Egypt. J. Bot. **Vol. 60**, No. 1, pp. 213-224 (2020)

Egyptian Journal of Botany
http://ejbo.journals.ekb.eg/

Characterization and Immobilization of a Novel Hyaluronidase Produced by *Streptomyces roseofulvus*

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MAXIMUM hyaluronidase production by *Streptomyces roseofulvus* S10 (LC314796) was attained when it was cultured in submerged fermentation process under favorable conditions, pH 5 at 40ºC for 6 days. Hyaluronidase was purified to its homogeneity by 9.2 fold with molecular weight of 97kDa under denaturing SDS- PAGE. Mg^{+2} exerted highly stimulatory effect on *S. roseofulvus* S10 hyaluronidase activity and was significantly reduced in presence of Mn^{+2}, Zn^{+2}, and EDTA. Optimum reaction was attained at pH 9 and the pH stability of enzyme ranged between 9-10 at 35°C. To protect the intrinsic activity and half-time of hyaluronidase, several carriers and immobilization of hyaluronidase were investigated. The immobilized enzyme had higher thermal stability than free one with T_m values; 46.1°C and 24.7°C, respectively. Maximum affinity of free and immobilized hyaluronidase was for hyaluronic acid followed by bovine albumin. Free enzyme had a high catalytic affinity of hyaluronic acid compared with immobilized enzyme. Our results demonstrated that *S. roseofulvus* S10 hyaluronidase was highly stable to pH and high temperature. These properties of long-term stability facilitate its wide range of applications.

Keywords: Hyaluronidase, Immobilization, Kinetic parameters, Purification.

Introduction

Hyaluronidases are a family of enzymes hydrolyzing hyaluronic acid (HA), which is composed of repeating disaccharide units, D-glucuronic acid and N -acetyl glucosamine (Assis et al., 2003). Hyaluronidases are classified into three different groups based on the enzyme reaction products (Meyer, 1971). The bacterial hyaluronidase (EC 4.2.2.1) is hyaluronate lyase type that catalyzes the degradation of HA by a β-elimination reaction across the β-1, 4 linkages to yield unsaturated disaccharide chains (Suzuki et al., 2002). The microbial hyaluronidases are reported to be obtained from several genera, including *Streptococcus* and *Streptomyces* (Suzuki et al. 2002).

Hyaluronidases are naturally found in mammals, insects, leaches and bacteria (Bertolami & Donoff, 1982). Several prokaryotic microorganisms produced hyaluronate lyases as *Streptomyces* (Laurent & Fraser, 1992), *Staphylococcus* (Ahmed, 2014) and *Streptococcus* (Mahesh et al., 2012) were different in substrate specificity**.** Different sources of hyaluronidases vary in their molecular weight, based on substrate specificity, pH optima and catalytic mechanism (Yingprasertchai et al., 2003).

Several reports indicated that there is a growing interest in the possible role of hyaluronan and hyaluronidase in numerous biological processes. Hyaluronidase is useful in the direct reduction of hyaluronic acid that was improperly placed during injection (Isman et al., 2008). It can act as an adjuvant, which accelerates and increases the absorption and dispersion of injected drugs (Yingprasertchai et al., 2003). Thereby hyaluronidase increases the membrane permeability in tissues, reduces the viscosity and renders the tissues more readily permeable to injected fluids (spreading effect) (Duterme et al., 2009). Also, the action of various chemotherapeutic agents in patients was enhanced by degradation of hyaluronic acid with hyaluronidases (Desoize &

#Corresponding author email: fifi.reda133@yahoo.com
Received 16/11/2018; Accepted 6/10/2019
DOI: 10.21608/ejbo.2019.6242.1248
Edited by: Prof. Dr. Wael S. El-Sayed, Faculty of Science, Ain Shams University, Cairo, Egypt.

Jardillier, 2000). Hyaluronidases are presupposed to facilitate penetration and decreasing interstitial fluid pressure, permitting anticancer agents to reach malignant cells. Moreover, it has been proposed that hyaluronidases may itself have intrinsic anticancer activity as spreading factor (Shuster et al., 2002; Chang, 2002). Also, Abdel-Aziz et al. (2019) and Saeed et al. (2020) reported that *Streptomyces globisporus* BU2018, *Ulva lactuca* and *Ulva fasciata* have distinct active metabolites being a promising source of antimicrobial and antiproliferative compounds that can be used effectively in pharmaceutical drug industry.

Interestingly, the enzyme may be recognized as a foreign agent by the body and potentially has immunogenic responses. Also, relatively high concentrations of the enzyme are needed for treatment to be clinically effective. These levels cause a wide range of toxic effects on several organs in patients. To overcome these problems, immobilized enzyme derivatives have been prepared on various supports for extracorporeal treatment (Gombotz et al., 1985). The enzymes immobilization on supportive materials have contributed largely to the success of diagnosis and enzyme therapy approaches. The recent progress in biological science has revealed many types of therapeutic proteins which able to regulate various cell functions (Costa et al., 2004). Immobilized enzymes are already being used in medical applications for clinical diagnosis and also for intra- and extracorporeal enzyme therapy (Minshull et al., 2004). As in parallel method, Gombotz et al. (1985) and Stecher et al. (1999) prepared immobilized L-asparaginase enzyme derivatives on various supports for extracorporeal treatment to overcome on several serious side effects. Blood can be passed over the immobilized enzyme, thus depleting the asparagine supply needed by the cancer cells. Due to hyaluronidase is considered to be a powerful clinical spreading factor as well as anticancer agent, the present work aimed to isolate a new hyaluronidase producing organism. Purification, characterization and immobilization of hyaluronidase enzyme were also investigated.

Materials and Methods

Isolation and identification of hyaluronidase producing organism

Actinobacteria were isolated from cultivated soils in Kafer El-Sheikh Governorate, Egypt according to Johnson et al. (1959). Each pure isolate was tested for hyaluronidase production on screening medium (modified Starch nitrate agar medium with addition of 0.2% hyaluronic acid). Preliminary assay of enzyme production was detected by appearance of clearing zone around colonies.

The most potent hyaluronidase producing isolate was identified according to identification keys (Kämpfer, 2006). Identification was molecularly confirmed by the analysis of 16S rRNA gene sequence (Altschul et al., 1997).

Hyaluronidase production by S. roseofulvus S10

The selected strain *S. roseofulvus* S10 was cultivated in Starch-nitrate broth medium supplemented with 2g/L hyaluronic acid. Enzyme production was tested under different culture conditions as different temperatures (25-60°C), different fermentation periods (3-8 days); different pH-values (pH 4-11); different carbon (sucrose, xylose, maltose, glucose, fructose, dextrose, starch and Lactose) and nitrogen (NaNO3, urea, asparagine , peptone, treptone, beef extract, casein, yeast, gelatin, NH_4Cl) sources. The enzyme activity (U/ml) and protein content (mg/ml) of crude enzyme were determined as mentioned below.

Hyaluronidase assay

Hyaluronidase was assayed by the method described by Tam & Chan (1985). Hyaluronidase activity was determined by reduction of turbidity. One ml of enzyme solution was mixed with 1ml of hyaluronic acid solution (1%) in the presence of 0.05M sodium phosphate buffer with 0.05M NaCl. This mixture was incubated at 30min at 37°C. Thereafter, 2.5ml of acidified albumin reagent (1%w/v) bovine serum albumin (BSA) fraction–V in 0.5M sodium acetate buffer was added. The mixture was shaken to insure complete mixing and incubated for 10min at 37°C. The reduction in turbidity was measured using spectrophotometer at 600 nm**.** One unit of enzyme (U/ml) is based on the change in turbidity at 600 nm under suitable conditions.

Determination of protein

Protein concentration was determined by the method of Lowery et al. (1951) using bovine serum albumin (Sigma chemical Co.) as a standard.

Purification of hyalurodinase

The crude enzyme preparation from three

liters of *Streptomyces roseofulvus* S10 culture was subjected to slow addition of 70% ammonium sulphate with continuous stirring at 4°C as mentioned by Reda et al. (2018a). The mixture was left overnight at 4°C, then centrifuged for 15min at 10000rpm at 4°C. The precipitated protein was dissolved immediately in a minimum volume of 0.01M phosphate buffer at pH 7.0. The pellets were dialyzed against phosphate buffer pH 7 using cellulose bag**.** The enzyme preparation was concentrated against polyethylene glycol crystals (PEG). The dialyzate was fractionated by DEAE-Cellulose and gel-filtration chromatography techniques.

Determination of molecular weight

The molecular weight of the purified hyalurodinase was carried out by SDS-PAGE according to the method of Laemmli (1970).

Physicochemical properties of hyalurodinase

The physicochemical properties of purified *S. roseofulvus* hyalurodinase such as optimum pH, pH stability, reaction temperature and activator/ inhibitor metal ions were determined (Reda et al., 2018b).

Immobilization of hyalurodinase

Different methods of hyaluronidase immobilization were described by Reda et al. (2018b). The different immobilization carriers as silica gel, Ca-alginate, agar–agar and polyvinyl alcohol (PVA) were tested on purified enzyme from *S. roseofulvus* S10. Immobilization efficiency (%) was expressed by the specific activity (U/mg protein) of immobilized hyaluronidase per specific activity of the soluble enzyme.

Kinetic and stability of free and immobilized hyaluronidases

The kinetic parameters of free and immobilized hyaluronidases as Michaelis-Mentel constant (Km) and maximum velocity (Vmax) were estimated using different concentrations of hyaluronic acid and bovine albumin substrates (10-50mM). Vmax and Km were calculated from Lineweaver-Burk plot.

The thermal stability of the free and Ca-alginate immobilized hyalurodinase was assessed by pre-incubation of enzyme without substrate at various temperatures (50, 60 and 70 ºC) using 0.1M phosphate buffer for different incubation periods (20-120min). The residual enzyme activity was determined intervally after 20, 40, 60, 80, 100 and 120min, for each temperature. The enzyme activity was assessed as described before.

To study the impact of storage period on hyaluronidase activity, the enzyme was stored without substrate for period extended to 90 days at -4°C. The relative stability was determined after 15, 30, 45, 60, 75 and 90 days (Sandeep et al., 2015).

Statistical analysis

Data are the mean of three replicates (n= 3) ± standard error. The attained data were examined statistically using one way analysis of variance (ANOVA). All statistical analysis were carried out using the software SPSS, version 16 (Spiegel, 1975). The significant differences among treatments were at 0.05 level of significance according to Duncan's test.

Results and Discussion

Hyaluronidases are a family of enzymes that degrade hyaluronic acid (hyaluronan). The degradation of hyaluronic acid by hyaluronidases can increase the membrane permeability (Csoka et al., 2001). So, hyaluronidases are important drug diffusion factors. The passage of antibiotics from the circulation into the synovial fluid can be accelerated by hyaluronidases and this improves the systemic bioavailability of proteins (Bocci et al., 1986; Csoka et al., 2001). In the present study, fifteen actinobacteria cultures were isolated from cultivated soil and preliminary screened for their ability to produce hyaluronidase on screening medium containing hyaluronic acid. Among 15 isolates, isolate no. 10 was selected as the highest producer organism for hyaluronidase production. This isolate was identified depending upon its morphological, biochemical and molecular characteristics. BLAST analysis indicated that the selected isolate showed 98% similarity with *Streptomyces roseofulvus* and identified as *Streptomyces roseofulvus* S10 with accession number LC314796 (Fig. 1). To optimize hyaluronidase productivity by *S. roseofulvus* S10*,* certain physical and nutritional parameters were achieved. The maximum hyalurodinase production by *S. roseofulvus* S10 was attained after 6 days and pH5 at 40°C (Fig. 2 A, B, C). The highest production of hyaluronidase and biomass for the tested strain was determined in culture supplemented with 1% starch and casein as the

best carbon and nitrogen sources, respectively (Fig. 2 D, E). These results are in agreement with Furqan et al. (2014), who reported that the maximum hyaluronidase activity and biomass of *S. aureus* was achieved when incubated at 37°C and pH 7 in presence of starch and yeast extract as carbon and nitrogen sources, respectively. Changes in temperature above or below optimum temperature leads to enzymatic denaturation and losing its three dimensional structure due to breaking of hydrogen bond and other covalent bonds (Bayramoğlu & Arica, 2008). Also, the pH value of the medium affects the solubility of the materials in the medium as well as the ionic state of materials interaction of hyaluronic acid in the growth of bacteria and in various metabolic events (Parente & Ricciardi, 1999).

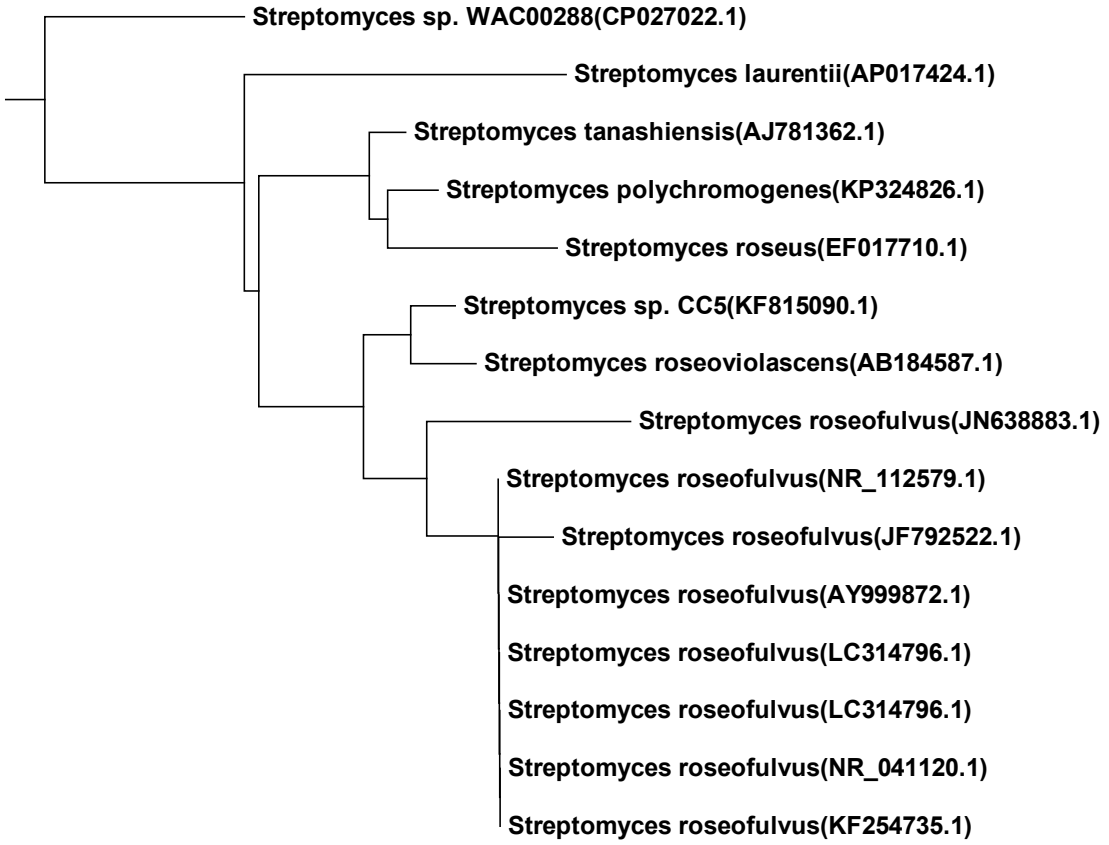
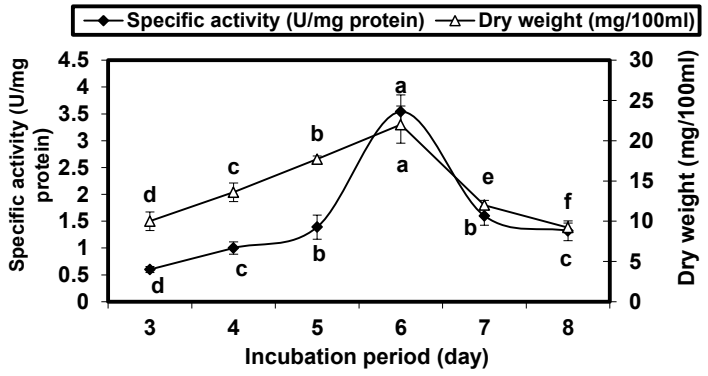


Fig. 1. Phylogenetic analysis of *Streptomyces roseofulvus* S10.



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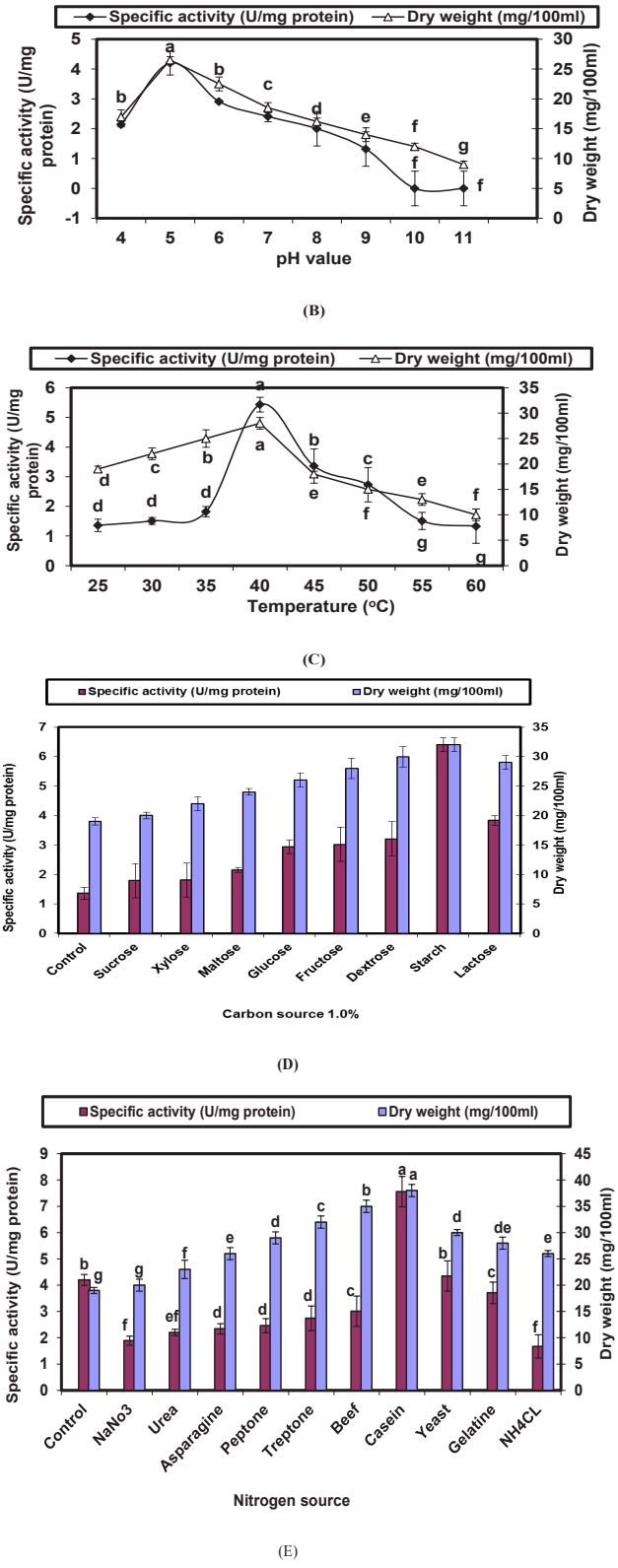


Fig. 2. Factors affecting the growth and the production of hyaluronidase by *Streptomyces roseofulvus* S10. (A) Effect of different incubation periods, (B) Effect of different pH values, (C) Effect of different incubation-temperature °C, (D) Effect of different carbon sources and (E) Effect of different nitrogen sources [*Values represent the mean of three replicates. Different letters indicate statistical differences at P< 0.05 by Duncan's test. Error bars are standard deviation of the mean].

Hyaluronidase was purified to apparent homogeneity from the liquid state cultures of gel filtration by about 34.2 Umg^{-1} protein and 9.2 fold with 31.4 yield (Table 1). Kaneko et al. (1967) reported that *Streptomyces hyalurolyticus* hyaluronidase had a specific activity of 18,400Umg^{-1} protein yielding 9.27% of the total amount presented by CM cellulose column chromatography and Sephadex G_{100} gel filtration. Guo et al. (2014) reported that the purity of hyaluronidase produced by *Bacillus* sp. A50 was 21 fold of the starting culture medium and a final yield of 25.38% after salting out, ion-exchange and gel filtration chromatography. Bakke et al. (2010) reported that *Penicillium* sp. HAase-PP was purified by a combination of anion exchange chromatography, ammonium sulfate precipitation, and hydrophobic interaction chromatography.

The molecular weight of the purified hyaluronidase was estimated by denaturing SDS-PAGE with 97kDa (Fig. 3). Hynes & Walton (2000) reported that the molecular weights of two enzymes from *Streptomyces* sp. were 77 and 84kDa.

Some physicochemical properties of the purified hyaluronidase were detected. Concerning the effect of different metal ions on purified hyaluronidase of *S. roseofulvus* S10*,* it could be observed that, Mg^{2+}exerted the highest stimulatory (122%) effect to occupy the first rank among all tested compounds followed by Ca^{+2} and Ba^{+2}. The enzyme activity was decreased by Na^{+}, Zn^{+2}, and Mn^{+2} and the metal chelating agent EDTA decreased the enzyme activity (Fig. 4 A). Our results are in accordance with Guo et al. (2014) who reported that metal ions including Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+} and Ba^{2} have positive effects on the hyaluronidase activity of *Bacillus* species and $CaCl_2$ solution which gave the highest activity. The activity of HAase-*B* was decreased by metal ion chelators (EDTA, EGTA and DFO), up to 100mM.

The optimum pH value of the maximum enzyme activity of *S. roseofluvus* S10 (3.91U/ml) was 9 and the pH stability within range 9 to 10 (Fig. 4 B, C). These results indicated that the enzyme has a broad basic pH range. Kaneko et al. (1967) revealed that the hyaluronidase from *S. hyalurolyticus* was most stable at pH range 4-11 and hyaluronidases from *Streptococcus hemalyticus, S. aureus* and *Cl. welchii* were most stable at pH range 5-8.

Moreover, Fig. 4 D shows that the optimum reaction temperature for maximum hyaluronidase activity (4.99U/ml) was recorded at 35°C. Also, Guo et al. (2014) reported that an optimal temperature of *Bacillus* sp.A50 hyaluronidase was attained at 44°C.

The immobilized enzymes onto solid insoluble supports can be reused in continuous processes and facilitate the economic recovery of the enzyme after the reaction without any significant loss to its biochemical properties (Singh et al., 2013). The present work was extended to elucidate the immobilization of hyaluronidase on solid carriers (Table 2). The maximum enzyme immobilization yield (67.2%) was observed with Ca-alginate followed by agar-agar and Silica gel respectively. The lowest immobilization yield was detected using polyvinyl alcohol. The physical adsorption of enzyme via polyvinyl alcohol PVA exhibited a relatively lower activity, comparing to the entrapment methods. These results revealed that, entrapment immobilization of hyaluronidase was the most potent method, among the various tested mechanisms. Ca-alginate enzyme was used for further comparative characterization of *S. roseofulvus* S10. Ortega et al. (1998) reported that entrapped β-glucosidase of *Saccharomyces cerevisiae* in alginate and polyacrylamide retained its activity of about 66.0%. Ahmed et al. (2013) reported that *Aspergillus niger* β-glucosidase immobilized onto sponge showed the highest immobilized yield compared with its immobilized in agarose.

The thermal stability of free and immobilized hyaluronidase from *S. roseofulvus* S10 was evaluated by its pre-incubation without substrate at different temperatures (50, 60 and 70°C) for 20, 40, 60, 80, 90 and 120min. From the profile of thermal stability (Fig. 5), it was found that half-life time ($T_{1/2}$) and half-life temperature (Tm) were more increased by immobilization than free enzyme, while the thermal inactivation rate (Kr) values were more decreased upon immobilization than free enzyme. As well as, the acquired structural stabilizing enzyme was effected by immobilization to the free hyaluronidase which was clearly revealed from the half-life temperature (Tm). The Tm value for the free hyaluronidase and Ca-alginate hyaluronidase were 24.7 and 46.1°C, respectively. These results indicated that, the immobilized enzyme was highly stable in comparison to the free form (Ahmed et al., 2013; Reda et al., 2018 a,b). Ahmed et al. (2007) reported that the immobilized

S. cerevisiae α-Glucosidase on Sepabeads EC-EA exhibited a high degree of thermoprotection at 45°C for 1hr, whereas the free enzyme lost its original activity completely under these conditions. This is probably due to the formation of multiple covalent bonds between β-glucosidase and the support which reduce its conformational flexibility and thermal vibration. This process prevents the immobilized protein from unfolding and denaturing (Wang et al., 2009; Figueira et al., 2011).

The kinetic parameters of *S. roseofulvus* S10 hyaluronidase as Vmax, Km and Kcat were estimated using different concentrations of hyaluronic acid and bovine albumin substrates (10-50mM). From Lineweaver-Burk plot (Fig. 6), the kinetic parameters of free *S. roseofulvus* S10 hyaluronidase for hyaluronic acid (Vmax= 1.58U/mg and Kcat= 16.2min^{-1}) was more than the immobilized one (Vmax= 1.055U/mg and Kcat= 15.9min^{-1}). Meanwhile, Km of the immobilized enzyme (0.21mM) was more than the free (0.11mM) (Table 3). This indicated that the affinity of free enzyme to hyaluronic acid was decreased upon immobilization. Chang et al. (2008) reported that the Vmax of immobilized β-glucosidase on chitosan-clay composite is smaller than that of free enzyme. This is because the immobilization of enzyme on a carrier treated with glutaraldehyde reduced enzyme activity and may inhibit substrate diffusion to the enzyme. Ahmed et al. (2013) reported that Km value of the free and immobilized β-glucosidase of *A. niger* was 40.0 and 46.51mM, respectively.

Storage stability of *S. roseofulvus* S10 hyaluronidase in free and immobilized form is one of the major concerns. The relative stability of free and immobilized enzymes slightly decreased with increasing the storage periods at 4°C (Fig. 7). The immobilized enzyme was more stable than free enzyme and maintained 37% of its activity after 90 days of storage. The purified hyaluronidase enzyme from *Palamneus gravimanus* was found to be stable at -20°C for 2 months in the presence of 0.15M NaCl (Morey et al., 2006). Klein & Langer (1986) reported that an immobilized enzyme should have long-term stability and unaltered sensitivity and biological activity after attachment to the matrix compared with free enzyme when it was used as therapeutic purpose.

TABLE 1. Purification profile of hyaluronidase produced by *Streptomyces roseofulvus* S10.

Purification steps	Enzyme activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold	Yield (%)
1- Culture filtrate (crude extract)	81.6±2.16 a	22.0±0.5 a	3.7±0.097d	1±0.026 d	100±2.65 a
2- Precipitation by $(NH_4)_2SO_4$ (70%)	55.8±1.48 b	7.1±0.19 b	7.8±0.206 c	2.1±0.056 c	62.4±1.65 b
3-DEAE-Cellulose	37.2±0.98 c	2.5±0.07 c	14.9±0.394 b	4.0±0.106 b	45.6±1.21 c
4- Sephadex G100	25.6±0.68 d	0.75±0.02 d	34.2±0.905 a	9.2±0.243 a	31.4±0.83 d

- Data are the mean of three replicates ± standard error (n= 3).
- Values within each column marked with different letters indicate significant differences among treatments at 0.05 level of significance according to Duncan's test.

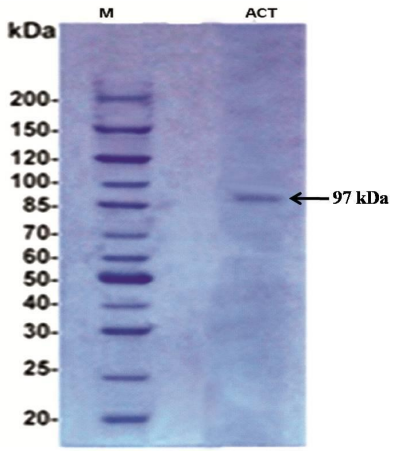
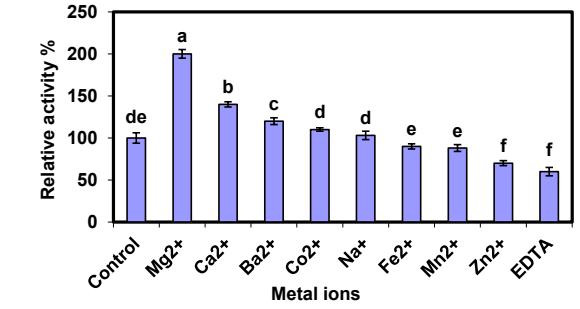


Fig. 3. SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) of purified hyaluronidase from *Streptomyces roseofulvus* S10, Lane M (marker protein), Lane ACT (purified hyaluronidase).



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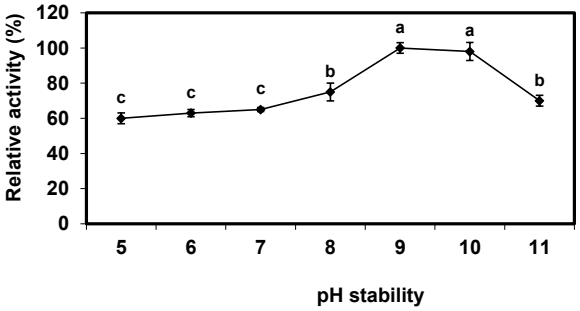

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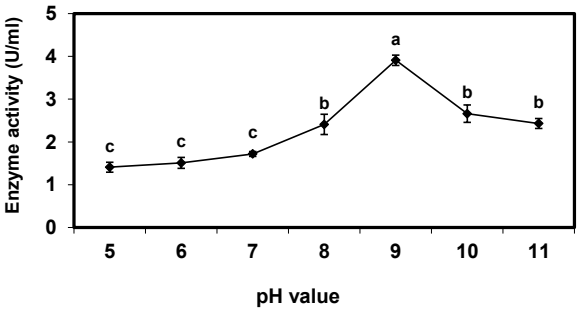

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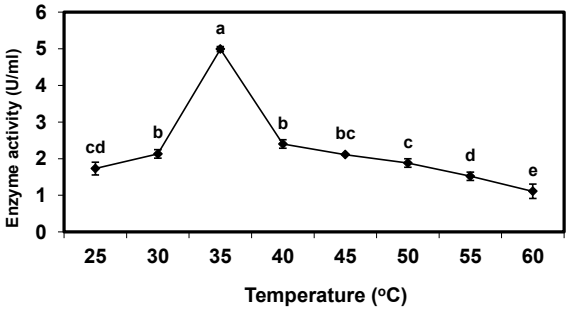

(D)

Fig. 4. Characterization of purified hyaluronidase produced by *Streptomyces roseofulvus* S10. (A) Effect of different metal ions. The enzyme was pre-incubated with different metal ions (10mM) separately for 30min before adding Hyaluronic acid. The relative activity was expressed as the percentage ratio of the activity after incubation at 37˚C for 30min, (B) pH stability. Stability of hyalurodinase was examined after pre-incubation of the enzyme for 2hrs at pH from 5.0-11.0. (C) Effect of different pH values (5-11) and (D) Effect of different temperatures (25-60˚C) [*Values represent the mean of three replicates. Different letters indicate statistical differences at P< 0.05 by Duncan's test. Error bars are standard deviation of the mean].

TABLE 2. Immobilization of the purified *Streptomyces roseofulvus* S10 hyaluronidase.

Immobilization Method	Carrier	Immobilized activity (U)	Bounded protein (mg)	Specific activity (U)	Immobilized yield %
Entrapment	Ca-Alginate	20.9±0.55 a	0.91±0.024 a	22.9±0.61 a	67.2±1.78 a
	Agar-Agar	18.1±0.48 b	0.83±0.022 b	21.8±0.58 a	63.9±1.69 a
Physical adsorption	Silica gel	12.2±0.32 c	0.74±0.019 c	16.5±0.44 b	48.4±1.28 b
Ionic binding	PVA	11.6±0.31 c	0.72±0.019 c	16.1±0.43 b	47.2±1.25 b

- Free hyaluronidase total protein content= 0.75mg total enzyme activity= 25.6U, specific enzyme activity= 34.2U/mg protein.
- Immobilization yield was expressed by the specific activity of immobilized enzyme by that of free enzyme x 100.
- Data are the mean of three replicates ± standard error (n= 3).
- Values within each column marked with different letters indicate significant differences among treatments at 0.05 level of significance according to Duncan's test.

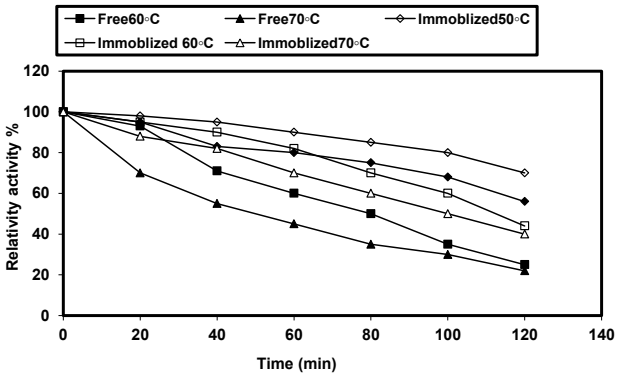

Fig. 5. Thermal stability profile of free and immobilized hyaluronidase [After incubation of enzyme in different temperature (50, 60 and 70ºC) at various periods (20-120min), the residual activity was determined by the standard assay method (Free enzyme (F): 50ºC: y= -0.6x + 100.54, 60ºC: y= -0.37x + 99.468, 70ºC: y= -0.5122x + 85.286; Immobilized enzyme (I): 50ºC: y= -0.312x + 101.61, 60ºC: y= -0.4071x + 100.12, 70ºC: y = -0.521x + 98.786].

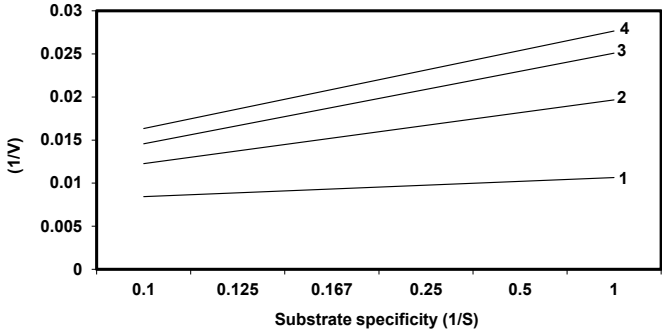

Fig. 6. Substrate specificity of free and immobilized *Streptomyces roseofulvus* S10 hyaluronidase [Km values (Lineweaver-Burk plot) were calculated by fitting the 1/S of substrate versus the 1/activity of the free enzyme; (1) hyaluronic acid: y= 0.096x + 0.538, (3) albumin bovine: y= 0.077x + 0.645; the immobilized enzyme;(2) hyaluronic acid: y= 0.116x + 0.692, (4) albumin bovine: y= 0.1152x + 0.645].

TABLE 3. Kinetics parameter of free and Ca-alginate immobilized hyaluronidase of *Streptomyces roseofulvus* S10.

Substrate	Free enzyme		Immobilized enzyme	
	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)	K_m (mM)
Albumin bovine	2.88±0.076*	0.17±0.005*	1.85±0.049*	0.33±0.009*
Hyaluronic acid	1.58±0.042	0.11±0.003	1.055±0.028	0.21±0.006

- Data are the mean of three replicates ± standard error (n= 3).
- * Indicates significant difference between treatments.

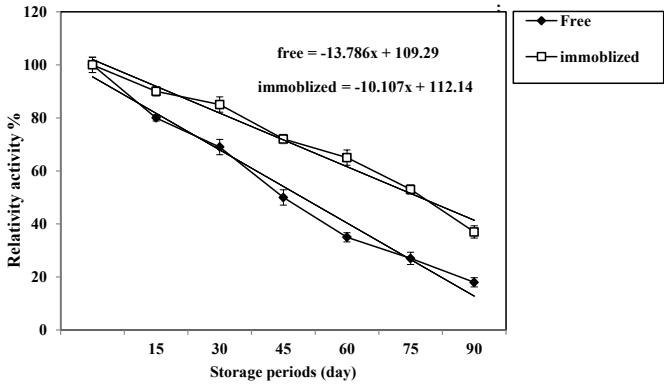

Fig. 7. Storage stability of free enzyme and immobilized hyaluronidase of *Streptomyces roseofulvus* S10.

Although purified enzymes are now available for some enzyme deficiency diseases, there are many problems in delivering the enzyme to the required site under such conditions. Cancer therapy based on the delivery of enzymes to tumor sites has advanced in several directions since antibody-directed enzyme/prodrug therapy was first described (Bagshawe et al., 1999). Therefore, these results indicate that the immobilized hyaluronidase enzyme can be utilized as an adjunct in cancer treatment, and to enhance penetration of chemotherapeutic drugs.

Conclusion

Finally, it could be concluded that both free and immobilized hyaluronidases from *Streptomyces roseofulvus* S10 (LC314796) exhibited a highly pH and thermal stability which facilitated their medical application as antitumor agents. To the best of knowledge, this is the first study declaring the immobilization of hyaluronidase and improved the stability of enzyme for next therapeutic purpose.

Acknowledgements: The authors would like to thank Dr. Wesam A. Hassanein and Dr. Ahmed A. Shindia for their revision of this study.

Conflict of interest: There is no conflict of interests regarding the publication of this paper.

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