Prospective Purification and Assay of Thrombolytic Protease from *Bacillus* sp. HSFI-10 Isolated from Sand Sea Cucumber for Antithrombotic Agent Development

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ABSTRACT
Thrombosis is a cardiovascular disorder due to the formation of a blood clot (thrombus), which can cause a blockage in a blood vessel. Such abnormality is responsible for more than millions of deaths per year in the world. In addition to cardiovascular disease, thrombosis can also occur in patients with the current pandemic era disease, Covid-19. *Bacillus* sp. HSFI-10 (*Holothuria scabra* fermented intestine-10) from a previous study has been described as a potential producer of thrombolytic crude protease, which can exhibit competitive thrombolytic activity. However, the purity and other properties of the purified form of the enzyme have not been reported. The remaining challenge is to enable strain HSFI-10 to be used as a source of an effective and low-cost alternative of the thrombolytic agent in the treatment of thrombosis. The purpose of this literature review is to highlight the most appropriate purification method for *Bacillus* sp. HSFI-10 protease dialysate and to recommend the appropriate assay according to the recommended purification method in the literature. The results suggested that both ion exchange and gel filtration chromatography (IEC and GFC) are the most prospective techniques for the purification of thrombolytic protease. These purification techniques should be carried out as a continuation of the previously concentrated thrombolytic protease of strain HSFI-10 obtained through precipitation using ammonium sulfate followed by dialysis processes. In addition, it was also revealed that the most prospective assays performed on purified proteases to be developed as antithrombotic agents are anticoagulant and platelet aggregation assays. In conclusion, the suggested techniques to purify the dialysate of HSFI 10 are both IEC and GFC. In addition to clot lysis activity assays, it is recommended that anticoagulant and antiplatelet assays are also performed to support the potential of *Bacillus* sp. HSFI-10 protease as an antithrombotic agent.

Keywords: *Antithrombotic agent, Bacillus* sp. *HSFI-10, Protease purification, Thrombolytic assays*

1. INTRODUCTION

Cardiovascular disease is caused by disorders of the heart and blood vessels such as myocardial infarction, stroke, and pulmonary embolism involving thrombosis in the veins resulting in blood clots due to pathological thrombus formation [1–4]. Thrombosis is a cardiovascular disorder due to the formation of a blood clot (thrombus), which can cause a blockage in a blood vessel. As reported by the American Heart Association

report, such abnormality is responsible for more than 17.3 million deaths per year in the world. In addition to cardiovascular disease, thrombosis can occur in patients with the current pandemic era disease, Covid-19. The hypercoagulable state in Covid-19 patients can increase the risk of thrombotic complications, especially venous thromboembolism [5]. A study reported by Ackermann et al. [7] showed that cases of micro-alveolar thrombosis mostly occur in Covid-19 patients who died [6]. The prevalence rate of thrombosis in Covid-19 cases is high, up to 79%, and causes a high mortality incidence [7-8].

Over-the-counter antithrombotic agents such as streptokinase, urokinase, tissue plasminogen activator (tPA), and Nattokinase are widely used to treat of thrombosis. However, these antithrombotic agents are costly and cause side effects such as allergic reactions, standard blood lysis, coagulation processes, and low fibrin specificity. Therefore, there is a need to look for new antithrombotic agents with no side effects, high specificity, purity, efficiency, and low cost [9-10].

Bacteria have largely contributed as a very potential source of antithrombotic protease enzymes. The potential for enzymes that can be developed as bacterial antithrombotic agents has been widely isolated, most of them from the Bacillus group. [11-13]. In recent years, many studies have confirmed the potential for antithrombotic effects due to the safety benefits of microbial proteases. The microbial origin of fibrinolytic enzymes is known to have few side effects. In addition, they offer low production costs, rapid growth, high substrate specificity, and abundant availability in nature [14-17].

An enzyme purification process is required to characterize the enzyme's structure's physical and biological properties [18-19]. The purity of bacterial fibrinolytic protease enzymes is strongly influenced by the purification technique used in the enzyme analysis [19-20]. Traditional purification steps with only ammonium sulfate precipitation (salting out) and ultrafiltration techniques have resulted in less specific enzyme activity [20-21]. More advanced enzyme purification steps can be performed by chromatographic techniques such as gel filtration chromatography, ion-exchange, affinity chromatography, high-performance protein liquid chromatography/FPLC, high-performance liquid chromatography/HPLC, and hydrophobic chromatography. The results of the enzyme purification operation can be evaluated by measuring the specific activity of the enzyme purification product [13, 16, 23-24].

In addition to the enzyme purification method, the use of contaminant-free purification tools is the other important consideration in enzyme purification so that the results obtained are accurate [24-26]. Based on the previous report, crude extract of the protease enzyme obtained from the HSFI10 bacterial isolate from the sea cucumber *Holothuria scabra* had antithrombotic activity, which was below 80% [27]. Another study showed a very high variation of in vitro blood clot lysis activity [90100%] of the thrombolytic proteases isolated from *Streptomyces radiopugnans*, a marine sponge microorganism. The results from the last study were obtained from enzymes purified by chromatography [28]. Another study revealed that in vitro assay results of a protease enzyme purified by ion-exchange chromatography can lyse blood clots up to 97% [26]. These studies showed that it is apparent that purified proteases tend to pose higher thrombolytic activities than crude ones do.

It is important to look for new sources of antithrombotic proteases with better properties, the surprising increase in CVD cases and death with thrombosis, and the low specificity of current over-the-counter antithrombotic agents. Current issues have led researchers to focus on identifying the high specificity and purity of thrombolytic proteases from various sources, including bacteria, as potential sources of antithrombotic therapy. This study evaluates the prospect of purification of thrombolytic proteases from HSFI-10 bacteria derived from sea cucumber (*H. scabra*) to obtain high-purity protease as antithrombotic agents, based on literature studies.

2. METHODS

Systematic literature was achieved by adopting formerly posted evaluation articles [29]. Primary literature was searched in the Pubmed database posted withinside the ultimate five years (2016-2020), discussing thrombolytic proteases and their purification. We also searched Medical Subject Title Headings [MeSH] in numerous combos such as bacterial fibrinolytic/ thrombolytic agents, bacterial proteases, fibrinolytic/ thrombolytic enzymes, fibrinolytic/ thrombolytic agents, and sea cucumber. Eligibility criteria, the identity of applicable studies, observe selection and manipulation of observe bias have been decided following the preceding procedure [30].

2.1. Study Eligibility Criteria

Preparatory steps were performed, including a study selection of procedures for the purification of the original fibrinolytic enzyme used in treating thrombosis worldwide. The data obtained were summarized to include the types of microorganisms that produce the enzymes and the sources. The study choices were based on the following selection criteria: [a] Subject-only study on a method for purifying fibrinolytic/thrombolytic enzymes developed as an antithrombotic agent. [b] Published in English and Indonesian. [c] A study that evaluates knowledge and practice of cardiovascular thrombotic treatment

worldwide due to actions to determine the range of bacteria as antithrombotic agents. The year of publication of this study was limited to the last five years of the search process. A summary of the use of chromatographic methods in the purification of fibrinolytic enzymes was performed to demonstrate the potential purity of the enzymes obtained as antithrombotic agents.

2.2. Identification of relevant studies

This literature had been done in 2 ways, particularly through the guide and non-guided searches in line with the preceding protocol [31]. The classes used withinside the look for applicable research had been done for two classes: 1. Bacterial protease enzymes. 2. Purification of fibrinolytic enzymes. In the primary category, digital literature searches and guide search thru the NCBI PubMed and Science Direct Database, in addition, to guide searches from the date of the book had been finished the use of the period MeSH—fibrinolytic enzyme purification; reviews; fibrinolytic enzymes; bacterial proteases; fibrinolytic proteases; and antithrombotic agents. All articles had been extracted through digital and guide seek from PubMed, Science Direct, and Google Scholar. Studies on this literature had been done for five years (2016-2020), research of fibrinolytic enzymes remoted in bacteria, research of purification of antithrombotic enzymes which have been done, and research of reporting of fibrinolytic enzymes as antithrombotic agents. In the second category, automated literature seek turned into done thru PubMed [National Biotechnology Information Center (accessed 2021) and Science Direct (Scopus 2021) in addition to a Google Scholar. With the assistance of the key phrases used withinside the seek method, which includes purifying fibrinolytic enzymes, fibrinolytic proteases, thrombolytic agents, fibrinolytic agents, and fibrinolytic enzyme-generating bacteria. Various key-word mixtures use `and', 'or'. The research excluded from this overview is research performed previous to 2016.

2.3. Study Selection

The authors (BPA, SNE, and DS) have uniquely identified literature that meets the selection criteria for this literature review. First, we evaluated the titles and summaries of the datasets generated by the search to determine which studies were ineligible and should be excluded according to the exclusion criteria. The full text articles of the rest of the study were considered to meet the selection criteria.

2.4. Study Bias Control

The assessment of bias or quality risk in this literature study included: [i] completeness of reporting information on the purification of antithrombotic protease enzymes, [ii] selective reporting of results, [iii] Choice of outcome measurement conflicts in antithrombotic proficiency testing, [iv] study design, and [v] study conduct using bacterial antithrombotic proteases used during the treatment of thrombosis. The aappropriate overall risk of bias was considered low if the overall criteria were met [30].

3. RESULTS AND DISCUSSION

This study was intended to evaluate prospective purification methods for thrombolytic proteases produced by bacterial strain HSFI-10. The next objective was to obtain recommendations on prospective assays to confirm the potential of protease the bacterial strain to be used as an antithrombotic agent based on literature studies. This literature observes the possibilities for the purification of protease from Bacillus sp. HSFI-10 to be advanced as an antithrombotic agent. It turned into anticipated that facts suggested that pure enzymes may have much higher thrombolytic activities. Such purification attempts turned into to assist the locating of secure and cheaper antithrombotic proteases with particular excessive interest for thrombosis remedy in Indonesia and the world.

Of 57 articles, 29 articles were found as the most relevant references for this literature review based on exclusion criteria set. Data from these 27 references were used to create tables (Table 1 and 2) to see the current trend of studies worldwide reporting antithrombotic sources in the last 5 years.

3.1. Source of microbial and non-microbial antithrombotic enzymes

Table 1 summarizes the non-microbial sources of purified fibrinolytic/ antithrombotic enzymes and the enzyme molecule size (kDa) available in recent years (2016-2020). Worms and plants dominate sources of antithrombotic enzymes reported in the last five years. As shown in Table 1, the molecular weight of the pure antithrombotic enzymes presented varied and was not influenced by the origin of the enzyme source. More specifically, the results showed that the molecular weight of the pure antithrombotic enzymes is reported to be in the range of 16-75 kDa. The studies are mostly reported from Asian countries (India, China, Korea, Thailand, and Indonesia). The molecular sizes of antithrombotic enzymes produced by non-microbial sources are ranged from 20 - 75 kDa. In this group, the largest size of the enzyme is produced by *E. resinifera* (a species of plant).

Table 2 summarizes the microbial reasserts of purified fibrinolytic/ antithrombotic, and the molecular enzyme size (kDa) suggested in the last five years (2016-2020). As displayed in Table 2, a review of

antithrombotic enzymes suggested within the ultimate five years from the microbial organization is ruled via bacteria. In addition, the molecular weight of the purified antithrombotic enzymes produced via way of microorganisms has been additionally varied, starting from 16 – 70 kDa [32-33]. The data in Table 2 may indicate the potential of purified bacterial proteases, especially HSFI10, isolated from sea cucumber animals to be developed as antithrombotic candidates. There have been only a few reports of purified bacterial enzymes from marine animals [9, 34-35]. Therefore, purification studies of the antithrombotic protease secreted by the isolate HSFI-10 provide insights into potentially new antithrombotic enzymes with unique molecular weights and improved activity and specificity. This review also summarizes the sources of bacterial thrombolytic enzymes that have been continuously studied in recent years (2016-2020).

Data in Tables 1 and 2 show that sources of bacterial antithrombotic enzymes have been found and identified primarily in fermented foods and soil. We limited only seafood products such as shrimp and marine animals as samples to isolate bacteria that produce antithrombotic enzymes. In Indonesia, the source of antithrombotic enzymes is often identified as coming from fermented foods.

Table 1. The non-microbial sources of purified antithrombotic enzymes reported in the last 5 years

No.	Source	Producing Species	Molecular weight (kDa)	Country	Reference
1.	Worm	*Pheretima* sp.	31	India	[34]
2.	Worm	*Lumbrineris nipponica*	28	Korea	[35]
3.	Worm	*Diopatra sugokai*	38	Korea	[36]
4.	Sea worm	*Sipunculus nudus*	28	China	[9]
5.	Plant	*Leucas indica*	35	India	[37]
6.	Tempe	N.A	75	Indonesia	[38]
7.	Plant	*Clerodendrum colebrookianum*	30	India	[39]
8	Plant	*Euphorbia resinifera*	60	Thailand	[40]

Note: N.A: not available

Table 2. The microbial sources of antithrombotic enzymes reported in the last 5 years

No.	Source	Producing Species	Molecular weight (kDa)	Country	Reference
1.	Soil	*Serratia* sp.	52	German	[12]
2.	Fungi	*Mucor subtilissimus*	N.A	Brazil	[23]
3.	Fungi	*Cordyceps militeris*	28	China	[33]
4.	Fungi	*Pleurotus ferulae*	20	Korea	[24]
5.	Yeast	*Pichia pastoris*	43	India	[1]
6.	Fungi	*Neurospora sitophila*	49	China	[10]
7.	Soil	*Mucor subtilissimus*	70	Brazil	[41]
8.	Seawater	*Chlorella vulgaris*	45	Brazil	[42]
9.	Sponges	*Streptomyces radiopugnans*	38	India	[28]
10.	Soil	*Bacillus cereus*	40	India	[43]
11.	Seawater	*Serratia marcescens*	43	India	[43]
12.	Fungi	*Streptomyces flaveolus* *Streptomyces galtieri*	16 41	Egypt Egypt	[45] [45]
13.	Oncom	*Stenotrophomonas* sp.	31	Indonesia	[46]
14.	Oncom	*Stenotrophomonas* sp.	NA	Indonesia	[46]
15.	Unspecified	*Bacillus cereus*	25	India	[47]
16.	Unspecified	*Bacillus tequilensis*	27	China	[48]
17	Doenjang	*Bacillus amyloliquefaciens*	27	Korea	[11]
18.	Shrimp Jeotgal	*Bacillus subtilis*	27	Korea	[50]
19.	Sea Squirt	*Bacillus velezensis*	27	Korea	[51]
20.	Douchi	*Bacillus subtilis*	29	China	[13]
21.	Douchi	*Bacillus amyloliquefaciens*	29	China	[52]

Note: N.A: not available

3.2. Properties of Bacterial Thrombolytic Enzyme

Table 3 summarizes the studies reported on different methods used for the purification of antithrombotic protease enzymes in different countries. The data in Table 3 summarized the study of purification processes used to purify thrombolytic protease enzymes in various countries. Table 3 showed that the specific activity of purified enzymes, in general, becomes markedly higher after chromatographic purification.

Table 3. Purification methods used in studies reporting the development of bacterial protease as antithrombotic agents**.**

Agents	Purification method	Specific activity (U/mg)	Recovery yield (%)	Reference
Fungi	Ammonium sulfate precipitation (0-20%), hydrophobic interaction chromatography (Phenyl Sepharose HP column), ion-exchange column chromatography (CM Sepharose FF), and gel filtration (Superdex 75 column).	1.467,4	5.80	[27]
Worm	Ammonium sulfate, ion exchange (DEAE Cellulose), and size exclusion chromatography (Sephadex G 50).	18,92	N.A	[28]
Worm	Ammonium sulfate precipitation, dialysis, affinity chromatography fast flow column (HiTrapTm), and ion-exchange [DEAE-Sepharose).	95,2	12.70	[31
Fungi	Anion and ion-exchange column (DEAE-Sepharose), gel filtration (Sephadex G50), and fast protein liquid columns (FPLC, HiPrep).	1.253,33	8.94	[22]
Worm	Ammonium sulfate precipitation (20-55%), affinity column (HiTrap Benzamidine AC), and ion-exchange column (Column HiTrap DEAE-Sepharose).	102	N.A	[34]
Soil	Ammonium sulfate precipitation, dialysis, and column chromatography (DEAE Sephadex).	493	14.90	[11]
Food	Ammonium sulfate precipitation (65%), chromatography column HiTrap Desalting, and chromatography HiTrap DEAE Sepharose.	N.A	N.A	[46]
Fungi	Ammonium sulfate precipitation (40%), hydrophobic interaction (Octyl Sepharose FF), ion-exchange column (Sp SepharoseHP), and gel filtration (Superdex 75).	415,6	2.20	[9]
Seawater	Ammonium sulfate precipitation, dialysis, and FPLC size exclusion chromatography.	1.033	19.38	[35]
Worm	Ammonium sulfate precipitation, hydrophobic interaction, Anion-exchange column, and gel filtration chromatography.	2.466,62	14.47	[8]
Bacterium	Ammonium sulfate precipitation, dialysis, gel filtration (FPLC), and Anion-exchange column chromatography (Macro Prep diethyl aminoethyl- FPLC).	14,532	22.20	[48]
Bacerium	Ammonium sulfate precipitation (75%), dialysis, ion exchange (Q-HP column), and size exclusion chromatography (Sephacryl-100).	329,76	42.70	[49]
Microalgae	Ion-exchange column chromatography (HiTrapTM DEAE FF)	1.834,6	4.00	[37]
Food	Ammonium sulfate precipitation (80%), ion-exchange column (DEAE-Sepharose), and gel filtration chromatography (Sephadex G-100 Column).	3.361	4.37	[38]
Sea animal	Affinity chromatography	N.A	N.A	[51]
Plant	Anion and cation-exchange column, gel filtration chromatography, and HPLC.	N.A	N.A	[36]
Fungi	Ammonium sulfate precipitation (0-20%), hydrophobic interaction chromatography (Phenyl Sepharose HP column), ion-exchange column chromatography (CM Sepharose FF), and gel filtration (Superdex 75 column).	1.467,4	5.80	[28]
Worm	Ammonium sulfate, ion exchange (DEAE Cellulose), and size exclusion chromatography (Sephadex G 50).	18,92	N.A	[29]
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Worm	Ammonium sulfate precipitation (20-55%), affinity column (HiTrap Benzamidine AC), and ion-exchange column (Column HiTrap DEAE-Sepharose).	102	N.A	[35]
Soil	Ammonium sulfate precipitation, dialysis, and column chromatography (DEAE Sephadex).	493	14.90	[11]
Food	Ammonium sulfate precipitation (65%), chromatography column HiTrap Desalting, and chromatography HiTrap DEAE Sepharose.	N.A	N.A	[47]
Fungi	Ammonium sulfate precipitation (40%), hydrophobic interaction (Octyl Sepharose FF), ion-exchange column (Sp SepharoseHP), and gel filtration (Superdex 75).	415,6	2.20	[9]
Seawater	Ammonium sulfate precipitation, dialysis, and FPLC size exclusion chromatography.	1.033	19.38	[36]
Worm	Ammonium sulfate precipitation, hydrophobic interaction, Anion-exchange column, and gel filtration chromatography.	2.466,62	14.47	[8]
Bacterium	Ammonium sulfate precipitation, dialysis, gel filtration (FPLC), and Anion-exchange column chromatography (Macro Prep diethyl aminoethyl- FPLC).	14,532	22.20	[49]
Bacterium	Ammonium sulfate precipitation (75%), dialysis, ion exchange (Q-HP column), and size exclusion chromatography (Sephacryl-100).	329,76	42.70	[50]
Microalgae	Ion-exchange column chromatography (HiTrapTM DEAE FF)	1.834,6	4.00	[38]
Food	Ammonium sulfate precipitation (80%), ion-exchange column (DEAE-Sepharose), and gel filtration chromatography (Sephadex G-100 Column).	3.361	4.37	[39]
Sea animal	Affinity chromatography	N.A	N.A	[52]
Plant	Anion and cation-exchange column, gel filtration chromatography, and HPLC.	N.A	N.A	[37]
Food	Ammonium sulfate precipitation (40-70%), column chromatography UNOsphere Q, gel filtration (Sephadex G-75 column), and HPLC.	11.274,4	12.73	[12]
Bacterium	Ion-exchange with Qsepharose (Fast flow), and Gel Filtration Chromatography (Superdex-75).	4671	18.20	[38]
Food	Ammonium sulfate precipitation (65%), cation-exchange column (DEAE-Sepharose fast flow column), and gel filtration column fast protein liquid chromatography (FPLC)-Superdex 75.	1.240,30	3.19	[51]
Sponges	Ammonium sulfate precipitation (0-85%), dialysis, ion-exchange column (Poros HQ), and gel filtration chromatography (Sepharose CL-6B).	3.891	35.00	[27]
Fungi	Ammonium sulfate precipitation (40-60%), and ion-exchange column chromatography (DEAE Sephadex A50).	N.A	N.A	[22]
Micro-fungi	Ammonium sulfate precipitation (10-80%), dialysis, Sepharose FF Column, and gel filtration column (Sephadex G50).	1.434,4 2.343,3	27.70 28.40	[39]
Plant	Kation-exchange column (CM Celulose), anion-exchange column (HiPrep), FPLC, and UHPLC.	N.A	N.A	[40]
Food	Gel filtration column (Sephadex G15), and Anion-exchange column chromatography (DEAE Sepharose).	1.180	11.20	[20]
Plant	Cation-exchange column (HiTrap Sepharose Fast Flow), and -hydrophobic interaction chromatography (Phenyl Sepharose HP column).	5,6	38.86	[41]
Soil	Ammonium sulfate precipitation (60%), dialysis, and ion-exchange column chromatography (diethyl amino ethyl cellulose)	876.36	21.90	[25]
Soul	Ammonium sulfate precipitation, ion-exchange column (DEAE Sephadex A50), and gel filtration chromatography (Superdex 75).	25,93	4.84	[42]

Agents	Purification method	Specific activity (U/mg)	Recovery yield (%)	Reference
Food	Ethanol precipitation, gel filtration P-100, and ion-exchange column chromatography (DEAE cellulose).	7.245	22.10 8.50	[43]
Soil	Ethanol precipitation, and gel filtration chromatography (Sephadex-G75).	28,627	33.11	[44]

Results found that the high purity of the enzyme gives it a relatively high specific activity (13). Several studies have reported that purified enzymes produced by chromatography can dissolve blood clots up to 80-100% [26, 28, 33, 43]. Other studies have reported that the purity level of bacterial-derived thrombolytic protease enzymes is strongly influenced by the techniques selected in enzyme purification [20-21]. Data from the past 5 years worldwide have shown that the most active thrombolytic protease reported from *Bacillus subtilis* isolated from Douchi in China was 11,274.4 U/mL obtained by purified UNOsphere Q column chromatography, Sephadex G75 filtration gel columns, and HPLC [52].

It is also worth noting that the main purification methods used in the obtained references are done step-by-step. Most purification is done with salt deposition as the first step, followed by dialysis and then further using chromatography. The evaluation of generated or purified enzymes is primarily based on measurements related to specific enzyme activity [13, 16, 23-24]. The results also showed that the specific activity of the enzyme produced from the enzyme purification using the chromatography method was very high. The high purity of the enzyme provides a fairly high specific activity and can dissolve blood clots up to 80-100% [25, 27-28]. Previous studies have reported that the purity of bacterial antithrombotic protease enzymes is highly affected by the purification techniques used to analyze the enzyme [19-20].

Based on the information extracted from Table 3, the purification of thrombolytic enzymes from marine sea cucumber (*H. scabra*) bacteria is an important step to detect its specific activity and increase its activity. It should improve the enzyme properties when used as an antithrombotic agent in treating thrombosis.

3.3. Prospect of Study on Purification of Thrombolytic Protease and Assays on Its Purified Product

The prospect of a study involving the purification of thrombolytic protease purified from bacteria HSFI-10 isolated from the fermented intestine of *H. scabra* as an antithrombotic agent is shown in Figure 1. Sand sea cucumber (*H. scabra*) is a biological protein-rich marine mammal that has been previously reported as a source of thrombolytic protease-producing bacteria [26]. Crude proteases of several isolates, including *Bacillus* sp. HSFI-10 showed a competitive thrombolytic activity against control Nattokinase [27, 53]. This is one of the important factors contributing to the prospect of an enzyme purified from *H. scabra* to be used as an antithrombotic agent with potentially better activities (Figure1)

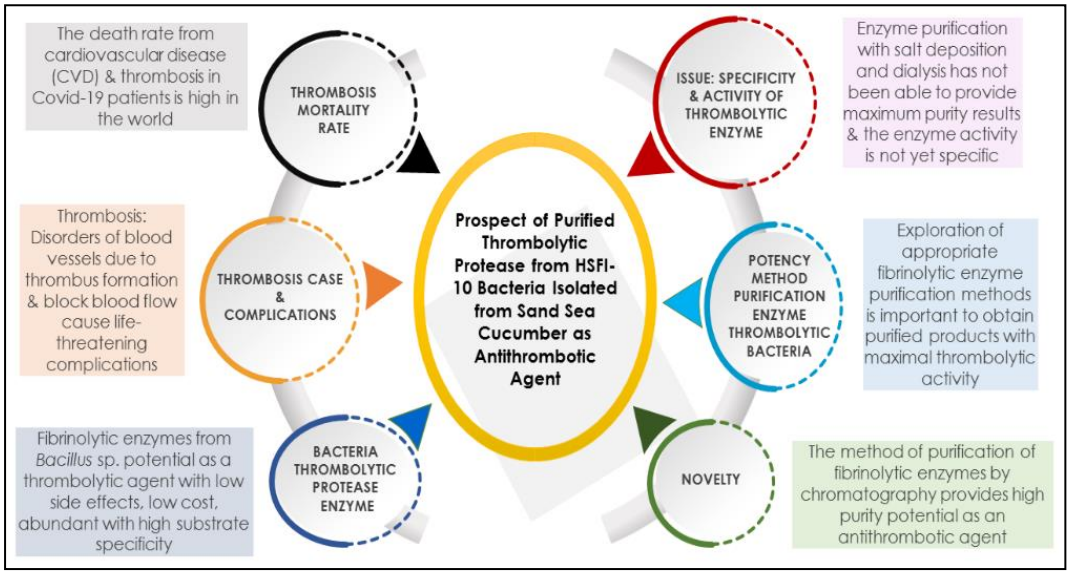

Figure 1. Factors contributing to the prospect of purified thrombolytic protease from *Bacillus* sp. HSFI-10 bacterium isolated from sand sea cucumber as an antithrombotic agent

This literature review highlight the purification of thrombolytic proteases from *Bacillus* sp. HSFI10 is an important work in improving enzyme activity and specificity. The information will advance knowledge of its potential as an antithrombotic agent in the treatment of cardiovascular diseases. The results of this literature review indicate that a purified product of the thrombolytic protease produced by *Bacillus* sp. HSFI-10 has not been reported. This means that the production of purified protease from *strain* HSFI10 offers novelty. Several purification strategies are recommended to obtain high-purity thrombolytic proteases from HSFI-10, including the use of column chromatography, gel filtration, and HPLC. Based on the results of this literature study, it can be concluded that:

1. Various proteases have been developed as antithrombotic agents from microbial and non-microbial sources worldwide, with molecular weights ranging between 16-75 kDa. This molecular weight measurement is often performed to ensure that specific activity increases with each step of enzyme purification.
2. The purified product of thrombolytic protease from any sea cucumber has not been reported, so the production of purified protease from *Bacillus* sp. HSFI10 from sand sea cucumber *Holothuria scabra* as an antithrombotic candidate offers novelty.
3. As recommended, the purified thrombolytic protease product of high-purity HSFI10 isolates can be obtained by different purification strategies, including column chromatography, gel filtration, and HPLC.

AUTHORS' CONTRIBUTIONS

BPA and SNE designed the study as a whole, while ARS and SSD reviewed and summarized all publications obtained. ARS and SNE evaluated the creation of tables and figures, and also analysed the study's bias. Main text written by BPA and SNE. The first draft was written by NI, then edited and proofread by SNE.

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