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Screening, Purification, and Characterization of Fibrinolytic Enzyme-Producing Bacteria from Indonesian Fermented Foods

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Original Research Article

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Abstract: Fermented foods have been explored for their potentials as main sources of fibrinolytic enzymes. In this study, we did screening for fibrinolytic enzymes-producing bacteria isolated from several Indonesian fermented foods, including black tauco, salted tauco, dadih, cassava tape, brem, salted vegetable, fermented fish, terasi, tempe, oncom, fermented fish, tahu, and tempoyak. Fibrinolytic activity was directly determined by fibrin zymogram and fibrin-plating assays. Crude enzyme from selected isolate producing optimal fibrinolytic activity was produced, purified, and further characterized for its biochemical properties (optimum pH, optimal temperature, protease inhibitor, metal ion, and substrate specificity). Crude enzyme was purified using ammonium sulphate fractionation with 65% saturation, dyalisis using nitrocellulose acetate membrane (cut off 10 kDa), concentration with polyethylene glycol, followed by fractionation and purification using fast protein liquid chromatography with DEAE-Sepharose column. Our results demonstrated that a fibrinolytic enzyme (F7 eluate) was successfully isolated, purified, and characterized from the culture filtrate of TH-5 strain isolated from the black tauco. Spesific activity of F7 eluate was 15.421 U/mg with 45.6 purification fold compared to its crude enzyme. The optimum pH and temperature for enzyme activity of F7 eluate were 7 and 50^{o}C, respectively. Enzyme activity of F7 eluate was totally inhibited by addition of phenylmethylsolfonylfluoride (PMSF) and N-α-tosyl-L-lysine chloromethyl ketone (TLCK) inhibitors at final concentration of 1 mM, indicating that enzyme was grouped in serine protease family. Metal ions (K^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, and Cu^{2+}) at total concentration of 5 mM strongly reduced enzyme activity with residual activity less than 30%, indicating that enzyme catalytic of F7 eluate was significantly inhibited by the addition of the ions. Fibrin zymogram profile of F7 eluate resulted in single fibrinolytic band with estimated molecular weight of 36.2 kDa, and the enzyme had a specific characteristic to degrade both fibrin and fibrinogen substrates.
Keywords: Indonesian fermented foods, fibrinolytic enzyme, screening, purification, characterization.

INTRODUCTION

Fibrinolytic enzyme is a serine protease group that is capable to degrade fibrin clot in thrombus. In our modern society, life-threatening cardiovascular diseases and intravascular thrombosis is one of the main causes of death. Some commercial fibrinolytic enzymes have been widely used as therapeutical thrombolytic agents, such as urokinase, streptokinase, and tissue-type plasminogen activator (t-PA). Lumbrokinase, extracted and purified from *Lumbricus rubellus*, has also been developed as orally thrombolytic therapy since commercial fibrinolytic enzymes are used as plasminogen activators for intravenous administration [1]. Several fibrinolytic enzymes produced from *Bacillus* sp. screened from fermented foods have been reported [2-5]. Nattokinase was produced from *B. natto* in the traditional Japanese fermented food. Nattokinase enhanced fibrinolytic activity and stimulated t-PA by oral administration [6]. Research study on fibrinolytic enzyme screened from fermented food has also been focused on a construction and cloning of gene of fibrinolytic enzyme for scale up production [7].

In recent years, several reports of *Bacillus* genus isolated from various fermented foods in Asia region with potential fibrinolytic enzymes have been published, such as *B. amyloliquefaciens* DC-4 from douchi, a fermented soy food from China [8], *Bacillus* sp. CK from chungkookjang, a fermented soy sauce from Korea [9], *Bacillus* sp. DJ-2 strains and DJ-4 from doenjang, a Korean fermented soybean paste [10, 11], and *Bacillus* sp. KA38 from jeotgal, a fermented salted fish from Korea [4]. Our previous study also demonstrated that *B. pumilus* 2.g isolated from gembus, the Indonesian traditional fermented soybean cake exerted fibrinolytic activity [12]. Moreover, *B.*

licheniformis RO3 screened from red oncom made from the Indonesian fermented waste tofu also secreted fibrinogenolytic activity [13]. Thus, in this study, we investigated the potency of fibrinolytic enzyme-producing bacteria from several Indonesian traditional fermented foods. A potent bacterial isolate from selected fermented food that produced fibrinolytic enzyme could be a promising agent for thrombolytic therapy.

MATERIALS AND METHODS

Materials

There were 13 candidates of traditional fermented foods that had been screened for its proteolytic activity on skim milk media. Indonesian fermented foods, such as black tauco, salted tauco, dadih, cassava tape, brem, salted vegetable, fermented fish, terasi, tempeh, oncom, fermented fish, tahu, and tempoyak) were collected from traditional markets in Java and Sumatera regions (Indonesia). Meanwhile, natto as the reference of Japanese fermented food was obtained from supermarket in Japan.

Microorganism isolation and cultivation

Samples were diluted and blended in 50 mM Tris-Cl buffer (pH 7), then centrifuged at 7500 ×g and 4°C for 10 min to produce filtrate extract. Filtrate was cultured in Luria Bertani (LB) media and incubated at 37°C for 24 h. A 100 µL of cultured microbe was spread on skim milk agar (SMA) and incubated at 37°C for 48 h. Selected isolate that has clear zone was further cultivated in LB media supplemented with skim milk (2%). Crude enzyme was separated from cell mass using centrifuge at 7.500 ×g and 4°C for 10 min.

Enzyme assays and protein concentration

Fibrinolytic activity was directly assayed using fibrin zymogram and fibrin plate methods. Fibrin zymographic analysis was done based on slightly modification of Choi *et al*. method [14]. In this method, acrylamide in separating gel was copolymerized in bovine fibrinogen substrate (0,5%b/v in Tris-Cl pH 8) that was previously stimulated by bovine thrombin (10 NIH U/mL). Clear zone on separating gel after renaturation, digestion, staining and destaining treatments indicated fibrinolytic enzyme activity.

Fibrin plate were perfomed by modification of Astrup and Mullertz method using bovine fibrinogen (0,5%b/v in Tris-Cl pH 7) and bovine thrombin 50 NIH U/mL (in aquabidest) [15]. Urokinase and streptokinase were used as standard of commercial fibrinolytic enzymes. After incubation at 37ºC for 0-15h, clear zone formation in fibrin clot indicated fibrinolytic activity. Caseinolytic activity was spectrophotometrically assayed by using casein Hammarsten substrate according to Bergmeyer method [16]. One unit of protease was defenited as the amount of enzyme needed to produce one micromole tyrosine per minute at optimal condition. Protein content was tested with Bradford method using Bovine Serum Albumin Fraction V as protein standard [17].

Purification of enzyme

Crude enzymes were purified with some steps, i.e. fractionation with ammonium sulphate with 65% saturation, dyalisis using nitrocellulose acetate membrane with cut-off 10 kDa, and concentration using polyethylene glycol (6 kD). Enzyme concentrate were fractionated with anion exchange column in DEAE-Sepharose matrices by conducting fast protein liquid chromatography instrument. Enzyme fractions were eluted with stepwise elution using NaCl (0.2 and 0.5 M) in 50 mM Tris-Cl buffer (pH 8). Eluate fractions were further assayed for enzyme activity and protein content.

Determination of molecular weight

SDS-PAGE was performed according to the method of Laemmli using a 4% stacking and 12% resolving polyacrylamide gel [18]. Low molecular weight (LMW) marker was used as protein standards.

Optimal temperature and pH

The optimal pH for the proteolytic activity was determined by varying the pH of the reaction mixture between 4 and 12 in 50 mM universal buffer. Optimum temperature was determined under standard conditions at different temperature (27-80°C). The enzyme was incubated at each temperature for 10 min and the proteolytic activity was assayed using Bergmeyer method as previously described [16].

Effect of protease inhibitors

Enzyme was pre-incubated with 1 mM phenylmethylsolfonylfluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.01 mg/mL soybean trypsin inhibitor (STI), and 0,1 mM N-α-tosyl-L-lysine chloromethyl ketone (TLCK) at 4^{o}C for 1 h. The residual enzyme activity was determined using the caseinolytic assay as previously described.

Effect of metal ions

Enzyme solution was pre-incubated with 5 mM of K^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, and Cu^{2+} metal ions at 4^{o}C for 1 h. The residual enzyme activity was determined using the caseinolytic assay as previously described.

RESULTS AND DISCUSSION

Selection of candidate for fibrinolytic enzyme-producing bacteria from Indonesian fermented foods

Among 13 Indonesian fermented foods, proteolytic bacteria strains were successfully isolated from 8 samples. However, only bacteria strains from 4 samples (black tauco, tempoyak, salted vegetable, and natto) exerted fibrinolytic activity on fibrin plate results (Table 1). Natto as Japanese traditional fermented food was used as a reference with local fermented foods in Indonesia. Sumi *et al*. had screened *Bacillus natta* that

secreted nattokinase from the vegetable cheese natto [2]. Nattokinase is a 27 kDa fibrinolytic enzyme that has been applied for thrombolytic oral therapy in one decade. Few reports also demonstrated that *Bacillus* sp. strains isolated from several Korean traditional fermented foods, such as chungkookjang, deon-jang, and jeot-gal showed a potent fibrinolytic action *in vitro* [3, 5, 19].

Table-1: Fibrinolytic enzyme-producing bacteria isolated from several Indonesian traditional fermented foods

No	Indonesian fermented foods	Proteolytic activity on SMA media	Strain code	Fibrinolytic activity on fibrin plate assay
1	Black tauco	+	TH	+
2	Salted tauco	+	TB	-
3	Fermented fish	+	IP	-
4	Terasi	+	DxTy	-
5	Salted vegetable	+	SA	+
6	Tempeh	-	-	-
7	Tahu	-	-	-
8	Oncom	-	-	-
9	Natto*	+	NT	+
10	Tempoyak	+	TPK	+
11	Cassava tape	+	TP	-
12	Dadih	-	-	-
13	Brem	-	-	-

(*) Japanese fermented food

Our results demonstrated that black tauco was selected as candidate for screening and purifying its fibrinolytic enzyme-producing bacteria due to its potential fibrinolytic activity in the culture crude (Table 2). From 7 bacterial strains with code of THs isolated from black tauco, the TH-5 strain was selected for further purification and characterization of its fibrinolytic enzyme.

Table-2: Comparison of caseinolytic and fibrinolytic activities of crude enzymes produced by bacteria isolated from Indonesian traditional fermented foods

No	Local fermented foods	Strain code	Caseinolytic activity (U/mL)	Fibrinolytic activity (U/mL)
1	Black tauco	TH-5	0.127	-
2	Salted vegetable	SA-1	0.159	0
3	Tempoyak	TPK-2	1.474	0.596
4	Natto*	NT-1	0.226	-

(*) Japanese fermented food

Purification of enzyme

Crude enzymes were purified with some steps, i.e. precipitation with ammonium sulphate with 65% saturation, dyalisis using nitrocellulose acetate membrane with cut off 10 kDa, concentration with PEG 6 kDa, followed by fractionation using anion exchange chromatography with DEAE-Sepharose FF matrice. Chromatogram profile showed that there was single enzyme peak (F7 eluate) after step wise elution with NaCl 0.5 and 1.0 M with its specific activity of 15.421 U/mg (Figure 1).

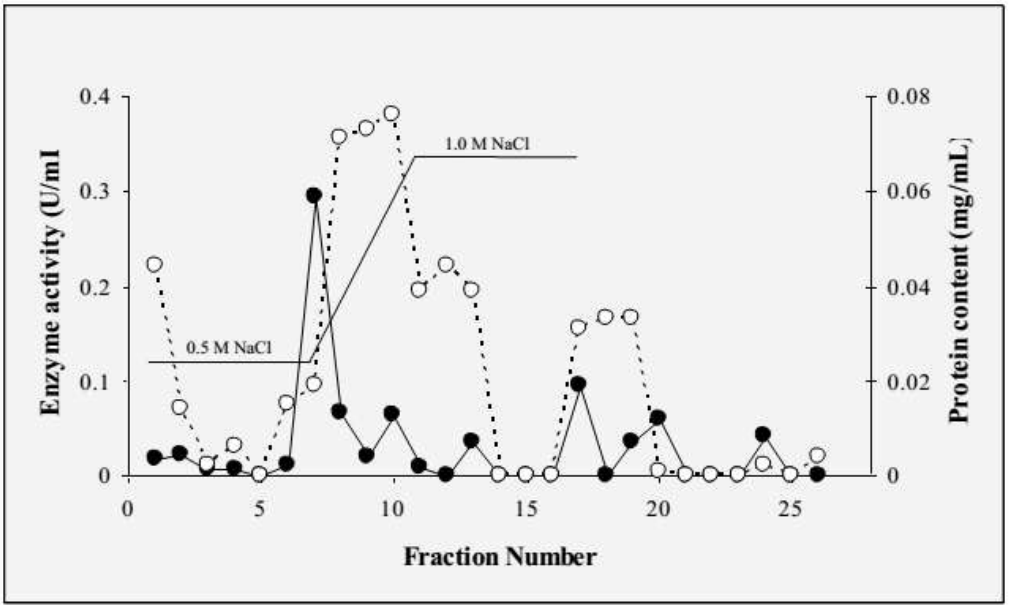

Fig-1: Chromatogram profile of enzyme produced by TH-5 strain isolated from black tauco using FPLC. DEAE-Sepharose column was washed with 25 mM Tris-Cl buffer (pH 8) and enzyme fractions were eluted using step wise gradient with NaCl 0.5 and 1.0 M in 25 mM Tris-Cl (pH 8). Signs of (-•-) and (-○-) indicated enzyme activity (U/mL) and protein content (mg/mL)

Table 3 showed that specific activity of F7 eluate, the purified enzyme from TH-5 strain isolated from black tauco, was 15.421 U/mg with a 45.6 purification fold compare to that of crude enzyme. Specific activity of enzyme was needed for measurement of enzyme purity. Thus, F7 eluate was further characterized for its fibrinolytic activity and biochemical properties.

Table-3: Purification steps of enzyme produced by TH-5 strain isolated from black tauco

Steps	Total activity (Unit)	Total protein (mg)	Spesific activity (U/mg)	Yield (%)	Purification fold (times)
Enzyme crude	8.1	24.20	0.338	100	1
Presipitate	1.76	3.60	0.489	21.73	1.4
Concentrate	1.24	1.11	1.117	15.31	3.3
Eluate (F7)	0.586	0.038	15.421	7.2	45.6

Fibrinolytic activity

Fibrinolytic activity of F7 eluate was tested by both fibrin plate and fibrin zymogram assays. Figure 2 showed that F7 eluate exerted a potent fibrinolytic activity compared to commercial fibrinolytic enzymes (streptokinase 250 U) after incubation at 37ºC for 1 h. Streptokinase had no fibrinolytic activity during 1 h incubation, indicating that the enzyme may need longer time for hydrolyzing the fibrin clot.

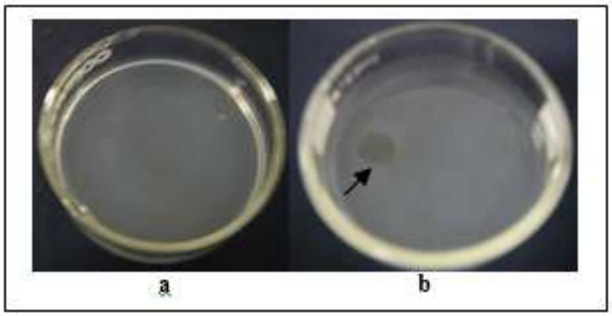

Fig-2: Fibrinolytic activity of F7 eluate (0.38 µg protein) and commercial streptokinase (250 U) on *in vitro* fibrin clot reaction: (a) before incubation, and (b) after incubation at 37°C for 1 h. F7 eluate was purified from crude enzyme produced by TH-5 strain isolated from black tauco. Sign of arrow showed fibrinolytic activity of F7 eluate

To determine the active fibrinolysis time, F7 eluate and commercial urokinase were further incubated at 37°C for 0-15 h. Fibrinolytic activity of F7 eluate was positively retained and stable after incubation for 15 h. Figure 3 demonstrated that the enzyme has a slightly work activity to degrade *in vitro* fibrin clot on dish. This data information is useful for enzyme application as oral or intravenous thrombolytic therapy in the future. Enzyme with longer fibrinolytic activity (>3 h) could be applied as a promising orally antithrombotic agent, such as lumbrokinase and nattokinase [1, 6, 20, 21].

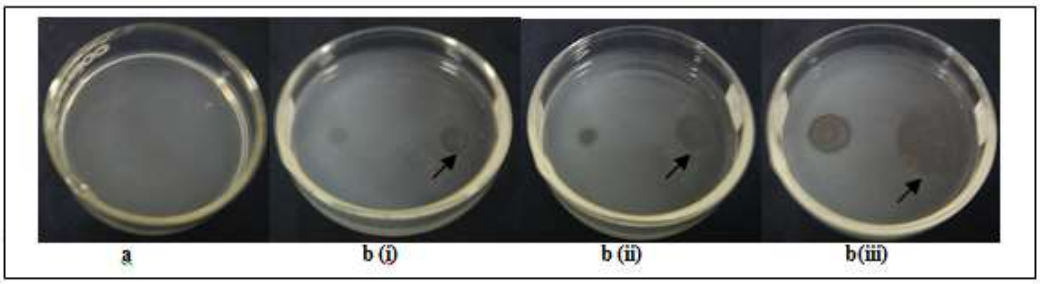

Fig-3: Fibrinolytic activity of F7 eluate (0,19 µg protein) and commercial urokinase (50 U) on *in vitro* fibrin clot reaction: (a) before incubation, and (b) after incubation at 37°C for (i) 1 h, (ii) 3 h, and (iii) 15 h. F7 eluate was purified from crude enzyme produced by TH-5 strain isolated from black tauco. Sign of arrow showed fibrinolytic activity of F7 eluate

Fibrin zymogram analysis was also conducted to support the potential fibrinolytic activity of F7 fraction. Our results revealed that F7 fraction had only a single fibrinolytic band with molecular weight of 36.2 kDa (Figure 4b). Enzyme purity was determined by SDS-PAGE analysis. There were two protein bands in F7 fraction with estimated molecular weight of 25.8 and 30.6 kDas (Figure 4a). Other fibrinolytic enzymes, lumbrokinase from *L. rubellus* has six fibrinolytic isozymes [1, 22]. Serine fibrinolytic protease from the dung beetles, *Catharsius molossus* has a molecular weight of 27 kDa [23]. Research study on a recombinant fibrinolytic enzyme from *Bacillus* sp. N18 reported that enzyme had an apparent molecular weight of 28 kDa [7]. Fibrinolytic enzyme from Korean snake venom was characterized by its molecular weight of 51 kDa [24].

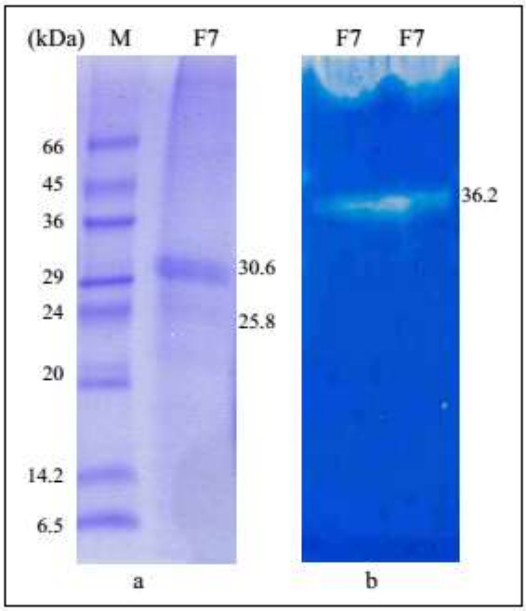

Fig-4: SDS-PAGE (a) and fibrin zymogram (b) profiles of F7 eluate. Lane a: LMW marker and F7 eluate (0.29 µg protein). Lane b: F7 eluate (0.29 µg protein). F7 eluate was purified from crude enzyme produced by TH-5 strain isolated from black tauco.

Optimal temperature and pH

The effects of temperature and pH on the enzyme activity of crude and F7 eluate were also determined. Figure 5 indicated that optimal pH of enzyme activity on crude enzyme and F7 eluate was reached in pH 7 (in universal buffer). The enzyme had still maintained its activity between 37°C and 65°C with an optimum at 50°C. In linier with this study, optimal

temperature and pH of fibrinolytic enzyme secreted by *B. amyloliquefaciens* screened from doenjang were 35-37°C and 6.3-6.5 [11].

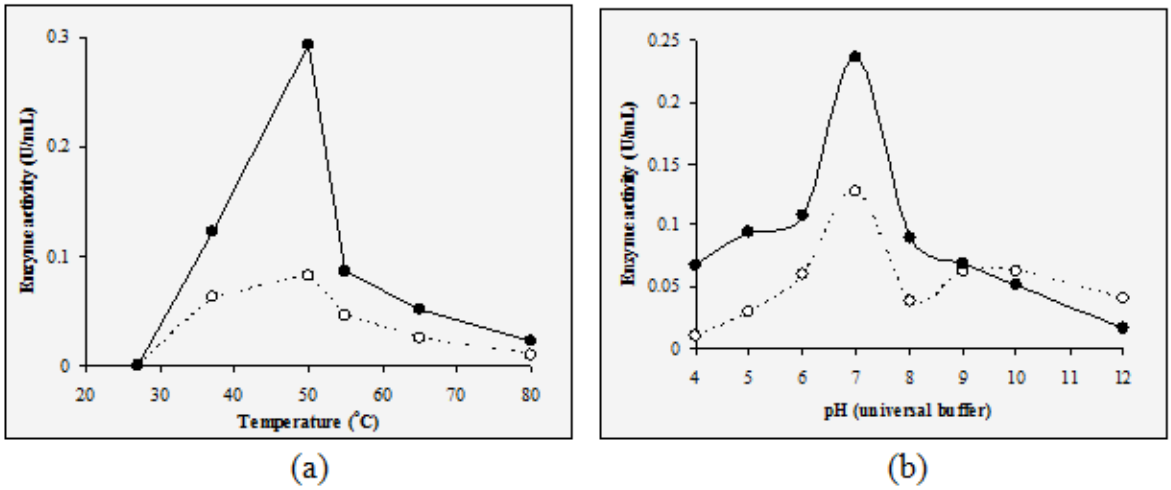

Fig-5: Optimal temperature (a) and pH (b) of enzyme activity: (-o-) crude and (-•) F7 eluate. F7 eluate was purified from crude enzyme produced by TH-5 strain isolated from black tauco

Effect of inhibitors

The effect of various inhibitors on enzyme activity of crude and F7 eluate was also measured. Figure 6 showed that PMSF and TLCK inhibitors, well-known inhibitor of serine protease, were found to strongly inactivate enzyme activity on crude and F7 eluate. Addition of STI inhibitor also reduced a half of enzyme activity on crude and F7 eluate. Meanwhile, EDTA, a metaloprotease inhibitor, did not affect enzyme activity. Thus, this data indicated that F7 eluate was grouped in serine protease family. Previous study stated that fibrinolytic enzymes from dung beetles and earthworms were also inhibited by typical serine protease inhibitors, such as PMSF, STI, TLCK, benzamidine, and aprotinin [22, 23].

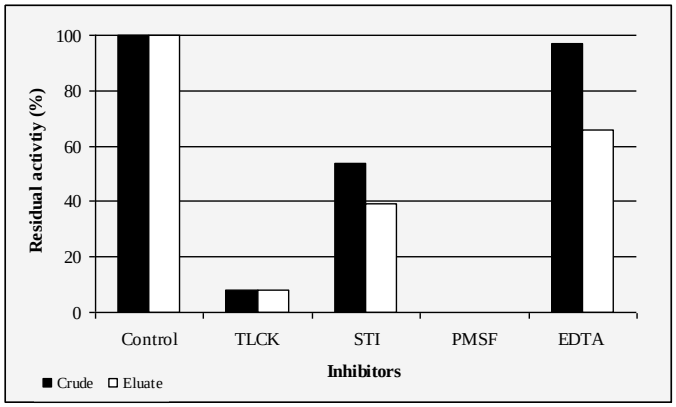

Fig-6: Effect of protease inhibitors on enzyme activity for crude and F7 eluate. F7 eluate was purified from crude enzyme produced by TH-5 strain isolated from black tauco

Effect of metal ions

Study on the effect of metal ions on enzyme activity of crude and F7 eluate was also determined. Some mono- (K, Na) and divalent ions (Mg, Mn, and Cu) at total concentration of 5 mM strongly decreased enzyme activity >70% compared to that of control (Fig. 7). It is indicated that metal ions were inhibitor agents for enzyme activity of crude and F7 eluate from TH-5 strain isolated from black tauco. Fibrinolytic enzyme from dung beetles was also inactivated by Cu^{2+} and Zn^{2+} ions [23].

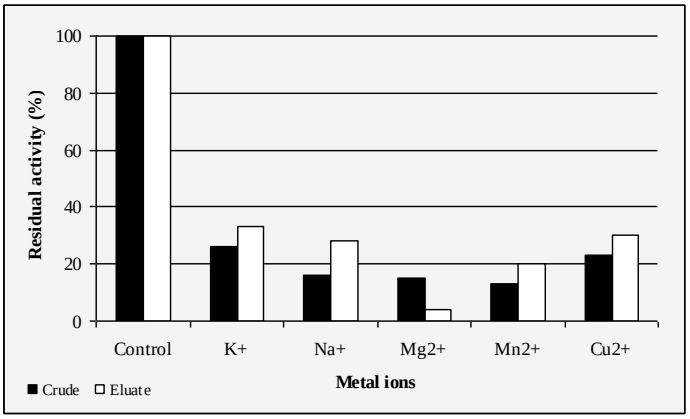

Fig-7: Effect of metal ions on enzyme activity for crude and F7 eluate. F7 eluate was purified from crude enzyme produced by TH-5 strain isolated from black tauco

CONCLUSION

A potential fibrinolytic enzyme with molecular weight of 36.2 kDa and specific activity of 15.421 U/mg has been isolated, purified, and characterized from crude enzyme produced by TH-5 strain isolated from the local fermented food of black tauco. The enzyme has fold purity of 45.6 times compared to that of its crude. The enzyme has optimum pH at 7 and optimum temperature at 50°C. The enzyme is grouped in serine protease and degrades both fibrin and fibrinogen substrates. These characteristics suggest that the enzyme may be orally applied for management of cardiovascular disease particularly preventing thrombosis.

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