Purification and characterization of a fibrinolytic enzyme from tempeh bongkrek as an alternative of thrombolytic agents

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Abstract. Tempeh is one of Indonesia's traditional foods that contain fibrinolytic enzymes. Tempeh bongkrek shows very strong activity among various tempeh. The fibrinolytic enzymes of bongkrek tempeh are obtained by steps of purification i.e. ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography. The fibrinolytic enzymes has been successfully purified with a yield of 4.37%, specific activity of 3361 U/mg and purification fold of 44.02. SDS PAGE analysis showed that the enzyme was purified in to single band with estimated molecular mass of 75.82 kDa. The purified enzyme has optimum pH of 7 and optimum temperature of 50°C and pH stability between pH 4 - 7 with temperature stability from 30°-50°C. The fibrinolytic activity is increased with addition of CaCl₂ but inhibited with CuSO₄, phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), and ethylenediaminetetraacetic acid (EDTA).

1. Introduction

Cardiovascular disease is a disease caused by heart and blood vessels disorders and is one of the most deadly diseases in the world. Based on the results of the report published by the World Health Organization (WHO) in 2011, an estimated 17.3 million people or 30% of the world’s deaths caused by cardiovascular disease [1]. In poor and developing countries, cardiovascular disease accounts for 80% of deaths in both women and men caused by heart attacks and strokes [2]. One of the things that can lead to heart disease and stroke are the occurrence of thrombosis. Thrombosis is a disorder of blood vessels that lead to the blockage of blood vessels [3]. Abnormalities caused by thrombosis can be treated with thrombolytic therapies that can be performed via injection and orally using agents containing urokinase, tissue plasminogen activator (TPA), and streptokinase. Although widely used, these agents have the effect of immunogenic [4], a short half-life, the price is relatively very expensive, specificity to fibrin is reocclusion, and requires a large of therapeutic dose [6].

In addition to the above thrombolytic agents, there is an enzyme that can break up blood clots that is fibrinolytic enzyme [7]. Fibrinolytic enzyme is a protease which has fibrinolytic activity that is able to degrade fibrin clot [8]. Based on several previous studies, the activity of fibrinolytic enzyme can be found in fermented food such as nattokinase in natto, katsuwokinase on Skipjack shiokara [8];
Subtilisin DJ-4 on Doen-Jang [9]; Douchifibrinolytic enzyme (DFE) on Douchi [5] and fibrinolytic enzyme on tempe gembus [10].

Since there are many variants of tempeh in Indonesia, we previously investigated the fibrinolytic activity of various tempeh that are produced in Malang and Batu, East Java using in vitro blood degradation methods (unpublished data). Among various tempeh i.e. tempeh bungkil, tempeh menjes and tempeh bongkrek, the tempeh bongkrek showed the highest fibrinolytic activity (2.11 FU/ml). Therefore, a more in-depth study of the purification and characterization of fibrinolytic enzymes derived from tempeh bongkrek is necessary. The understanding of the properties of the enzyme will open the possibilities of the enzyme to be an alternative for thrombolytic therapy for cardiovascular diseases and increase the economic value of tempeh.

2. Materials and Methods

2.1 Extraction of Enzyme from Tempeh Bongkrek

Five hundred (500) grams of tempeh sample was used and put into sterile plastic containing 500 mL of PBS buffer pH 7.4. Then the sample was homogenized using stomacher tool with medium speed for 3 min. The homogenate is centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was taken as a crude enzyme extract [12].

2.2 Ammonium Sulfate Precipitation

Ammonium sulphate was then added with 80% saturation level and stirred overnight at temperature of 0-4°C. After stirred overnight, the mixture was centrifuged at a rate of 10,000 rpm for 20 min at temperature of 4°C. The precipitate was resuspended in buffer Tris-CL 50mM pH 7 and then dialyzed against 20 volumes of the same buffer for 24 h at 4°C with four buffer changes [10,13].

2.3 Ion Exchange Chromatography

The dialyzed enzyme was chromatographed on a column of DEAE-Sepharose. The sample was loaded on to a column of DEAE-Sepharose (10 cm x 1 cm) equilibrated with 50 mMTris-Cl pH 7.0. The absorbed protein solution was eluted at a flow rate of 1 ml/min with a discontinuous gradient ranging 0.1 to 1 M NaCl dissolved in same buffer. The eluted fraction was collected in 4 ml per tube. The fraction measured at λ 280 nm and fibrinolytic activity of each fraction was measured with the fibrinolytic enzyme assay using spectrophotometry method [14].

2.4 Gel Filtration Chromatography

Gel filtration chromatography was carried out using a Sephadex G-100 Column (10 cm x 1 cm). The column was equilibrated with 50mM Tris-Cl pH 7.0. The flow rate was 1 ml / min. The eluted fraction was collected in tubes with a volume of 4 ml each. The fraction was measured at λ 280 nm and fibrinolytic activity of each fraction was measured.

2.5 Fibrinolytic Activity Assay

Fibrinolytic activity was carried out according to the method described by Chang et al. [15]. Briefly, 1.4 mL of 50mMTris-Cl pH 8.0 and 0.4 mL of 0.72% (W/V) fibrinogen solution were mixed and incubated at 37°C for 5 min. Then, 0.1 mL (20 U /mL) of thrombin was added and incubated at 37°C for 10 min. Then, 0.1 mL of sample was added and incubated for an additional 60 minutes. The reaction was stopped by addition of 0.2 M trichloroacetic acid (TCA). After centrifugation (8000 rpm for 10 min), the absorbance of supernatant at 275 nm was read and recorded. One unit of fibrinolytic activity (FU) is defined as the amount of enzyme required to produce an increase in absorbance equal to 0.01 in 1 min at 275 nm [15].
2.6 Characterization of The Fibrinolytic Enzyme

To determine the effect of pH on fibrinolytic enzyme activity with 0.1 ml fibrinolytic enzyme sample incubated at 50 mM acetate buffer (pH 4.0 - 5.0), 50 mM phosphate buffer (pH 6.0 - 7.0), 50mMTris-Cl buffer (pH 8.0 - 9.0) for 1 h at 37°C. Then the enzyme activity was determined by the fibrinolytic activity assay [12].

For thermal stability measurements, 0.1 ml of fibrinolytic enzymes were suspended in 50 mMTris-Cl (pH 7.0) and incubated in a water bath for 1 h at various temperatures (30–70 °C). After incubation at each temperature, the residual fibrinolytic activity was measured [12].

To determine the effect of metal ions and inhibitors on the activity of fibrinolytic enzyme, 0.1 ml samples were incubated in Tris-Cl (pH 7.0) containing 1mM metal ions (NaCl, MgCl₂, CaCl₂, CuSO₄) and 1mM inhibitor (phenylmethylsulfonyl fluoride (PMSF), SDS, ethylenediaminetetraacetic acid (EDTA), and HgCl₂) for 1 hour at temperature of 37°C. Then the residual enzyme activity was determined by the fibrinolytic activity assay [12].

2.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis

SDS-PAGE analysis was carried out using 12% polyacrylamide gel. Zyomogram analysis was performed as described by [16], using 12% of the SDS-PAGE gel containing 0.12% fibrinogen and 10NIH U/ml thrombin. After electrophoresis, the gel was washed with 0.05M Tris-Cl pH 7.8 and then immersed in the same buffer for overnight at temperature of 37°C. The enzyme activity was revealed as a colorless band with a blue background, after washed with 0.11M trichloroacetic acid and staining with Coomassie blue.

3. Results and Discussion

3.1 Purification of Fibrinolytic Enzyme from Tempeh Bongkrek

The purification of fibrinolytic enzyme from tempeh bongkrek is summarized in Table 1. The enzyme was purified to homogeneity by a three-step procedure, resulting in a yield of 4.37%, specific activity of 3.361 U/mg and purification fold of 44.02. The SDS-PAGE and zymography analysis showed that the enzyme is purified to a single band of fibrinolytic enzyme corresponding to a molecular mass of about 75.82 kDa (Figure 1). Previous studies have reported different molecular mass for different fibrinolytic enzymes: 70 kDa and 140 kDa for Bacillus licheniformis RO3 [11], 29.93 kDa for Bacillus subtilis [15], and 20 kDa for Bacillus pumilus 2 g [10].

| Table 1. Purification of Fibrinolytic enzymes. |
|-----------------------------------------------|
| Volume (ml) | Total Activity (U) | Total Protein(mg) | Specific Activity (U/mg) | Yield (%) | Purification Fold |
| Crude Extract | 500 | 1190 | 14425 | 0.082 | 100 | 1 |
| Ammonium Sulfate | 75 | 442.5 | 803.25 | 0.551 | 37.18 | 6.68 |
| DEAE - Sepharose | 28 | 175 | 127.60 | 1.372 | 14.71 | 16.63 |
| Sephadex G-100 | 8 | 45.36 | 14.32 | 3.631 | 4.37 | 44.02 |
3.2 Effect of Temperature on Enzyme Activity and Stability

The effect of temperature on enzyme activity was studied by incubating the enzyme in temperature of 10 to 70°C. The enzyme has optimum temperature of 50°C and stable at temperature up to 50°C. At temperature of 60°C, its activity is reduced as much as 40%. The enzyme is stable in temperature range of 30-50°C and rapid decrease in activity was observed at temperature above 60°C (Figure 2). Previous studies have reported in natto from Bacillus substilis RJAS19 was optimal at 50°C[15], in douchi from Bacillus substilis DC33 was optimal at 40°C^5, in Gembus from Bacillus pumilus was stable in temperature of less than 60°C[10].

Figure 1. The SDS Page and Zymogram of fibrinolytic enzyme produced by Tempe Bongkrek (M) Maker; (A) SDS-PAGE; (B) Zymography result; (1) Crude extract; (2) ammonium sulfate; (3) DEAE-Sepharose; (4) Sephadex. A fibrinolytic enzyme with an estimated molecular mass of 75.82 kDa was successfully purified into a single band of protein.

Figure 2. Effect of temperature on activity and stability of fibrinolytic enzyme from tempeh bongkrek. The enzyme has optimum temperature of 50°C and stable at temperature up to 50°C. At temperature of 60°C almost half of its activity is reduced.
3.3 Effect pH on Enzyme Activity and Stability

The effect of pH on the activity and stability of the enzyme was determined using buffers at various pH values. Fibrinolytic enzyme from tempeh bongkrek is optimal in pH 7 and stable in pH range of 4–7 (Figure 3). These results are similar with the results from previous reports. A fibrinolytic enzyme of meju fermented using Bacillus amyloliquefaciens MJ5-41 and the one from Bacillus pumilus isolated from Tempeh gembus [10] are optimum at pH 7. The pH stability is slightly different, other enzymes were reported have stability in pH range of 5 – 9 [9, 10, 16], but the purified enzyme from this work have more acidic resistance.

![Figure 3. Effect pH on stability fibrinolytic enzymes](image)

3.4 Effect of metal ion on the activity of fibrinolytic enzyme

Effect of various metal ions was observed (Figure 4). Calcium ion of CaCl₂ could increase the fibrinolytic enzyme activity. It showed that calcium ion has significant role to the enzyme. On the other hand, the Cu decreased the enzyme activity. It was reported that Cu can bind to sulphydryl group which caused the fibrinolytic enzyme inactive [10]. In this research, the addition of PMSF decreased the activity of fibrinolytic enzyme > 60%. PMSF is specific inhibitor for serine proteases, so it is possible that fibrinolytic enzyme of tempeh bongkrek is also a serine protease [17], but this prediction needs further study. Serine Protease is a group of proteolytic enzymes which had active group (OH) from serine amino acid. PMSF which contained sulfonyl group would hammer on irreversible competitive basis toward this enzyme with the reaction on serine OH group in the active side [16].
4. Conclusion
The fibrinolytic enzymes have been successfully purified with a yield of 4.37%, specific activity of 3.361 U / mg and purification fold of 44.02. SDS PAGE combined with zymography analysis showed that the enzyme was purified in to single band with an estimated molecular mass of 75.82 kDa. The purified enzyme showed optimum pH of 7 and optimum temperature of 50°C and showed its pH stability between pH 4-7 with temperature stability from 30-50°C. The fibrinolytic activity is increased with addition of CaCl₂ but inhibited with CuSO₄, phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), and ethylenediaminetetraacetic acid (EDTA). Effect of EDTA showed that metal ion is important for the activity. The fibrinolytic enzyme isolated and purified from this work are able to degrade blood clod that indicated the enzyme can be applied for thrombosis therapy related to cardiovascular disease.

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