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Thrombolytic Activity of Fibrinolytic Enzyme from Black Soybean Tempeh (*Glycine Soja Sieb. Et Zucc*) Fermented by *Rhizopus Oligosporus* FNCC 6010.

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ABSTRACT

Tempeh is a famous Indonesian traditional fermented food. It is made by various soy and legumes as raw materials. In this research, black soybean (*Glycine soja* sieb. et zucc) is used as a substrate for fermentation process by fibrinolytic enzyme producing *Rhizopus oligosporus* FNCC 6010. Optimization of the fermentation process was performed by temperature and inoculums concentration. The tempeh fibrinolytic enzyme (TpFE) was extracted from the tempeh by water and centrifuged to separate supernatant from solid materials. Ammonium sulfate was used for partially the TpFE purification. The enzyme activity was tested by fibrin plate. It was found that the TpFE activity from the supernatant is 2,745 U/ml with a protein concentration of 10.358 mg/ml and specific activity is 0.265 U/mg. Furthermore, increasing concentrations of ammonium sulfate for partial purification was affected the TpFE activity. The higher concentration of ammonium sulfate is the lower the enzyme activity. The maximum activity of fibrinolytic was obtained at a temperature of 45 ºC. The highest activity of the fibrinolytic enzymes (22,851 U/mL) was achieved at pH 5,5. Addition of Ca^{++}, Co^{++} and Mn^{++} in the fermentation media lead to increase the TpFEactivity, 23.653 U / mL, 22.262 U / mL and 21.566 U / mL respectively. Trombolitic activity in vitro clot lysis results in a decrease to 97.6%

Keywords: Black soybean tempeh, *Rhyzopus oligosporus*, Thrombolytic activity, fibrinolytic enzyme

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INTRODUCTION

The fibrinolytic enzymes of microbial origin that are able to dissolve endogenous thrombi in vivo were reported. Mechanism action of the fibrinolytic enzymes as thrombolytic was also studied special focus on the structure and character of the enzymes. Streptokinase, for example, is produced by b-hemolytic streptococci and exerts its enzyme action indirectly by activating plasminogen. On the other hand, staphylokinase is produced by *Staphylococcus aureus* by stoichiometric complexation with plasmin (ogen) that activates other plasminogen molecules. Serrapeptase is a different fibrinolytic enzyme produced by entero bacterium *Serratia* sp. E-15 with multiple functions including fibrin degradation. In addition, nattokinase is one of the fibrinolytic enzymes, produced by *Bacillus* natto, derived from fermented food [1]. On the other hand, the available thrombolytic agents are very expensive and cause side reactions effects such as increasing the risk of unwanted bleeding internally in the intestine channel when used orally.

Thrombolysis therapy with thrombolitic agents, such as alteplase, anistreplase, streptokinase, urokinase and tissue plasminogen activator (t-PA) is often used to degrade the clot formed in vascular disease. Therefore, the agent thrombolitic still continues to be developed to obtain a therapeutic agent with a lower risk of side effects as well as a relatively affordable cost [2]. Research on isolation and screening activity of fibrinolytic enzymes from many microorganisms as agents of thrombolysis has been reported. Fibrinolytic activity on various fermented foods from Korea (Chungkookjang and Doen-jang, Jeot-gal); Japan (natto) has been studied. Tempeh soybeans fermented by Fusarium sp has also been studied to produce proteolytic enzymes that have fibrinolytic activity [1]. Tempeh is Indonesian traditional food that is processed through fermentation technology by using soybean and *Rhizopus* spp. [3]. The soybean tempeh fermented by *Rhizopus* spp. could be able to produce proteolytic enzymes that are potential as fibrinolytic agent [4]. Most of the tempeh made with basic ingredients of soy, but can also be made from a wide variety of beans such as black soybean (*Glycine soja* sieb.et zucc), koro bean (Mucuna pruriens), red bean (*Phaseolus radiatus*), and tunggak bean (*Vigna unguiculata*). Peng et al. (2003) [5] have managed to do a purification of fibrinolytic enzymes of *Bacillus amyloliquefaciens* DC-4 from dochi, the traditional food of Chinese origin made of soy. In addition, purification of fibrinolytic enzyme nattokinase from *Bacillus subtilis* TKU007 has been carried out by [6]. In his research, nattokinase obtained through purification with a 3-stage procedure that precipitation with ammonium sulfate. This research, the thrombolytic activity of TpFE extract from black soybean (*Glycine soja* sieb. et zucc) fermented by *Rhizopus oligosporus* FNCC 6010 has been investigated. The performed a partial purification and characterization of the influence of pH, temperature, and metal activator has been performed. In addition to testing the thrombolytic activity using clots lysis.

MATERIAL AND METHODS

Materials

Fibrin, thrombin, fibrinogen and plasmin (all from human plasma), nattokinase, and tyrosine (Sigma-Aldrich, St. Louis, MO, USA). Sephadex 50 (Pharmacia, Uppsala, Sweden). All other chemicals and reagents used are analytical grade.

Microorganism and maintenance of culture

Rhizopus oligosporus FNCC 6010 was derived from Microbiology Laboratory, PAU Pangan Gizi Gadjah Mada University and grown on potato dextrose agar slants at 30^{o}C for 3-5 days and stored at 4^{o}C. The stock culture was revived on fresh agar slants medium for the whole experiment.

Collection of legume for tempeh production

The black soybean (*Glycine soja* sieb. et zucc) was obtained from Indonesian Legumes and Tuber Crops Research Institute in Malang, East Java, used as raw material for tempeh production.

Preparation of tempeh [7]

Producing tempeh from black Soybean (*Glycine Soja* Sieb . Et Zucc) fermented by the *Rhizopus oligosporus* FNCC 6010 spores obtained a compact mass texture and homogenous structure as well as taste, smell and distinctively flavored tempeh. Yellowish white, but white color evenly on the surface is due to the presence of mycelium growing on the surface of the seed legume after 44 hours incubation (Figure 1). The tempeh texture can be determined by identification of dense surface mycelia. If the mycelia were lush, it indicates that the texture of tempeh has a compact, and vice versa. *Rhizopus oligosporus* FNCC 6010 is obligate aerobes, so the availability of oxygen absolutely is rate determinate factor for the fermentation process. Incubation for the tempeh preparation was carried out at Laminar flow cabinet to minimize contamination during the experiment. The specification of the tempeh produced was appropriate to national food standard.

Qualitative screening for proteolytic enzyme production

About 20,0 mg of tempeh extract was plated onto protein agar plates containing skim milk powder 100 mg, peptone 5g and agar 20g per liter at pH 5.0, incubated at 37°C for 24 h. A clear zone of skim hydrolysis has appeared from protease producing samples. The diameter of the clear zone was observed and measured.

Qualitative screening of fibrinolytic enzyme [8]

In a 90 mm petri dish, 5 mL of 0.5% w/v fibrinogen from bovine plasma in 50 mM Tris-HCl buffer (pH 7.8) mixed with 5 ml of 2% (w/v) agarose and 0,1 ml of thrombin (100 NIH U/ml). Petri dish is allowed to stand for 30 minutes at room temperature to form a layer of white clouded fibrin clots. Half of the petri dish is heated at 85^{o}C for 30 minutes to deactivate plasminogen (plasminogen petri dish free fibrin obtained) and the remaining is considered rich fibrin plasminogen. Furthermore, 10 µl of each crude enzyme extract was dropped carefully on surface media. The petri dish was incubated at 37oC and then observed every 6 hours for 18 hours. The fibrinolytic activity was observed as observed by measuring the clear transparent zone. The clear transparent zone indicate degradation of fibrin and its diameter is proportional to the TpFE potency. All experiments were performed three times.

Effect of pH

Optimization of medium pH was performed by maintaining the pH using various buffer solutions such as glisin-HCl (pH 2.0-3,5), acetic 0,05 M (pH 3,5-5,5), phosphate 0,05 M (pH 5,5-8,5), and glisin-NaOH 0,05 M (pH 8,5 - 12,0). Each media was inoculated by culture and grown for 24 hours at optimum temperature. The culture was filtered and the filtrate was centrifuged at 10,000 rpm for 10 min and supernatant was assayed for the fibrinolytic enzyme activity.

Effect of temperature

The optimization of temperature, the activity of the fibrinolytic enzyme was observed at various temperatures (37- 80^{o}C) in 0.05 M phosphate buffer solution pH 7.0.

Effect of inoculum volume

The volume of inoculum used to optimise the enzymes production was arranged at 0.5, 1.0, 1.5, 2.0 mL. The black soybean was depped in water for 16 hours, incubation was done at 30^{o}C for 36 hours.

Effect of metal ion [9]

The effect of metal ion was performed by incubating 100 uL enzyme solution added by 100 uLmetal ion solution until the final concentration of 1 and 5 mM at room temperature for one hour. The enzyme activity was assayed quantitatively. The metal ion used were KCl, NaCl, $CaCl_2$, $CuCl_2$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$ and $FeCl_3$.

Extration of TpFE enzyme

About 150 grams dried tempeh (by adding liquid nitrogen) were cut in the shape of cubes, put in a mixer with filters and add 250 mL of buffer phosphate 0.05 M pH 7,0 solution. The homogenate was destroyed and poured into the tube, centrifuged at 4^{o}C, 9000 rpm for 10 minutes. The supernatant was separated and lyophilized to gain powder. The powder dissolved in 20 mL of pH 7.0 phosphate buffer solution.

Purification of TpFE by ammonium sulfate fractionation

Fractionation of 0 % - 30 %. Supernatant obtained from crude extract was concentrated by adding ammonium sulfate with varying degrees of saturation.The mixture was stirred on an ice bath up to dissolved, added by 3,28 g of ammonium sulfate, centrifugated 6000 rpm at 4°C in centrifuge refrigerator for 10 min. The supernatant was taken for treatment of ammonium sulfate. Fractionation of 30 % - 50 %. The first precipitate dissolved with phosphate buffer pH 7.8 solution and continued by dialysis (cut off 12 kd) process. Fractionation of 30% - 40 %. The supernatant of 0 – 30% was stirred on an ice bath up to dissolved, added by 1,22 gram of ammonium sulfate. Fractionation of 40%-50%, 50%-60% and 60% - 80% were conducted with the same way as described above and added of ammonium sulfate 1,24, 3,26 and 3,50 g respectively.

Process of dialysis with cellophane tube

Firstly, the cellophane tubes were prepared for each precipitate, and cut into 15 cm and washed by soaking in water at 80 °C for 3 hours. Furthermore, the end of the cellophane was tied with a binder. Secondly, a solution of 0.05 M phosphate buffer pH 7.0 prepared in Beaker glass. Each of the precipitates of fraction 0-30%, 30-40%, 40-50%, 50-60% and 60-80% was dissolved into 10 mL of 0.05 M phosphate buffer pH 7.0and then each solution was put into a cellophane tube and tied the other end to form a bag. Each cellophane bag containing the enzyme solution soaked into Beaker glass containing phosphate buffer, stirred with a stirrer for 12 hours in a 4 °C ice bath. Replacement of phosphate buffer solution was performed every 4 hours. Activity test was carried out on each of enzyme solution obtained from dialysis process.

Fibrinolytic enzyme activity assay:

The activity of TpFE was assayed by measuring the α-amino nitrogen liberated. A mixture consisting of 50 uL enzymes, 500 uL of 100 mM Tris-HCl buffer solution at pH 4.5, 100 uL of 3% fibrin substrate and coupled to 1 ml of distilled water was incubated at 37 ºC for 1 hour, and then stopped by the addition of 200 uL of 20% trichloroacetic acid (v/v). After centrifugation, 0.5 ml of the supernatant was added to 1 ml of ninhydrin reagent (0.5 ml of 1% ninhydrin in 0.5 M citrate buffer, pH 5.5, 0.2 ml of the buffer, and 1.2 ml of glycerol) and boiled for 10 minutes. Four ml of distilled water was added to each sample and measured the absorbance at 570 nm and the increase in free amino groups was determined. Tyrosine is used as a standard. One unit of proteolytic activity was defined as 1µmol of the α-amino acids released per minute under the standard of testing conditions.

Thrombolytic activity assay (Blood Clot Lysis)

The clot lytic effect of the fibrinolytic enzyme was studied with an artificial clot in vitro. The blood was collected from healthy mice with written informed consents. The artificial blood clot was made by spontaneous coagulation in the centrifuge vials. Then, different doses (100, 200, and 300 units) of the fibrinolytic enzyme were added. Nattokinase (250 units) was used as the positive control; buffered saline solution was used as the negative control. These were incubated at room temperature for 25, 50 and 75 min analyzed. After incubation, fluid obtained was removed and tubes were again weighed to observe the difference in weight after clot disruption. The difference obtained in weight taken before and after clot lysis was expressed as a percentage of clot lysis. The test was repeated five times.

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RESULTS AND DISCUSSION

Preparation of *Rhizopus oligosporus* FNCC 6010 inoculums

Rhizopus oligosporus taken from stock culture was transferred to fresh potato dextrose slant agar media, incubated for 72 hours at 30°C. Regeneration of cells successfully produced white and can be turned into a blackish color/dark, and when viewed from the surface of the fibers such as cotton. The *Rhizopusoligosporus* spore was collected by added 10 mL saline solution and the transmittant of the culture suspension measurement was performed to obtain reproducible condition. Number of cells used for fermentation processes was 10^7 CFU/mL

Preparation of tempeh

Producing tempeh from black Soybean (*Glycine Soja* Sieb . Et Zucc) fermented by the spores of *Rhizopus oligosporus* FNCC 6010 obtained a compact mass texture and homogenous structure as well as taste, smell and distinctive flavored tempeh. Yellowish white, but white color evenly on the surface is due to the presence of mycelium growing on the surface of the seed legume after 44 hours incubation (Figure 1). The tempeh texture can be determined by identification of dense surface mycelia. If the mycelia was lush, it indicates that the texture of tempeh has a compact. *Rhizopus oligosporus* FNCC 6010 is obligate aerobes, the availability of oxygen absolutely is rate determinate factor for the fermentation process. Incubation for the tempeh preparation was carried out at Laminar flow cabinet to minimize contamination during the experiment. The specification of the tempeh produced was appropriate to national food standard (Table 1).

Figure 1: Black Soybean (*Glycine Soja Sieb.Et Zucc*) tempeh produced from *Rhizopus oligosporus* FNCC 6010 fermented at room temperature for 44 hours.

Table 1: Tempe test results are compared with the Indonesian National Standard (SNI)

No	Criteria	Result	Requirements (SNI) (SNI)
1	Organoleptis		
1.1	Smell	Normal	Normal, specific
1.2	Color	Normal	Normal
1.3	Flavor	Normal	Normal
2	*Moisture content* (w/w)	55,38±1,7 %	Max 65 %
3	Ash content (w/w)	1,40±0,1 %	Max 1.5 %
4	Fat content	2,99±0,13	Min 10 %
5	Protein content (N x 6.25)	27,3±1,6 %	Min 16 %
6	Fiber content(w/w)	2.29±0.4 %	Max 2.5 %
7	Metal contaminant		
7.1	Cadmium (Cd)	Not	Max 0.2 mg/kg
7.2	Lead (Pb)	Not	Max 0.25 mg/kg
7.3	Tin (Sn)	Not	Max 40 mg/kg
7.4	Mercuri (Hg)	Not	Max 0.03 mg/kg

8	Arsenic (As)	Not	Max 0.25 mg/kg
9	Microbial contamination		
9.1	Coliform	Negative	Max 10 apm/g
9.2	*Salmonella* sp	Negative	Negative/25 g

Qualitative screening for proteolytic enzyme activity

It was found that the tempeh extract produced clear zone sizes that were proportional to *Rhizopus oligosporus* growth size, while some colonies, even if their colony sizes were relatively small, they have exceptionally big clear zones around them (Figure 2). To equalize this, a parameter called relative halo size was determined by the following equation:

$$Relative\ clear\ zone = \frac{d\ clear\ zone - d\ colony}{d\ colony} \text{x100}$$

In which d clear zone is diameter formed around the colony (mm), while d colony is the diameter of the colony (mm).

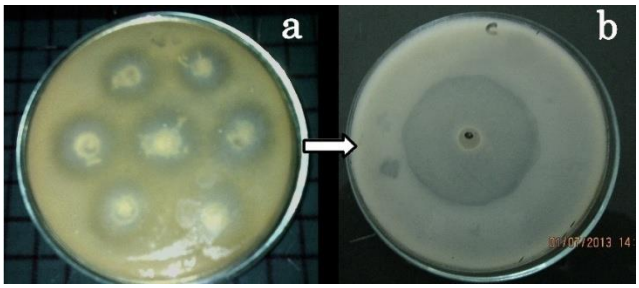

Figure 2: Proteolytic activity of *Rhizopus olygosporus* FNCC 6010 spores on skim milk agar plates (a) and proteolytic activity of a slice of the tempeh fermented by *Rhizopus olygosporus* FNCC 6010 (b)

Qualitative screening for fibrinolytic enzymes activity

Fibrin plate test conducted on agarose-fibrin media containing fibrinogen mixed with thrombin and metilen blue. It was found that the mixture of fibrinogen and thrombin formed cloudy white color on agarose media. Turbid white blobs are called fibrin. Samples (5 uL) containing fibrinolytic enzyme caused fibrin in the plate degraded to form clear zones. Observations were made after 3 hours treatment. The radius of a clear zone will be enlarged after more than 3 hours. Within 3 hours of the screening test indicate that the black soybean tempeh (*Glycine Soja* Sieb. et Zucc) produced hydrolyzed regions diameter was larger compared to other legumes. Preliminary qualitative screening showed that the *Rhizopus oligosporus* FNCC 6010 exhibited proteolytic activity as well as the tempeh produced by the fungi (Figure 3). The ability of proteolytic enzymes production was differ depending on the media used. The media will also cause a variety of legumes degraded by*Rhizopus oligosporus* producing different proteolytic enzyme activity. Screening of bacteria and fungi that produce fibrinolytic enzyme reported by [10] have successfully produced ninety-four fibrinolytic enzyme - producing bacterial colonies using soybean substrate. Likewise, Kumaran et al. 2011 has screened the enzyme on Ganoderma lucidum.

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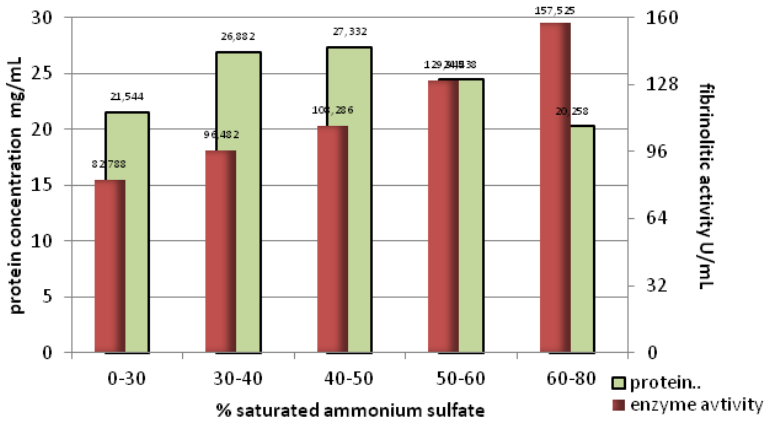

Figure 3: Profile of fibrinolytic enzyme activity and total protein in the precipitate (pellet) on a variety of percent saturation of ammonium sulfate with phosphate buffer pH 7.

Enzyme extraction and partial purification

Enzyme divided into intracellular and extracellular enzymes. This will give the effect of extraction and purification methods. Enzyme extraction process can be done by homogenization, centrifugation, filtration, or their combination. The first stage of extraction is the addition of phosphate buffer pH 7.2 and homogenized by destroying at 4 °C so that it becomes a suspensionSeparation of supernatant and pellets was done by filtration. Furthermore, centrifuged at low temperatures 4 °C with a speed of 9,000 rpm for 10 minutes. Centrifugation carried out at low temperatures with the aim of maintaining the enzyme remains active. Then the pellet was extracted again. The resulting supernatant was added buffer solution, allowed to stand for 48 hours to obtain the cell-free supernatant of crude extract fibrinolytic enzymes. Enzymes have been extracted in the supernatant still form an aqueous solution with a low enzymatic activity and still contains many impurities. From the experimental obtained the crude extract of fibrinolytic enzyme activity of 0,030 U / mL and the specific activity of 0.025 U / mg. Thus, the concentration needs to be done through the use of ammonium sulfate precipitation of crude extract to the saturation of the enzyme with different concentrations ranging from the lowest saturation concentration of 0-30, 30-40, 40-50, 50-60, and 60-80 %. Precipitation with ammonium sulfate was based on salting out mechanism, so there is competition between ammonium sulfate ions with ion binding protein in water. The higher concentration of ammonium sulfate, the greater density of ionic ammonium sulfate is compared to the protein. The result showed that water molecules are bound to the greater salt. Decreased water molecules in hydrophobic amino acid residues caused attractive forces between molecules of protein increase when compared to the attractive forces between the protein molecules with water molecules so that the protein will precipitate. During the salting-out process, salt concentration must be kept so as not decrease in the solution, so that no precipitation occurs simultaneously between purified proteins and undesired proteins.In this study, ammonium sulfate precipitation aims to separate between the fibrinolytic enzyme with another enzyme partially purified from the crude enzyme, thus fibrinolytic enzymes have higher activity than the crude extract.

The fibrinolytic enzyme activity was greater in precipitate compared to the supernatant (Fig. 3 and Fig. 4). The addition of ammonium sulfate of 0-30% saturation obtained the fibrinolytic enzyme activity in the supernatant 2,745 U/ml with a protein concentration of 10.358 mg /ml and a specific activity of 0.265 U/mg. Increasing concentrations of ammonium sulfate lead to decrease the enzyme activity in the supernatant, 0.905 U/ml with a protein concentration 4.719 mg/ml (Fig. 4) and the specific activity of 0.191 U/mg at a concentration of 80% saturation.

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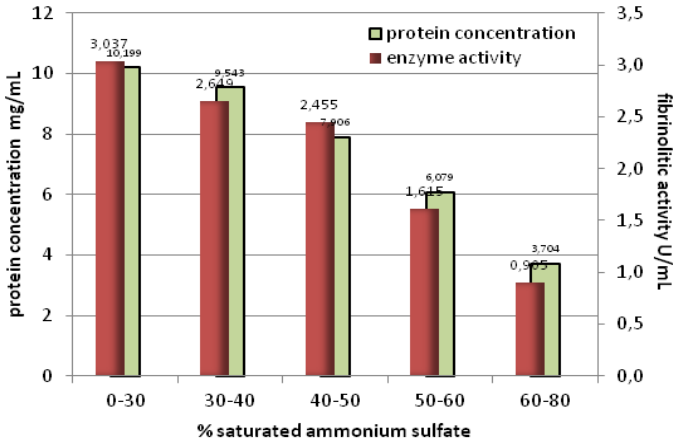

Figure 4: Profile of fibrinolytic enzyme activity and total protein in the supernatant on a variety of percent saturation of ammonium sulfate with phosphate buffer pH 7

The fibrinolytic enzyme activity decreased in the supernatant indicates that the protein including fibrinolytic enzyme was precipitated by the addition of ammonium sulfate. The increasing saturation of 80% makes the fibrinolytic enzyme activity increased up to a maximum of 157.454 U / ml with a protein concentration has been precipitated 20.258 mg/ml (Fig. 3) and the specific activity of 7,772 U/mg.

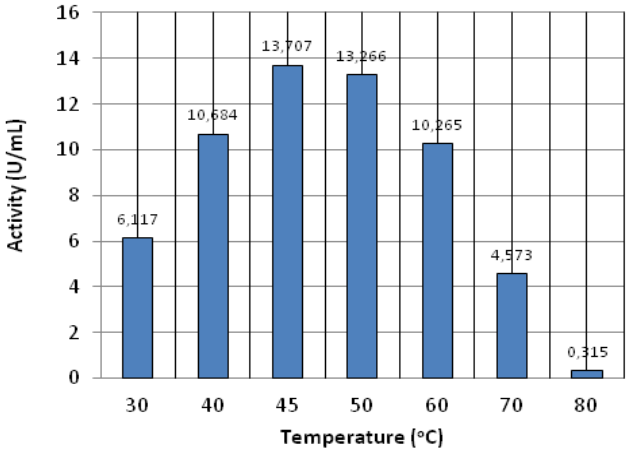

Figure 5: Profile of fibrinolytic enzyme activity influenced by temperature with phosphate buffer pH 7.

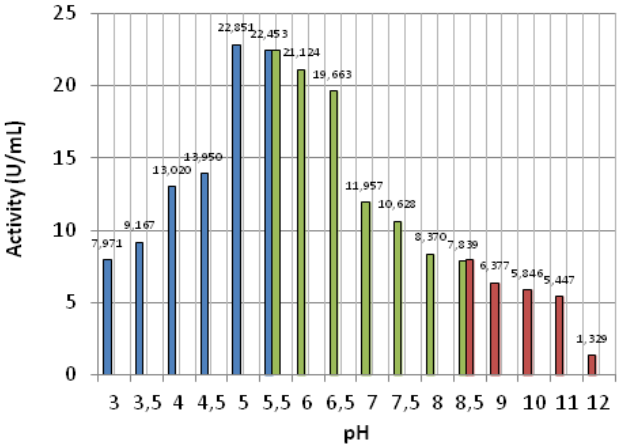

Figure 6: Profile of fibrinolytic enzyme activity influenced by pH. glycine-HCl buffer (pH 3.0 to 3.5), 0.05 M acetate buffer (pH 3.5-5.5), 0.05 M phosphate buffer (pH 5.5 to 8.5), and glycine-NaOH buffer 0.05 M (pH 8.5 to 12.0).

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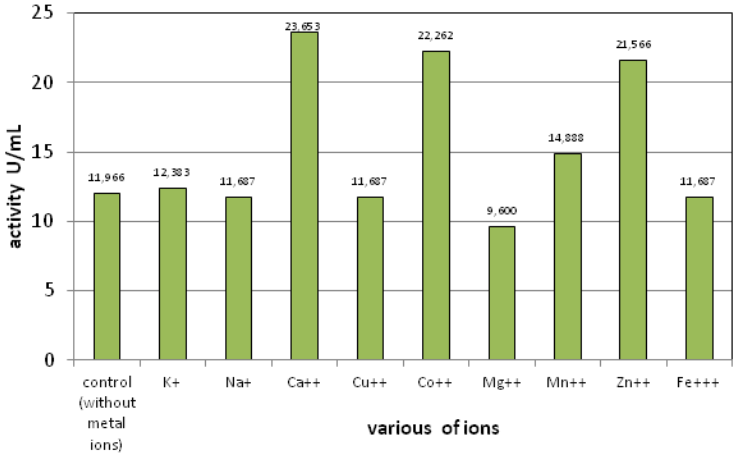

Figure 7: Profile of fibrinolytic enzyme activity in a variety of metal ions KCl, NaCl, $CaCl_2$, $CuCl_2$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$ and $FeCl_3$ concentrations of 0.05 mM respectively.

Effect of temperature

Temperature variation is used to determine the optimum temperature is a fibrinolytic enzyme in the range of 30 º C to 80 º C. To reduce the possibility of interference residual peptides formed during the degradation of the substrate protein (fibrin) then after enzymatic reaction of acetic acid is added trichloroacetic acid. The precipitate was separated by centrifugation 9000 rpm 4°C. Increasing the temperature of the chemical reaction rate will be faster.in Figure 7, the maximum activity is achieved at a temperature of 45º C with phosphate buffer pH 5.5 for 10 minutes. The enzyme activity reached 13,707 U / ml. The increase in activity of the enzyme is due to the kinetic energy resulting vibrational motion, translation and rotation of the enzyme and the substrate so that the enzyme substrate interaction becomes larger and faster. At temperatures above 50º C (above the optimum temperature), fibrinolytic enzyme activity was gradually be decreased due to protein denaturation. Enzymes are protein molecules that can be denatured at high temperatures as well. The catalytic activity gradually increased as the temperature rises to the limit and the higher temperatures resulting in increased damage to the enzyme. As research on the enzyme activity of fermented solid fibrinolytic SSF (solid state fermentation) of the fungus *Rhizopus chinensis* 12, *Pleurotus eryngii* and *Pleurotus sajor - caju* . Fibrinolytic enzyme activity decreased after the temperature of 50°C [12][13], [14].

Fibrinolytic enzymes from *Bacillus firmus* NA - 1 isolated from Natto , a traditional Japanese fermented food [15] and from *Bacillus* sp strain CK 11-4 isolated from Chungkook -jang , a traditional food of Korea [16], has been reported to be relatively stable at a temperature of 40 º C. While the fibrinolytic enzyme isolated from *Bacillus subtilis* LD - 8547 is reported to have activity between 35° C and 65° C with optimum activity at a temperature of 50°C . The fibrinolytic enzyme is very stable at a temperature of 45°C after 60 min incubation and showed 82 % and 11 % residual activity respectively at a temperature of 60°C and 70°C after 60 minutes,but at 75°C for 10 minutes, fibrinolytic enzyme activity is lost. Performed on the lyophilized enzyme and stored at 4° C, its activity did not change after 2 months and after freezing then performed dissolution, fibrinolytic activity remained stable [10].

Effect of pH

Most of the enzyme showed optimal activity at pH between 5 and 9. Changes in the structure of the enzyme will affect the rate of reaction. The relationship between the activity of the enzyme with a concentration of hydrogen ion showed that the equilibrium between the denaturation of enzymes at high or low pH and its effect on the condition of the enzyme charge, the substrate, or both. At the time a certain medium pH changed, will result in changes in enzyme form. Not only on the enzyme, the pH level can also change the nature of the charge and form of the substrate. If only there was a slight change in its pH change

the structure of enzymes and its substrate to reversible (irretrievable). But significant changes that would make the pH level and in denaturing, its substrate to an enzyme so that it can perform its function normally in the enzyme will be changed. The enzyme has an amino group and a carboxyl group. Both of these groups are affected by the strength of the hydrogen ions that will result in the catalytic and enzymatic changes of conformation. These changes will be indicated when enzyme activity was treated at the pH range is between 3 to 12.

The research was carried out the determination of the optimum pH of the fibrinolytic enzyme to partial purification results. These characteristics are needed because, the enzyme has many functional groups that can be ionized so that it changes the pH will change the conformation of the enzyme, the binding of the substrate and the catalytic power of the functional groups on the active enzyme. Changes that may occur is the maximum rate of the enzyme, changes in the stability of the enzyme. It looks that have the highest activity of fibrinolytic enzymes to achieve optimal at pH 5, is 22,851 U/mL and at pH 5.5 still showed its activity despite the ability of the enzyme to hydrolyze started having decreased. Then fibrinolytic enzyme activity is continuing to decrease after pH 7 to 12.

The interaction between the fibrinolytic enzyme with the solvent will result in ionization occurs on the active site. If the pH is low or below the optimum pH or pH levels above pH optimum, it will be directly in contact with the active site, the result will be a rapid decrease in enzyme activity. Beyond the optimum pH, lower fibrinolytic enzyme activity due to characterize of the enzyme protein molecules that have positive and negative charges. Ionization of amino acids in the active site of the enzyme is such that it allows the electrostatic interaction between the enzyme active site to the substrate to form the enzyme-substrate complex. Based on Figure 6, note that the fibrinolytic enzyme extracted from black soybean tends to work in an acidic environment. In a study carried out by Cui et al. (2008) [17] at *Cordyceps militaristic* fungus that the fibrinolytic enzyme activity reached an optimum at pH 6. It is very different from other fibrinolytic enzymes have been reported. In general, like the nattokinase most active in the alkaline pH range [18]; [19]; [20]. Moreover, the enzyme is very stable in the pH range 5.0-8.0, 37 ° C for 1 hour, but above pH 8.0, the stability of the enzyme decreased very rapidly.

Effect of metal ions

Fibrinolytic enzymes in their activities need additional cofactors. Most enzymes require non protein co-factors for catalysis. The co-factor is usually as trace elements with small concentrations are able to increase the activity. The metal salts of inorganic compounds such as potassium, sodium, calcium, copper, cobalt, magnesium, manganese, zinc or Fe. Usually, as inorganic salts produce an effect on the apoenzyme to form an active holoenzyme. During the time that others can be as co-substrate cofactors that bind covalently to the enzyme.

In this study, all metal ions derived from the chloride, not others. It is avoided that the ionization is always uniform in the same salt. In the implementation of the cation is added to a solution of the enzyme so that it gets the same concentration of 0.05 mM at the time of fibrinolytic enzyme activity assay. The addition of Ca^{++}, Co^{++} and Mn^{++} indicates that fibrinolytic enzyme activity 23.653 U / mL, 22.262 U / mL and 21.566 U / mL respectively was greater more than control or other metal ions (Fig. 7). Increasing activity of this enzyme could be due to the presence of inhibitors of the enzyme removes the enzyme solution (1), change the surface tension of the enzyme (2), replace other metal ions in the enzyme active site (3) or may also change the conformation of the enzyme (4). During the time that other metal ions did not produce an effect on or even decrease the activity of the fibrinolytic enzyme. Increasing activity of fibrinolytic enzyme from *Armillaria mellea* by Ca^{++} to 126.4% has been reported by [21].

Thrombolytic activity assay

The lyophilized crude enzyme was characterized and assayed for thrombolytic activity. Table 2 showed the thrombolytic activity at 250Ul and 500 µl concentration of the crude enzyme expressed as average of decreasing of blood clot after 25s, 50s, and 75s treatment (Fig. 8).

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Table 2: Blood clot observation on thrombolytic activity of crude enzyme from black soybean tempeh extract

Vol. of crude enzyme	Lead time (min.)	Average of blood clot weight (g)				% average of decrease
		Before leading	After leading	Difference	% of decrease	
250 µL	25	0.129	0.032	0.097	75.100	75.10
	50	0.136	0.017	0.119	87.148	87.79
	75	0.140	0.016	0.124	88.873	89.05
500 µL	25	0.122	0.009	0.113	92.490	92.58
	50	0.127	0.004	0.123	96.325	96.66
	75	0.127	0.004	0.123	97.367	97.46
Black soybean solution in saline	25	0.118	0.108	0.010	8.077	8.16
	50	0.095	0.088	0.007	6.160	7.70
	75	0.070	0.064	0.006	5.264	8.77

Each experimental was performed five times.

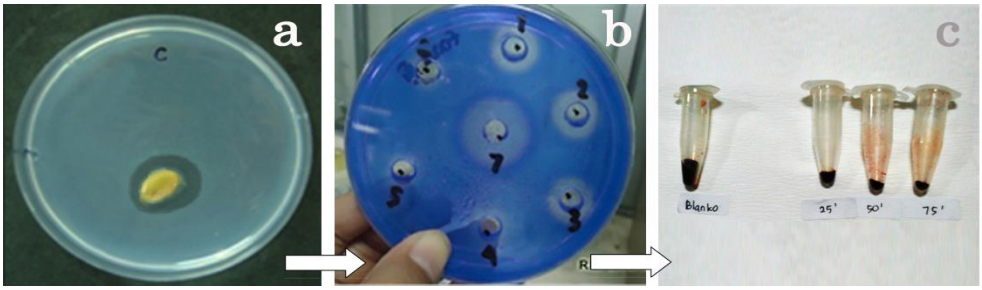

Figure 8: Fibinolytic activity of black soybean tempeh on fibrin plates (a); That exhibited fibrinolytic activity on artificial fibrin agarose methylene blue medium (b) and Thrombolytic activity of the tempeh extract observed at 25s, 50s, and 75s in vitro (c).

CONCLUSION

Rhizopus oligosporus FNCC 6010 showed fibrinolytic enzyme activity in the media fibrin plate, Black soybean tempe fermented *Rhizopus oligosporus* FNCC 6010 showed fibrinolytic activity and thrombolytic. Characteristics of fibrinolytic enzyme leads to the nature of the aspartic protease is based on an optimum pH 5.5 and optimum temperature of 45^{o}C. Characters fibrinolytic enzyme lead to the nature of the metal, which is influenced by the activator ion Ca^{++}, Co^{++} and Zn. Trombolitic activity in vitro clot lysis results in a decrease to 97.6%

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