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by Nurrahman Nurrahman

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Prospects of fibrinolytic proteases of bacteria from sea cucumber fermentation products as antithrombotic agent

Hayatun Fuad[1], *Nur* Hidayati[1], *Sri* Darmawati[1,2], *Hendra* Munandar[3], *Ayu* Rahmawati Sulistyaningtyas[2], *Nurrahman* Nurrahman[2], *Aditya* Rahman Ernanto[2], *Dewi* Seswita Zilda[4], *Widjanarka* Widjanarka[5], and *Stalis* Norma Ethica[1,2*]

[1]Medical laboratory Science Study Program, Universitas Muhammadiyah Semarang, Jl. Kedungmundu No.18, Semarang, Jawa Tengah, 50273 Indonesia
[2]Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang, Jl. Kedungmundu No.18, Semarang, Jawa Tengah, 50273 Indonesia
[3]Balai Bio Industri Laut-LIPI (Laboratorium Ilmu Pengetahuan Indonesia), Lombok, Nusa Tenggara Barat, Indonesia
[4]Research and Development Center for Marine and Fishery Product Processing and Biotechnology, Ministry of Maritime Affairs and Fisheries of Indonesia, Indonesia
[5]Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Diponegoro, Jl. Prof. Sudarto No.13, Tembalang, Semarang, Jawa Tengah 50275, Indonesia

Abstract. Cardiovascular disease is among the largest contributors of premature mortality in the world caused by inflammation of blood vessels. The abnormalities provoke thrombus formation or thrombosis blocking blood vessels leading to strokes, heart attacks and coronary artery diseases. Increasing percentage of cardiovascular cases and deaths due to thrombosis has attracted researchers to look for newer thrombolysis agents. Commonly used drugs to treat thrombosis has been limited due to various side effects. Therefore, the search for sources of safer and cheaper fibrinolytic enzymes for handling thrombolysis continues. This study aimed to evaluate potentials of fibrinolytic protease of bacteria isolated from fermented seafood (sea cucumber) products as antithrombotic agents. Information was initially gathered from scientific publications identified using web-based tools including PubMed (National Center for Biotechnology Information), Science Direct (Scopus) and Web of Science (Thomson Reuters) using combinations of search terms including "fibrinolytic enzyme protease", "endopeptidase", "fermented food", "sea cucumber", "thrombolysis therapy," "thrombolytic agent," "fibrinolytic bacteria," "fibrinolysis," "protease producing bacteria," "fibrin degradation," "holothurians," etc. We also searched for these terms in national and international organization technical reports and databases. This literature review reveals the prospects of fibrinolytic protease enzymes from bacteria from fermented seafood, particularly sea cucumber as novel antithrombotic agents.
Keywords: Cardiovascular disease, fermented seafood, fibrinolytic protease, thrombotic agent

1 Introduction

Cardiovascular disease (CVD) is leading cause of disability and premature all over the world due to the formation of thrombus in the walls of blood vessels causing heart disease such as stroke, heart attack and coronary artery disease. Increasing percentage of cardiovascular cases and deaths due to thrombosis worldwide has attracted researchers to look for agents that are more recent in degrading thrombus [1–5].

Common drugs that have been used to treat thrombosis include urokinase, tissue type plasminogen activator (t-PA) and streptokinase. The drugs work by activating plasminogen and then transforming plasminogen into plasmin. This action will degrade fibrin. However, the use of these agents has been limited due to various side effects including high price, half-life issues, allergy risks and administration problem. It has urged researchers to keep seeking for fibrinolytic enzymes with better characteristics for thrombolysis treatment [2].

In the last decades, many thrombolytic agents have been identified, studied and characterized from various sources such as earthworms, snakes, fungi, and bacteria. Bacteria have the potential to produce economically valuable enzymes with several advantages, including faster growth, scale of cell production will be more easily increased, production conditions do not depend on the season, short-time required, low cost, high activity, safe and not toxic and easily manipulated genetically [4, 6, 7]. Therefore, research about anti thrombolytic agents from new fibrinolytic protease producing bacteria must be done because more effective and efficient anti thrombolytic agents are still needed.

Fibrinolytic proteases in fermented seafood products other than shrimp and fish, i.e. sea cucumber, has not been reported. Sea cucumbers, which belong to the class *Holothuroidea*, have been known to possess high commercial value and have long been used for food and

* Corresponding author: snorma@unimus.ac.id

medicine with impressive profile of valuable nutrients including proteins and minerals [8]. Therefore, it is important to conduct research on the fibrinolytic activity of bacterial strains which is isolated from sea cucumber fermentation products is interesting to do [9]. This work aimed to evaluate prospect of bacterial fibrinolytic protease enzyme from fermentation products of seafood (especially sea cucumber) as an antithrombotic agent based on literature review.

2 Methods

This systematic review was carried out to determine prospect of developing anti thrombolytic agents from fibrinolytic protease of bacteria isolated from fermented sea cucumber. It is expected that the obtained anti thrombolytic agents can be used as an alternative medicine with better economic value, high activity and safer to deal with cardiovascular disease in Indonesia and the world. Eligibility criteria, identification of relevant studies, study selection, and control research bias were determined following previous procedures [10].

2.1 Eligibility criteria for study

First, types of thrombolytic agents that have been used in thrombosis treatment throughout the world were summarized, including the types of bacteria producing them. Study selection was based on the following inclusion criteria: [i] studies with limited subjects for treatment applied to cardiovascular disease; [ii] published in English; [iii] studies that evaluate the knowledge and practice of treatment of cardiovascular disease throughout the world as a result of measuring to find out how widespread the use of bacteria as anti-thrombolytic agents; [iv] observational study. The publication date was limited to the last 10 years considered in the search strategy.

A summary of the use of fibrinolytic protease bacteria as anti-thrombolytic agents in Indonesia was carried out to indicate the potential of using fibrinolytic protease bacteria as thrombolytic agent. The selection of studies was carried out based on the following inclusion criteria: [i] evaluation studies using knowledge and practice of using fibrinolytic protease bacteria in the treatment of cardiovascular disease as an outcome measure in Indonesia. [ii] subjects related to treatment using fibrinolytic protease bacteria in Indonesia are applied for cardiovascular disease; [iii] published in both languages namely Indonesian and English; [iv] observational studies. As part of the search strategy, the year of publication is also limited to the last 10 years.

2.1.1 Identification of relevant studies

This literature review was carried out in both manual and non-manual ways based on previous protocol [10-11]. A search for relevant studies was conducted in 2 categories: 1. *Enzyme fibrinolytic proteases from fermented foods*. 2. Purification and characterization of fibrinolytic protease enzymes. For the first category, electronic literature and initial manual searching through PubMed and Science Direct Database as well as manual searches regardless of publication date were carried out using the term MESH-- fibrinolytic enzyme protease; enzymes; endopeptidases; seafood; fermentation; thrombolysis therapy; sea cucumber; holothurian. Full text of all articles was extracted by electronics and manual searches from PubMed and Science Direct. The studies released from this review were: [i] studies conducted after 2010 [ii] studies of fibrinolytic protease enzymes isolated from groups of bacteria in fermented products in Indonesia; [iii] reviews; [iv] study of important fibrinolytic enzymes in thrombolysis [v] study of fibrinolytic enzyme-producing bacteria [vi] study of fibrinolytic enzyme-producing bacteria in food products. For the second category, computerized literature searches through PubMed (National Center for Biotechnology Information 2019), Science Direct (Scopus 2019) and Web of Science (Thomson Reuters 2019), as well as manual searches regardless of date of publication are carried out using the MESH query for the treatment of thrombolytic or fibrinolytic protease enzymes from food fermented products. A variety of keywords are used in search strategies including thrombolysis therapy, fibrinolytic protease enzymes, fibrinolytic protease-producing bacteria, fibrin degradation. Various keyword combinations are created using 'and', 'or' as Boolean operators. The relevant subject matter experts and selected study authors were also contacted to obtain missing or unclear data whenever it was considered important. The studies excluded from this review were: [i] studies conducted before 2010 [ii] patents.

2.1.2 Study selection

Three authors (HF, SNE, and HM) independently identified literature that fulfil the inclusion criteria in this review. Full text articles from the remaining studies taken fulfil the inclusion criteria. Observational studies were filtered using the STROBE checklist

2.1.3 Control research bias

The following issues were included in the risk of bias or quality of assessment in this systematic review: (i) completeness of reporting information on the fibrinolytic protease enzymes produced by bacteria in food fermentation products, (ii) selective yield reporting, (iii) selection of outcome measures [practice test the ability of thrombosis agents using fibrinolytic protease bacteria applied during cardiovascular treatment in Indonesia], (iv) research design, and (v) inter-conflict conflict in conducting research. When the overall criteria were met, the risk of a reasonable bias overall was regarded low.

2.1.4 Data collection and extraction

This review had been carried out based on the established guidelines submitted by Choice Reporting Items for Systematic Review and META Analysis

[PRISMA] [12]. Four writers (ARS, SD, DSZ, & NH) were responsible for extracting data from research. From each study, the predetermined data extracted included the research design, sample size, treatment of cardiovascular disease among study subjects around the world, and knowledge related to the use of microbial fibrinolytic protease-producing enzymes to deal with various cardiovascular diseases.

3 Literature review

3.1 Importance of fibrinolytic enzymes in thrombolysis

Table 1 summarises cases of Cardiovascular disease (CVD) and reported mechanism of treatment by drugs in the last decade. From the data, it can be seen that thrombolysis/ fibrinolysis treatment is still widely used to date. The known mechanism of the treatment basically utilizing drugs targeting the degradation of fibrin by plasmin activation.

Table. 1 Current CVD cases and related treatment reported in the last decade

Case	Mechanism of Treatment	Region	Source, Year
Stroke, thrombosis, embolism, atherosclerosis	Hydrolysing fibrin	Indonesia	[1]
Heart & cerebrovascular disease	Hydrolysing fibrin	Vietnam	[13]
Heart attack & stroke	Dissolving fibrin lumps	China	[14]
Coronary heart disease & atherosclerosis	Blocking thrombus	China	[15]
Myocardial infarction &coronary heart disease	Hydrolysing fibrin	China	[16]
Myocardial infarction & thrombosis	Hydrolysing fibrin	Indonesia	[17]
Stroke & coronary artery disease	Dissolving fibrin clots	Vietnam	[4]
Thrombosis	Hydrolysing fibrin	Taiwan	[18]
Myocardial infarction	Hydrolysing fibrin	Korea	[19]
Heart attack & coronary thrombosis	Breaking down fibrin	India	[20]
Myocardial infarction	Degrading fibrin	China	[21]
Myocardial infarction, thrombosis & embolism	Preventing thrombus formation	Korea	[22]
Heart disease & stroke	Hydrolysing fibrin	Indonesia	[2]
Thrombus & vascular cardiovascular disease.	Hydrolysing fibrin	China	[23]
Myocardial infarction & ischemic heart disease	Hydrolysing the α, β & γ chains	Korea	[24]
Myocardial dial infarction & stroke	Converting plasminogen	India	[25]
Pulmonary embolism, myocardial infarction	Degrading fibrin	China	[26]
Acute myocardial infarction & brain infarction	Dissolving fibrin inside blood	Korea	[27]
Myocardial infarction & cardiovascular infarction	Degrading α & β chains	Korea	[28]

3.2 Study of bacterial diversity isolated from seafood fermented products

Studies of bacterial diversity isolated from fermented food products have been conducted in many countries, although the frequency is not as often as that isolated from other ingredients. Diversity of bacteria in fermented foods is found, most of bacteria has identified as bacteria *Bacillus* sp. It could be seen from Table 2 and 3 that group of bacteria that produce fibrinolytic protease enzymes applied to cardiovascular disease mostly use *Bacillus* sp. species bacteria. Based on 19 literatures reviewed, all of the bacteria producing fibrinolytic protease enzymes reported from all over the world in the last decade were mostly used in the treatment of cardiovascular disease (Table 2).

3.3 Fibrinolytic protease enzymes from bacteria isolated from fermentation products

Studies about diversity of fibrinolytic protease enzymes from bacteria isolated from fermented foods have been carried out in many countries although their frequency is not as often as that isolated from other ingredients. For example, Stephani (2017) reported about bacterial populations that produce fibrinolytic protease enzymes in red oncom products is carried out in Indonesia [1]. The results showed that the bacteria isolated from red oncom with fibrin degradation potential consisted of *Bacillus licheniformis*, *B.cereus*, *B.licheniformis*, dan *B. cereus*. Meanwhile, molecular analysis of a potential bacterial thrombolytic agent from Douchi-China was successfully carried out by Hu (2019) [16]. The study conducted genetic diversity analysis based on the bacterial 16S rRNA gene.

Table. 2 Biodiversity study on protease producing bacteria from various fermented products in the last decade

Fermented Food Product	Isolated Bacteria	Source, Year
Soy Meju	*B. amyloliquefaciens* MJ5-41	Jo *et al.*, 2011 [28]
Fermented Chickpeas	*B. amyloliquefaciens*	Wei *et al.*, 2011 [23]
Soy Meju	*B. amyloliquefaciens* MJ5-41	Jo *et al.*, 2011 [28]
Red bean Natto	*B. subtilis*	Chang *et al.*, 2012 [18]
Soy Douchi	*B. subtilis* LD-8547	Yuan *et al.*, 2012 [15]
Soy & cheese Doufuru	*B. subtilis*	Chen *et al.*, 2013 [14]
Salted fish & shrimp Terasi	*B. coagulans*	Prihanto *et al.*, 2013 [29]
Soy Douchi	*B. amyloliquefaciens* DC-4	Zhang *et al.*, 2013 [26]
Soy Cheonggukjang	*B. amyloliquefaciens* CB1	Heo *et al.*, 2013 [27]
Soy Gembus	*B. pumilus* 2.g	Afifah *et al.*, 2014 [2]
Fermented Indian rice	*B. cereus* IND1	Vijayaraghavan & Vincent, 2014 [25]
Shrimp Terasi	*Lactobacillus plantarum*	Chalthoniyah *et al.*, 2015
Fermented shrimp paste	*B. weihenstephanensis, Bacillus* sp.	Anh *et al.*, 2015 [4]
Soy Cheonggukjang	*B. subtilis* HK176	Jeong et al, 2015 [24]
Green chili, soy Miso	*B. amyloliquefaciens* FZB42	Huy *et al.*, 2016 [13]
Soy Oncom	*Bacillus* spp..	Nurlufar *et al.*, 2016 [17]
Soy Doenjang	*Bacillus* spp.	Jeon *et al.*, 2016 [30]
Soy Doenjang	*B. amyloliquefaciens* RSB34	Yao *et al.*, 2017 [31]
Fermented rice	*B.* sp. IND6	Almalki *et al.*, 2017 [32]
Red oncom, fermented soybean/ tofu dregs	*Bacillus* spp. & *Stenotrophomonas* sp.	Stephani *et al.*, 2017 [1]
Flour Dosa Batter	*B. amyloliquefaciens* MCC2606	Devanaj, 2018 [20]
Small shrimp Jeotgal	*B. subtilis* JS2	Yao *et al.*, 2018 [9]
Soy fermented Oncom	*B. megaterium*	Lestari *et al.*, 2018 [33]
Soy Gembus	*Pseudomonas stutzeri* ISTD4	Inayatul *et al.*, 2018 [34]
Soy red Oncom	*B. thuringiensis* IRODI	Safitri *et al.*, 2018 [35]
Soy red Oncom	*S. hominis*	Harun *et al.*, 2018 [36]
Soy Douchi	*B. subtilis* DC27	Hu *et al.*, 2019 [16]
White shrimp Rusip	*S. cohnii* IRLV5	Japri et al., 2019 [37]
White shrimp Rusip	*S. epidermidis*	Fazri *et al.*, 2019 [38]

The diversity of bacteria in fermented foods brings the flexibility to find groups of fibrinolytic bacteria that produce various enzymes that are able to degrade fibrin. By conducting biodiversity study, it is expected that the bacterial group could be selected, tested and then used as a new antithrombotic agent.

3.4 Fibrinolytic enzymes producing bacteria

Table 3 summarised biodiversity studies conducted to isolate proteolytic bacteria from various fermented foods in various countries. It was found that majority of proteolytic bacteria isolated from fermented foods belong to *Bacillus* group. It is also confirmed by data in Table 2 and 3 that there are no other fermented sea food products other than fish (catfish) and shrimp used as samples to isolate fibrinolytic enzyme producing bacteria.

Based on the summarised data, study aiming at production of fibrinolytic enzymes from bacteria isolated from fermented sea cucumber as a rich protein source is potential to be done. It offers novelty, yet potential considering that reports about sea cucumber fermentation could not been found during this review process. A scheme showing potential of fibrinolytic protease enzymes of bacteria from sea cucumber fermentation products as antithrombotic agent is presented in Figure 1.

Table. 3 Fibrinolytic enzyme producing bacteria from food

Species	Application	Food Sample	Country	Source, Year
B. amyloliquefaciens	Thrombolytic agent	Chickpeas	China	[23]
B. amyloliquefaciens MJ5-41	Thrombolytic agent	Meju	Korea	[28]
B. subtilis	Thrombolytic agent	Natto-red bean	Taiwan	[18]
B. subtilis LD-8547	Thrombolytic agent	Douchi	China	[15]
Bacillus subtilis	Thrombolytic agent	Doufuru	China	[14]
B. coagulans	Fibrinolytic agent	Fermented Fish	Indonesia	[29]
B. amyloliquefaciens DC-4	Fibrinolysis agent	Douchi	China	[26]
B. amyloliquefaciens CB1	Fibrinolysis agent	Cheonggukjang	Korea	[27]
B. pumilus 2.g	Thrombolytic agent	Gembus	Indonesia	[2]
Bacillus sp.	Thrombolytic agent	Fermented shrimp paste	Vietnam	[4]
B. cereus IND1	Thrombolytic agent	Indian rice	India	[25]
B. subtilis HK176	Thrombolytic agent	Cheonggukjang	Korea	[24]
Bacillus sp.	Hemolytic agent	Doenjang	Korea	[30]
B. amyloliquefaciens FZB42	Thrombolytic agent	Green chili miso	Vietnam	[13]
Bacillus sp. & *Stenotrophomonas* sp.	Thrombolytic agent	Oncom	Indonesia	[17]
Stenotrophomonas sp.	Thrombolytic agent	Oncom	Indonesia	[1]
Bacillus sp. IND6	Thrombolytic Agent	Fermented rice	Saudi Arabia	[32]
B. amyloliquefaciens RSB34	Thrombolytic agent	Doenjang	Korea	[31]
B. subtilis JS2	Fibrinolysis agent	Saeu jeotgal	Korea	[31]
B. amyloliquefaciens MCC2606	Thrombolytic agent	Soy	India	[20]
B. subtilis DC27	Thrombolytic agent	Douchi	China	[16]

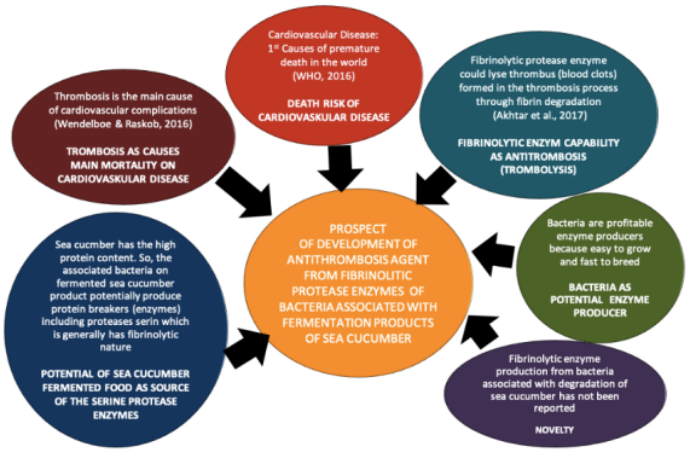

Fig. 1. Prospects for the production of antithrombotic agents in the form of protease enzymes from the results of proteolytic bacterial secretion in the fermented sea cucumber.

4 Conclusion

Based on results of this review, majority of proteolytic bacteria isolated from fermented food belong to *Bacillus* group. Most bacteria producing fibrinolytic proteases applied in CVD treatment are also from *Bacillus* group. Studies about fibrinolytic protease enzymes of bacteria from sea cucumber fermentation products offers novelty and discovery of new proteolytic enzymes potential to be used in CVD treatment.

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