Original Article

BIOCHEMICAL ISOLATION AND CHARACTERIZATION OF HYALURONIDASE ENZYME FROM VENOM OF EGYPTIAN HONEY BEE *APIS MELLIFERA LAMARCKII*

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Abstract

The hyaluronidase enzyme has been used in many such fields of medicine as ophthalmology, orthopaedia, internal medicine, gynecology, surgery, oncology and dermatology. In this study, the hyaluronidase enzyme was purified and characterized for the first time from Egyptian bee venom homogeneously using DEAE-cellulose and Sephacryl S-300 columns. Bee venom hyaluronidase specific activity was 411.7 units/mg protein with 49.9% yield and 3.23-fold purification. The molecular weight of the purified bee venom hyaluronidase native form was 37 kDa. The purified enzyme was found homogeneous on native PAGE and SDS-PAGE, with two congruent subunits of 18.4 kDa and isoelectric point (pI) of 8.6-8.8. The enzyme was found to be stable over a wide range of temperature (20-60°C) and pH (4.5-6.5), and its optimum activity at 37°C, pH 5.4 and 0.15 M NaCl. K^m for bee venom hyaluronidase was 0.029 mg/ml hyaluronic acid and its activity was elevated in presence of $MgCl_2$ and $ZnCl_2$ and lowered in presence of $FeCl_2$. Heparin inhibited the hyaluronidase enzyme noncompetitively with a *Ki* value of 2.9 units heparin and one binding site on the enzyme molecule.

Keywords: bee venom, characterization, hyaluronidase, purification

INTRODUCTION

Bee keeping in Egypt has been an important type of farming since ancient Egyptian. Bee venom products are important in the pharmaceutical industry and drug formulations. The venom produced by the Egyptian honey bee (*Apis mellifera lamarckii*) has a high biological activity and toxicity and is the best form for potential pharmacological sources (Kokot et al., 2009). Apitherapy is an alternate kind of therapy which utilizes honey bee constituents especially bee venom in treating various human illnesses. Bee venoms can be introduced into the human body manually through injection or by immediate bee sting. It includes many energetic molecules including peptides and enzymes which have a beneficial ability in the treatment of inflammations, different nervous diseases, cancer, antiviral activities and human immune disorders (Wehbe et al., 2019). Hyaluronidase, found in all kinds of animal venoms, hydrolyzes hyaluronic acid, one of the major components of extracellular matrix, and causes local tissue damage (Girish et al., 2004; Kemparaju, & Girish, 2006; Senff-Ribeiro et al., 2008; Bordon et al., 2015).

Hyaluronidase is the major allergen in the venoms of bees, hornets, wasps and scorpions that stimulate lethal systemic IgE-intermediated anaphylactic responses in humans (Kolarich et al., 2005). In microbes, hyaluronidases are virulence agents included in pathogenesis and disease advancement brought on by pathogens. The hyaluronidase degradation of the host tissue components facilitates the

infestation of pathogens. Increased permeability of tissues appears to play a principle role in wound infections, meningitis, pneumonia and bacteremia (Matsushita & Okabe, 2001; Makris et al., 2004). Additionally, hyaluronidase increases membrane permeability, so therapeutically it is used to enhance injected fluid absorption and excess-fluid resorption, increase local anesthesia effectiveness and decrease tissue destruction in subcutaneous and intramuscular injection (Farr et al., 1997).

The hyaluronidase of sperms plays an important role in fertilization success in humans and mammals (Klocker et al., 1995). Mammalian sperm hyaluronidase is located on the sperm surface, where it facilitates the penetration through the ovum and is also necessary for fertilization (Zheng et al., 2001). It can be used to decrease myocardial infarction (Muckenschnabel et al., 1996). Hyaluronidase may have essential anticancer impacts and may repress tumor growth, treatment with it was stated to block tumor cells invasion to lymph node in T cell lymphoma (Zahalka et al., 1995). It was used as a chemotherapeutic-drug additive for anticancer activity augmentation (Baumgartner, 1998). The hyaluronidases of bee venom preparations have been used successfully in medicine combined with anticancer drugs (Moga et al., 2018) and for scar resorption in treating wounds and burns of skins and mucosa surfaces (Krylov, 1995).

There have been studies on the hyaluronidase from the venom of the honey bee (Gmachl & Kreil, 1993; Topchiyeva & Mammadova, 2016), the scorpion *Palamneus gravimanus* (Morey, Kiran, & Gadag, 2006), the funnel web spider *Hippasa partita* (Nagaraju, Devaraja, & Kemparaju, 2007), the Egyptian horned viper *Cerastes cerastes* (Wahby et al., 2012), the social wasp *Polybia paulista* (Justo Jacomini et al., 2013) and the spider *Vitalius dubius* (Sutti et al., 2014). Given the limited information regarding Egyptian bee venom components and its important roles, the purpose of this study is the purification and characterization of hyaluronidase from Egyptian bee venom for uses in various therapeutic, cosmetic and pharmaceutical applications.

MATERIAL AND METHODS

MATERIALS

Venom Collection

The colonies of honeybees *Apis mellifera lamarckii* (Egyptian subspecies) were obtained from the Asuit governorate and the venom was extracted from 500 forager workers. The bees were caught at the colony entry and immobilized through rapid freezing at -20°C. Individuals were dissected; sting device and venom reservoir were removed and disrupted in tube with 2.5 ml H_2O, followed by five-minute centrifugation at 12000 xg at 4°C. The supernatants were saved and designated as the crude venoms extracts.

Chemicals

Hyaluronic acid, cetyltrimethylammonium bromide, alcian blue, phenymethylsulfonylfluoride (PMSF), 1,4 dithiothreitol (DTT), 1,10 phenanthroline, sephacryl S-300, bovine serum albumin (BSA), blue dextran, gel filtration kits for molecular weight marker, (DEAE-cellulose) diethylaminoethyl cellulose and standard markers mixture *pI* 3.6 - 9.3 for isoelectric focusing (IEF) were products of Sigma Chemical Company. Marker proteins for molecular weight SDS were the product of Pharmacia Fine Chemicals Company. Other chemicals were of analytical grade.

METHODS

Assay of hyaluronidase enzyme activity

The activity of hyaluronidase is measured turbidometrically according to Pukrittayakamee et al. (1988). The assay mixture consisted of 0.5 ml 0.2 M acetate buffer, pH 5.5 containing 0.15 M NaCl, 50 mg hyaluronic acid and venom extract. It was incubated for fifteen minutes at 37°C then stopped by adding 1 ml of 2.5% cetyltrimethylammonium bromide in 2% NaOH and absorbance was read at 400 nm. Turbidity reducing units (TRU) were defined as the amount of hyaluronidase necessary for hydrolyzing 50% of hyaluronic acid. One unit was the amount of hyaluronidase that caused 50% reduction in turbidity.

Hyaluronidase activity staining on polyacrylamide gels

Samples were electrophoresed on 7% native-PAGE that co-polymerized with 0.17 mg/ml of hyaluronic acid. Following electrophoresis, gel was incubated in 20 mM Tris-HCL buffer, pH 7.5, containing 150 mM NaCl for three hours at 37°C, followed by incubation in 100 mM sodium acetate buffer pH 4.0 overnight at 37°C. Gels were then stained for two hours at room temperature with 0.5% alcian blue in 3% acetic acid and destained in 70% acetic acid. The activity in gel was detected by a lack of color in the digested area against a blue background distinctive for undigested hyaluronic acid (Guntenhöner, Pogrel, & Stem, 1992).

Purification of hyaluronidase enzyme from bee venom

DEAE-cellulose column chromatography

Crude venom extract underwent chromatography on DEAE-cellulose column (12 cm x 2.4 cm i.d.) equilibrated previously with 0.02 M Potassium phosphate buffer pH 7.4. The adsorbed proteins were eluted with NaCl stepwise gradient (0 - 1 M) in the preceding buffer at 60 ml / hr flow rate. Fractions of 3 ml volumes were collected and those containing hyaluronidase activity were combined and concentrated.

Sephacryl S-300 column chromatography

The DEAE-cellulose concentrated proteins of the peak including hyaluronidase activity were loaded to a Sephacryl S-300 column (142 cm X 1.75 cm i.d.) that equilibrated with 0.02 M Potassium phosphate buffer pH 7.4, two ml fraction volumes were collected with 30 ml / hr flow rate.

Electrophoretic analysis

Electrophoresis was performed using 7% PAGE as described by Smith (1969). SDS-PAGE was carried out with 12% PAGE as described by Laemmli (1970). The hyaluronidase molecular weight was determined by SDS-PAGE according to Weber & Osborn (1969). Isoelectric focusing was performed as described by O'Farrell (1975). Proteins were stained with 0.25% Coomassie Brilliant Blue R-250.

Protein determination

Protein determination utilizing Coomassie Brilliant Blue G-250 relied on the existence of Coomassie dye in two different color forms, red and blue. On dye-protein binding, the red color was converted to blue. 0.5 ml of the protein reagent (Coomassie dye) was added to 0.5 ml dH20 and the protein sample. The absorbance was recorded at 595 nm against a blank control. A calibration curve was constructed using bovine serum albumin (BSA) as a standard protein (Bradford, 1976).

RESULTS

Purification of hyaluronidase from bee venom

The starting specific activity of hyaluronidase in the crude venom extract was 127.43 units / mg protein. One peak containing the hyaluronidase activity was resolved and eluted from the DEAE-cellulose column by equilibration buffer (Fig. 1a). The bee venom hyaluronidase specific activity of the pooled fraction of DEAE-cellulose peak increased 1.84 fold over crude venom with a recovery of 84.6%. Then, the chromatography of this DEAE-cellulose concentrate on Sephacryl S-300 column gave one hyaluronidase activity peak (Fig. 1b) with the elevation of specific activity to 411.7 units / mg protein showing 3.23 fold and 49.9% yield (Tab. 1). The molecular weight of the bee venom hyaluronidase enzyme was determined from its elution volume using the gel filtration column as 37 kDa.

Electrophoretic analysis of purified bee venom hyaluronidase

Purification steps samples, crude venom, DEAE-cellulose and Sephacryl S-300 portions were electrophoresed on 7% native PAGE. A single protein band matched with the hyaluronidase activity band emphasized the hyaluronidase purity (Fig. 2a and 2b). Electrophoretic analysis of purified hyaluronidase on SDS-PAGE was compared to the protein markers and its subunit molecular weight was determined to be 18.4 kDa

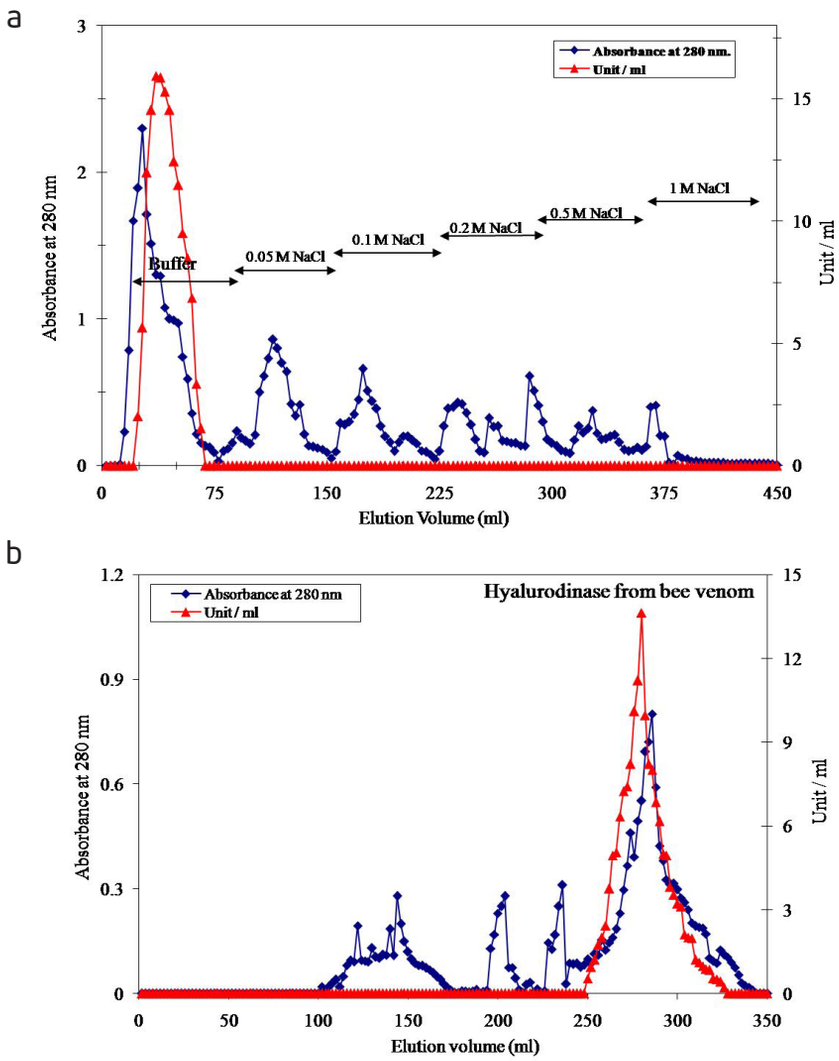

Fig. 1. (a): A typical elution profile for the chromatography of the crude honey bee venom on DEAE-cellulose column (12 cm x 2.4 cm i.d.) previously equilibrated with 0.02 M potassium phosphate buffer, pH 7.4. (b): A typical elution profile for the chromatography of the concentrated pooled DEAE-cellulose fractions containing hyaluronidase enzyme activity on Sephacryl S-300 column (142 cm x 1.75 cm i.d.) previously equilibrated with 0.02 M potassium phosphate buffer pH 7.4. The blue line indicates the protein (Absorbance at 280 nm) present on the left vertical axis, while the red line indicates the hyaluronidase activity (Unit / ml) present on the right vertical axis.

Table 1.

A typical purification scheme of honey bee venom hyaluronidase

Purification steps	Total protein (mg)	Total Activity (unit)	Specific Activity	Yield (%)	Fold Purification
Crude honey bee venom	27.2	3466.2	127.43	100	1.0
DEAE-Cellulose					
Hyalurodinase (0 M NaCl)	12.5	2932.8	234.62	84.6	1.84
Sephacryl S-300					
Hyalurodinase	4.2	1729.1	411.7	49.9	3.23

*One unit of hyaluronidase enzyme activity is identified as the amount of enzyme required to cause 50% turbidity reduction as one unit of international standard preparation. Turbidity reducing units (TRU) are expressed as the amount of enzyme required to hydrolyze 50% of the hyaluronic acid.

*Specific activity is expressed as units / mg protein.

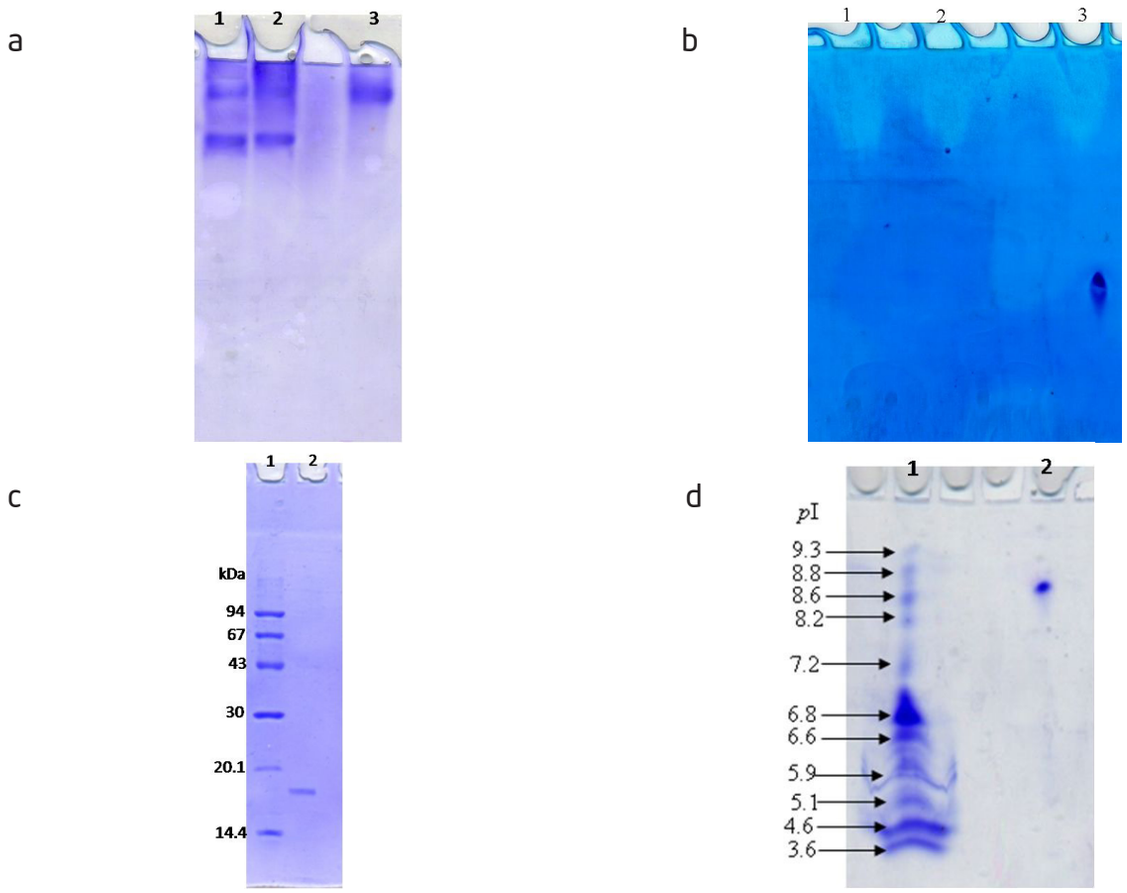

Fig. 2. (a) Electrophoretic analysis of hyaluronidase protein pattern of the different purification steps on 7% native polyacrylamide gel: (1) crude venom, (2) DEAE-cellulose fraction and (3) Sephacryl S-300 purified fraction of honey bee venom hyaluronidase. (b) Electrophoretic analysis of hyaluronidase isoenzyme pattern of the different purification steps on 7% native polyacrylamide gel: (1) crude venom, (2) DEAE-cellulose fraction and (3) Sephacryl S-300 purified fraction of honey bee venom hyaluronidase. (c): Subunit molecular weight determination by electrophoretic analysis of purified hyaluronidase on 12% SDS-polyacrylamide gel: (1) molecular weight marker proteins and (2) purified hyaluronidase. (d): Isoelectrofocusing; (1) Isoelectric point (*pI*) marker proteins and (2) the purified hyaluronidase.

(Fig. 2c). Isoelectrofocusing of bee venom hyaluronidase showed a single molecular species with an isoelectric point (*pI*) of 8.6 - 8.8 as calculated from a calibration curve (Fig. 2d).

Effect of pH, temperature, NaCl concentration and substrate concentration

The pH effect on the activity of purified hyaluronidase was examined using 0.02 M sodium acetate buffer, pH (3.2 - 5.6) and Tris-HCl buffer, pH (5.8 - 8.6). It displayed the optimum activity at pH 5.4 (Fig. 3a). The temperature's effect on hyaluronidase enzymatic activity determined after incubating the enzyme at different temperature values (20-100°C). The optimum hyaluronidase activity was attained at a temperature of 37-40°C with a relatively sharp decrease at 60-70°C and complete inactivation at 85°C (Fig. 3b). The NaCl effect on hyaluronidase enzymatic activity was determined after pre-incubating the enzyme with different concentrations (0 - 0.5 M) of NaCl. The optimum hyaluronidase enzymatic activity was attained at 0.15 M NaCl and was enhanced in the presence of 0.1 - 0.2 M NaCl and inhibited in 0.25 - 0.5 M NaCl (Fig. 3c). The Lineweaver-Burk plot of the reciprocal of the reaction velocity (1/V) and substrate concentration (1/[S]) was constructed (Fig. 3d) detecting the K^{m} value as 0.029 mg/ml of hyaluronic acid.

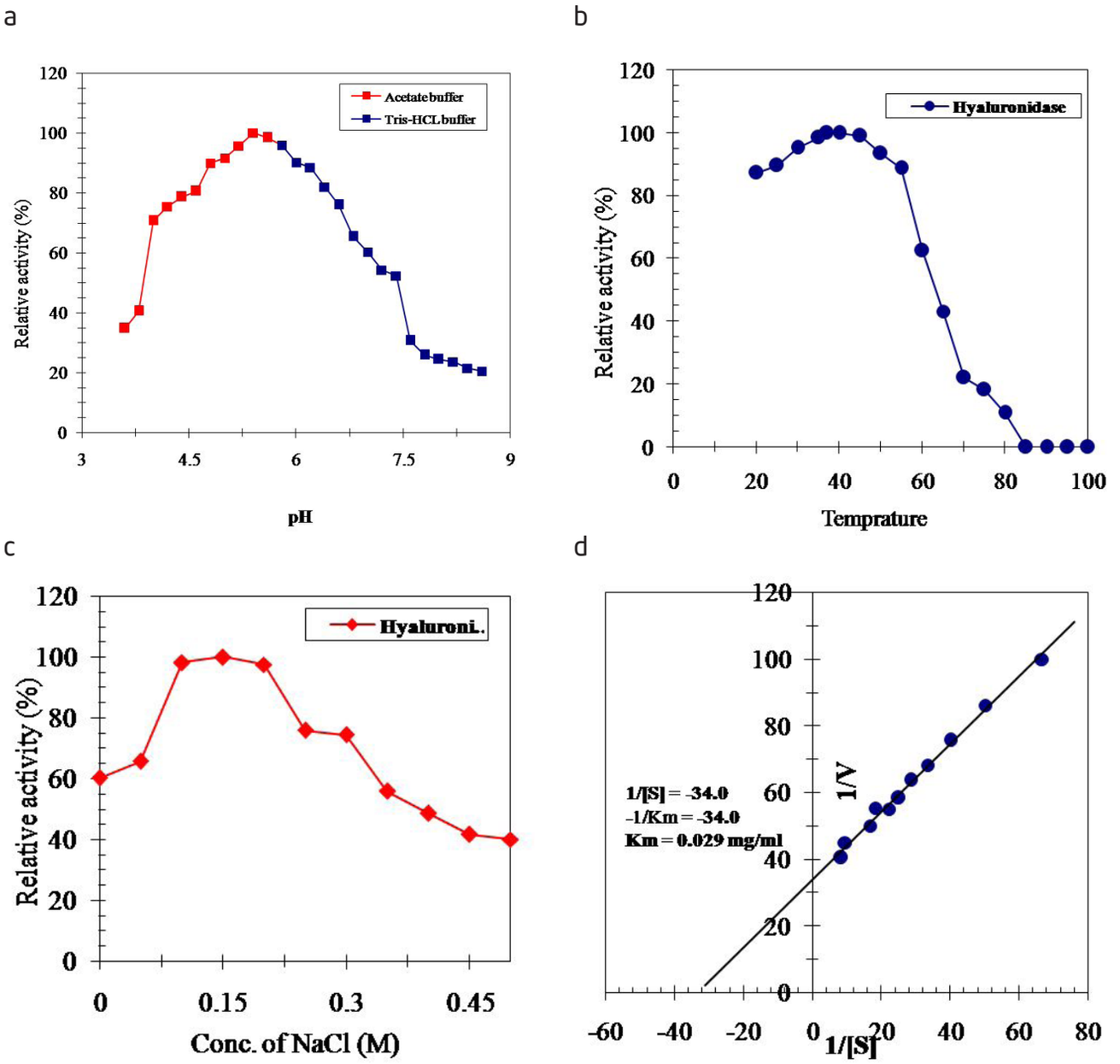

Fig. 3. (a) Effect of pH on the purified honey bee venom hyaluronidase using 0.02 M sodium acetate buffer, pH (3.2 - 5.6) and Tris-HCl buffer, pH (5.8 - 8.6). (b) Effect of temperature on the purified honey bee venom hyaluronidase. (c) Effect of NaCl concentration on the purified honey bee venom hyaluronidase. (d) Lineweaver-Burk plot relating the reciprocal of the reaction velocity of the purified hyaluronidase to hyaluronic acid concentration in mg/ml.

Effect of cations and inhibitors

The effect of divalent cations on the activity of hyaluronidase was examined after a pre-incubation with 2 and 5 mM solutions of every cation at 37°C. A control without cation was considered to be 100% activity. $ZnCl_2$ and $MgCl_2$ elevated hyaluronidase activity of while $CoCl_2$, $FeCl_2$ and $NiCl_2$ inhibited it (Tab. 2). The different inhibitors' effect on the hyaluronidase activity was examined after pre-incubating with 2 and 5 mM solutions of every inhibitor or five and ten units of heparin at 37°C. A control without inhibitor was considered to be 100% activity. Heparin, 1,10 Phenanthroline, β-Mercaptoethanol, Citric acid, EDTA and Urea were inhibitors for bee venom hyaluronidase activity (Tab. 3).

Kinetics of hyaluronidase inhibition by heparin

How different concentrations heparin affect hyaluronidase activity is examined (Fig. 4a). There was a linear relationship between the heparin

Table 2.

Effect of divalent cations on honey bee venom hyaluronidase

Reagent	Final Concentration (mM)	Residual activity (%)
Control	-----	100.0
$CaCl_2$	2.0	96.4
	5.0	89.9
$CoCl_2$	2.0	49.7
	5.0	28.1
$CuCl_2$	2.0	98.0
	5.0	93.1
$FeCl_2$	2.0	18.8
	5.0	11.2
$MgCl_2$	2.0	109.0
	5.0	115.0
$MnCl_2$	2.0	44.5
	5.0	20.7
$NiCl_2$	2.0	52.4
	5.0	27.5
$ZnCl_2$	2.0	112.0
	5.0	118.0

concentration and the inhibition of hyaluronidase activity. In the Hill plot, log Vi / Vmax – Vi values were plotted against log [I] values of the heparin and a straight line was obtained with a slope of 0.91 (Fig. 4b). The type of inhibition of hyaluronidase by heparin was examined by using Lineweaver-Burk plot (Fig. 4c). A constant amount of hyaluronidase activity was measured in the presence and absence of three various heparin concentrations with varying substrate concentrations. The plot indicated that the inhibition of hyaluronidase by heparin was non-competitive. The determination of *Ki* for hyaluronidase inhibition by heparin was obtained graphically from the reciprocal plots made at a series of different inhibitor concentrations and the slopes of these lines were graphed against the concentrations of inhibitor (Fig. 4d), and the *Ki* determined from the intercept of the X axis of the plot to be 2.9 units for heparin.

DISCUSSION

Hyaluronidase has many such medical applications as in embryo implantation, surgery, wound healing and drug delivery. This study presents a simple method for hyaluronidase purification from Egyptian honey bee *Apis mellifera lamarckii* venom using DEAE-cellulose and Sephacryl S-300 columns. This method is easier and faster than the method used by Kemeny et al, (1984) which is difficult and requires a large number of chromatographic steps. The specific activity of purified hyaluronidase enzyme is elevated to 411.7 units/mg protein which represents 3.23 fold over crude venom with 49.9% yields (Tab. 1).

Various specific activities and purification folds were reported; hyaluronidase from venom of the honey bee *Apis mellifera* L. *caucasica* of 103.1, 102.4, 99.5, 92.3, 81.0 and 69.5 units/mg (Topchiyeva & Mammadova, 2016), hyaluronidase from venom of the scorpion *Palamneus gravimanus* of 6411.7 units/mg, 25.6- purification fold and 39.2% yield (Morey, Kiran, & Gadag, 2006), from venom of the *Hippasa partita* (funnel web spider) of 37.67 units/mg, 20.4- purification fold and 8.10% yield (Nagaraju, Devaraja, & Kemparaju, 2007), from venom of the Egyptian horned viper *Cerastes cerastes* of 555 units/mg, 3.60- purification fold and 14.3% yield (Wahby et al., 2012) and from venom of the spider *Vitalius dubius* of 36 units/mg, 4- purification fold (Sutti et al., 2014). The purity of the hyaluronidase enzyme was analysed on 7% native PAGE which revealed a homogeneous preparation indicating

Table 3.

Effect of various inhibitors on honey bee venom hyaluronidase

Reagent	Final Concentration (mM)	Inhibition (%)
Control	-----	0.0
1,10 Phenanthroline	2.0	72.8
	5.0	85.1
β-Mercaptoethanol	2.0	37.6
	5.0	48.4
Citric acid	2.0	50.9
	5.0	69.3
Dithiothreitol (DTT)	2.0	15.6
	5.0	23.3
EDTA	2.0	52.2
	5.0	71.8
Heparin	5 U	47.85
	10 U	85.73
Hydrogen peroxide (H_2O_2)	2.0	30.2
	5.0	43.6
Iodoacetamide	2.0	21.3
	5.0	29.1
Potassium cyanide (KCN)	2.0	12.4
	5.0	15.8
Sodium azide (NaN_3)	2.0	16.5
	5.0	22.1
Phenyl methyl sulfonyl fluoride (PMSF)	2.0	26.7
	5.0	31.8
Reduced glutathione	2.0	8.20
	5.0	12.4
Sodium dodecyl sulphate	2.0	6.20
	5.0	9.60
Urea	2.0	78.3
	5.0	89.9

the tentative purity of enzyme preparation (Fig. 2a). The molecular weight of hyaluronidase was determined by Sephacryl S-300 column to be 37 kDa and appeared on SDS-PAGE as one band of 18.4 kDa revealing that bee venom hyaluronidase is a dimeric protein consisting of two similar subunits (Fig. 2b).

Various molecular weights for hyaluronidases were reported; 41 kDa for venom of the honeybee *Apis mellifera* (Gmachl & Kreil, 1993), 33 kDa for venom of the Egyptian horned viper (Wahby et al., 2012), 39 kDa for venom of the social wasp *Polybia paulista* (Justo Jacomini et al., 2013), 42 kDa for venom of the funnel web spider (Nagaraju, Devaraja, & Kemparaju, 2007), 43 kDa for venom of the spider *Vitalius dubius* (Sutti et al, 2014) and 52 kDa for venom of the scorpion *Palamneus gravimanus* (Morey, Kiran, & Gadag, 2006). The isoelectric point (*pI*) was determined to be 8.6-8.8 for purified bee venom hyaluronidase (Fig. 2c) which was similar to the hyaluronidase of social wasp venom at 8.14 (Justo Jacomini et al, 2013). The purified bee venom hyaluronidase optimum activity was displayed at pH 5.4 (Fig. 3a) similar to that of web spider venom hyaluronidase at pH 5.8 (Nagaraju, Devaraja, & Kemparaju, 2007). The optimum activity for purified hyaluronidase was

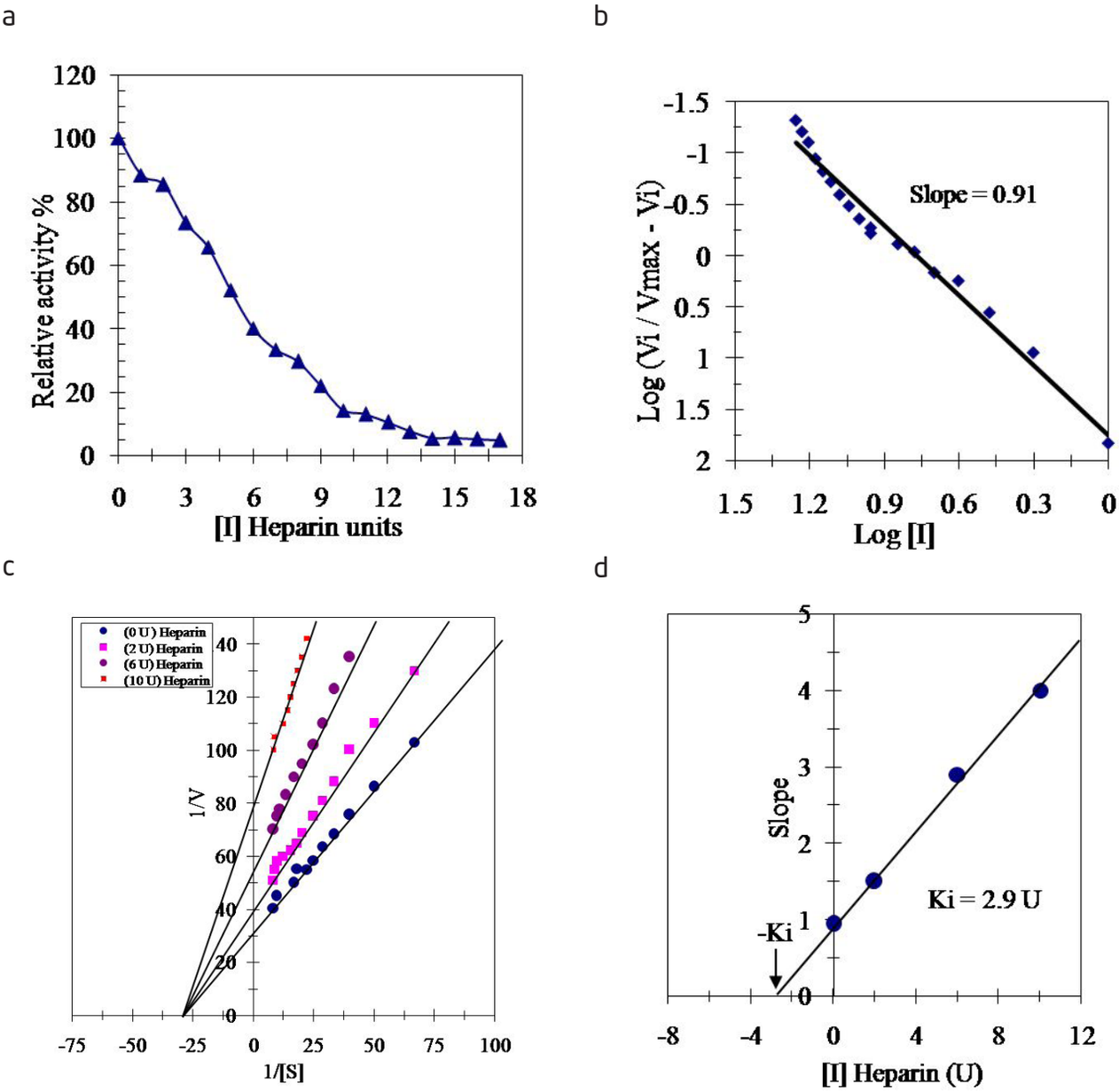

Fig. 4. (a) Inhibition of the purified honey bee venom hyaluronidase by varying concentrations of Heparin. (b) Hill plot for inhibition of the purified hyaluronidase by varying concentrations of Heparin where Vmax is the enzyme activity in absence of inhibitor, Vi is the enzyme activity in presence of inhibitor and [I] is inhibitor concentration in units of Heparin. (c) Lineweaver-Burk plots showing the type of inhibition of the purified honey bee venom hyaluronidase by Heparin. The activity of the purified hyaluronidase was measured with varying concentrations of the substrate hyaluronic acid in absence and presence of three various concentrations of Heparin. (d) Determination of the inhibition constant (*Ki*) value for the inhibition of the purified hyaluronic acid by Heparin. The plotted slope values were determined from the lines of the reciprocal plots of the different inhibitor concentrations.

attained at temperature of 37°C (Fig. 3b). This is consistent with hyaluronidase from venoms of scorpion *Palamneus gravimanus* (Morey, Kiran, & Gadag, 2006), funnel web spider *Hippasa partita* (Nagaraju, Devaraja, & Kemparaju, 2007) and spider *Vitalius dubius* (Sutti et al, 2014). The heat stability of purified bee venom hyaluronidase was up to 40°C. The optimum activity for purified hyaluronidase was reached at a NaCl concentration of 0.15 M. The activity enhanced with 0.1 - 0.2 M NaCl and inhibited with 0.25 to 0.5 M NaCl (Fig. 3c). In contrast, hyaluronidase from *Hippasa partita* (funnel web spider) venom displayed optimum activity at 0.30 M NaCl

(Nagaraju, Devaraja, & Kemparaju, 2007). The purified hyaluronidase activity was raised in the presence of $MgCl_2$ and $ZnCl_2$.

In contrast $FeCl_2$ was found to be a potent inhibitor of hyaluronidase activity, while $CoCl_2$ and $NiCl_2$ were moderate inhibitors (Table 2) which is similar to the Egyptian horned viper *Cerastes cerastes* venom hyaluronidase (Wahby et al, 2012). The purified hyaluronidase K^m value was 0.029 mg/ml of hyaluronic acid (Fig. 3d). Hyaluronidase from *Palamneus gravimanus* (Indian black scorpion) venom K_m value was 0.047 mg/ml of hyaluronic acid (Morey, Kiran, & Gadag, 2006), and hyaluronidase from Egyptian horned viper *Cerastes cerastes* venom K_m was 0.020 mg/ml of hyaluronic acid (Wahby et al., 2012). EDTA and 1,10 phenanthroline inhibited bee venom hyaluronidase activity affirming the containment of the enzyme with a metal cofactor. Dithiothreitol and β-mercaptoethanol also inhibited hyaluronidase revealing the importance of the SH group for the enzyme activity. The bee venom hyaluronidase was inhibited with PMSF indicating the involvement of a serine residue in the enzyme active site (Tab. 3). Heparin was the most potent inhibitor for purified bee venom hyaluronidase activity which is completely inhibited by 18 units of heparin (Fig. 4a). One binding site is deduced on the purified hyaluronidase molecule for heparin (Fig. 4b). Similarly hyaluronidase from Egyptian horned viper *Cerastes cerastes* is completely inhibited by 15 units of heparin (Wahby et al., 2012). Heparin inhibited bee venom hyaluronidase noncompetitively as long as it heparin did not bind to the enzyme at the catalytic site (Fig. 4c). The *Ki* value for the inhibition of hyaluronidase by heparin is 2.9 units (Fig. 4d). This is in accordance with the hyaluronidase studies reported by (Maksimenko et al., 2001).

In conclusion, this study presents for the first time a reproducible and simple procedure for the purification of well characterized hyaluronidase from Egyptian honey bee venom. The characterizations of the purified enzyme established the optimum conditions for its activity and will help in its uses in different applications with maximum efficiency.

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REFERENCES

Baumgartner, G. (1998). The impact of extracellular matrix on chemoresistance of solid tumors-experimental and clinical results of hyaluronidase as additive to cytostatic chemotherapy. *Cancer Letters*, *131*(1), 85-99.

Bordon, K. C., Wierel, G. A., Amorim, F. G., & Arantes, E. C. (2015). Arthropod venom hyaluronidases biochemical properties and potential applications in medicine and biotechnology. *Journal of venomous Animals and Toxins including Tropical Diseases*, *22*, 21, 43. DOI: 10.1186/s40409-015-0042-7

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248-254.

Farr, C., Menzel, J., Seeberger, J., & Schweigle, B. (1997). Clinical pharmacology and possible applications of hyaluronidase with reference to Hylase "Dessau". *Wiener Medizinische Wochenschrift, 147*, 347-355.

Girish, K. S., Mohankumari, H. P., Nagaraju, S., Vishwanath, B. S., Kemparaju, K. (2004). Hyaluronidase and protease activities from Indian snake venoms: neutralization by *Mimosa pudica* root extract. *Fitoterapia*, *75*(3-4), 378-380. DOI: 10.1016/j.fitote.2004.01.006

Gmachl, M. & Kreil, G. (1993). Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm. *Proceedings of the National Academy of Sciences. USA, 90*, 3569-3573.

Guntenhöner, M. W., Pogrel, M. A., & Stem, R. (1992). A substrate-gel assay for hyaluronidase activity. *Matrix*, *12*(5), 388-396. DOI: 10.1016/s0934-8832(11)80035-1

Justo Jacomini, D. L., Campos Pereira, F. D., Aparecido dos Santos Pinto, J. R., dos Santos, L. D., da Silva Neto, A. J., Giratto, D.T., ... Brochetto Braga, M. R. (2013). Hyaluronidase from the venom of the social wasp *Polybia paulista* (Hymenoptera, Vespidae): Cloning, structural modeling, purification, and immunological analysis. *Toxicon*, *15*(64), 70-80. DOI: 10.1016/j.toxicon.2012.12.019

Kemeny, D. M, Dalton, N., Lawrence, A. J., Pearce, F. L., Vernon, C. A. (1984). The purification and characterisation of hyaluronidase from the venom of the honey bee, *Apis mellifera*. *European Journal of Biochemistry*, *139*(2), 217-23. DOI: 10.1111/j.1432-1033.1984.tb07997

Kemparaju, K., & Girish, K. S. (2006). Snake venom hyaluronidase: a therapeutic target. *Cell Biochemistry and Function*, 24, 7-12. DOI: 10.1002/cbf.1261

Klocker, J., Sabitzer, H., Raunik, W., Wieser, S., & Schumer, J. (1995). Combined application of cisplatin, vindesine, hyaluronidase and radiation for treatment of advanced squamous cell carcinoma of the head and neck. *American Journal of Clinical Oncology*, *18*(5), 425- 428. DOI: 10.1097/00000421-199510000-00013

Kokot, Z. J., Matysiak, J., Kłos, J., Kędzia, B., & Hołderna-Kędzia, E. (2009). Application of Principal Component Analysis for evaluation of chemical and antimicrobial properties of honey bee (*Apis mellifera*) venom. *Journal of Apicultural Research*, *48*(3), 168-175. DOI: 10.3896/IBRA.1.48.3.04

Kolarich, D., Leonard, R., Hemmer, W., & Altmann, F. (2005). The N-glycans of yellow jacket venom hyaluronidases and the protein sequence of its isoform in *Vespula vulgaris*. *FEBS Journal*, *272*(20), 5182-5190. DOI: 10.1111/j.1742-4658.2005.04841.x

Krylov, V. N. (1995): *Bee venom. Properties*, receipt, use: Novgorod, 224.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature, 227*, 680-685.

Makris, G., Wright, J. D., Ingham, E. I., & Holland, K. T. (2004). The hyaluronate lyase of *Staphylococcus aureus* - a virulence factor? *Microbiology*, *150*(6), 2005-2013. DOI: 10.1099/mic.0.26942-0

Maksimenko, A. V., Petrova, M. L., Tischenko, E. G., & Schechilina, Y. V. (2001). Chemical modification of hyaluronidase regulates its inhibition by heparin. *European Journal of Pharmaceutics and Biopharmaceutics, 51*(1), 33-38. DOI: 10.1016/s0939-6411(00)00136-3

Matsushita, O., & Okabe, A. (2001). Clostridial hydrolytic enzymes degrading extracellular components. *Toxicon*, *39*(11), 1769-1780. DOI: 10.1016/s0041-0101(01)00163-5

Moga, M. A, Dimienescu, O. G., Arvătescu, C. A., Ifteni, P., Pleş, L. (2018). Anticancer Activity of Toxins from Bee and Snake Venom- An Overview on Ovarian Cancer. *Molecules*, *23*(3), 692-713. DOI: 10.3390/molecules23030692

Morey, S. S., Kiran, K. M., & Gadag, J. R. (2006). Purification and properties of hyaluronidase from *Palamneus gravimanus* (Indian black scorpion) venom, *Toxicon, 47*(2), 188-95. DOI: 10.1016/j.toxicon.2005.10.014

Muckenschnabel, I., Bernhardt, G., Spruss, T., & Buschauer, A. (1996). Hyaluronidase pretreatment produces selective melphalan enrichment in malignant melanoma in nude mice. *Cancer Chemotherapy and Pharmacology, 38*(1), 88-94. DOI: 10.1007/s002800050452

Nagaraju, S., Devaraja, S., & Kemparaju, K. (2007). Purification and properties of hyaluronidase from *Hippasa partita* (funnel web spider) venom gland extract. *Toxicon*, *50*(3), 383-393. DOI: 10.1016/j.toxicon.2007.04.007

O'Farrell, P. H. (1975). High resolution two-dimentional electrophoresis of proteins. *Journal of Biological Chemistry*, *25*(10), 4007-4021.

Pukrittayakamee, S., Warrel, D.A., Deasakorn, V., Mc Michael, A.J., White, N.J., Bunnag, G.D. (1988).

The hyaluronidase activities of some Southeast Asian snake venoms. *Toxicon*, *26*(7), 629-637. DOI: 10.1016/0041-0101(88)90245-0

Senff-Ribeiro, A., Henrique Da Silva, P., Chaim, O.M., Gremski, L.H., Paludo, K.S., Bertoni Da Silveira, R., ... Veiga, S.S. (2008). Biotechnological applications of brown spider (*Loxosceles genus*) venom toxins. *Biotechnology Advances, 26*(3), 210-218. DOI: 10.1016/j.biotechadv.2007.12.003

Smith, I. (1969). *Acrylamide gel disc electrophoresis*. In "Electrophoretic techniques" (Edited by Smith, I.) pp. 365-515, Academic press, New York.

Sutti, R., Tamascia, M. L., Hyslop, S., & Rocha-E-Silva, T. A. (2014). Purification and characterization of a hyaluronidase from venom of the spider *Vitalius dubius* (Araneae, Theraphosidae). *Journal of Venomous Animals and Toxins including Tropical Diseases, 20*(2), 1-7. DOI: 10.1186/1678-9199-20-2

Topchiyeva, A. & Mammadova F. Z. (2016). The seasonal activity of hyaluronidase in venom of a honey bee (*Apis mellifera* L. *caucasica*) in various regions of Azerbaijan. *Journal of Entomology and Zoology Studies, 4*(4), 1388-1391.

Wahby, A. F., Mahdy, el-SM., El-Mezayen, H. A., Salama, W. H., Abdel-Aty, A. M., Fahmy, A. S. (2012). Egyptian horned viper *Cerastes cerastes* venom hyaluronidase: Purification, partial characterization and evidence for its action as a spreading factor. *Toxicon*, *60*(8), 1380-1389. DOI: 10.1016/j.toxicon.2012.08.016

Weber, K., & Osborn, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry*, *244*(16), 4406- 4412.

Wehbe, R., Frangieh, J., Rima, M., El Obeid, D., Sabatier, J., Fajloun, Z. (2019). Bee venom: overview of main compounds and bioactivities for therapeutic interests. *Molecules, 24*, 2997. DOI:10.3390/molecules24162997

Zahalka, M. A., Okon, E., Gosslar, U., Holzmann, B., Naor, D. (1995). Lymph node (but not spleen) invasion by murine lymphoma is both CD44- and hyaluronate-dependent. *Journal of Immunology*, *154*(10), 5345-5355.

Zheng, Y., Deng, X., Zhao, Y., & Martin-DeLeon, P.A. (2001). SPAM1 (PH-20) mutations and sperm dysfunction in mice with the Rb (6.16) or Rb (6.15) translocation. *Mammalian Genome, 12*(11), 822-829. DOI: 10.1007/s00335-001-1008-3