WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 9, 1726-1736. Research Article ISSN 2277– 7105

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF A NOVEL FIBRINOLYTIC PROTEASE FROM *STAPHYLOCOCCUS AUREUS* MTCC 902

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Article Received on
02 July 2015,

Revised on 26 July 2015,
Accepted on 20 Aug 2015

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ABSTRACT

Fibrinolytic enzymes are protease enzyme that helps in treating cardiovascular diseases. Microorganisms are easily available and less expensive source of fibrinolytic enzyme. Fibrinolytic protease (FP31) was purified from Staphylococcus aureus (MTCC 902) by sequential steps of precipitation by ammonium sulphate, Sephadex G- 75 and DEAE –Sephadex A- 50. After the purification of enzyme the activity was enhanced to 40.32 fold and 9.47% recovery was achieved. The optimum temperature was 40 ºC and the optimum pH was found to be 8. Activity was significantly blocked by EDTA, pefabloc and EGTA. All this indicates purified enzyme as a serinemetallo protease with application in thrombolytic therapy.

KEYWORDS: Fibrinolytic enzyme, Cardiovascular diseases, Sephadex G-75, thrombolytic therapy, DEAE-Sephadex A-50.

INTRODUCTION

Fibrinolytic enzyme helps in treating ischemic heart disease, peripheral vascular diseases, myocardial infarction, valvular heart diseases, high blood pressure, stroke and arrhythmias like cardiovascular diseases that are the major reason for the global death. According to WHO, non- communicable diseases (NCDs) are responsible for 52% of global death under the age of 70 years in 2012. NCDs includes four major diseases namely, chronic respiratory diseases, cancer, cardiovascular diseases (CVDs), and diabetes. People died from CVDs were 37%, cancer was 27% ,diabetes was 4% and chronic respiratory diseases were responsible for 8% of death while other NCDs were 23% responsible for global death.[1]

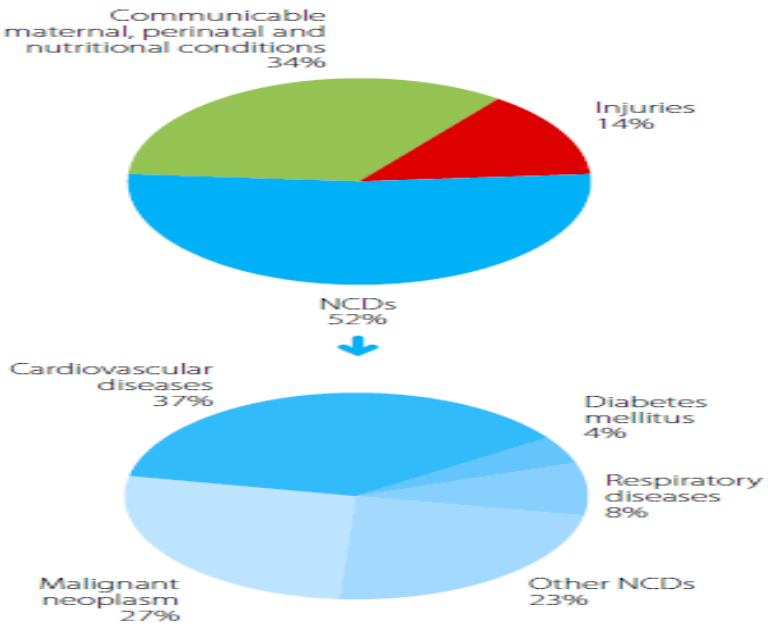

Fig. 1. Proportion of global deaths below age of 70 years estimated in 2012

Block buster drugs like statins are used to treat CVDs. Statins inhibits the HMG-CoA reductase enzyme for the formation of cholesterol. But use of statins have many side-effects like rhabdomyolysis, muscle pain, weakness, memory loss, forgetfulness, memory impairing, kidney, brain and liver failure.[2] Anticoagulants or blood thinners like heparin which bears anti-thrombin activity suppress activity of thrombin and activates anti- thrombin III. It is given by continuous intravenous infusion. Heparin has side effects like hemorrhage, osteoporosis, hypersensitivity, alopecia and thrombocytopenia. Anti-platelet drugs like aspirin, clopidogrel, dipyridamole and ticlopidin used to treat CVDs but its side-effects can lead to other diseases like bone marrow suppression, in particular leucopenia.[3]

To prevent all these side- effects of all above drugs fibrinolytic enzyme can be used as a therapeutic agent to treat CVDs which does not have any side-effects. These enzymes works by dissolving cross linked fibrin. According to the working mechanism thrombolytic agents are divided into two categories, one in which plasmin protein that directly degrade fibrin and second is plasminogen activator which activates plasminogen into active plasmin (fig. 2).[4]

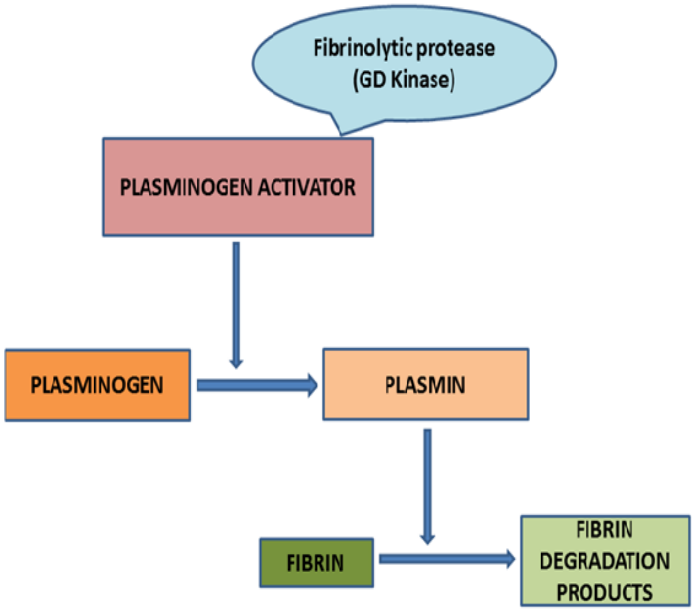

Fig 2: Schemetic representation of fibrinolysis.

Fibrinolytic protease enzyme are the protease enzyme that degenerate the enzymes by scission of peptide bonds to yield polypeptide to dipeptides to peptones to amino acids. Intracellular proteases helps in the consumption of proteins containing nutrient, activation of zymogen, liberation of protein, self- digestion, growth of spore and other physiological phenomena.[5] Plants, animals and microorganisms are the sources from which proteases can be extracted. Proteases from plants source includes bromelain, ficin, keratinase and papain. Papain is extracted from *Carica papaya.* Bromelain is from the stalk and fruit of pineapples and ficin from the latex of *Ficus glabrata* fig tree.[6] From animals examples of protease enzymes are protaminase, trypsin, chymotrypsin, pepsin and renin. Protaminase is obtained from bovine pancreas, sea lions and pigs, while trypsin and chymotrypsin, both known as proteinase from pancreas, are produced by acinar cells of pancreas and secreted in the form of zymogen each known as trypsinogen and chymotrypsinogen. Pepsin (E.C. 3.4.4.1) can be obtained from gastric mucosal cells of pig and renin (E.C. 3.4.4.3) from lamb stomach.[6] Among microbes reported to be intracellular protease producers include *Clostridium perferingens* tip a.[8] and *Streptococcus saliarius* subsp. Thermophiles,[10] Protease that binds to membrane is the least studied protease. It was reported to be produced by *Escherichia coli.*[7] and *Lactococcus lactis* subsp. cremoris AMI.[9]

Thrombolytic agents such as t-PA and urokinase are expensive, exhibit low fibrin specificity, and have undesired side effects such as allergic reaction, resistance to repercussion and gastrointestinal bleeding.[16] Due to this reason safer and cheap thrombolytic agents from

microorganisms like bacterias are used to produce thrombolytic agents. Here staphylococcus aureus (MTCC 902) has been cultured to isolate this enzyme which is a serine metallo protease enzyme.

2. MATERIALS AND METHODS

2.1 Bacterial and culture condition

Nutrient broth for culturing bacteria and composition for modified fermenting media at pH 8 includes:- fructose (1 %), peptone (1 %), di-Potasium hydrogen orthophosphate (0.02 %), calcium chloride (0.004 %), ammonium nitrate (0.05 %), magnesium sulphate (0.002 %), biotin (5 µg/ml), calcium carbonate (10 µg/ml).

2.2 For rupturing cell wall

Lysis buffer at pH 7.3 includes 140 mM sodium chloride (0.81 %), 2.7 mM potassium chloride (0.02 %), 10mM di-Sodium hydrogen orthophosphate (0.141 %), 1.8 mM Potassium di-hydrogen orthophosphate (0.024%), lysozyme (100 µg/mL).

2.3 Enzyme purification

Staphylococcus aureus (MTCC 902) was cultured for 24 hrs in nutrient broth of 100 ml at 37 ºC. 10% (v/v) of above culture was then added as an inocula to modified fermenting media of 1 Liter and incubated for 48 hrs. After incubation centrifuge the media at 4 ºC for 10,000 rpm for 15 minutes. Pellet was collected and then mixed with lysis buffer and then homogenised at 1000 rpm and centrifuged at 4 ºC for 15 min for 10,000 rpm. The supernatant was precipitated by adding solid $(NH_4)_2SO_4$ at 70% saturation. After precipitation centrifugation was done at 10,000 rpm for 15 min at 4 ºC. The supernatant was subjected to dialysis against 10mM Tris HCl buffer (pH 7.5) for 1hr at 4 ºC. After this the enzyme was then subjected to Gel filtration on Sephadex G-50 (120cm × 1cm). 10mM phosphate buffer was used as an elution buffer and the flow rate was adjusted to 15ml/hr. The active fractions that showed high specific values were pooled, concentrated and then subjected to anion exchange chromatography using DEAE Sephadex A-50 as a matrix (24cm × 2 cm) against 25 mL NaCl of 0.01M to 1.0 M. The flow rate was adjusted to 30ml/hr. After this protein concentration was determined and assay was done.[4, 11]

2.4 Analysis of protein concentration

Taking bovine serum albumin as standard method the protein concentration was established by Lowry's method.

2.5 Enzyme activity assay

With some modifications in the method of Oda and Murao.[12] assay of enzyme activity was done. Azocasein solution (0.05 ml) containing azocasein of 2mg/ml in 10 mM Tris HCl buffer of pH 7.5 mixed with sample of enzyme (total volume of reaction mixture was 0.15 ml) and incubated with at 45 ºC, 20 min. After this the reaction was stoped by adding 0.06 ml of 10% (w/v) trichloroacetic acid and incubating it for 10 min in ice water. It was then centrifuged for at 4 ºC, for 20 min. for 10,000 rpm. After centrifuging the supernatant was obtained to which supernatant of 0.15 ml was mixed with FC reagent (0.33 M) of 0.3 ml and (10% w/v) 0.45 ml Na_2CO_3 solution. Incubate it for half an hour and after incubation the optical density was taken at 660 nm using UV–Visible Spectrophotometer. Tyrosine was taken as reference standard. One unit of activity of enzyme was defined as the amount of enzyme required to release 1 mg tyrosine from azocasein in one min.

2.6 Fibrinolytic assay

The fibrin plate method of Astrup and Mulertiz.[13] with slight modifications was used to determine fibrinolytic assay. The agarose gel of fibrin consist agarose (1.2%), fibrinogen (4.5mg /ml) and thrombin (0.45 U/ml). It was then poured Bio-Rad mini gel sandwich and allowed for setting for half an hour at room temperature. After this 10 µl of enzyme sample with different concentration (of final volume 0.5-4 µg per well) was poured in well of 3.5 mm diameter in gel. Incubation was done at 37 ºC and measuring the lytic circle around the well after 16 hrs. Fibrinolytic activity was defined as the lysed area per µg of protein (mm^2 /µg),[14] For positive control plasmin was used.

2.7 Analysis by electrophoresis

SDS-PAGE (Sodium dodecylsulfate polyacrylamide gel electrophoresis) was carried out using polyacrylamide resolving gel of 10% and stacking gel of 5 % by to the Laemmli method.[15] For reference molecular marker of low weight (Bio-Rad) was used For staining gel Coomassie Brilliant Blue R-250 and destaining solution containing methanol, glacial acetic acid and distilled water in ratio 1:1:8 (by vol.) respectively.

2.8 For pH and temperature effect

Temperatue and pH effect can be done by using azocasein for 20 min at 45ºC under the assay condition. Standard buffers like citric acid – sodium phosphate of pH 2 to 7, tris –HCl from pH 8 to 9 and $NaHCO_3$ from pH 9 to 11 were used to find out pH effect. After this incubation

of enzymes with different buffer for 3 hrs at one degree celcius was done and residual activity of enzyme was established at pH 8 by taking initial activity as 100 %.

Temperature effect was established at range from 0 to 80 ºC and pH 8. After this the aliquots of enzyme at pH 8 were incubated at different temperatures for 15 to 90 min. The relative activities were measured and relative activity of enzyme was established by considering initial activity as 100%.

2.9 For metal ion and inhibitors effect

Sample were incubated at room temperature with different inhibitors and metal ion for half an hourand their residual activity was established. Divalent metal ions effect like $CoCl_2$, $FeSO_4$, $MgCl_2$, $ZnCl_2$, $CuCl_2$, and inhibitors like as ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacitic acid (EGTA), ethylenediamine tetraacetic acid (EDTA), pefabloc, phenylmethylsulfonyl fluoride (PMSF) and aprotinin were found out.

3. RESULTS

3.1 Enzyme purification and molecular weight determination

Fibrinolytic protease purification from the supernatant of Staphylococcus aureus MTCC 902 culture is summarized in Table 1. By three-step procedure the enzyme was purified to homogeneity (Table 1, Fig. 3, 4), resulting in 9.47% activity recovery and 40.32 -fold purification. In SDS-PAGE analysis purified enzyme shows a single band having 31 kDa molecular weight (Fig. 5).

Table 1: Summarizing the purification of Fibrinolytic Protease from Staphylococcus aureus (MTCC 902)

Purification Step	Total Protein (mg)	Total activity (U)	Specific Activity (U/mg)	Fold Purification	% Yield
Crude	11,900	14,783	1.24	1	100
70% Ammonium sulphate	8,400	12,200	1.45	1.16	82.53
Sephadex G-50	3,600	6,500	1.80	1.45	43.96
DEAE A-50	29	1,400	50	40.32	9.47

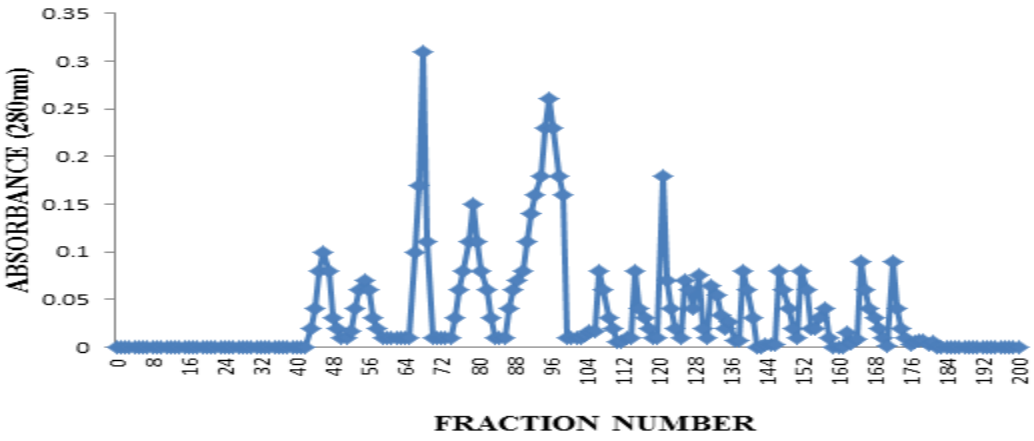

Fig. 3 : Gel filtration Chromatography using Sephadex G- 50 as a matrix

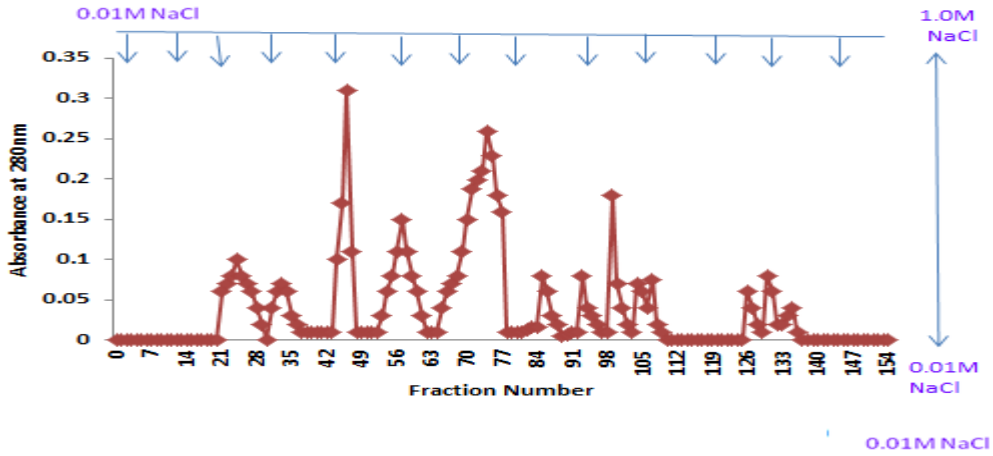

Fig 4: Anion Exchange Chromatography using DEAE Sephadex A- 50 as a matrix and elution buffer of 0.01M NaCl to1M NaCl

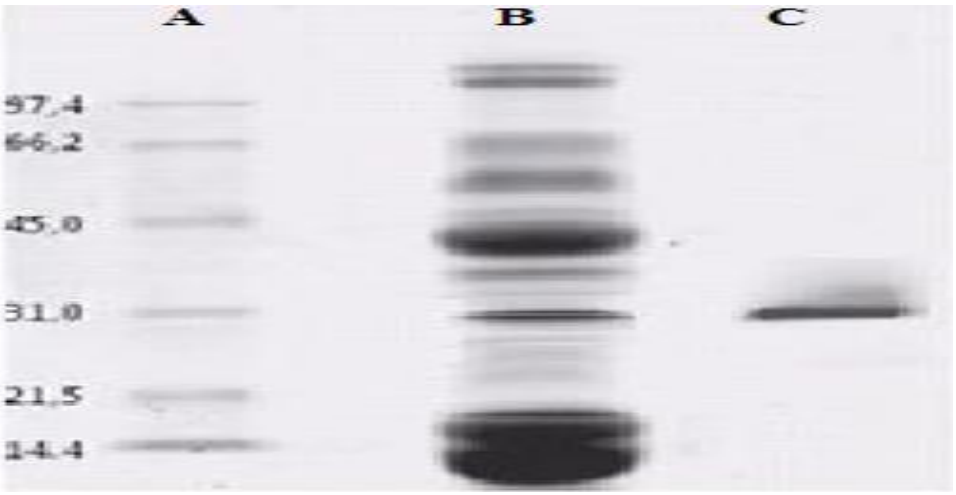

Fig 5: SDS PAGE – fibrinolytic protease. Lane A shows molecular marker, Lane B shows crude and Lane C shows purified enzyme.

3.2 pH and temperature effect determination

The stability and activity of purified sample of enzyme were highly influenced by temperature and pH. Fibrinolytic protease activity was highest in between pH 8 (Fig. 6). Furthermore, fibrinolytic protease activity was highest at 40ºC (Fig.7)

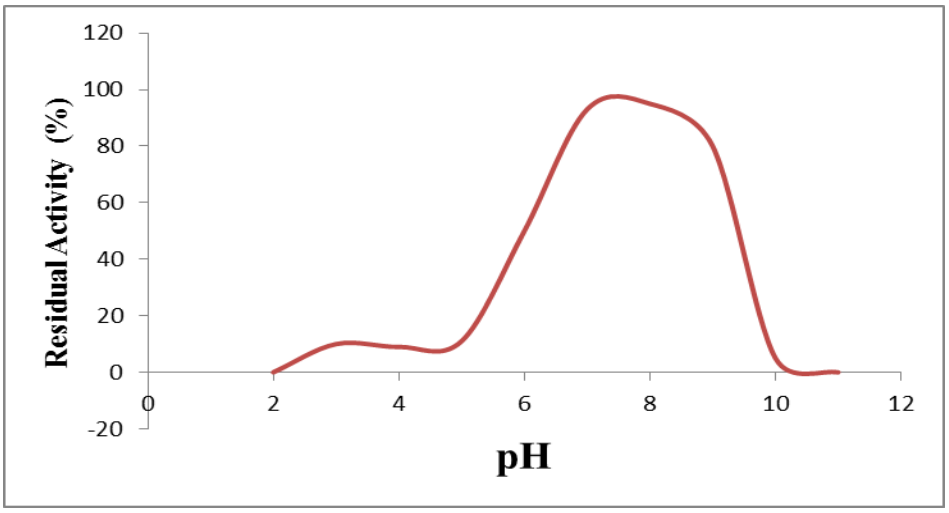

Fig 6 : Effect of pH

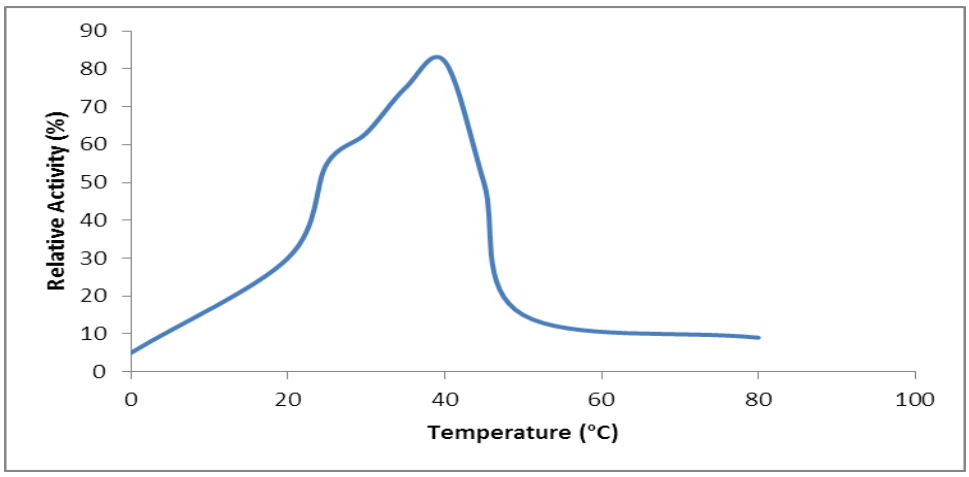

Fig. 7: Effect of Temperature

3.3 Metal ions and inhibitors effect determination

Residual activity was slightly enhanced by the involvement of $CaCl_2$ and $MgCl_2$ .The activity was supressed by $CoCl_2$ and $ZnCl_2$ and completely inhibited in the presence of $CuCl_2$ and $FeSO_4$. Aprotinin and PMSF slightly inhibited the activity . Pefabloc, EDTA and EGTA significantly inhibited the activity (Table 2).

Table 2: metal ions and inhibitors effect on purified enzyme

Metal ions or Inhibitor	Concentration	Residual Activity
None		100
$CaCl_2$	2mM	109 ± 2.3
$MgCl_2$	2mM	105 ± 2.6
$CoCl_2$	2 mM	42.0 ± 3.0
$CuCl_2$	2 mM	1.8 ± 3.1
$FeSO_4$	2 mM	0
$ZnCl_2$	2 mM	34 ± 2.3
EDTA	1mM	8.0 ± 3.2
EGTA	1mM	18.7 ± 2.8
PMSF	30µM	88.9 ± 3.2
Aprotonin	2.5mM	96.1 ± 2.3
Pefabloc	4mM	51± 2.3

3.4 Fibrinolytic activity

Fibrin plate method was analysed for hydrolysis of fibrin by purified sample of enzyme. Fig. 8. depicts that enzyme sample have bigger clear zone than that of equal amount of (µg) plasmin. The clearance area of purified enzyme sample after 16 h incubation at 37 ºC was established to be 120.6±3.4 mm^2/µg, which is 1.38-fold more than that produced by plasmin of 87 ±2.7 mm^2/µg.

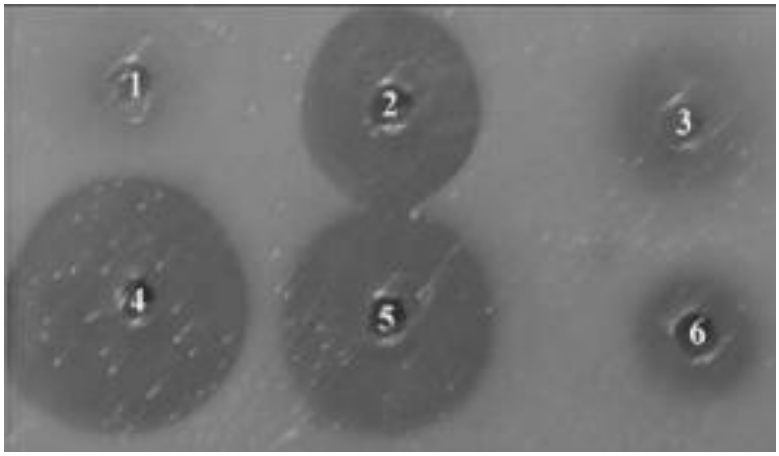

Fig 8 : Fibrinolytic activity of purified enzyme. In well 1 to 6 (3.5 mm diameter) were:1. buffer 3µg as a Negative control, 2. plasmin 0.5 µg as positive control and well 3 to 6 is purified sample of 4, 3,1 and 0.5 µg, respectively.

4. DISCUSSION

Cardiovascular diseases are the major reason of global mortality rate,[1] and can be treated by using thrombolytic agents i.e. fibrinolytic enzyme. The typical thrombolytic agents i.e t-PA and urokinase are very costly and exhibit low fibrin specificity, and have undesired side

effects such as allergic reaction, resistance to repercussion and gastrointestinal bleeding,[16] therefore microbial source have attracted much more medicinal interest . Here in this report the purification and biochemical characterisation of a novel fibrinolytic protease from S. aureus (MTCC 902) is described. The enzyme was purified by various chromatographic techniques on Sepharose G- 50 and DEAE Sephadex A – 50. The molecular mass of purified enzyme was estimated to 31 kDa approximately by SDS PAGE. The specific activity of purified enzyme was an increase of 40.32 fold with activity recovery of 9.47% than crude enzyme extract. The residual activity of enzyme was slightly inhibited by PMSF and aprotonin and however Ca^{2+} and Mg^{2+} ions slightly enhanced the activity. This dipicted that the purified enzyme is a serinemetallo protease enzyme. The optimum temperature and optimum pH for the purified enzyme was 40 ºC and pH 8 respectively. The purified enzyme degraded fibrin clot by fibrinolysis more efficiently than plasmin. This enzyme can be further developed as potential thrombolytic agent as it is highly active and stable in moderate pH and temperature.

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