Holding Company for Biological Prducts—Vaccines and Drugs (VACSERA), Egypt.

2.2. Chemicals

A number of fine chemicals were purchased from Sigma Chemical Co. (USA). BLUeye prestained protein ladder with broad molecular masses from 10-245 kDa were obtained from GeneDirex. The Sephacryl S-200 and CM-Sepharose resins were acquired from GE-Healthcare Life Sciences (Sweden).

2.3. Antisera

Hyper-immune antisera against venoms of Egyptian *Echis pyramidum*, *Echis coloratus*, Cerastes *cerastes*, *Cerastes vipera*, *Naja nigricollis* and *Naja haje* were previously prepared in rabbits and stored at -20 °C.

2.4. Hyaluronidase activity

Hyaluronidase activity was determined by a turbidimetric method [10]. The mixture contained 100 µl of hyaluronic acid (0.5 mg/ml) and enzyme to give a final volume of 0.5 ml of 0.2 M sodium acetate, pH 5.5 containing 0.15 M NaCl was incubated for 15 min at 37°C. The reaction was terminated by addition of 1 ml of CTAB (2.5% of cetyl-trimethyl ammonium bromide dissolved in 2% NaOH). The turbidity after 10 min was monitored at 400 nm. One unit activity corresponded to the amount of enzyme that produced a 50% reduction in turbidity under standard assay conditions.

2.5. Purification of hyaluronidase from *Echis pyramidum* venom

The purification protocol was performed using 50 mM sodium acetate buffer, pH 5.5 for column equilibration and protein elution. The lyophilized *E. pyramidum* venom (100 mg/ml) was centrifuged at 10,000 rpm for 10 min at 4 °C to remove the debris.

a. Size exclusion chromatography: The venom supernatant was applied to a Sephacryl S-200 column (1× 90 cm) and proteins were eluted by the same buffer. 4 ml fractions were collected at a rate of 24 mlh^{-1}. The absorbance of each fraction was recorded at 280 nm for protein and hyaluronidase activity was assayed using hyaluronic acid (substrate) as previously mentioned and read at 400 nm.

b. Cation exchange chromatography: The fractions having hyaluronidase activity were pooled and loaded in a CM-Sepharose column and the non-adsorbed proteins were washed by 50 mM sodium acetate buffer, pH 5.5. The adsorbed proteins (1 ml/min) were eluted by the same buffer containing serial concentrations of NaCl of 0.05 M, 0.1M, 0.2 M and 0.3 M. The fractions of each molarity were monitored at 280 nm and hyaluronidase activity was performed and monitored at 400 nm. The fractions exhibiting hyaluronidase activity were collected, concentrated and stored for characterization study at -20 °C.

2.6. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The preparation of acrylamide cross-linked with bis-acrylamide at a concentration of 30% and the electrophoresis in the Mini-Protean II Dual-Slab Cell (Bio Rad, USA) under standard parameters was carried out as described by Laemmli [11]. The obtained Epy-Hase was resolved in 10% SDS-PAGE in the absence and presence of denaturing agent (β-mercaptoethanol). The resolving gel was stained by Coomassie brilliant blue (CBB R-250) for 1 h. Excess destaining was performed with a methanol and acetic acid solution. The pre-stained protein ladder was used as a reference for molecular mass estimation.

2.7. Protein content determination

The protein was quantified according to Bradford [12] using Coomassie brilliant blue (CBB G-250) as a developing reagent and unknown protein was calculated from the graph of different concentrations of bovine serum albumin (1 mg/ml) versus absorbance at 595 nm.

2.8. Biochemical characterization of purified hyaluronidase enzyme

To investigate the properties of the purified Epy-Hase enzyme, the turbidmetric assay described by Pukrittayakamee et al. [10] was performed with slight modifications.

The optimum pH was examined by using several assay buffers of different pHs at concentrations of 0.2 M containing 0.15 M NaCl as follows: sodium citrate (3–4), sodium acetate (4-6), sodium phosphate (6-7), and Tris-HCl (7-9). The reaction mixture was incubated at 37 °C for 15 min and the enzymatic activity was read at 400 nm. The pH stability was determined by incubating the enzyme alone for 15 min in several pH

buffers (4–9) as previously prepared, followed by the adjustment of the pH of reaction mixture to 5.5 and the reaction was done as previously mentioned and the enzymatic activity was monitored.
The optimum temperature was tested by changing the assay temperature from 20 to 100 °C instead of 37 °C for 15 min and the hyaluronidase activity was determined. The thermal stability was monitored by incubating the enzyme alone at several temperatures (20–100 °C) for 15 minutes, then cooling on ice prior to the addition of substrate, and the activity was examined under the conditions previously described.
The optimal NaCl concentration on the enzymatic activity was examined individually by incubating the enzyme reaction mixture in buffer containing serial concentrations of NaCl ranging from 0.05 M to 0.5 M, and the enzymatic activity was monitored under assay conditions.

a. Inhibition assays: The investigation of various metal ions and inhibitors was assessed by pre-incubation of enzyme with several metal solutions (150mM) or inhibitors (5 mM) for 15 min at 37 °C before the addition of substrate, and the assay was performed under the described conditions. The inhibition activity was calculated by considering the enzyme alone as 100% activity.

b. Substrate specificity: The preference of the purified enzyme was evaluated by varying the substrates used in the assay (chondroitin sulfate A, B, C and D) at the same concentration and comparing them with hyaluronic acid (100% activity). The relative activity was calculated for each substrate.

c. K_m and V_{max} estimation: The assay was performed using serial concentrations of hyaluronic acid mixed with enzyme (5 U) under the assay conditions described above. The Lineweaver-Burk graph was made to figure out the values of K_m from the X axis (substrate concentration gives the half maximum velocity) and V_{max} from the Y axis (the maximum velocity of the reaction).

2.9. Immunological studies

To evaluate the cross-neutralization of purified enzyme (Epy-Hase) with different antisera, the purified enzyme was pre-incubated individually with antisera against several vipers (*Echis pyramidum*, *Echis coloratus*, *Cerastes cerastes*, *and Cerastes vipera*) and elapids (*Naja haje* and *Naja nigricollis*) venoms at a dilution of 1:10 for 30 min at 37 °C. Then, the reaction was performed as previously mentioned [10], and the enzymatic activity was recorded. The venom alone was used as a control.

2.10. Statistical Analysis

The results were presented as the mean ± SD (standard deviation) for three independent experiments. Statistical comparisons between groups using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test were made; the significance was indicated at $p < 0.05$ (CoStat version 6.3).

3. Results and discussion

Venom hyaluroniadses are considered the factors that facilitate the venom toxins to spread inside the body of the victim and potentiate the enzymatic activity of venom components. Hyaluronidases are present in elapids, vipers, and colubrids but in varied concentrations and with different structural and functional characteristics [13, 14]. Structural and functional variation of venom components in the same species and among species was observed in several studies, besides differences in the yield of enzymes due to geographical location, sex, age, and feeding of snakes of the same species [15]. In this sense, in a previous study by Wahby et al. [16], we purified and characterized the biological activity of Egyptian *Cerastes cerastes* hyaluronidase.

3.1. Hyaluronidase purification

In our current paper, the chromatographic purification of hyaluronidase from Egyptian *Echis pyramidum* venom was achieved in two consecutive steps. Four protein peaks with hyaluronidase activity detected in the second peak were obtained from size exclusion chromatography on a Sephacryl S-200 column (Fig 1A). The specific activity of pooled fractions (Hase) was estimated to be 240 U/mg over the whole venom of 80 U/mg, with a recovery of 54% and 3-fold purification (Table 1). The same protocol was performed in our previous study [16] to isolate Egyptian horned viper hyaluronidase, which showed a higher specific activity of 555 over 154 U/mg of crude venom with similar recovery% and fold purification of 51% and 3.6, respectively. The fractions detecting hyaluronidase activities after pooling were separated on a cation exchange column of CM-sepharose, and the active peak was eluted at 0.2 M NaCl with a specific activity and enzymatic yield of 610 and 38.1%, respectively, and a purification factor of 7.6 (Fig 1B and Table 1). In contrast, a higher specific activity of 4000 U/mg with 26-fold purification was achieved in Egyptian *Cerastes cerastes* hyaluronidase purification [16]. Similarly, a yield of 42.5% and a purification factor of 8.6 were obtained from the separation of hyaluronidase *Naja melanoleuca* cobra venom [17]. On the other hand, hyalronidases isolated from *Vitalius dubius* spider and *Tityus serrulatus* scorpion venoms demonstrated a yield of nearly 5% [18, 19], whereas a yield of 40% was obtained from the

Table 1. Purification of hyaluronidase from Egyptian *Echis pyramidum* venom.

Column	Total protein (mg)	Total activity (U)*	Specific activity (U/mg)	Recovery protein	Recovery Activity	Fold purification
Whole Epy venom	100	8000	80	100.0	100.0	1.0
Sephacryl S-200 Hase	18	4320	240	18.0	54.0	3.0
CM-Sepharose EpyHase	5	3050	610	5.2	38.1	7.6

*One unit activity is expressed as the amount of enzyme that hydrolyzes 50% of concentration of hyaluronic acid in turbidity under standard assay conditions.

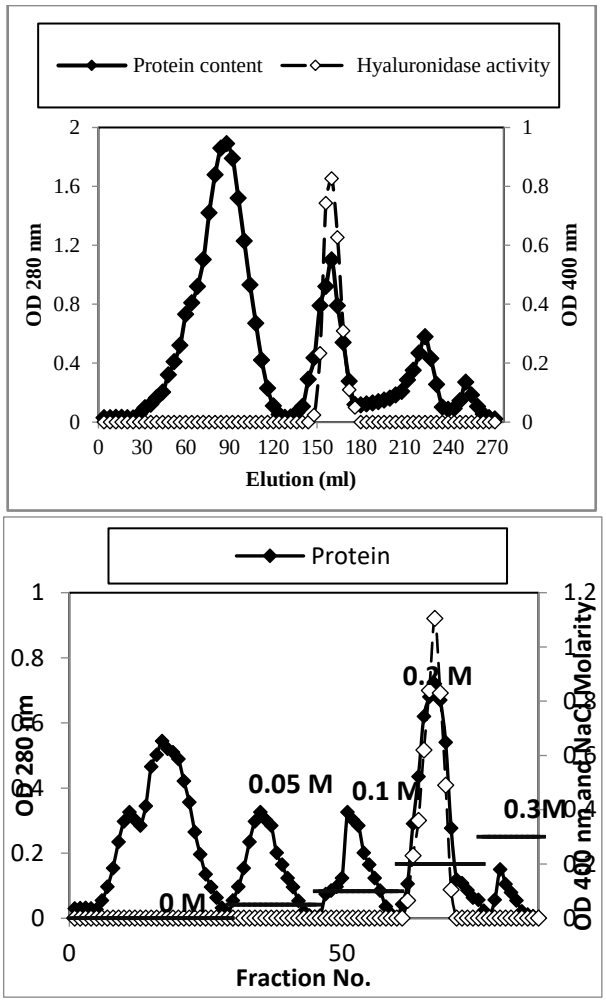

Fig 1. Chromatographic profile of Purified *Echis pyramidum* hyaluronidase on different separation columns. (A) Gel filtration of venom (100 mg protein) dissolved in 50 mM of sodium acetate buffer pH 5.5; elution was done by the same buffer on an equilibrated Sephacryl S-200 column (1 × 90 cm) with a flow rate of 24 ml/h and 4 ml volume fractions. (B) CM-Sepharose cation exchange of pooled hyaluronidase fractions (18 mg protein) with the same elution buffer containing different concentrations of NaCl molarities (0-0.3) and a flow rate of 1 ml/min.

separation of hyaluronidase from *Palamneus gravimanus* scorpion venom [20].

3.2. Molecular mass estimation

One isoform of 48 kDa was estimated for the isolated E. pyramidum hyaluronidase under reduction by β-mercaptoethanol on 10% SDS-PAGE; the same molecular mass was obtained without heating the enzyme (Fig 2). Our finding is in the range of the reported molecular mass of venom hyaluronidases of 33–110 kDa [8]. Correspondingly, the molecular masses of the hyaluronidases of Egyptian *Cerastes cerastes*, *Naja melanoleuca* and *Naja naja* snakes were 33, 54 and 70 kDa, respectively [16, 17, 21].

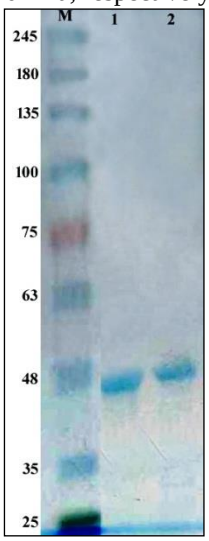

Fig 2. The 10% SDS-PAGE of 25 µg Echis pyramidum hyaluronidase under reducing (1) and non-reducing agent (2). BLUeye pre-stained protein ladder (10-245 kDa), M.

3.3. Biochemical characterization of *E. pyramidum* hyaluronidase

a. pH effect: The optimal pH of the purified enzyme was 5.5, and the enzymatic activity remained stable in the pH range of 4 to 6 (Fig 3). This matches with the reported optimal pH of snake venom hyaluronidases in the range of 3–7 [8]. The same optimal pH was reported for *C. cerastes* hyaluronidase [16] and slightly differed from *Naja melanoleuca* and *Vitalius dubius* hyaluronidases, estimated to be 6 [17, 18]. Our results may be explained by the favored degradation of hyaluronic acid by the enzyme at a pH range of 4-6,

which demonstrated that this enzyme is in the neutral active class [16].

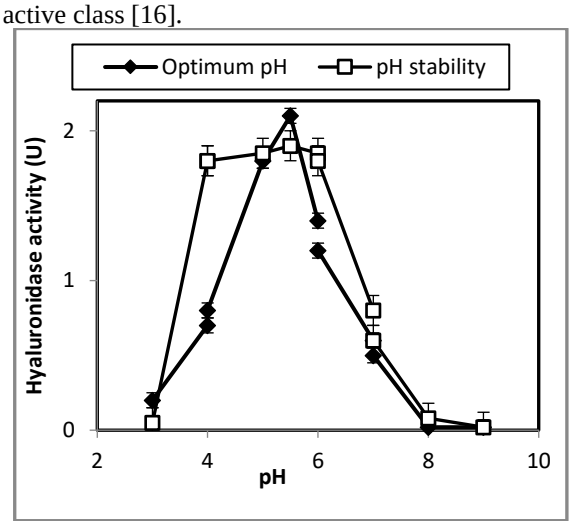

Fig 3. The pH effect on the enzymatic activity of *E. pyramidum* hyaluronidase. 0.2 M of buffers with different pHs as follows: sodium citrate (3-4), sodium acetate (4-6), sodium phosphate (6-7) and Tris-HCl (7-9). The points described means±SD (number of experiments=3).

b. Temperature effect: The enzymatic activity of the examined hyaluronidase in this study was raised with increasing the incubation temperature, with a maximal hyaluronidase activity recorded at 40 °C. After that, the activity was reduced with the increase in temperature and completely lost its activity at 90°C (Fig 4). In addition, the enzyme was stable up to 60 °C and totally lost its activity in the range of 70 to 100 °C (Fig 4). These results may be due to the denaturation of the enzyme structure with heating over 60 °C. These findings nearly match with the hyaluronidase presented in C. cerastes, *Agkistrodon contortrix*, *Vitalius dubius* and *Tityus serrulatus* venoms [16, 18, 19, 22].

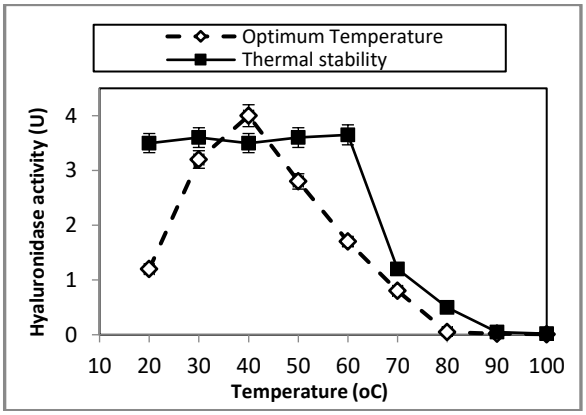

Fig 4. Temperature effect on the enzymatic activity of *E. pyramidum* hyaluronidase. The data described mean±SD of three separate reads.

c. NaCl concentrations effect:

Different molarities of NaCl concentrations (0.05–0.5 M) were added to the assay buffer, and the enzymatic activity increased with the increase of NaCl, with the highest activity recorded at 0.15 M, whereas concentrations above 0.2M had an inhibitory effect on the Epy-Hase enzyme, as shown in Fig 5. The same optimal value indicated for the hyaluronidases of Egyptian horned viper [16] and Indian cobra [21], whereas *A. blomhoffi* hyaluronidase exhibited its maximum at 0.3 M NaCl [23]. Regarding activity reduction in the absence of NaCl, the salt concentration added to the buffer is required to both diminish turbidity interference and stabilization and catalytic activation of the enzyme.

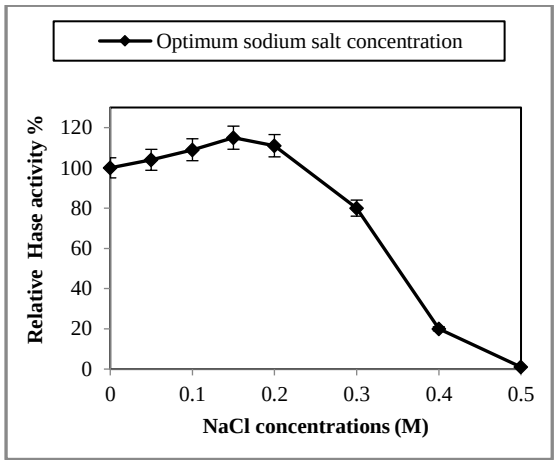

Fig 5. NaCl concentrations effect on the enzymatic activity of *E. pyramidum* hyaluronidase. Enzyme in the absence of NaCl was considered as 100% activity. The data present the means ± SD of three experiments.

d. Metal ions effect:

Further evaluation of the specific activity of the purified Epy-Hase enzyme using both selected mono- and divalent metal ions (0.15M) and inhibitors is summarized in Fig 6. The results observed that except Na^{+}, all divalent ions reduced the enzyme activity, particularly Fe^{+2}, Zn^{+2}, Cu^{+2}, Mn^{+2} and Co^{+2} (100% inhibition). K^{+}, Ca^{+2} and Mg^{+2} slightly inhibited the enzyme activity by 15, 34 and 40%, respectively (Fig 6). In addition, EDTA, o-phenanthroline and urea (5 mM), and heparin (10 U) had an inhibitory effect on the activity of the examined enzyme (Fig 6). In contrast, PMSF slightly reduced the enzyme activity. The results are somewhat in agreement with the other reported hyaluronidases [16, 24]. The findings may be attributed to metals and inhibitors interacting with the enzyme surface or substrate.

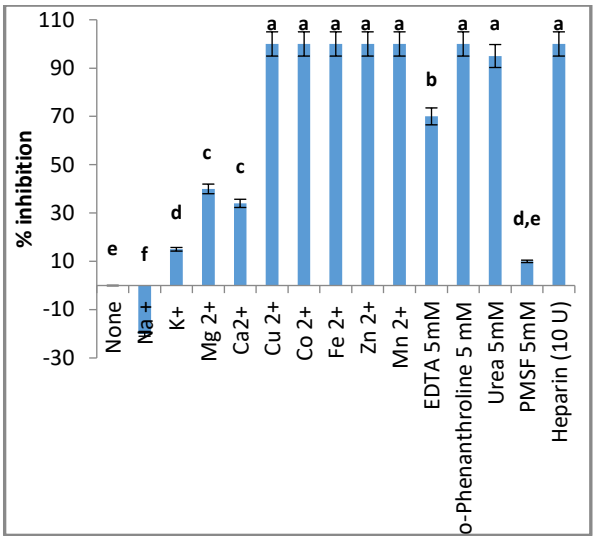

Fig 6. Selected metals (150 mM), inhibitors (5 mM) and heparin (10 units) effect on the activity of the tested enzyme. Experiments were made in triplicate and the data described as Means± SD. [a-e] Means have different letters are significantly different depending on Duncan's multiple range test.

e. Specificity towards different substrates:

In order to evaluate the preferential activity of enzyme toward the substrates, the obtained enzyme had 100% activity against hyaluronic acid alone and gave a negative effect towards the tested chondroitin sulfate salts (0% activity) (Table 2). The same specificity was observed with *C. cerastes* [16], *Agkistrodon contortrix* [22] and *Hippasa partita* [24] hyaluronidases, which are in agreement with most snake venom hyaluronidase [8], whereas *V. dubius* spider hyaluronidase exhibited a low catalytic activity with chondroitin salt [18]. In contrast, bovine testicular hyaluronidase exerts a positive activity to all chondroitin salts [8, 16]. The switch in specificity may be attributed to the change in amino acid in the catalytic position on the surface of the enzyme.

Table 2. Specificity of *E. pyramidum* hyaluronidase towards substrates

Substrates (50µg)	Relative Activity *
Hyaluronic acid	100%
Chondroitin sulfate A	0%
Chondroitin sulfate B	0%
Chondroitin sulfate C	0%
Chondroitin sulfate D	0%

*The activity with hyaluronic acid represents 100% activitiy

f. K_m and V_{max}: A further study of the action of serial concentrations of hyaluronic acid with the tested enzyme was made to investigate the K_m and V_{max} values. The K_m and V_{max} were estimated to be 20 µg/ml and 40 µg/min from the Lineweaver-Burk linear plot (Fig 7). The same affinity for hyaluronan was recorded for *C. cerastes* hyaluronidase and much higher affinity than *Crotalus durissus terrificus* hyaluronidase (164.5 µg/ml) [16, 25]. On the other hand, scorpions and spiders hyaluronidases signifying a comparatively low affinity of the hyaluronan for the enzyme active site [18–20].

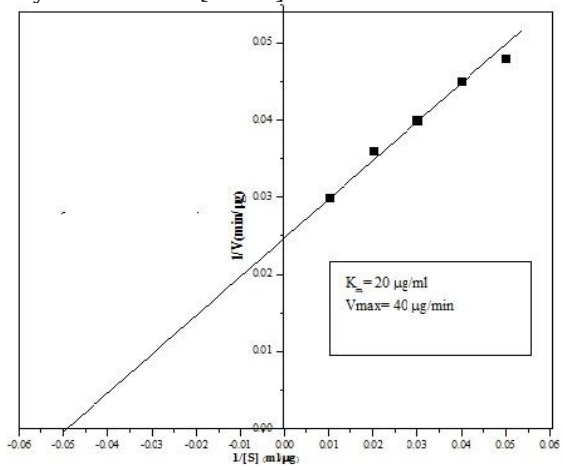

Fig 7. Linear Lineweaver-Burk plot of *E. pyramidum* hyaluronidase toward hyaluronic acid.

3.4. Cross-neutralization studies

The neutralizing action of the enzymatic activity of *E. pyramidum* hyaluronidase (Epy-Hase) by the antisera of venoms of several Egyptian snakes was examined and summarized in Fig 8. The results revealed a 100% neutralization of hyaluronidase activity by the corresponding *E. pyramidum* antisera in a dilution of 1:10. In addition, the neutralization of enzyme was decreased to a lesser extent with the other antisera of venoms of Egyptian snakes *E. colaratus*> *C. cerastes*> *C. vipera*> *N. haje*, whereas antisera of *N. nigricollis* venom exerted no neutralization in the same dilution (Fig 8). The findings demonstrated the shared epitopes presence in the tested Egyptian vipers venoms compared to spitting cobra detected no cross-reactivity with *E. pyramidum* hyaluronidase enzyme.

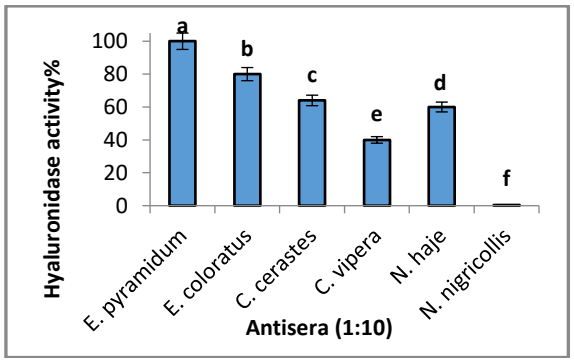

Fig 8. Neutralizing action of E. pyramidum hyaluronidase (5U) with the antisera of selected Egyptian vipers and elapids venoms at a dilution of 1:10. Three separate experiments represent means ± SD.

4. Conclusions

In our study, a novel hyaluronidase (Epy-Hase) was purified from Egyptian *Echis pyramidum pyramidum* venom and investigated for its biochemical and immunological properties. The purified enzyme has a molecular mass of 48 kDa and is highly active towards hyaluronic acid. Its activity was enhanced by Na+ ions, while other divalent ions inhibited it. In addition, the purified *E. pyramidum* hyaluronidase shares immunological determinants among the tested species, particularly vipers rather than elapids.

Overall, this study highlights the importance of characterization of enzymes from different Egyptian species and opens up the possibilities for the potential neutralization of different venoms by hyaluronidase inhibitors.

Conflicts of interest

There are no conflicts of interest to declare.

Formatting of funding sources

There are no specific grants from any funding agencies. All facilities provided by National Research Centre.

Acknowledgments

The author deeply thanks the Mol. Biology department, National Research centre for facilitate financial support to conduct the current study.

References

1. Gopalakrishnan M., Vinod K.V., Dutta T.K., Shaha K.K., Sridhar M.G. and Saurabh S., Exploring circulatory shock and mortality in viper envenomation: a prospective observational study from India. QJM: An International Journal of Medicine 111: 799-806 (2018).
2. Maduwage K. and Isbister G.K., Current treatment for venom-induced consumption coagulopathy resulting from snakebite. PLOS Negl. Trop. Dis. 8: e3220 (2014).
3. Xie C., Slagboom J., Albulescu L.O., Bruyneel B., Still K.B.M., Vonk F.J., Somsen G.W., Casewell N.R. and Kool, J., Antivenom neutralization of coagulopathic snake venom toxins assessed by bioactivity profiling using nanofractionation analytics. Toxins 12: 53 (2020).
4. Matias M.S., de Sousa B.B., da Cunha Pereira D.F., Dias E.H.V., Mamede C.C.N., de Queiroz M.R., Silva A.C.A., Dantas N.O., Soares A.M., de Oliveira Costa J. and de Oliveira F., BaltDC: purification, characterization and infrared spectroscopy of an antiplatelet DC protein isolated from Bothrops alternatus snake venom. J Venom. Anim. Toxins Incl. Trop. Dis. 23: 36 (2017).
5. Oliveira A.L., Viegas M.F., da Silva S.L., Soares A.M., Ramos M.J. and Fernandes P.A., The chemistry of snake venom and its medicinal potential. Nat. Rev. Chem. 6: 451-469 (2022).
6. Kini R.M. and Koh C.Y., Metalloproteases affecting blood coagulation, fibrinolysis and platelet aggregation from snake venoms: Definition and nomenclature of interaction sites. Toxins (Basel) 8: 284 (2016).
7. Slagboom J., Kool J., Harrison R.A. and Casewell, N.R., Haemotoxic snake venoms: Their functional activity, impact on snakebite victims and pharmaceutical promise. Br. J. Haematol. 177: 947–959 (2017).
8. Silva de França F. and Tambourgi D.V., Hyaluronan breakdown by snake venom hyaluronidases: From toxins delivery to immunopathology. Front Immunol. 14: 1125899 (2023).
9. El-Yamany M.F., Samy E.M., Salama W.H., Shaaban E.A. and Abd El-Latif H.A., Gamma irradiated protease from Echis pyramidum venom: A promising immunogen to improve viper bites treatment. Toxicon 188: 108-116 (2020)
10. Pukrittayakamee S., Warrell D., Desakorn V., McMichael A., White N. and Bunnag D., The hyaluronidase activities of some Southeast Asian snake venoms. Toxicon 26: 629–637 (1988).
11. Laemmli U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685 (1970).
12. Bradford M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal.Biochem.72: 248–254 (1976).
13. Kang T.S., Georgieva D., Genov N., Murakami M.T., Sinha M., Kumar R.P., Kaur P., Kumar S., Dey S., Sharma S., Vrielink A., Betzel C., Takeda S., Arni R.K., Singh T.P. and Kini R.M., Enzymatic toxins from snake venom: structural characterization and mechanism of catalysis. FEBS J. 278: 4544-76 (2011).
14. França J.B., Cologna C.T., Pucca M.B., Bordon K.C., Amorim F.G., Anjolette F.A., Cordeiro F.A., Wiezel G.A., Cerni F.A., Pinheiro-Junior E.L., Shibao P.Y., Ferreira I.G., de-Oliveira I.S., Cardoso I.A. and Arantes E.C., Minor snake venom proteins: structure, function and potential applications. Biochim. Biophys. Acta Gen. Subj. 1861: 824–838 (2017).
15. Girish K., Jagadeesha D., Rajeev K. and kemparaju K., Snake venom hyaluronidase: An evidence for isoforms and extracellular matrix degradation. Mol. Cell. Biochem. 240: 105–110 (2002).
16. Wahby A.F., Mahdy E.M.E., EL-mezayen H.A., Salama W.H., Abdel-Aty A.M. and Fahmy A.S, Egyptian horned viper Cerastes cerastes venom hyaluronidase: Purification, partial characterization and evidence for its action as a spreading factor. Toxicon 60: 1380-1389 (2012).
17. Shanono I.M., Muhammad Y.Y., Kankara A.I. and Kurfi, B.G., Isolation and enzymatic characterization of the snake venom hyaluronidase from Naja melanoleuca (Forest cobra) venom. J. entomol. zool. stud. 7: 1248-1252 (2019).
18. Sutti R., Tamascia M.L., Hyslop S. and Rocha-E-Silva, T. A., Purification and characterization of a hyaluronidase from venom of the spider Vitalius dubius (Araneae, Theraphosidae). J Venom. Anim. Toxins Incl. Trop. Dis. 20: 2 (2014).
19. Pessini A.C., Takao T.T., Cavalheiro E.C., Vichnewski W., Sampaio S.V., Giglio J.R. and Arantes E.C., A hyaluronidase from Tityus serrulatus scorpion venom: isolation, characterization and inhibition by flavonoids. Toxicon 39: 1495–1504 (2001).
20. Morey S.S., Kiran K.M. and Gadag J.R., Purification and properties of hyaluronidase from Palamneus

gravimanus (Indian black scorpion) venom. Toxicon 47: 188–195 (2006).
21. Girish K.S., Shashidharamurthy R., Nagaraju S., Gowda T.V. and Kemparaju K., Isolation and characterization of hyaluronidase a "spreading factor" from Indian cobra (Naja naja) venom. Biochimie 86: 193-202 (2004).
22. Kudo K. and Tu A.T., Characterization of hyaluronidase isolated from Agkistrodon contortrix contortrix (Southern copperhead) venom. Arch. Biochem. Biophys. 386: 154–162 (2001).
23. Zhong D., Meng Q., Li J., Wang Y., Zhang X., Wang S. and Zhu H., A hyaluronidase from the snake venom of Agkistrodon blomhoffii ussurensis of Changbai Mountain: isolation and characterization. Int. J. Biol. 2: 171-180(2010).
24. Nagaraju S., Devaraja S. and Kemparaju K., Purification and properties of hyaluronidase from Hippasa partita (funnel web spider) venom gland extract. Toxicon 50: 383-393 (2007).
25. Bordon K.C.F., Perino M.G., Giglio J.R. and Arantes E.C., Isolation, enzymatic characterization and antiedematogenic activity of the first reported rattlesnake hyaluronidase from *Crotalus durissus terrificus* venom. Biochimie 94: 2740-48 (2012).