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PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF NOVEL FIBRINOLYTIC PROTEASE PRODUCED BY MESOPHILIC *BACILLUS CIRCULANS GD*25

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ABSTRACT: A novel fibrinolytic protease from *Bacillus circulans* GD25 was isolated and characterized for the fibrinolytic activity. The fibrinolytic protease was purified by ammonium sulphate fractionation (70%) and subjected to chromatographic methods like Sephadex G-50, DEAE –Sephadex A-50 columns. The purified enzyme has an approximately 38 kDa in size by SDS-PAGE and gel filtration. Optimum activity was at 35ºC and the enzyme was highly active over a wide range of pH from 7.0-9.0 with an optimum at pH 8.0. It exhibited stronger fibrinolytic protease activity. The activity was slightly enhanced by Ca^{2+} and Mg^{2+} whereas $Zn^{2+,}$ $Cu^{2+,}$ Co^{2+} and Fe^{2+} suppressed the activity. The fibrinolytic protease activity was totally lost in the presence of PMSF, suggesting that the purified enzyme is a serine metallo-protease with potential application in thrombolytic therapy

INTRODUCTION: Proteases are proteolytic enzymes which catalyze the hydrolysis of proteins based upon their structures or properties of the active site. There are several kinds of proteases such as serine, metallo, carboxyl, acidic, neutral and alkaline proteases. Proteases are industrially important due to their wide applications in leather processing, detergent industry, food industries, pharmaceutical and textile industry etc [1, 2].

Proteases are obtained from plants, animal organs and microorganisms, with the majority obtained from microbial sources. Currently, a large proportion of commercially available proteases are derived from bacteria and fungi.

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Selection of the right organism plays a key role in obtaining high yield of desirable enzymes. On the other hand, it is a well-known fact that extra cellular proteases production in a microorganism is greatly influenced by media components, especially carbon and nitrogen sources and metal ions along with other growth parameters.

Cardio vascular such as high blood pressure, acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, stroke etc. are the primary causes of death. CVDs are the number one cause of death globally: more people die annually from CVDs than from any other cause [3].

Throughout the world, according to data of World Health Organization (WHO) an estimated 17.3 million people died from CVDs in 2008, representing 30% of all global deaths[3]. Of these deaths, an estimated 7.3 million were due to coronary heart disease and 6.2 million were due to stroke [4].

Low and middle income countries are disproportionally affected: over 80% of CVD deaths take place in low- and middle-income countries and occur almost equally in men and women [3]. The number of people, who die from CVDs, mainly from heart disease and stroke, will increase to reach 23.3 million by 2030 [3-5]. CVDs are projected to remain the single leading cause of death [5].

The fundamental pathophysiological process related to the devastating problem in the formation of fibrin (blood clot) which adheres to the unbroken wall of blood vessels. Fibrin is normally formed from Fibrinogen by the action of Thrombin (E C 3.4.21.5). It is sliced by plasmin (E C 3.4.21.7) which is activated from Plasminogen by tissue plasminogen activator. In balanced condition, fibrin clots are hydrolysed by plasmin to avoid thrombosis in blood vessels.

However, in unbalanced condition, as a result of pathophysiological disorders, the clots are not hydrolysed and thus, thrombosis occurs. Streptokinase is typical thrombolytic agents used for therapeutic purposes. Based on working mechanism, thrombolytic agents are of two types, one is plasminogen activator and urokinase which activates plasminogen into active plasmin to degrade fibrin and the other is plasmin like proteins which directly degrade fibrin.

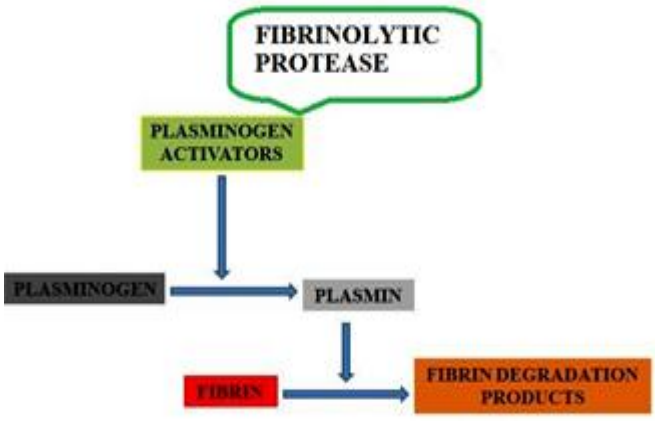

FIG.1: SCHEMATIC REPRESENTATION OF FIBRINOLYSIS

Moreover, on the basis of catalytic mechanism, microbial fibrinolytic enzymes are classified into three types, serine protease (eg. Nattokinase), Metallo protease (eg. *Armillaria* mellea metallo protease), mixture of both serine and metallo protease (eg.Protease from *Streptomyces*).

Despite widespread uses, thrombolytic agents such as t-PA and Urokinase are expensive. They exhibit low fibrin specificity and have undesired side effects such as gastrointestinal bleeding, resistance to repercussion and allergic reactions. Therefore, continuous efforts have been focused in the search of safer and less expensive thrombolytic agents from diverse sources.

Recently fibrinolytic enzymes with potential thrombolytic application have been purified from various sources such as fermented food, earthworms, mushrooms, snake venom and microbial sources. From microbial sources, bacteria, actinomyces, other fungi and algae are reported to produce fibrinolytic enzymes. Recombinant strepto kinases with reduced immunogenicity have been produced. A mutant streptokinase that lacks the C terminal 42 amino acids was found to be less immunogenic than the native molecule [6]. One chemical modification has involved complexing streptokinase with PEG [7-9], primarily for reducing immunogenecity.

Streptokinase variants with one or more of the normal amino acids residues replaced by others have been prepared in attempts to enhance plasminogen activation [10]. Some of the modified variants displayed an enhanced stability. The preferred variant had lys 59 replaced with or glutamic acid residue. Streptokinase derivatives having platelet glycoprotein binding domains [11], these derivations produced higher local concentrations of plasmin in vivo when compared to unmodified streptokinase.

Plasminogen is a glycoprotein of molecular weight 90 kDa, which is synthesised in the liver. It is converted enzymatically by PAs to the fibrinolytic enzyme; plasmin cuts away its covalently cross linked α-chain protuberences. The rather open mesh like structure of a blood clot gives plasmin relatively free access to the polymerised fibrin molecules thereby facilitating clot lysis.

Plasmin, a plasma serine protease that specifically cleares fibrins triple standard coiled coil segment and plasmin is formed through the proteolytic cleavage of the 86 kDa Zymogen plasminogen, a protein that is homologous to the zymogens of the blood clotting cascade.

PA is present in the tissue (t - pA) in plasma and in urine (UK). t-PA is localized in the vascular endothelium of veins, capillaries and pulmonary arteries and in the microsomal fraction of cells. t-PA is released into the blood stream in response to number of stimuli including ischaemia, vasoactive drugs and exercise. Released activator is inactivated rapidly in the blood stream by complexing to tissue plasminogen activator inhibitors (PA IS) and has a half-life of about five minutes. The major tissue PAIS are synthesised in the liver and in the vasculsar endothelium but about 30% of the total is probably megakaryocyte derived and is stored in platelets and granules.

MATERIAL AND METHODS:

Chemicals: Thrombin, bovine fibrinogen, fibrin, plasminogen, agarose, acrylamide, bis-acrylamide, sodium dodecyl sulfate, TEMED, ammonium per sulphate, phenylmethylsulfonyl fluoride, DIFP, EGTA, Ethyl methyl sulphonate, Ethidium bromide and protein markers were purchased from Sigma Chemicals CO.USA. All other chemicals and analytical reagents were purchased from Hi-media, India, unless stated otherwise.

Bacterial strain and Culture conditions: The bacterial strain of *Bacillus circulans* GD25 isolated from soils of various regions in Bangalore was identified by standard method for bacterial identification [12]. Stock cultures were maintained in nutrient broth medium with 70% glycerol; cultures were preserved at -20^{o}C [13]. The inoculum was prepared by transferring a loopful of stock culture (*Bacillus circulans* GD25) to 100ml of sterile nutrient broth stock medium, then incubated it overnight at 35^{o}C on a rotary shaker with 200 rpm, before being used to inoculation [14]. A stock suspension was prepared and adjusted to 7×10^{3} cell/ml^{-1}.

Production of Fibrinolytic Protease: The Fibrinolytic Protease production was carried out in a 2 liter fermentor (New Brun Swick), containing 1L modified production medium composed of (gm/l): fibrin, 2; NH_4NO_3,0.05; $KHPO_4$, 1.0 and $(NH_4)_2SO_4$ at pH 8. A 10% (v/v) level of inoculums was added. The fermentation was carried out at 35^{o}C for 48h. After the completion of fermentation the whole fermentation broth was centrifuged at 10,000 rpm at 4^{o}C and the clear supernatant (crude enzyme) was subjected to recovery and purification process.

Ammonium Sulfate Fractionation and Dialysis: All purification steps were performed at 4^{o}C. The crude enzyme was subjected to ammonium sulfate fractionation, at different concentrations ranging from 30-80% saturation [15]. The precipitates so obtained were suspended in cold saline and tested for fibrinolytic activity and total protein content. The salting out concentration of crude enzyme was at 70% on the basis of enzyme activity. To obtain complete precipitation of the crude enzyme, the remaining harvest fluid was subjected to ammonium sulfate precipitation at 70% saturation.

The precipitate so formed was separated by centrifugation (10000 g) for 15 min., again suspended in cold saline solution (100ml) and dialyzed [16] in cold against 1L of 20mM Tris-HCl buffer, pH 8 for 24 hrs. After dialysis, the solution was centrifuged and supernatant obtained was designated as fraction-I, and subjected to gel filtration chromatography.

Gel Filtration Chromatography on Sephadex G-50: Gel filtration chromatography was carried out using a Sephadex G-50 Column (120cm× 1.0 cm) [17]. The column was equilibrated with 10 mM Tris-HCl buffer, pH 8 [18]. The flow rate was 1ml/6min. The fractions collected were determined for its total protein concentration and fibrinolytic enzyme activity. The active fraction obtained was pooled together, concentrated by lyophilization and used as purified fibrinolytic enzyme for subsequent studies.

Anion Exchange Chromatography on DEAE Sephadex A-50: The dialyzed enzyme was chromatographed on a column of DEAE Sephadex A-50. The sample was loaded on to a column of DEAE Sephadex A-50 (24cm× 2.0 cm) equilibrated with 20mM Tris-HCl buffer, pH 8 [18]. The absorbed protein solution was eluted at a flow rate of 1ml/6min with a discontinuous gradient ranging from 0.2M to 1.0M of NaCl dissolved in same buffer. Major peaks of fibrinolytic activity were observed in fractions 66 to 74 (FA-I), 116 to 124 (FA-II) and fraction 146 to 164 (FA-III). The fractions collected were determined for its total protein concentration and fibrinolytic activity.

The active fractions 146 to 164 (FA-III) with high fibrinolytic activity were dialyzed and used for further studies.

Fibrinolytic Protease Assay: Fibrinolytic protease activity was carried out according to the method described by Greenberg [19]. The reaction mixture contained 8 mg bovine fibrin, 500µl enzyme in Phosphate buffer (0.05mM, pH 8) in a total volume of 1mL. This mixture was incubated for 30 min at 37^{o}C in a water bath. The reaction was stopped by adding 0.5mL of 15% cold trichloro acetic acid (TCA). The mixture was centrifuged at 3,000 rpm for 10 min to remove precipitated fibrin. To 0.5mL of acid soluble filtrate 2.5mL of 0.3 N sodium hydroxide and 2.9% (w/v) sodium carbonate was added, followed by 0.75mL of Folin's phenol reagent. The mixture was incubated for 25 min at room temperature and the color developed was read at 660 nm. The above said procedure was followed with heat killed enzyme and kept as blank. Tyrosine was used as a standard. One unit of enzyme activity was calculated as the amount of enzyme which releases 1µmol of tyrosine/min under the specified reaction conditions.

Protein Determination:

Total Protein Concentration (Lowry Method): Lowry method was carried out by using bovine serum albumin (BSA) as standard. Serum Albumin solution was prepared in increasing concentration for the Lowry Assay standard curve. The total protein concentration was determined by using spectrophotometer at the wavelength of 680nm. Various protein concentrations were determined based on the standard curve [20].

Electrophoretic analysis: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 5% stacking gel and a 12% polyacrylamide resolving gel according to the method of Laemmli [21]. A low molecular weight marker (Biorad) was used as reference proteins. Gel was stained with Coomassie Brilliant Blue R-250 and destained with a solution containing methanol: glacial acetic acid: distilled water = 1:1:8 (by vol.).

TABLE 1: SUMMARY OF THE PURIFICATION OF FIBRINOLYTIC PROTEASE FROM *BACILLUS CIRCULANS GD25*

Purification Step	Total Protein (mg)	Enzyme activity Uml^{-1}	Total activity (U)	Specific activity U/mg	Fold Purification	% Yield
Crude extract	370	300	150,000	0.81	1	100
70% ammonium sulfate saturation	55	140	70,000	2.55	3.15	46.6
Sephadex G-50	13.5	94	47,000	6.96	8.59	31.3
DEAE- Sephadex A-50	1.5	32	16,000	21.33	26.33	10.06

Effect of Temperature on Enzyme activity and Stability: The optimum temperature for enzyme activity was determined by keeping the purified enzyme in 20 mM phosphate buffer (pH 8) for 30 min at various temperatures (20, 35, 40, 50, 60, 70, and 80°C). Stability of Fibrinolytic Protease was also investigated by measuring the residual activity after incubating the enzyme solution at 20-80°C for 30 min in 20 mM phosphate buffer (pH 7.8). All experiments were conducted three times.

Effect of pH on Enzyme activity and Stability: The optimal pH of the enzyme was determined between pH 3.0-12.0 using the following buffer systems: 10 mM citric acid buffer (pH 3.0- 5.0), 0.05 M sodium phosphate buffer (pH 6.0-7.0), 0.05 M Tris-HCl (pH 8.0), 0.05 M glycine-NaOH (pH 9.0-10.0), 0.1 M sodium phosphate buffer (pH 11.0), and 0.05 M hydroxyl-chlorite buffer (pH 12.0), respectively. The pH stability in the range of 3.0-12.0 was examined by incubating the enzyme solution for 30 min at 35°C with the different buffers, and then the residual activity at pH 8 was determined. All experiments were conducted three times.

Effect of metal ions and inhibitors: The protease samples were incubated with various metal ions and inhibitors for 30 min at room temperature and the residual activity was measured. Effect of divalent metal ion such as $CaCl_2$, $MgCl_2$, $CuCl_2$, $ZnCl_2$, $FeSO_4$ and $CoCl_2$ and inhibitors such as ethylenediamine tetraacetic acid (EDTA), EGTA DIFP and phenylmethylsulfonyl fluoride (PMSF) on protese activity was checked.

RESULTS:

Purification of Fibrinolytic Protease from *Bacillus circulans GD25*: The purification of fibrinolytic protease from the culture supernatant of *Bacillus circulans GD25* is summarized in **table 1**. The enzyme was purified to homogeneity by a three step procedure (**Table 1 and fig. 4 and 5**), resulting in 26.33 fold purification and 10.06 percent activity yield. An SDS-PAGE analysis gave a single band of fibrinolytic protease corresponding to a molecular weight of 38 kDa (**fig. 2**).

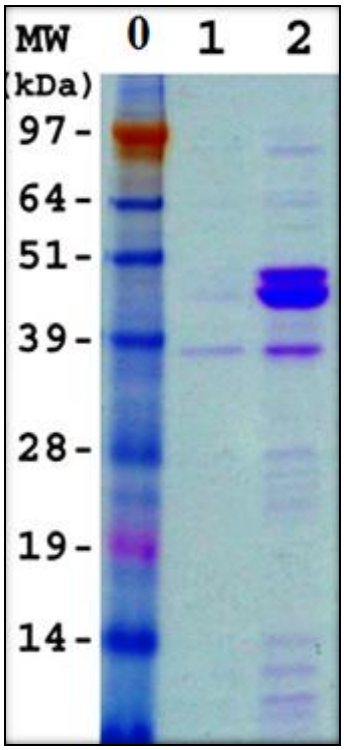

FIG. 2: SDS PAGE –FIBRINOLYTIC PROTEASE
(0: Marker, 1: Purified Enzyme, 2: Crude enzyme)

Determination of Optimum pH and Temperature of Purified Enzyme: The activity (%) of purified enzyme and stability can be seen in **figure 3**. It showed optimum pH of purified enzyme is 8 and stable in the pH range of 6.5-8.0 in case of 2hr. preincubation and at 20h. Preincubation the enzyme was stable in pH range 6.5-8.0.

The activity (%) of purified enzyme at various temperatures can be seen in figure 3b. The recorded optimum temperature of purified enzyme was 35^{o}C.

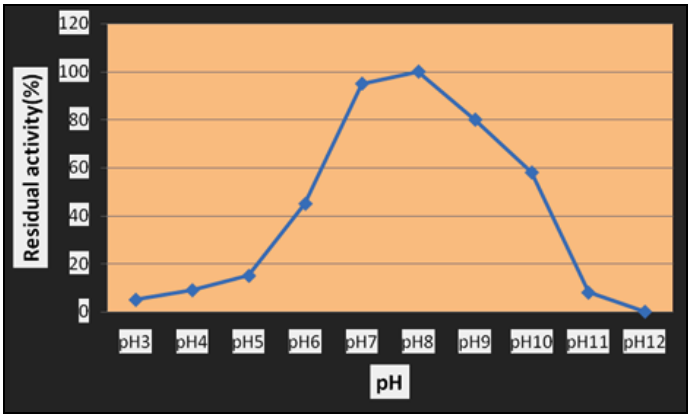

FIG. 3A: EFFECT OF pH

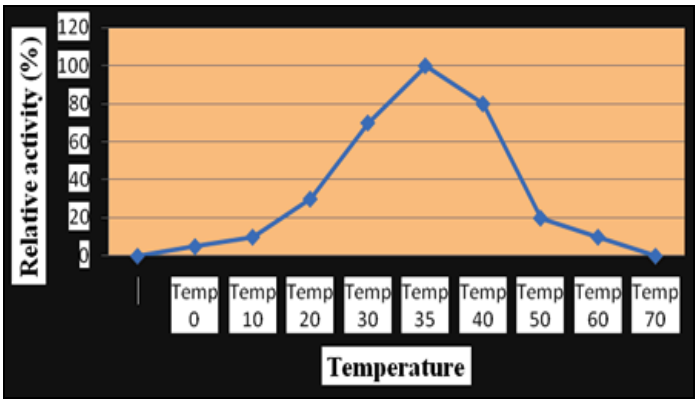

FIG. 3B: EFFECT OF TEMPERATURE

Effect of Inhibitors and Metal ions on purified enzyme: The activity (%) of purified enzyme at various inhibitors, phenylmethane sulfonyl fluoride (PMSF), Di isopropyl fluorophosphate (DIFP), EDTA and EGTA can be seen in table.2. PMSF was able to inhibit the enzyme almost completely. The data indicates that Ca^{2+}, Mg^{2+} and Na^{+} have slight stimulatory effect activation of the enzyme may be due activation of metal ions while other ions have slight inhibitory effect on enzyme. These results suggest, the metal ions apparently activate the enzyme.

Table 2: Effect of metal ions and protease inhibitor

Metal ion or Inhibitor	Concentration	Residual activity %
None		100
CaC12	2 mM	101 ± 2.6
MgC12	2 mM	96 ± 2.7
CoC12	2 mM	31.5 ± 3.0
DIFP	2 mM	1.1 ± 2.6
CuCl2	2 mM	1.6 ± 3.1
FeSo4	2 mM	1.2 ± 3.1
ZnCl2	2 mM	24 ± 2.3
EDTA	1 mM	8.9 ± 3.5
EGTA	1 mM	13.9 ± 2.6
PMSF	5 mM	0

DISCUSSION: The fibrinolytic enzymes play an important role in decreasing blood viscosity which in turn, strikes at the root of arteriosclerosis and atherosclerosis as well as by penetration. Thus they are useful mainly in curing cardiovascular diseases such as heart attack, atherosclerosis and stroke [22-24]. Fibrinolytic enzymes also have an industrial application in detergent production as they produce a variety of both extracellular and intracellular proteases [25-27]. They are obtained from many sources including microorganisms. The fibrinolytic enzymes from *Bacillus sp.* have great interest as thrombolytic agents because of their use being safer and efficiency in fibrinolysis process including plasmin activation [28-32].

The enzyme was purified to electrophoretic homogeneity by combination of chromatographic steps on Sephadex G-50 and DEAE Sephadex A-50. Molecular mass of the purified enzyme was estimated to be approximately 38 kDa by SDS-PAGE. With respect to the effect of pH and temperature, the fibrinolytic protease showed optimum activity at pH8 and temperature of 35ºC respectively. The activity was slightly enhanced by Ca^{2+} and Mg^{2+} whereas $Zn^{2+,}$ $Cu^{2+,}$ Co^{2+} and Fe^{2+} suppressed the activity. The fibrinolytic protease activity was totally lost in the presence of PMSF, suggesting that the purified enzyme is a serine metallo-protease with potential application in thrombolytic therapy.

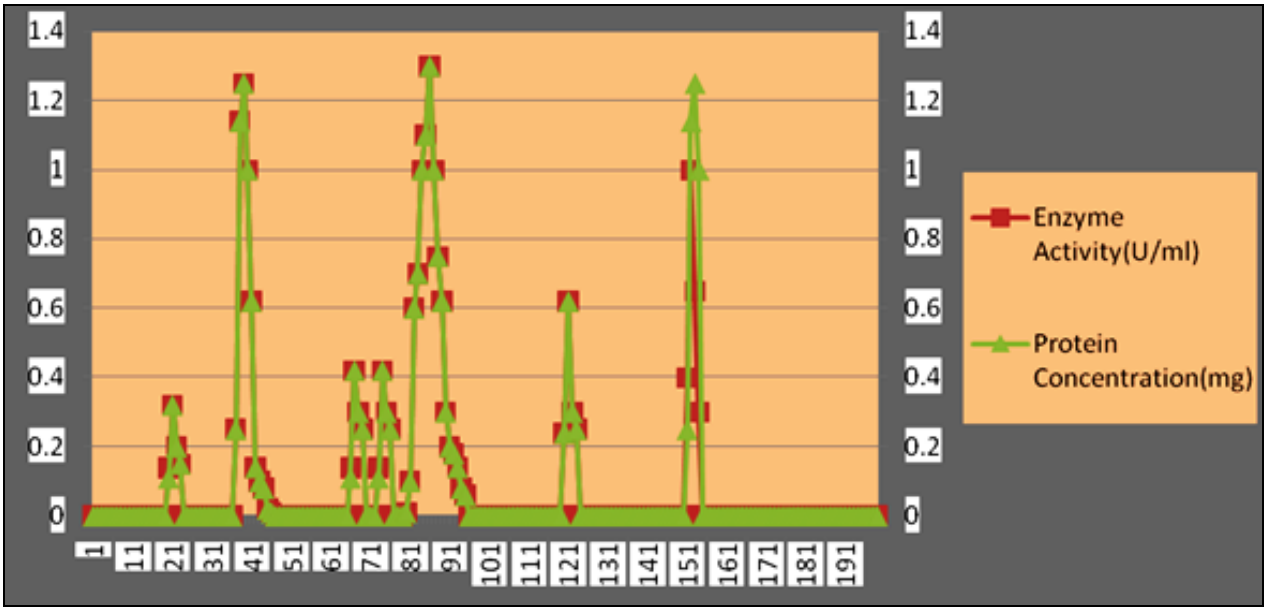

FIG. 4: GEL FILTRATION CHROMATOGRAPHY ON SEPHADEX G-50

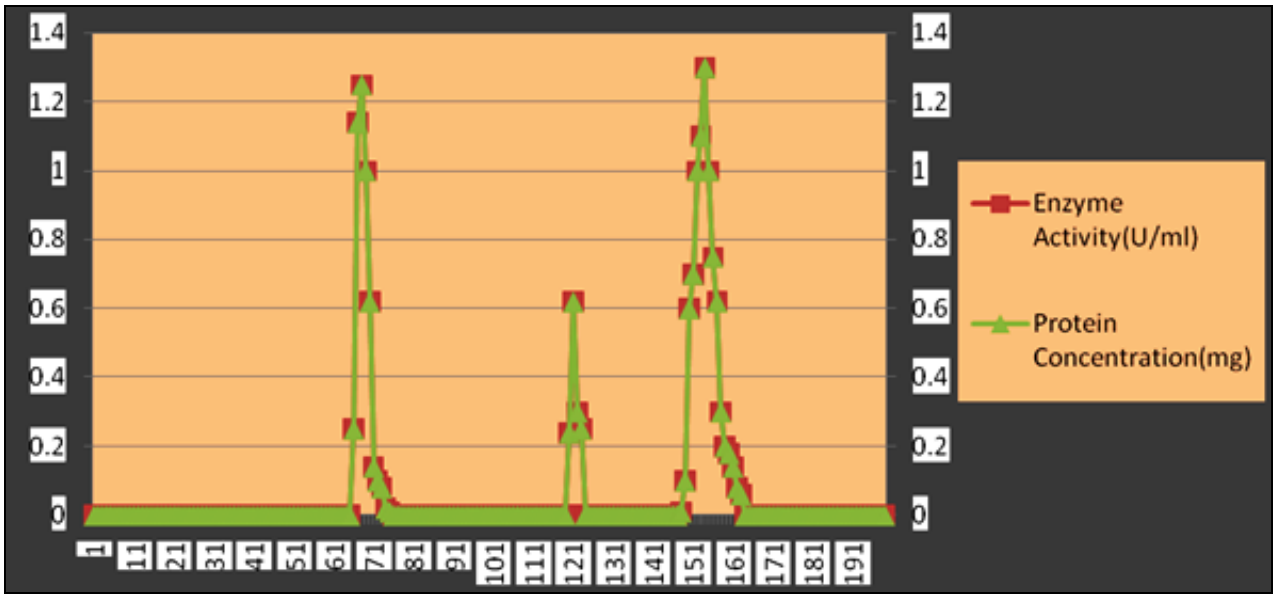

FIG. 5: ANION EXCHANGE CHROMATOGRAPHY ON DEAE SEPHADEX A-5

CONCLUSIONS: In the present study, we report the purification and characterization of a new fibrinolytic protease from *Bacillus circulans* GD25, which may be a good candidate in pharmaceutical industry of blood clot busters which may enable treatment of acute isochaemic stroke by removing fibrinogen from the circulation and treatment of occlusive thrombi by dissolving blood clot. Based on the above results and discussion the following conclusions are made: The analysis of SDS-PAGE indicates the molecular weight of fibrinolytic protease is 38 kDa, depending on the relative mobility.

The purified enzyme specific activity was an increase of 26.33 fold than crude enzyme extract. The purified enzyme has optimum pH 8.0. The enzyme was stable from pH7 to 9 even after 24 h, preincubation. The optimum temperature for purified enzyme activity was 35^{o}C. Fibrinolytic protease inhibitors like PMSF and EDTA showed inhibitory effect. However Ca2+ and Mg2+ ions have slight stimulatory effect. However it is reasonable to assume the promising nature of this enzyme for other commercial applications.

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