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Original Research Article

Food Origin Fibrinolytic Enzyme With Multiple Actions

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ABSTRACT

Many health-related problems such as cardiovascular diseases are associated with the formation of excessive clot in the blood (thrombus). Approaches in cardiovascular disease treatment are preventing the formation or removing the thrombus. The present thrombolytic agents can be classified as plasminogen activators, fibrinolytic enzyme which directly degrades fibrinogen or fibrin and heparin type which act as thrombin inhibitor. Recently, microbial fibrinolytic enzymes of food origin receive more attention that leads to escalating efforts to explore traditional fermented foods as the natural sources. We have successfully isolated microorganism from Indonesian fermented soybean/tofu dregs "*Oncom*" that secretes fibrinolytic enzyme. The microorganism identified as *Stenotrophomonas* sp. is unique because most of the reported fibrinolytic microorganism belongs to *Bacillus* sp. This isolate was found to produce extracellular fibrinolytic enzyme which could degrade fibrinogen and fibrin directly as determined by fibrinogen zymography and fibrin plate methods. More importantly, the 30-kD purified enzymes was found to demonstrate not only fibrin and fibrinogen degradation capabilities, but also acted as thrombin inhibitor as determined using specific substrates for thrombin. This is the first report of a fibrinolytic enzyme that demonstrates additional synergistic activities. This finding accentuates the importance of further development of the enzyme into a powerful agent to treat the thrombus-related disease effectively.

1. Introduction

A variety of diseases and health problems are influenced by reactions that maintain a balance between blood coagulation and anticoagulation. Coagulation process produces fibrin-containing blood clots which is formed from fibrinogen by thrombin catalysis (Lioudaki and Ganotakis 2010; Voet and Voet 1990; Wang *et al.* 2006). Fibrin can be dissolved by fibrinolytic enzymes such as plasmin, which is normally activated from the nonactive plasminogen by a tissue-type plasminogen activator (tPA) (Collen and Lijnen 2004; Nakajima *et al.* 1993; Wang *et al.* 2011). This process maintains blood flow at vascular injury sites and is an important component of the normal haemostatic responses. Disturbances in the anticoagulation process can lead to accumulation of fibrin in the blood vessels and results as thrombosis conditions which usually lead to myocardial infarction and other cardiovascular diseases (Collen and Lijnen 2004; Kim *et al.* 1996). Researches are continuously pursued to find fibrinolytic agents, plasminogen activators and thrombin inhibitors which are safe and can work efficiently.

tPA is a serine protease which catalyses the conversion of plasminogen to plasmin, a major enzyme responsible for breakdown of fibrin in the blood clots. Plasminogen activators, such as tPA, urokinase, alteplase and reteplase are used in the clinical medicine to treat embolic and thrombotic strokes (Dubey *et al.* 2011; Duffy 2002; deMers 2012). Thrombin has many important functions in the clotting pathway leading to formation of the insoluble fibrin clots, so it is a good target for anticoagulants drugs. Natural antithrombin (AT) is a small protein molecule of 58 kDa that inactivates several enzymes of the coagulation system. ATs are required not only for blood disease disorder, arterial and deep vein thrombosis or coronary syndromes, but also during sepsis conditions which is known to activate the coagulation system and induces intravascular fibrin coagulation associated with increased mortality. Some of the clinically approved antithrombotic drugs include natural protein AT purified from human blood,

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recombinant human AT, hirudin peptides and heparin and its derivative anticoagulant. Their mode of actions range from hydrolysing AT or bind at specific sites of the thrombin molecules, resulting in the inactivation of the thrombin action (Fareed *et al.* 1999; Li *et al.* 2004).

Microbial fibrinolytic enzymes from food origin attracted much more medical interest lately. Many indigenous fermented foods have the ability to prevent thrombosis. A number of potent fibrinolytic enzymes have been isolated and characterized from fermented food products, such as Korean *Chungkook-Jang* (Kim *et al.* 1996), Japanese Natto (Wang *et al.* 2009), Chinese Douchi (Wang *et al.* 2006) and Indonesian Tempe (fermented soybean; Kim *et al.* 2006; Sugimoto *et al.* 2007). The fibrinolytic enzymes obtained from different microorganisms were mostly of the genus *Bacillus*.

We have successfully isolated a microorganism from Indonesian fermented soybean/tofu dregs "*Oncom*" that secretes fibrinolytic enzyme. The microorganism was identified as *Stenotrophomonas* sp., which is unique because most of the reported fibrinolytic microorganism belongs to *Bacillus* sp. We had confirmed safety of the extracellular protein enzyme of the isolate 11 (*Stenotrophomonas* sp.) using cell culture and experimental rat; in addition, the thrombus degrading effect of the enzyme had been also tested using experimental rats (Nailufar *et al.* 2016). This study showed that the purified extracellular fibrinolytic enzyme has multiple activities, namely degrading fibrinogen and fibrin directly as well as acting as AT. This is the first report on a fibrinolytic enzyme that demonstrates additional synergistic activities. This finding accentuates the importance of further development of the enzyme into a powerful agent to treat the thrombus-related disease effectively.

2. Materials and Methods

2.1. Microorganisms

Bacterial isolates used as source of fibrinolytic enzymes were obtained and screened from the red *oncom*, a fermented soybean/tofu dregs. Identification of the specific isolate was based on 16s rDNA gene sequence analysis.

2.2. Fibrinolytic enzyme production

The medium used for cultivation of the bacterial isolate and enzyme production was described by Miyaji *et al.* (2005), which consisted of 0.5% (w/v) casein, 0.5% (w/v) glucose, 0.6% (w/v) $Na_2HPO_4.2H_2O$, 0.2% (w/v) yeast extract, 0.1% (w/v) KCl and 0.01% (w/v) $MgSO_4.7H_2O$. pH of the medium was adjusted to 8.1 with 0.1 M sodium carbonate buffer. The cultivation was performed aerobically in a shaker incubator at 120 rpm for 48 h. The crude enzyme was obtained as supernatant following centrifugation at 9000*g*, 4°C for 30 min.

2.3. Fibrin plate assay

The fibrinolytic activity was measured using the standard fibrin plate method (Astrup and Mullertz 1952) with modification. In a petri dish (diameter of 85 mm), 7.3 mL of 0.5% w/v agarose and 200 μL of 1 mg/mL bovine thrombin were mixed gently, 2.5 mL of 1% w/v bovine fibrinogen was added and mixed to induce a solid fibrin formation. The fibrin plate was incubated at 37°C after 10 μL of the enzyme sample was dropped onto the disc (6 mm) previously placed on the surface of the fibrin agar. Diameter of the clearing zone areas were measured, and indicated as fibrinolytic activity of the enzyme.

2.4. Fibrin degrading unit

Fibrinolytic activity was measured using fibrin degradation assay described earlier (Hua *et al.* 2008). As much as 0.4 mL of 0.72% fibrinogen was placed in a test tube with 0.1 mL of 245 mM phosphate buffer (pH 7) and incubated at 37°C for 5 min. Then, 0.1 mL of a 20 U/mL thrombin solution was added. The solution was incubated at 37°C for 10 min, 0.1 mL of diluted enzyme solution was added, and incubation was continued at 37°C. This solution was shaked again after 20 and 40 min incubation. At 60 min, as much as 0.7 mL of 0.2 M trichloroacetic acid was added and mixed. The reaction mixture was centrifuged at 15,000*g* for 10 min. Then, 1 mL of the supernatant was collected and the absorbance at 275 nm was measured. In this assay, 1 unit (fibrin degradation unit, FU) of enzyme activity is defined as a 0.01 increase in absorbance at 275 nm/min.

2.5. Protein determination

Protein concentration was determined by Lowry's method using bovine serum albumin as a protein standard (Lowry *et al.* 1951).

2.6. Ammonium sulphate precipitation

Ammonium sulphate was added to the crude enzyme solution (free extracellular supernatant) at 4°C with continuous stirring over night to get saturation percentage of 65%, then centrifuged at 16,000 *g* at 4°C for 15 min. The precipitate was collected and dissolved in a half amount of 0.2 M phosphate buffer pH 7.5 from the initial volume. The dissolved enzyme was desalted against the same buffer using HiTrap Desalting column.

2.7. Enzyme purification

Protein solution from the desalting process was loaded onto HiTrap DEAE Sepharose column (GE Healthcare) equilibrated with 20 mM phosphate buffer, pH 7.5. The column was washed with the same buffer and the proteins were eluted using the same buffer containing NaCl (0–1.0) M by a stepwise gradient of 0.15 M, 0.35 M, 0.50 M and 1.0 M NaCl at a flow rate of 1 mL/min.

2.8. Thrombin inhibition assay

Chromozym TH (Roche Applied Science) used for thrombin activity assay was dissolved in redistilled water. The reaction was performed by adding 2.8 mL of Tris buffer (0.05 M, pH 8.3), 227 mM NaCl and 0.3 mL Chromozym TH (1.9 mM) into the cuvette. The enzyme sample was added at 0.1 mL, and the absorbance at 405 nm was observed. Changes in absorbance: ΔA/min was calculated from the linear curve. Under these conditions, the enzyme activity (U/mL) can be calculated as 3.077 × ΔA/min by following the instruction manual.

2.9. SDS-PAGE and zymogram

SDS-PAGE was performed using Laemmli's method (Laemmli 1970) with 12% separating gel and 4% stacking gel. The gel was run at 70 V, 400 mA for 2 h. The protein bands were then visualized by staining the gel with Coomassie blue and the apparent molecular mass of the protein was calculated using low molecular weight standard protein markers (GE Healthcare).

3. Results

Oncom is a traditional fermented soybean/tofu dregs. The soy protein soft material used for the basic ingredient comes from the bean paste left during tofu making, and this made the typical soft texture in the red *oncom*. The red colour is due to the metabolism product of the fungi, indicating high content of beta carotene. Indonesian people recognized *Oncom* as a traditional food rich in vitamin B and has ability to reduce cholesterol. We used *Oncom* to screen for fibrinolytic bacteria and found one of the potent isolates identified as *Stenotrophomonas* sp. The extracellular enzyme was capable of degrading fibrinogen and fibrin substrates. In our previous study, we had confirmed safety of the extracellular protein

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