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Full paper

Purification of a Fibrinolytic Enzyme from *Bacillus* Sp. Isolated from Budu

Nurulhanis Ahmad Sanusi[a], Haryati Jamaluddin[a]*

[a]*Department of Biological Sciences, Faculty of Biosciences and Bioengineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia*

*Corresponding author: haryati@fbb.utm.my

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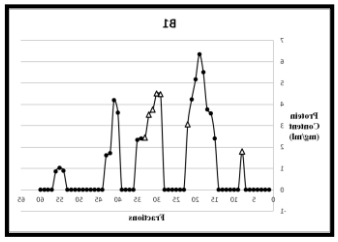
Graphical abstract



Abstract

Bacillus sp. strain B1 producing wild type fibrinolytic enzyme was isolated from Budu. The fibrinolytic enzyme was collected from the supernatant of *Bacillus* sp. strain B1 culture broth and purified to electrophoretic homogeneity through a combination of various purification schemes, which include ammonium sulphate precipitation, followed by anion exchange chromatography using DEAE-Sepharose Fast Flow and gel filtration chromatography on Sephadex G-75 column. During ammonium sulphate precipitation screening, it was observed that the crude enzyme from *Bacillus* sp. strain B1 precipitated at 40% and 50% of ammonium sulphate saturation respectively. The fibrinolytic enzyme was purified 58.5-fold with a final yield of 0.51%. The specific activity was determined to be 1.17 Units/mg using plasmin as standard and the final total protein content was 8.58 mg/ml. After the successive purification steps, the estimated molecular mass of fibrinolytic enzymes from strain B1 was estimated via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE analysis showed a single band at 45 kDa corresponding to the purified fraction with fibrinolytic activity.

Keywords: *Bacillus* sp.; Fibrinolytic enzyme; fermented food; chromatography

Abstrak

Spesis *Bacillus* B1 yang menghasilkan enzim fibrinolitik daripada makanan yang ditapaikan dikultur di dalam larutan nutrien untuk proses penyesuaian. Enzim fibrinolitik yang dikumpul daripada supernatan spesis *Bacillus* B1 ditulenkan sehingga mencapai kehomogenan menerusi kombinasi skim penulenan termasuk pemendapan ammonium sulfat, dialisis, dan diikuti oleh teknik kromatografi menggunakan kolum DEAE-Sepharose Fast Flow iaitu kromatografi penukaran anion serta kromatografi gel penapisan menggunakan kolum Sephadex G-75. Semasa pemeriksaan pemendapan ammonium sulfat, keputusan eksperimen menunjukkan enzim mentah daripada spesis *Bacillus* B1 termendap pada ketepuan ammonium sulfat sebanyak 40% dan 50%. Enzim fibrinolitik daripada spesis *Bacillus* B1 ditulenkan sebanyak 58.5 kali dengan hasil akhirnya sebanyak 0.51%. Spesifik aktiviti enzim ini ditentukan sebanyak 1.17 Unit/mg dengan menggunakan plasmin sebagai standard serta jumlah kandungan akhir bagi protin yang ditulenkan ialah 8.58 mg/ml. Selepas beberapa turutan proses penulenan, anggaran jisim molekul bagi enzim fibrinolitik ini telah ditentukan dengan menggunakan kaedah natrium dodecyl sulfat polyacrylamide gel elektrophoresis (SDS-PAGE). Menerusi analisis SDS-PAGE ini, satu jalur yang mewakili anggaran jisim molekul bagi pecahan enzim yang ditulenkan iaitu 45 kDa..

Kata kunci: Spesis *Bacillus*; enzim fibrinolitik; makanan yang ditapaikan; kromatografi

1.0 INTRODUCTION

Fibrinolytic system is a hydrolysis process of fibrins which are primary protein component of a blood clot, formed from fibrinogen via proteolytic action of thrombin. This system is also involved in tissue repair, macrophage function, ovulation and malignant transformation (Nordenhem, 2006). Fibrin which is the final product in blood clotting plays a vital role in health and healing, but sometimes it can also lead to inappropriate clots in blood vessels (Ref?).

This problematic clotting can lead to serious disease to human such as myocardial infarction and stroke (Kotb, 2011). The blood clots cause various types of thrombosis which is a major disease that causes increasing number of human death every year. According to Lopez *et al*., (1995), there is estimated around 1000,000 patients in US alone affected by lung blood clots annually. Cardiovascular disease (CVD) cause high rate of death in the world. About 17.5 million people died from cardiovascular disease in 2005 as mentioned by World Health Organization (Deng *et al*., 2009). According to Deng and co-workers (2009), almost 20 million people will die from CVDs mainly from heart disease and stroke by 2015. The thrombolytic agents; plasmin-like proteins such as nattokinase and lumbrokinase can directly degrade the fibrin fibres in the blood clot (Peng *et al*., 2005) and

plasminogen activators, such as tissue-type plasminogen activator (t-PA) and urokinase act as enhancer to activate plasminogen into active plasmin in order to degrade fibrins. Unfortunately, these agents are often expensive, thermolabile and can produce undesirable side effects (Mine *et al*., 2005).

Because of the promising benefits of fibrinolytic enzymes isolated from fermented food sources, many researchers have extensively explored new sources of fibrinolytic enzymes in the form of Asian fermented food product. One of the important microorganisms that can produce fibrinolytic enzyme is *Bacillus* sp. This bacterial strain have been found to effectively produce extracellular and intracellular proteases including nattokinase enzyme isolated from *Bacillus natto* which was first discovered by Sumi and his team in 1987. Proteases produced by *Bacillus* sp. has been identified to be effective in treating thrombolytic therapy. Therefore, many previous researches have shown to successfully isolate and purify the fibrinolytic enzyme from *Bacillus* sp. For examples, *Bacillus* sp. strain CK 11-4 was discovered from traditional Korean fermented-soybean sauce, named Chung-Kook-Jang (Kim *et al*., 1996), *Bacillus subtilis* DC33 was isolated from Chinese traditional Douchi (Wang *et al*., 2006), and *Bacillus licheniformis* KJ-31 isolated from Korean traditional Jeot-gal (Hwang *et al*., 2007). In this research, fibrinolytic enzyme from the wild type *Bacillus* sp isolated from "budu", a local Malaysian fermented.was successfully purified to homogeneity.,

■2.0 PROBLEM STATEMENT

Thrombolytic agents such as tissue plasminogen activator (t-PA) and urokinase have been further investigated to be used as thrombolytic therapy in medical treatment. These activators are of human origin, but it was observed that these thrombolytic agents can cause excessive bleeding and recurrence at the site of the residual thrombosis besides having a short half-life (Wang *et al*., 2006). Despite their widespread use, scientists have concluded that these agents have undesirable effects as well as very costly to produce. Therefore, further research needs to be carried out in order to find another fibrinolytic enzyme source which has the potential to be developed for therapeutic use.

■3.0 EXPERIMENTAL WORK AND METHODOLOGY

Bacillus sp. producing fibrinolytic enzyme, B1 isolated from Malaysian fermented sauce called, budu (. Human plasma fibrinogen (Cat# 341576) and thrombin from bovine (Cat# 605157) were purchased from the Calbiochem. Plasmin from human plasma (10 602 361 001) [5U] and plasminogen from human serum (10 874 477 001) were purchased from ROCHE, DEAE Sepharose™ Fast Flow was product of Amersham Pharmacia Biotech AB (Sweden), Sephadex G-75 was product of Sigma-aldrich, Perfect Protein Markers (Novagen, 80030-960), Broad range protein molecular weight markers (Promega, Cat#V8491), Sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) set was purchased from Bio-Rad Co. (Richmond, CA, USA). All other reagents were analytical grade commercially available.

3.1 Bacterial Culture Condition for Enzyme Production

A single colony of *Bacillus* sp. strain B1 was inoculated into nutrient broth (NB)as seed culture and were placed in a shaker incubator at 37°C for 12 hours. After cultivation process, 10% (v/v) inoculums were transferred into the fermentation medium which was broth (NB) and shaken at 37°C for 36 hours at 210 rpm.

3.2 Crude Enzyme Preparation

After 36 hours of fermentation , thecell cultures of *Bacillus* sp. strain B1 was harvested by centrifuging at 4°C, 10000g, for 30 minutes. The supernatant was then collected and used as crude enzyme for the next purification steps.

3.3 Enzyme Assay

3.3.1 Fibrinolytic Activity

Fibrinolytic activity was identified by using two types of fibrin plate methods. There were plasminogen-free fibrin plate method and plasminogen-rich fibrin plate method (Astrup and Mullertz, 1952). Plasminogen-free fibrin plate was prepared by mixing the fibrinogen solution containing 5ml of 0.6% (w/v) bovine fibrinogen in 50 mM potassium phosphate buffer (pH 7.4), 100 µl of thrombin solution (42 units/ml), and 5ml of 2% (w/v) agarose. This plate was heated at 80°C for 30 minutes mainly to eliminate other fibrinolytic factors (Hua *et al*., 2008). Meanwhile, plasminogen-rich fibrin plate was made up by the addition of 200 µl of 5 units of plasminogen in a petri dish, but not heated as plasminogen-free fibrin plate. 20 µl of enzyme solution was gently dropped onto this hole. The fibrin plate was then incubated at 37°C for 12 hours. The fibrinolytic activity was determined by measuring the diameter of clear zone/ holozone formed. The units of the enzyme activities were determined according to the standard curve using plasmin as a standard.

3.4 Protein Determination

3.4.1 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 750 nm. Various protein concentrations were determined based on the standard curve.

3.4.2 Protein Molecular Weight Determination (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE))

The molecular weight of the enzyme was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), according to research done by Laemmli (1970). SDS-PAGE was carried out using 12% gradient polyacrylamide gel and 4-5% acrylamide stacking gel at 4°C (Jeong *et al*., 2001). The gel sheet was stained with 0.25% Coomassie brilliant blue (CBB) according to Merril (1990).

3.5 Purification of Fibrinolytic Enzyme

3.5.1 Screening of Ammonium Sulphate Precipitation

All purification steps were performed at 4°C. The crude enzyme was mixed with ammonium sulphate powder in order to fractionate the supernatant, up to 30-60% saturation (Balaraman and Prabakaran, 2007). Screening of ammonium sulphate precipitation was carried out by mixing the crude enzyme with 10 parts of ammonium sulphate saturation which were 10% (w/v), 20% (w/v), 30% (w/v), 40% (w/v), 50% (w/v), 60% (w/v), 70% (w/v), 80% (w/v), 90% (w/v), and 100% (w/v). The mixture was

left to stand for 10 to 30 minutes at 4°C with constant stirring. After that, the mixture was centrifuged at 10000 rpm at 4°C for 30 minutes. The precipitated formed was re-dissolved in 20 mM Tris-HCI buffer, pH 8.4. Dialysis was then carried out in order to remove the salt formed. The total protein concentration and fibrinolytic enzyme activity were determined via Lowry method and fibrin plate respectively.

3.5.2 Anion Exchange Chromatography on DEAE-Sepharose FF Column

The dialyzed enzyme was loaded on a DEAE-Sepharose column equilibrated with 20 mM Tris-HCI buffer, pH 8.4 (Peng *et al*., 2003). The absorbed protein solution was eluted with stepwise elution buffer, ranging from 0.2 M to 1.0 M of NaCI dissolved in same buffer, 20 mM Tris-HCI, pH 8.4 at rate of 60 ml/hour. The fractions collected were determined for its total protein concentration and fibrinolytic enzyme activity. The active fractions were pooled for the next purification step.

3.5.3 Gel Filtration Chromatography on Sephadex G-75 Column

Gel filtration chromatography was carried out using a Sephadex G-75 column (Jo *et al*., 2011). The column was equilibrated with 10 mM Tris-HCI buffer, pH 7.8 (Peng *et al*., 2003). The flow rate was 60 ml/hour. The fractions collected were determined for its total protein concentration and fibrinolytic enzyme activity. The active fraction obtained was pooled and kept in freezer at -20°C to be used as purified fibrinolytic enzyme for subsequent studies. All the active fractions from each purification steps were loaded on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the molecular weight of protein of interest.

■4.0 RESULTS AND DISCUSSION

4.1 Bacterial Culture

B1 taken from glycerol stock was successfully grown on nutrient agar plate and wascultured in nutrient broth medium for acclimatization processes. The results of B1 grown on nutrient agar were shown in **Figure 4.1**. The streaking plate was done twice in order to make sure the single colony obtained was not contaminated with the other microorganisms before inoculation into the fresh culture medium.

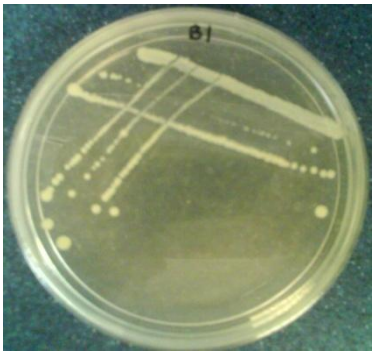


Figure 4.1 Growth of *Bacillus* sp. strain B1

4.2 Enzyme Assay

4.2.1 Fibrinolytic Enzyme Activity

The fibrinolytic enzyme activity for bacteria strain B1 was tested on two types of fibrin plates which were plasminogen-free fibrin plate and plasminogen-rich fibrin plate. After 24 hours incubation, clear zone/holo formed on both fibrin plates which indicated fibrinolysis of the fibrin plates for each different stages of purification steps (Table 4.2.1(b)). The summary of holozone diameter and enzyme activity for each of purification steps were shown in Table 4.2.1(a).

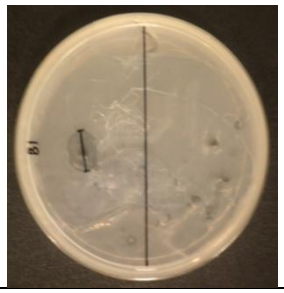
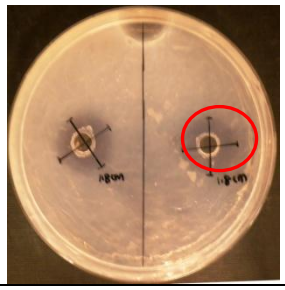
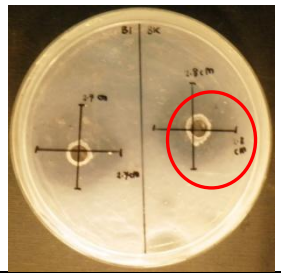
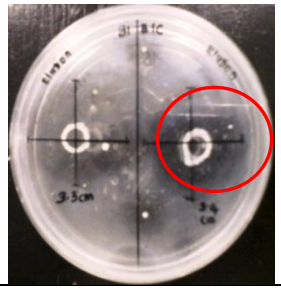
Table 4.2.1(a) Diameter of holozone and enzyme activity for each purification steps

Purification Step	B1	
	Holozone Diameter (cm)	Enzyme Activity (U/ml)
Crude Enzyme	1.3	1.98
Ammonium Sulfate Precipitation	1.8	2.73
DEAE-Sepharose Fast Flow	2.7	4.10
Sephadex G-75	3.3	5.01

The results obtained showed that the diameter of clear zone/holo on the fibrin plate for supernatant of bacteria B1 culture was 1.3 cm corresponding to 1.98 Units/ml activity. The fibrinolytic activity was slightly enhanced in plasminogen-rich fibrin plate compared to plasminogen-free fibrin plate. After the culture supernatant was subjected to ammonium sulphate fractionation, precipitated protein from strain B1 showed 1.8 cm of clear zone diameter on fibrin plate which corresponds to 2.37 Units/ml of enzyme activity.

Meanwhile, the active fraction of protein sample having fibrinolytic activity after elution from anion exchange chromatography on DEAE-Sepharose column gave higher reading of enzyme activity compared to the crude enzyme and ammonium sulphate fractionation. Up to this purification step, the enzyme activity for strain B1 was increased from 2.37 Units/ml to 4.10 Units/ml. This reading indicated that the fibrin around the well in fibrin plate was effectively degraded by the enzyme. The concentrated and pooled fraction eluted on Sephadex G-75 column formed 3.3 cm of clear zone on fibrin plates which corresponds to 5.01 Units/ml of fibrinolytic enzyme activity.

Table 4.2.1(b) Clear zone formed on fibrin plate of strain B1 indicated by the red circle

Step	Figure
Crude enzyme	
Ammonium Sulfate Precipitation	
Anion Exchange Chromatography (DEAE-Sepharose Fast Flow)	
Gel Filtration Chromatography (Sephadex G-75)	

4.2.2 Total Protein Concentration

The summary of the total protein concentration for each purification step was shown in Table 4.2.2. Based on the result, crude enzyme concentration was estimated at 89.35 mg/ml. The total protein content of the culture supernatant decreased when the crude enzyme were subjected to ammonium sulphate precipitation. The concentrated protein sample was determined to be 13.83 mg/ml of the total protein content.

The concentrated protein sample from ammonium sulphate fractionation was then further purified using anion exchange chromatography on DEAE-Sepharose Fast Flow column. The active fractions eluted from DEAE-Sepharose column was measured for protein content and fibrinolytic activity. About 4.521 mg/ml of protein was in the active fraction of strain B1. The lowest protein content was achieved when the active fractions eluted from DEAE-Sepharose column was loaded onto Sephadex G-75 column. The active fractions eluted from gel filtration chromatography was pooled, concentrated and measured for their total protein content. The total protein content after gel filtration chromatography was 4.290 mg/ml.

Table 4.2.2 Protein concentration for each purification steps

Purification Step	Protein Concentration (mg/ml)
	B1
Crude Enzyme	89.35
Ammonium Sulfate Precipitation	13.83
DEAE-Sepharose Fast Flow	4.521
Sephadex G-75	4.290

4.3 Ammonium Sulphate Precipitation Screening

Ammonium sulphate precipitation screening was carried out to determine the best percentage of ammonium sulphate saturation which gave the highest fibrinolytic activity. The result for ammonium sulphate saturation on the precipitation of fibrinolytic enzyme from *Bacillus* sp. strain B1 was shown in Table 4.3.

Based on the result, the crude enzyme did not precipitate at 10% ammonium sulphate saturation. Thus, there were no results for total protein content and fibrinolytic enzyme activity which indicated that the protein did not tend to aggregate and salt out of culture solution due to low ammonium sulphate concentration. However, the fibrinolytic enzyme activity was increased at 40% and 50% of ammonium sulphate concentration. This indicated that the fibrinolytic enzyme precipitated between 40% and 50% of ammonium sulphate saturation (Vengadaramana *et al*., 2011).

The crude enzyme from strain B1 precipitated at 40% ammonium sulphate and gave the highest specific activity which was 1.803 Units/mg. When compared to the crude enzyme, the specific activity of precipitated protein was increased by 10 times with 40% ammonium sulphate.

Table 4.3(a) Screening for ammonium sulphate saturation (in percentage) for precipitation of fibrinolytic enzyme from *Bacillus* sp. strain B1

Ammonium Sulphate (%)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)
Crude Enzyme	3425.10	594	0.173
10	ND	ND	-
20	332.10	546	1.644
30	363.00	639	1.760
40	404.40	729	1.803
50	425.10	639	1.503
60	425.10	639	1.503
70	476.70	594	1.246
80	632.10	594	0.939
90	673.50	501	0.744
100	807.90	501	0.620

4.4 Purification of the Fibrinolytic Enzyme

4.4.1 Wild Type Bacillus sp. Strain B1

The wild type fibrinolytic enzyme was purified from the culture supernatant of *Bacillus* sp. strain B1. The crude enzyme from supernatant containing 89350 mg of protein was collected after harvesting the cell from the *Bacillus* sp. strain B1 culture. After the supernatant was subjected to fractional ammonium sulphate precipitation, the crude enzyme precipitated at 40% of ammonium sulphate saturation. The yield of fibrinolytic enzyme was 4.14% with specific activity 0.20 Units/mg and was purified 10 fold compared to the crude enzyme. The precipitated protein contained 414.90 mg of total protein, which was lower that the total protein of crude enzyme (89350 mg). The precipitated protein was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to estimate the molecular weight of fibrinolytic enzyme.

Table 4.4.1 Purification schemes of the fibrinolytic enzyme of wild type *Bacillus* sp. Strain B1

Purification Steps	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Crude Enzyme	1000	1980	89350	0.02	1	100
Ammonium Sulfate Precipitation	30	81.90	414.90	0.20	10	4.14
DEAE-Sepharose FF	8	32.80	36.17	0.91	46	1.66
Sephadex G-75	2	10.02	8.58	1.17	59	0.51

The dialyzed protein sample from ammonium sulphate precipitation was then loaded on anion exchange DEAE-Sepharose Fast Flow column. As shown in elution profile of fibrinolytic enzyme from *Bacillus* sp. strain B1 on DEAE-Sepharose FF (Figure 4.4.1(a)), there were five peaks formed according to the elution order. There were only two peaks corresponding to 0.2 M and 0.6 M of NaCl elution buffer which contained the fibrinolytic enzyme activity. Among all the fractions, fraction 8, 22, 29, 30, 31, 32, and 33 had fibrinolytic activity. These fractions were selected for SDS-PAGE, only fractions 29 and 30 showed clear bands on SDS gel also indicating higher protein concentration.

Due to low fibrinolytic activity of fraction 8, 22, 31, 32, and 33, only fractions 29 and 30 which contained high specific fibrinolytic activity were selected for further purification steps. The fractions 29 and 30 were pooled and this pooled fibrinolytic enzyme showed 0.91 Units/mg enzyme activity, containing 36.17 mg protein. Up to this purification step, it was observed that the specific activity of fibrinolytic enzyme was slightly increased from 0.20 to 0.91 Units/mg protein (Table 4.4.1). During this step, the sample protein was purified 45.5 fold which was higher than the crude enzyme with 1.66% yield.

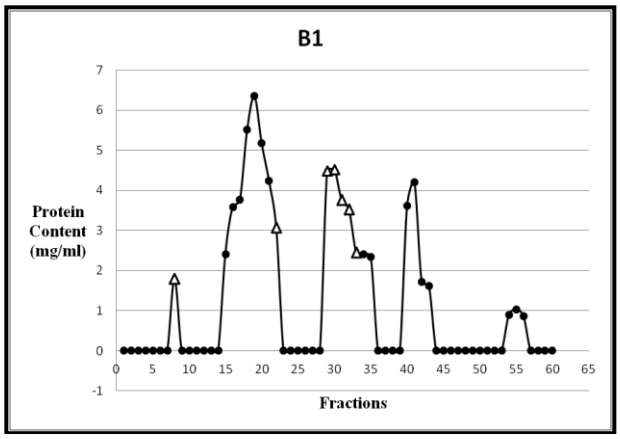


Figure 4.4.1(a) Elution profile of fibrinolytic enzyme from *Bacillus* sp. strain B1 on DEAE-Sepharose FF:( ) protein content (mg/ml) and ( ) fraction contain fibrinolytic activity

The wash-out protein fraction through DEAE-Sepharose Fast Flow column was subjected to SDS-PAGE. There were three bands of proteins observed (Figure 4.4.1(c)). The active fractions with fibrinolytic activity collected through DEAE-Sepharose column were then loaded onto the gel filtration chromatography on Sephadex G-75 column. There were 30 fractions collected during this gel filtration chromatography except fraction 1 and 29 which did not contain the protein content (Figure 4.4.1(b)).

The elution of fibrinolytic enzyme solution by Sephadex G-75 gel filtration yielded two major peaks, P1 and P2. However, only the second peak, P2, was noted to have fibrinolytic activity and shown by the fraction 22 and 23 among 30 fractions. Fraction 22 and 23 were concentrated using viva spin and then were subjected to SDS gel electrophoretic separation to determine the purity of enzyme. Only fraction 23 appeared on SDS-PAGE and migrated as a double protein band which indicated that this fibrinolytic enzyme was successfully separated from the other non-targeted protein and contaminants. The final specific activity of eluted protein increased from 0.91 to 1.17 Units/mg. The wild type fibrinolytic enzyme was purified 58.5 fold with 0.51% yield and the total protein content was 8.58 mg.

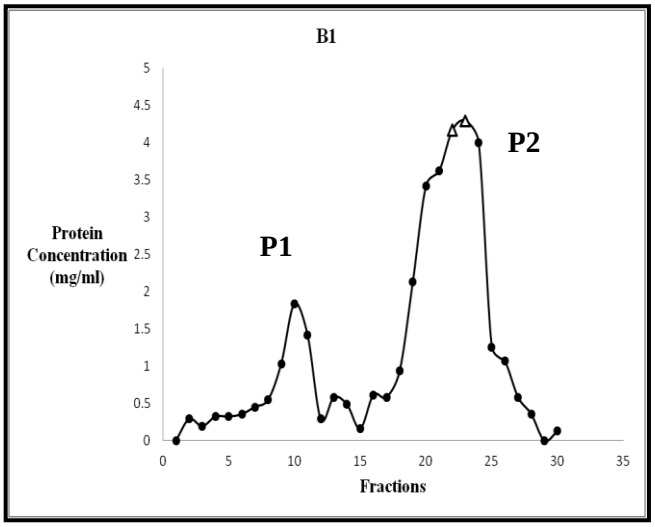


Figure 4.4.1(b) Elution profile of gel filtration for the chromatography of DEAE-Sepharose fractions on a Sephadex G-75 column ( ) protein content (mg/ml) and (fraction contain fibrinolytic activity

Based on SDS-PAGE analysis, it was shown that the estimated molecular weight of target fibrinolytic protein was 45 KDa (Figure 4.4.1(c)). Previous work on purification and characterization of fibrinolytic enzymes from bacterial sources reported that the purified fibrinolytic enzyme has a molecular weight within the range of 20-45KDa (Sumi *et al.*, 1987, Kim *et al.* 1996, Jeong *et al.*, 2001, Peng *et al.*, 2003, Balaraman and Prakabaran, 2007, Hwang *et al.*, 2007, Do *et al.*, 2011). This is an indication the fibrinolytiv acitivity of the purified fraction was conferred by the purified protein. In conclusion, the fibrinolytic enzyme obtained from bacillus sp. Which was isolated from feremented food exhibits a strong fibrinolytic activity which has the potential to be developed as therapeutic agents for the treatment of thrombolytic related diseases.

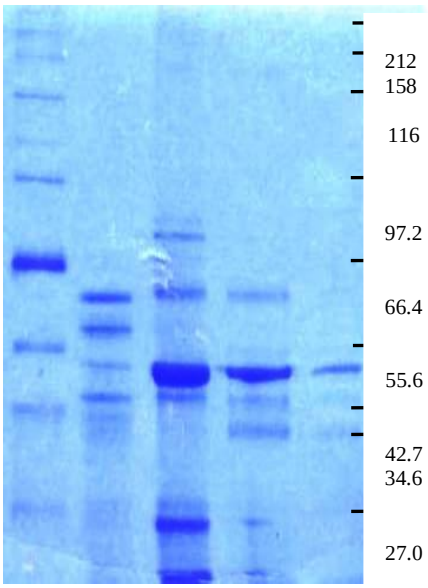


Figure 4.4.1(c) Polyacrylamide gel electrophoresis analysis of purified fibrinolytic enzyme from *Bacillus* sp. strain B1. **M**: molecular weight marker in kDa**. Lane 1**: crude enzyme. **Lane 2**: concentrated enzyme from ammonium sulphate precipitation steps. **Lane 3**: eluted proteins through DEAE-sepharose fast flow column. **Lane 4**: purified fibrinolytic enzyme from DEAE-anion exchange column. **Lane 5**: purified fibrinolytic enzyme from Sephadex G-75 column

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