PURIFICATION, CHARACTERIZATION, AND EVALUATION OF FIBRINOLYTIC ACTIVITY OF STAPHYLOKINASE FROM LOCALLY ISOLATED *STAPHYLOCOCCUS AUREUS* GH38

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ABSTRACT

This study was aimed to purify, characteristic, and fibrinolytic activity of staphylokinase (SAK), is an enzyme activates plasminogen to form plasmin, which digest fibrin clots that cause thrombosis clot. Staphylokinase was purified from local isolate *Staphylococcus aureus* GH38 by ammonium sulfate precipitation at 70% saturation followed by ion exchange chromatography (CM-Cellulose) with a purification fold 2.73, and 1.53, and recovery 72.1, and 33.11% in wash and elution steps respectively. The partially purified enzyme was high activity at 40°C with pH 7 and the enzyme retained 100% of its activity at 35°C with pH 7. The activity of an enzyme increased by its treatment with calcium and sodium chloride, while the activity affected when incubated with mercury, silver, and iron chloride. The enzyme have high effective against thrombus (blood clot), which encourage the use of enzyme in the treatment as therapeutic agent to remove clots formed in the human body.

Keywords: Thrombosis, Ion exchanger, Chemical compounds

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تنقية، توصيف وتقييم النشاط الحال للخثرة للستافلوكاينيز المنتج من العزلة المحلية *Staphylococcus aureus* GH38

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المستخلص

تهدف هذه الدراسة الى تنقية وتوصيف إنزيم الستافلوكاينيز حيث يعد من الانزيمات المنشطة للبلازمينوجين وتكوين البلازمين الذي يعمل على تحليل الفايبرين المسببة للخثرة (الجلطة). نقي الستافلوكاينيز من العزلة المحلية لبكتريا *S.aureus* GH38 بخطوتي الترسيب بكبريتات الامونيوم بنسبة تشبع 70% والتبادل الايوني باستعمال المبادل الايوني كاربوكسي مثيل سليلوز (CMC) وتم الحصول على عدد مرات تنقية 2,73و 1.53 وحصيلة انزيمية 72.1% و33.11% لخطوتي الغسل والاسترداد على التوالي. اتصف الانزيم المنقى جزئيا بفعالية عالية في درجة حرارة 40م ورقم هيدروجيني 7 واحتفظ الانريم بكامل فعاليته عند درجة حرارة 35م ورقم هيدروجيني 7. أزدادت فعالية الانزيم بمعاملته مع كلوريدات الكالسيوم والصوديوم بينما تأثرت فعاليته عند حضنه مع كلوريدات الزئبق والفضة والحديد. امتلك الانزيم فعالية تحللية عالية تجاه خثرة (جلطات الدم) مما يشجع استغلال الانزيم في العلاج (Therapeutic agent) لأزالة الجلطات المتكونة بجسم الانسان.

كلمات مفتاحية: الجلطة، كروماتوغرافيا التبادل الايوني، مركبات كيميائية

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INTODUCTION

Staphylokinase (SAK) known as one of the staphylococcal extracellular proteins, converts plasminogen, a precursor of proteolytic enzyme, into an active enzyme, as well it a plasminogen activator protein, which a small protein molecule, made up of 136 amino acids belong to the family of staphylococcal proteins. It was secreted by many *Staphylococcus aureus* strains as one of virulence factor (29). Staphylokinase currently used in clinical medicine as a therapeutic agent in the treatment of thromboembolic blockages, including coronary thrombosis (5, 10). The main reason for selecting the enzyme because staphylokinase could be relatively inexpensive when compared to other thrombolytic agents and an alternative cure against cardial blood clot (11). Apparently, the only limitation with this thrombolysis it was bacterial origin that could raise undesired immune responses (21). This study was aimed to partial purification and characterization of staphylokinase from locally isolate of *Staphylococcus aureus* GH38 as well as therapeutic agent to remove blood clots (14).

MATERIALS AND METHODS

Isolation and screening for staphylokinase producing *Staphylococcus aureus* GH38

The bacterial strain, *S. aureus* GH38 isolated from a patient suffering from burns was used as the source for enzyme production. The isolated strain was characterized by cultural, morphological and biochemical tests. *S. aureus* GH38 was screened for staphylokinase production by submerged fermentation.

Enzyme precipitation by ammonium sulfate

The crude enzyme supernatant was fractionated with ammonium sulfate by 30%, 40%, 50%, 60%, 70%, and 80% saturation at 4°C, the enzyme solution was mixed gently with ammonium sulfate for 45 min. Then was centrifuged at 10,000 rpm for 20 min., the supernatant was discarded and the precipitate was dissolved in 10 ml of 0.1 M phosphate buffer solution (1). The staphylokinase product from the bacterial culture was preliminary concentrated by salt precipitation and then purified by ion exchange chromatography.

Ion exchange chromatography

The partially purified enzyme was then applied to cation exchange chromatography column packed with CM-Cellulose (15×2.5 cm). Phosphate buffer 5mM (pH 7.0) was used in wash step. The enzyme solution was passed through the column at a flow rate 30 ml/ hr, 3 ml for each fraction. The bound proteins were eluted with a linear gradient of NaCl (0-1.0 M) in 5mM phosphate buffer.

Determination of staphylokinase activity

Staphylokinase activity was determined using casein digestion method, which based on the amount of enzyme that caused an increase of 0.01 in the absorbance at 275 nm. within one min (25).

Estimation of protein by Bradford method

The total protein content of fibrinolytic enzymes were determined by spectrophotometer at 595 nm. Bovine serum albumin (BSA) was used as standard protien (7).

Characterization of partially purified enzyme

Effect of pH on the activity and stability of enzyme

The optimum pH values on partially purified SAK was determined by casein as substrate prepared in acetate buffer (pH 4, 4.5, 5, 5.5, and 6), phosphate buffer (pH 6.5, 7, and 7.5), and Tris-HCl buffer (pH 8, 8.5, and 9).

pH stability of an enzyme was also determined, equal volume from partially purified enzyme was mixed with the different buffers at a ratio of (1:1) and incubate the mixture in a water bath at 40 ˚C for 15 min, then the samples were transferred directly to an ice bath. Followed with all activity measurement procedures to determine the residual activity % (15).

Effect of temperature on the activity and stability of enzyme

Optimum temperature was determined by measuring the partial purified staphylokinase activity in different temperature range 30-60°C. Temperature stability was also measured by incubating the partially purified enzyme at different temperatures (30-70°C), and then the samples were transferred directly to an ice bath. Followed with all activity measurement procedures to determine the residual activity % (15).

Effect of some chemical compounds on enzyme activity

The effects of some divalent cations on the activity of SAK enzyme were also analyzed. KCl, $CaCl_2$, $CoCl_2$, $MnCl_2$, $MgCl_2$, $HgCl_2$,

NaCl, AgCl, $FeCl_3$, ethylene diamine tetraacetic acid (EDTA) , 2-Mercaptoethanol, and phenylmethyl sulfonyl fluoride (PMSF) were used in this study at concentration 5, and 10mM (27).

Blood clot lysis

In vitro blood clot lysis method used to determine the fibrinolytic activity of the purified SAK was confirmed by modified Holmstrom method. The clot was degraded by two methods; a slide method by a drop of blood (100µl) on slide was left for 45 min followed by dropping 100µl enzyme (22), and glass test tubes method done by placing the blood sample (200µl) left at room temperature for 45 min. After that, the blood clot was treated with purified enzyme using different units of enzyme: 0.175U, 0.35U, 0.525U and 0.7U (24).

RESULTS AND DISCUSSION

Purification of staphylokinase

Staphylokinase from *Staphylococcus aureus* GH38 was partially purified with ammonium sulfate precipitation its considered as an important methods for purification of enzymes (17). The results observed that 77.88% of SAK enzyme was precipitated with purification fold 2.92 in the saturation ratio of 70%. Followed by loaded onto a CM-Cellulose ion-exchanger. Ion exchange chromatography is a technique used to separate organic compounds based on their charge, size, shape and their solubility (19). This procedure involves a mobile phase and a stationary phase. Proteins contain many ionizable groups on the side chains of their amino acids as well as their amino and carboxyl- termini (16). CM- Cellulose is a weak cation exchanger; it will bind to the opposite charge of the protein of interest (13). The presence of one peak of protein and activity in wash step as shown in Figure 1 with a purification fold 2.73 and yield reached to 72.1%, and two peaks of protein with one peak for activity in elution step with a purification fold 1.53 and yield reached to 33.11% as given in Table 1. The presence of activity peak in wash and elution steps indicated that the staphylokinase from *S.aureus* GH38 isolate had isoenzymes which have different net charge and pI; in another word, the staphylokinase purified from wash step had a negative charge similar to ion exchange resin and had a positive charge at elution step. In a previous study, separation of staphylokinase from *Staphylococcus aureus* VITSDVM7 was precipitated with 75 % $(NH_4)_2So_4$ and showed maximum level of enzyme activity on which specific activity 1035 U/mg protein and purified on CM-cellulose column; the purified staphylokinase revealed its corresponding purification fold of 1.5 with a total yield of 12.8% (18). In addition, it was agreed with Adam *et al*. (3) used CM-Cellulose for purification of SAK from *Staphylococcus* spp. with purification 1.5 fold and 12.8%.

Table 1 Summary of the purification steps of staphylokinase by local isolate of *S.aureus* GH38

Purification steps	Volume (ml)	Total activity (U)	Total Protein (mg)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Crude enzyme	50	293.5	0.75	391.33	1	100
Ammonium sulfate precipitation (70%)	10	228.6	0.2	1143	2.92	77.88
Ion exchange chromatography (CMC) (Wash)	30	211.5	0.198	1068.1	2.73	72.1
Ion exchange chromatography (CMC) (Elution)	18	97.2	0.162	600	1.53	33.11

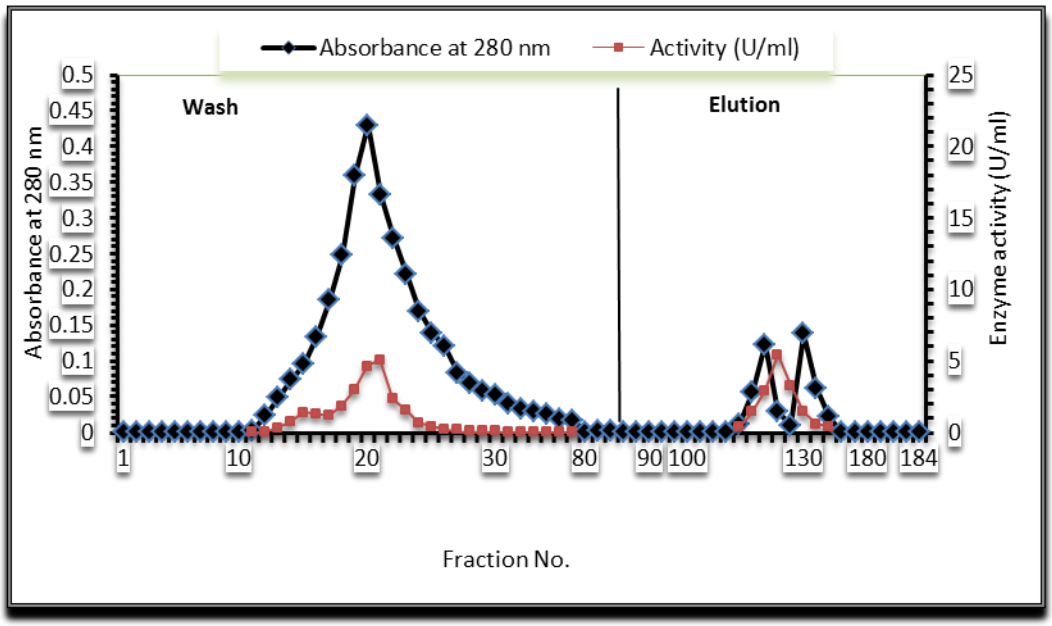


Figure 1. Ion exchange chromatography for staphylokinase purification from *S.aureus* by using the CM-Cellulose column (2.5×15) cm equilibrated with phosphate buffer (5mM, pH 7.0), eluted with a phosphate buffer with NaCl gradient (0-1) M in phosphate buffer inflow rate 30ml/hr. 3ml for each fraction

Characterization of staphylokinase

Effect of pH on the activity and stability of enzyme

The activity was measured at different pH. It was inferred that staphylokinase has the best activity in pH ranged between 6.5 to 7.5, with maximum enzyme activity at pH 7.0 on which enzyme activity was 7.3 U/ml as shown in Figure 2. It can be concluded from these results, that the staphylokinase activity at neutral or nearly basic pH values was higher than the activity at nearly acidic or alkaline values and this variation return mainly to the influence of pH on enzyme activity through the ionization of groups in the enzyme active site, or the ionization of groups in the substrate, or by influencing the conformation of the enzyme or the substrate. Our results are consistent with the report of the wild type SAK Vesterberg (26); the optimal pH value for the SAK activity was 7.0. For determination the pH stability the remaining activity was determined as above mentioned, it was noticed that pH ranged between 6.5-7.5 were the optimum pH for staphylokinase stability, the enzyme was retained 96% of its activity in pH 6.5 while retained 100% of its activity in pH 7.0, about 94% for pH 7.5. The activity was decreased away either side of the optimum pH values, the residual activities were 70% for pH 5.0 and pH 5.5 as in Figure 3. The enzyme activity was very low at nearly acidic pH, approximately half of activity was lost at pH 4.0, 4.5, and below 50%, residual activity was recorded. The results may donate a conclusion that the staphylokinase of *S.aureus* GH38 is more stable in nearly alkaline pH. In general, this lowering in enzymatic activity at pH values away from the optimum condition may be due to the effect of pH on enzyme structure that leads to denaturate of enzyme molecule or a change in the ionic state of the enzyme active site. Nguyen and Quyen (23) also found the same result that purified SAK from *S. aureus* showed pH stability at a pH range of 7 to 9.

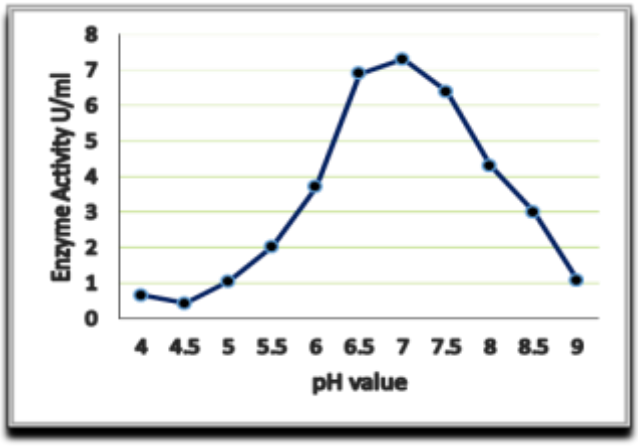


Figure 2. Effect of different pH values (4.0-9.0) on partially purified staphylokinase activity from local isolate *S.aureus* GH38 using casein as a substrate

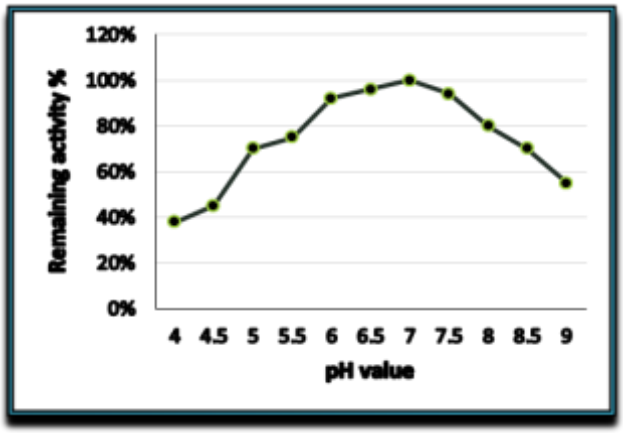


Figure 3. Effect of different pH values (4.0-9.0) on the stability of partially purified staphylokinase from a local isolate of *S. aureus* GH38

Effect of temperature on the activity and stability of the enzyme

The optimum temperature of SAK activity were determined by measuring the activity at different range of temperature 30- 70°C. Results in Figure 4 indicated that increasing in staphylokinase activity by enhancing the temperature when it reached to the highest value at 40˚C was 7.65U/ml productivity, because of increasing the movement energy of the molecules. Whereas the decline in the enzymatic activity by temperatures over 50˚C a result of the denaturation of protein structure and changes in the active sites which leads to loss of the enzyme activity. Then it began to decrease with increasing temperature until it reached 1.0 U/ml at 60˚C due to the increase of the clash between the enzymatic molecules sharing in the reaction with the substrate. This result similar to Nguyen and Quyen (23) when they showed that SAK from *S. aureus* has an optimum temperature of 30 to 37°C and was active at a broad temperature range from 20 to 45°C with the activity of over 93% in comparison to the optimal activity, and was stable at a temperature range from 25 to 50°C. Moreover, the remaining activity was then determined and the results presented in Figure 5, it was inferred that the enzyme was maintained its activity at temperatures ranged between 30-45°C, then the activity began to decrease with increasing temperature although at 50°C about 80% of the activity remained. Higher temperatures showed a sharp decrease in the stability, the enzyme retained 30% of the initial activity at 65˚C, whereas, at 70˚C there was no remaining activity indicating loss of enzyme activity. Most enzymes are more stable at low temperatures therefore; they are stored at low temperatures. The decline in staphylokinase activity at a temperature degree more than 55˚C belongs to its sensitivity against high temperature, reflecting the temperature effect on the 3D structure of the protein by damaging R-groups of amino acids that results to denaturation of protein and losing its activity. The thermal stability of *S.aureus* GH38 staphylokinase was close to Nguyen and Quyen (23), where they reported that the fibrinolytic enzyme produced from *S.aureus* QT08 was stable when incubated for 30 min. at temperatures from 30-40°C.

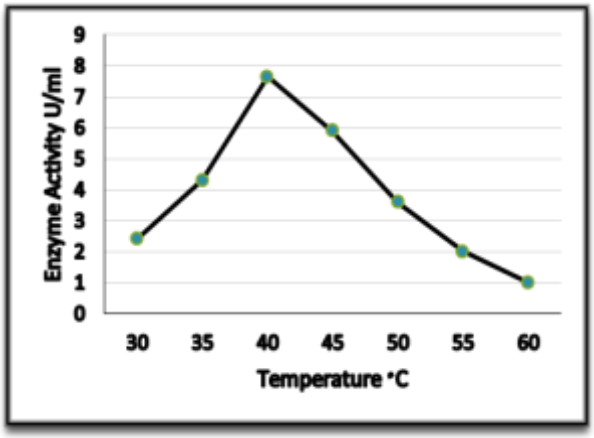


Figure 4. Effect of different range of temperatures (30-60) ˚C on partially purified staphylokinase activity from *S.aureus* GH38 at pH 7.0 using casein as a substrate

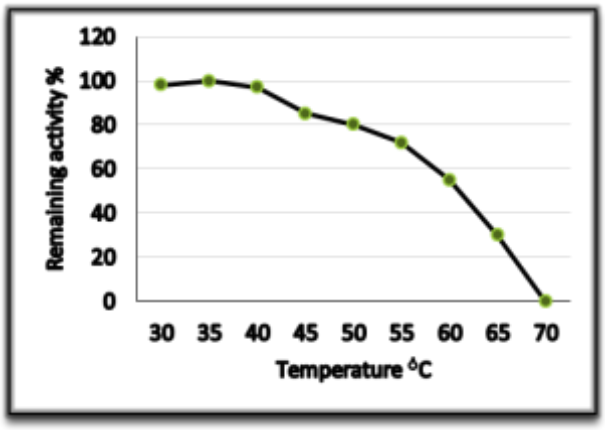


Figure 5. Effect of different ranges of temperature (30-70) ˚C in *S.aureus* GH38 staphylokinase stability at pH 7 using casein as a substrate

Effect of Some Chemical Compounds on the Enzyme Activity: In this study, purified SAK was incubated with 5 mM and 10mM of various metal ions, EDTA, 2-Mercaptoethanol, and PMSF, respectively at 37˚C for 30 min (2). The remaining activity was then determined as shown in Table 2. The effect of metal ions on

the fibrinolytic activity depends on the origin of the enzyme, in general, the presence of KCl, $CaCl_2$, $MnCl_2$, $MgCl_2$, and NaCl were observed to enhance enzyme activity to levels above their original activity (control value) at 5 and 10mM. This may mean that staphylokinase of *S.aureus* GH38p needs ions as a cofactor. Other ions $CoCl_2$, $HgCl_2$, AgCl and $FeCl_3$ decreased the enzyme activity, it was also noted that the enzyme was completely inhibited by $HgCl_2$ and $FeCl_3$ at 10mM. Complete inhibition of enzyme by $HgCl_2$ indicated the presence of SH groups in the enzyme active site leading to oxidize them by $HgCl_2$. From the results mentioned, it can be concluded that metallic chlorides vary in their effect on staphylokinase depending on the type of ion and concentration, and this may give some knowledge about the inhibition or activation of *S.aureus* GH38 staphylokinase by cation .In addition, the effect of EDTA and PMSF showed an inhibitory effect. It was found that the enzyme showed a marked decline in enzyme activities along with an increase in the concentration of the enzyme inhibitors in the two cases and in 10mM from PMSF the enzyme lost complete activity. Which confirmed that the enzyme was from metalloenzymes that the divalent ions formed important part from induced activity, the EDTA works as chelating agent and draw the divalent ions existing in the active site and form complexes lead to inhibit of enzyme activity. In addition, the inhibition of the enzyme by PMSF suggests that the purified enzyme was a serine protease. Also, reduction by 2-Mercaptoethanol is one of the most common agents used for disulfide reduction because cleavage of a single disulfide bond Fujimura *et al*. (12). Present results concord many of the earlier studies on the fibrinolytic enzyme. Dubey *et al*. (9) the purified an enzyme of bacterial sources was stimulated by $MgCl_2$, $CaCl_2$, and $MnCl_2$, but inhibited by $HgCl_2$, PMSF and EDTA. Also Afifah *et al*. (4) also found that the fibrinolytic enzyme was competitively inhibited by EDTA and PMSF while activated by 5mM $MgCl_2$ and 5mM $CaCl_2$.

Fibrinolytic activity of staphylokinase

Blood clot degradation by staphylokinase was determined by dissolving a human blood clot. The coagulated drop in a slide method was digested by 100µl of the partially purified staphylokinase at 37°C for 90 min figure (6A). The blood clot lytic was also assayed in the glass test tubes as in figure (6B). A human blood clot was incubated with the enzyme, and clot degradation was analyzed. Treatment of 0.175U, 0.35U, 0.525U, and 0.7U staphylokinase digested the clot; clot lysis was not shown in the control. In addition, SAK at higher doses digested the blood clot effectively and are dose-dependent Vijayaraghavan *et al*. (28). From the results of the present study, it can be concluded that the staphylokinase purified from *S.aureus* GH38 display excellent fibrinolytic activities in vitro. Similar results were also reported with other microorganisms like *Bacillus subtilis* (8), *Schizophyllum commune* (20), *Streptococcus* sp. (6).

Table 2. Effect of some chemical compounds on staphylokinase activity purified from *S.aureus* GH38

Agents	Remaining activity% (5mM)	Remaining activity% (10mM)
Control	**100**	**100**
KCl	**140**	**133.4**
$MnCl_2$	**103**	**107.9**
$CaCl_2$	**159**	**171.2**
$MgCl_2$	**150**	**137.9**
NaCl	**144**	**121.4**
$CoCl_2$	**52**	**55**
$HgCl_2$	**32.3**	**8.4**
AgCl	**20.06**	**0.9**
$FeCl_3$	**13.1**	**0**
2-mercaptoethanol	**21**	**11**
EDTA	**66.6**	**30**
PMSF	**24**	**0**

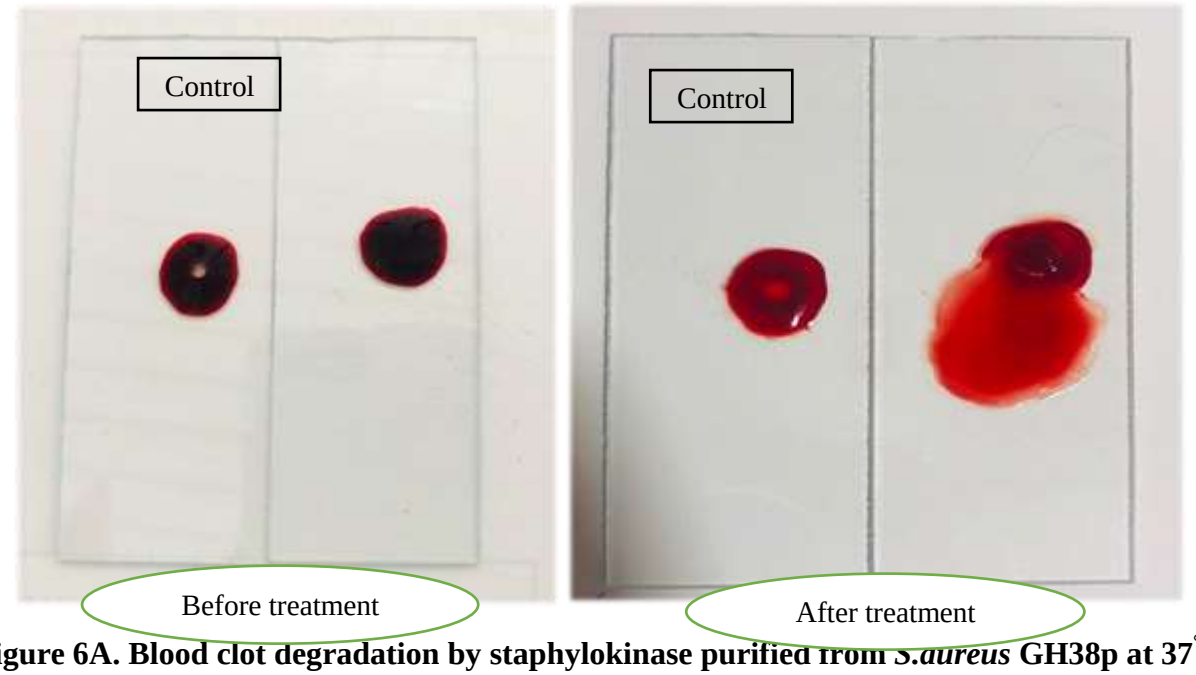
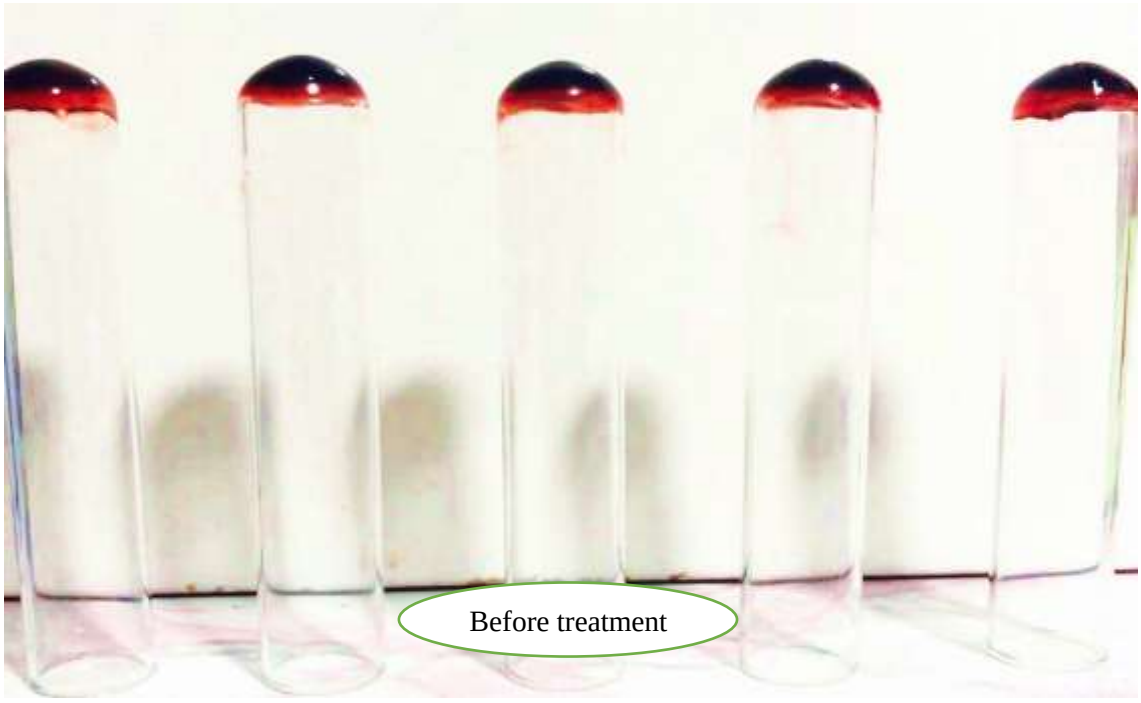


Figure 6A. Blood clot degradation by staphylokinase purified from *S.aureus* GH38p at 37°C after 90 min



Control A B C D

Figure 6B. Effect of partially purified staphylokinase from *S.aureus* GH38 on human blood clot using different enzyme concentrations (A: 0.175U, B: 0.35U, C: 0.525U, D: 0.7U

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