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# The Importance of Purification and Activity Analysis of the Purified Product of Thrombolytic Protease from *Bacillus* sp. HSFI-12– A Review

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**ABSTRACT**
Mortality and morbidity of Cardio-vascular diseases (CVDs) have been the major issue in the group of non-communicable diseases worldwide. However, the existing antithrombotic drugs to combat CVD still have many shortcomings in terms of price and safety. *Bacillus* sp. HSFI-12 had been previously reported as a highly potential producer of thrombolytic protease, in which crude protease has competitive clot lysis or thrombolytic activity. However, the purity and other characteristics of the protease in its purified form have not been reported. The remained task is to ensure that *Bacillus* sp. HSFI-12, as a source of thrombolytic protease, can be used as an alternative of effective treatment with lower cost to combat thrombosis in cardiovascular diseases. This review aims to provide a highlight the most suitable purification methods to be applied to the protease dialysate of *Bacillus* sp. HSFI-12 and recommending appropriate assays following the recommended purification methods. Results of this review showed that both ion exchange and gel filtration chromatography (IEC and GFC) methods are suggested to be used in the purification step as a continuation of the dialysis process of HSFI-12 protease previously concentrated using ammonium sulfate. In addition, the results of the review found that the most common assays conducted on purified protease developed as antithrombotic agents are anticoagulation and antiplatelet assays. In conclusion, the purification and activity analysis of the purified product of thrombolytic protease from *Bacillus* sp. HSFI-12 using chromatographic methods is important to do. This review recommended that aside from anticoagulant and antiplatelet assays, thrombolytic activity tests on HSFI-12 protease dialysate are also important to do to support the protease's characteristics an antithrombotic agent.

***Keywords:*** *Bacillus sp. HSFI-12, Chromatography, Enzyme purification, Thrombosis, Thrombolytic protease.*

## 1. INTRODUCTION

Cardiovascular disease (CVD) is among the primary causes of death worldwide triggered by thrombosis. Thrombosis is the formation of blood clots containing fibrin aggregates in the blood vessels causing block of blood flow. Such blockage could cause heart and brain failures for not receiving an adequate oxygen supply [1,2]. The imbalance of the hemostasis system will result in pathological abnormalities that cause spontaneous bleeding because the blood cannot clot and the formation of thrombus due to excessive blockage. The presence of a thrombus will trigger vascular diseases including myocardial infarction, heart attck, brain embolism

 

(stroke), and various other vascular diseases. In addition, the thrombus also causes tears in the tissue and swelling of the arteries [3,4].

Therefore, treatment therapy is needed to overcome the abnormality using antithrombotic drugs such as anticoagulants and antiplatelets. Blood clots can be treated with antithrombotic drugs and can be eliminated through a fibrinolysis process involving hydrolysis by plasmin. However, the existing antithrombotic drugs still have many shortcomings in terms of high prices and serious side effects, so new drugs, which are more affordable, and safe are needed [5].

Due to the high rate of cardiovascular disease morbidity and mortality, special attention is required to develop more cost-effective therapies. *Bacillus* sp. has been widely recognized for its activity as a thrombolytic and antiplatelet agent and is a potential producer of fibrinolytic proteases. A member of the bacterial genus, namely *Bacillus* sp. HSFI-12 has been reported to be able to produce crude extracts of protease enzymes with the thrombolytic activity that competes with the antithrombotic drug Nattokinase. The isolate was originated from "Rusip" fermented product of the intestine organs of sea cucumber, *H. scabra*, collected from seawater of Kodek Bay, West Nusa Tenggara Island, Indonesia [6-7].

*Bacillus* sp. HSFI-12 was recognized in a previous study as a highly potential producer of thrombolysis protease. Crude protease of HSFI-12 isolated was found to have competitive clot lysis activity [6]. However, the purity of the protease has not been reported. The remained task is to ensure that *Bacillus* sp. HSFI-12, as a source of thrombolytic protease, can be used as an alternative of effective treatment with lower cost to combat thrombosis in cardiovascular diseases. However, the purity and other characteristics of the protease in its purified form have not been reported.

To develop the dialysate extract of the thrombolytic protease previously obtained from HSFI-12 strains, purification should be conducted and the product should be purified and further tested using *in vitro* and *in vivo* approaches. Without purification the antithrombotic activity of the enzyme could not be accurately determined. This article aims to provide a literature review on the importance of purifying the crude extract of the protease enzyme in *Bacillus* sp. HSFI-12 and recommending appropriate purification methods to further enhance its proteolytic activity.

## 2. METHODOLOGY

A literature search was first carried out from the literature databases such as PubMed, Science Direct, Pro-quest and Google Scholar. The screened articles were those published between 2011-2021 on the role of thrombolysis and its mechanisms in cardiovascular disease. In particular, the search was advanced on bacterial enzymes developed as antithrombotic agents involving purification methods. Searching for subject titles in several combinations including "cardiovascular disease", "cardiovascular drugs and therapy", fibrinolytic proteases, "antithrombotic proteases", "bacterial protease", "fibrinolysis mechanism", "antithrombotic agent", "purification method", purified enzyme test", "antithrombotic activity" and their derivatives.

### *2.1. Criteria set for Relevant Studies*

Study screening was based on inclusion criteria set as follows: [i] Subjects associated with the purification of thrombolytic protease enzymes in cardiovascular treatment; [ii] antithrombotic enzymes produced by bacteria [iii] enzyme purification studies and antithrombotic assays as a measure in determining pure enzyme characteristics; [iv] reported in Bahasa Indonesian or English; [v] observational studies as a search plan with the age of publication set to the last 10 years.

### *2.2. Study Selection*

Following guidelines from Polanin et al. (2019), independent studies were conducted to identify studies, which met the inclusion criteria applied in this review. Both titles and abstracts from the records generated by the search were carefully identified and analyzed to determine which unsuitable sources should be excluded adhering to the exclusion criteria. Articles (full-text) of the remaining studies were also taken and evaluated whether or not they meet the inclusion criteria [8].

### *2.3. Bias Control Assessment*

In this review, the following issues are included in the risk of tendency or quality assessment: completeness of reporting facts on the urgency of purification of thrombolytic bacterial proteases, choice of outcome measures (practical test of the capacity of thrombotic agents applied in cardiovascular treatment). When all of these criteria are fulfilled, the risk of possible bias is regarded low [9-10].

## 3. RESULT

This review summarized the purification methods and types and sources of bacterial proteases reported in the development of antithrombosis agents that have been reported in the last 5 years (during 2016-2020). The aim was to determine the most suitable purification methods to be applied to the dialysate product of protease originated from *Bacillus* sp. HSFI-12 to obtain the purified form of the enzyme with better antithrombotic characteristics supporting its role as a candidate of the antithrombotic agent.

**Table 1.** Chromatographic methods used to purify bacterial proteases developed as antithrombotic agents reported by various studies worldwide in the last 5 years

| Bacterial species | Chromatography type of method | Country | References |
|---|---|---|---|
| *Bacillus tequilensis* | Ion Exchange<br>Size Exlusion | China | Xin *et al.,* 2018 [11] |
| *Bacillus cereus* | DEAE-Cellulose Ion Exchange<br>Gel Filtration | India | BKM *et al.,* 2018 [12] |
| *Bacillus* sp. | DEAE-Cellulose Ion Exchange<br>Gel Filtration | Malaysia | Jimat *et al.,* 2017 [13] |
| *Triticum aestivum* | Ion Exchange<br>Gel Filtration | Turkey | Altin *et al.,* 2017 [1] |
| *Bacillus cereus* | Ion Exchange | Indonesia | Junaidia *et al.,* 2017 [2] |
| *Bacillus* sp. | DEAE-Cellulose Ion Exchange | China | Yu *et al.*,2019 [3] |
| *Geobacillus toebii* | DEAE-Cellulose Ion Exchange<br>Gel Filtration | Tunisia | W *et al.*,2016 [4] |
| *Bacillus* sp. | Column (unspecified) | India | Putatunda *et al.,* 2019 [18] |
| *Bacillus subtilis* | Ammonium Sulphate Precipitation<br>DEAE-Cellulose Anion Exchange | Turkey | Nur *et al.,* 2019 [19] |
| *Bacillus subtilis* | Ammonium Sulphate Precipitation<br>DEAE-Cellulose and sephadex G-100 Ion Exchange | India | Sujatha A *et al.,* 2018 [20] |
| *Geobacillus* sp. | Ammonium Sulphate Precipitation<br>Ion Exchange | Indonesia | Iqbalsyah *et al.,* 2019 [21] |
| *Bacillus subtilis* | Ammonium Sulphate Precipitation<br>Ion Exchange<br>Gel Filtration | Pakistan | M *et al.,* 2018 [5] |
| *Bacillus amyloliquefaciens* | DEAE-Cellulosa Anion Exchange<br>Gel Filtration | China | Hu *et al.*, 2020 [6] |
| *Bacillus subtilis* | Column<br>Gel Filtration<br>High Performance Liquid | China | Hue *et al.,* 2019 [24] |

Table 1. Summarizes the chromatographic types of methods used to purify bacterial proteases developed as antithrombotic agents reported in various studies worldwide in the last 5 years (2016-2020). In Table 1 the trend of data displays that the chromatographic-based purification methods widely used are dominated by Ion Exchange Chromatography (IEC) and Gel Filtration Chromatography (GFC). In Table 1, the trend of data also reveals that all of the identified studies reporting the purification process on antithrombotic bacterial proteases are dominated by Asian Countries Including China, Japan, India, and Indonesia. Those from Europe are only represented by one country (Turkey). Interestingly, proteases subjected to the purification process are those mainly from *Bacillus* sp. [11-24].

Supporting the finding based on Table 1, there are several reasons why IEC is often selected in the purification process of antithrombotic proteases (usually done after ammonium deposition and dialysis process). First, IEC could separate ionizable molecules based on charge property differences. Its strong resolving capability, wide applicability (especially for proteins and enzymes), large sample handling capacity, moderate cost, and ease of scaling and automation have made it among the most widely used and versatile liquid chromatography methods found today [25-27]. On the

**Tabel 2.** Studies in the last 5 years reporting *in vitro* antithrombotic assays and associated instrumentation on bacterial enzymes following purification process using chromatographic methods

| No | Antithrombotic assays | Instruments | References |
|---|---|---|---|
| 1 | *In vitro* Anticoagulant Activity (PT, aPTT, TT) | Automated Coagulation Analyzer Diagnostica Stago STart Hemostasis Analyzer | Frias *et al.,* 2021 [7] |
| 2 | *In vitro* Anticoagulant Activity (PT, aPTT) | Automated Coagulation Analyzer Sysmex | Sukati *et al.,* 2021 [8] |
| 3 | Antiplatelet Activity (Platelet Adhesion & Platelet Aggregation) | Collagen Type IV Coated Strip | Sukati *et al.,* 2021 [8] |
| 4 | *In vitro* Anticoagulant Activity (PT, aPTT, TT) | Automated Coagulation Analyzer Diagnostica Stago STart Hemostasis Analyzer | Bougetef *et al.,* 2020 [9] |
| 5 | Thrombin Clotting Time (TCT) | ACL 200 Coagulation Analyzer | Pepe *et al.,* 2016 [10] |
| 6 | *In vitro* Antiplatelet Activity | Spektrofotometer UV Vis | Rohmah *et al.,* 2020 [11] |
| 7 | *In vitro* Anticoagulant Activity (CT, PT, aPTT) | Lee-White | Rohmah *et al.*, 2020 [11] |
| 8 | *In vitro* Anticoagulant Activity, (PT, aPTT) | Siemens Fibrintimer II Optical Coagulometer and Thromborel Kits | Bijak et al., 2019 [12] |
| 9 | *In vitro* Anticoagulant Activity (PT, aPTT) | Commercial Kits | Gogoi *et al.,* 2019 [13] |
| 10 | *In vitro* Anticoagulant Activity (PT, aPTT, TT) and FB | Automated Coagulometer Sysmex CA-1500 | Choi *et al.,* 2013 [14] |
| 11 | *In vitro* Anticoagulant Activity (aPTT dan PT) | Sysmex CA-50 Semi-automated Coagulation | Wang *et al.,* 2018 [15] |
| 12 | *In vitro* Anticoagulant Activity (PT, aPTT, TT) | Diagnostoc Stago | Krichen *et al.,* 2018 [16] |
| 13 | *In vitro* Anticoagulant Activity (PT, aPTT, TT) | Humaclot duo coagulometer and human reagent kits | Rehman *et al.,* 2019 [17] |
| 14 | Platelet Aggregation | Optical lumi-aggregation system | Sachin *et al.,* 2021 [18] |
| 15 | *In vitro* Anticoagulant Activity | Lee-White and Blood Smear Eustrek | Fuad *et al.,* 2021 [42] |
| 16 | *In vitro* Anticoagulant Activity | Lee -White | Krishnamurthy *et al.,* 2017 [43] |
| 17 | *In vitro* Anticoagulant Activity | Lee-White and Blood Smear Eustrek | Rusyiana *et al.,* 2021 [44] |
| 18 | *In vitro* Anticoagulant Activity | Lee-White | Tian *et al.*, 2015 [22] |
| 19 | *In vitro* Anticoagulant Activity | Lee-White | Mahajan *et al.,* 2012 [23] |

other hand, GFC is also preferred because it can perform the separation of different molecules with high yields, and is carried out with a special design to keep the activity and stability of the desired molecule without reducing its resolution [28]. Both IEC and GFC are not only the most widely reported in the literature, yet most suitable to be used to increase the activity of a proteolytic enzyme in general [16-19].

Table 2. Summarizes antithrombotic assays and used instrumentation on bacterial enzymes following purification using chromatography methods process [29-39]. Data in table 2 interestingly shows that regardless of the widely varied instrumentation used, types of antithrombotic assays are grouped into 3 main categories: *In vitro* anticoagulant activity and antiplatelet activity assays. *In vitro* Anticoagulant Activity assays include PT

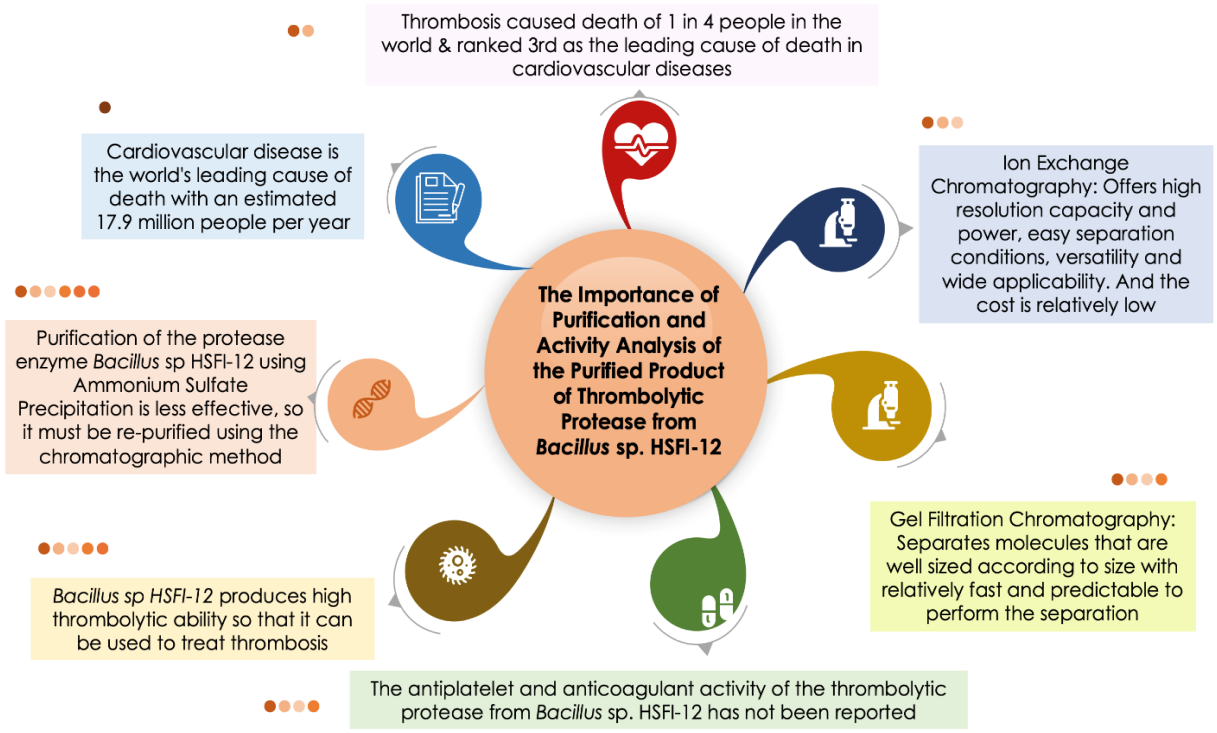


**Figure 1** Summarized factors contributing to the importance of purification and activity analysis of the purified product of thrombolytic protease from *Bacillus* sp. HSFI-12

(Prothrombin Time), aPTT (Activated Partial Thromboplastin Time), TT (Thrombin Time). Meanwhile Platelet Activities assays encompasses Platelet Adhesion and Platelet Aggregation. These *in vitro* aPTT, PT, and TT tests calculate the time elapsed from activation of the coagulation cascade [40].

It is known that antithrombotic characteristics encompass anticoagulant, antiplatelet and thrombolysis activities [41]. So, to develop an antithrombotic agent from proteases, it is also important to conduct thrombolytic assays on the purified product of the enzyme aside from anticoagulation and antiplatelet activities. This should also be applied to thrombolytic protease produced by isolate HSFI-12 if the aim is to develop it as an antithrombotic agent.

The mortality and morbidity of CVD as well as the death risk of thrombosis in CVD has motivated many researchers to search for better antithrombotic agents in terms of price, availability and side effects. Figure 1 summarizes factors contributing to the importance of purification and activity analysis of the purified product of thrombolytic protease from *Bacillus* sp. HSFI-12.

As schemed in Figure 1, the development of an antithrombotic agent using protease from *Bacillus* sp HSFI-12 offers a possible novelty in cardiovascular medicine therapy. The purification and activity analysis of the purified product of thrombolytic protease from *Bacillus* sp. HSFI-12 using IEC and GFC has not been reported, thus they are suggested to do to enhance the enzyme's activities. Anticoagulant and antiplatelet activity assays on the same product have also not been reported, yet they are mostly reported by successful studies worldwide in the last 5 years. Thus, they are also required to do. In addition to this, however, to support the protease's characteristics as an antithrombotic agent, thrombolytic activity tests on the purified HSFI-12 protease dialysate should be done.

Both ion exchange and gel filtration chromatography (IEC and GFC) methods are suggested to be used in the purification step as a continuation of the concentration process of HSFI-12 protease dialysate as obtained in the previous study. In addition, this study suggested the importance of thrombolytic assays aside from anticoagulant and antiplatelet activity assays, which are often reported, to fully support the characteristics of HSFI-12 protease dialysate if developed as an antithrombotic agent.

## AUTHORS' CONTRIBUTIONS

NI and SNE conceived the original idea, NI and MAA screened and summarized all obtained literatures. AHM evaluated the generation of tables and schemes, as well as analysed the bias of the study. The main text was written by NI and SNE. The manuscript was initially written by NI, and the improved and revised by SNE.

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