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Systematic review: How to obtain Fibrinolytic Enzymes from Fungi

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Abstract

Fibrinolytic complications such as stroke and peripheral occlusive disease due to vascular occlusion are significant causes of poor prognosis and death. Given the ability of mushroom fibrinolytic enzymes to dissolve blood clots and prevent side effects, their use as a potential thrombolytic therapy has attracted great interest. Serine protease, metalloprotease, and serine metalloprotease are protease enzymes that can affect the fibrinolytic mechanism by dissolving blood clots and increasing blood flow. This systematic review aimed to disclose all information regarding the production, purification, and characterization of fibrinolytic proteases from fungi. Searches were conducted on online media databases, including Google and journal sites (NCBI, PubMed, and others, using the keywords "(Fibrinolytic Enzymes) OR (Fibrinolytic Proteases) AND (Fungi or Mushrooms)" from 2012-2023. This systematic review observed reviews from the purification and characterization steps, indicating the need for attention to the manufacturing process and enzymatic applications. The production of these enzymes by fungi is associated with developments in pharmaceutical applications.

Keywords: Enzymes; Fibrinolytic; Fungi; Purification; Characterization

1. Introduction

Hemostasis is spontaneously stopping bleeding from blood vessels damaged or due to breaking or tearing of blood vessels. If there is damage to blood vessels, physiologic hemostasis responds to this damage, which involves several components: the vascular system, platelet system, coagulation system, and fibrinolysis system[1]. The efficiency of fibrinolysis is greatly influenced by the coagulation structure, fibrinogen isoforms, and polymorphisms, the rate of thrombin formation, the reactivity of thrombus-associated cells such as platelets, and the overall biochemical environment. Regulation of the fibrinolytic system, such as the coagulation cascade, is carried out by various cofactors, receptors, and inhibitors[2]. Formation of a fibrin clot is an essential process in the regulation of the hemostatic as well as the coagulation cascade.

Several things can affect failure in the hemostatic process caused by several disorders that can cause various other health problems such as cardiovascular disease, stroke, and others[3]. Several thrombolytic agents often used to treat thrombosis include urokinase, streptokinase, and tissue plasminogen activator. The contents of these various drugs still have some side effects, while in terms of price, they are still quite expensive and uneconomical. This has prompted several studies to obtain safer compounds with lower prices for the treatment of diseases related to the mechanism of fibrinolysis[4]. The classifications of fibrinolytic enzymes are serine protease, metalloprotease, and serine

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metalloprotease. Protease enzymes affect the fibrinolytic mechanism by dissolving blood clots and improving blood flow. In fibrin degradation, this enzyme will undergo a cleavage process with direct or indirect plasmin activation[5]. This fibrinolytic enzyme can be obtained from various sources, including bacteria, fungi, insects, and fermented foods[6,7]. Although multiple sources of fibrinolytic enzymes have been discovered, only some are used in clinical and therapeutic applications due to drawbacks such as high production costs, low enzyme stability, or therapeutic side effects. However, discovering new fibrinolytic enzymes requires complex purification steps and exceptional characterization to provide insight into the diversity in obtaining fibrinolytic enzymes. This systematic review will summarize, derive, characterize, and present the function-generated fibrinolytic enzymes from various reference sources[5].

2. Methods

The preparation of this systematic review uses literature study techniques by finding sources or literature in the form of national and international journals from 1998 to 2019. In addition, in making this review, data search was done using online media, including Google and journal sites (NCBI, PubMed, and others.)

3. Results

3.1. Characteristics of enzyme

A summary of the main characteristics of the studies is presented in Table 1. As explained in the methodology, articles were selected from 1998 to 2019, and 16 reports were obtained. All studies resulted in the acquisition of fibrinolytic enzymes by fungi and some degree of enzyme purification and characterization. On the other hand, not all articles detail the name and class of enzymes.

Table 1 Characteristics of enzyme

Species	Enzyme name	Enzyme class	Mass	References
Armillaria mellea[8]	AMMP	Metalloprotease	21 kDa	Lee SY, et al. 2005
Cordycep militaris[9]	N/A	Serine Protease	32 kDa	Xiaolan Liu, et al. 2015
Cordycep militaris korea[10]	N/A	Serine Protease	34kDa	Choi D, et al. 2011
Cordycep sinensis[11]	CSP	Serine protease	31kDa	Li HP, et al. 2007
Flammulina velutipes[12]	FVP-1	Metalloprotease	37kDa	Se-Eun PARK, et al. 2007
Fomitella fraxinea[13]	FFP1 and FFP2	- FFP-1: Serine Protease - FFP-2: Metalloprotease	32kDa and 42kDa	Lee Jong-Suk, et al. 2006
Fusarium sp[14]	Fu-P	Serine metalloprotease	28kDa	Wu B, et al. 2009
Lyophyllum shimeji[15]	N/A	Serine metalloprotease	21kDa	Sung-Min Moon, et al. 2014
Mucor subtilissimus[16]	N/A	N/A	N/A	da Silva MM, et al. 2019
Paecilomyces tenuipes[17]	PTEFP	N/A	14kDa	Hoe Chang Kim, et al. 2011
Perenniporia fraxinea[18]	N/A	Metalloprotease	42kDa	Kim JS, et al. 2008
Pleurotus eryngii[19]	N/A	Serine protease	14kDa	Cha WS, et al. 2010

Pleurotus ostreatus[20]	N/A	Metalloprotease	12kDa	Hye-Seon Choi, et al. 1998
Rhizopus chinensis[21]	N/A	Serine protease	18kDa	Xiao-Lan L, et al. 2004
Tricholoma saponaceum[22]	TSMEP-1 and TSMEP2	Metalloprotease	18.147kDA and 17,947kDA	Kim JH, et al. 2001
Xylaria curta[23]	Xylarinase	Metalloprotease	33.76kDa	Meshram V, et al. 2016

3.2. Characteristics of enzyme purification

A summary of the characteristics of enzyme purification from various literature is presented in Table 2. All studies resulted in the characterization of the purification process of fibrinolytic enzymes by fungi. However, not all articles change the purification stages. One of the articles did not go into detail about the overall purification of the enzyme, and the other did not show the pI of the enzyme.

Table 2 Characteristics of enzyme purification

Species	Pre-Purification	Purification	Purification pH	PF	Specific Activity (U/mg)	pI
Armillaria mellea	Protein precipitation: ethanol	CM-cellulose Sephadex G-75 column Superdex 75 column	6.0	627	1097.5	N/A
Cordycep militaris	20% Ammonium sulfate precipitation	Hydrophobic Interaction Chromatography (HIC) - Phenyl Sepharose FF column Cation exchange Chromatography - CM-Sepharose FF Gel filtration chromatography - Superdex 75	7.4	41.3	1682	9.3 ± 0.2
Cordycep militaris korea	Ammonium sulfate was added to the supernatant up to 80% saturation	DEAE-Sepharose FF column HiLoad 16/60 Superdex 200 column FPLC HiLoad 16/60 Superdex 75 column	7.0	86.1	499.6	8.2
Cordycep sinensis	(NH4)2SO4 precipitation (0–20%)	Sephadex G-25 Phenyl Sepharose HP column CM Sepharose FF Superdex 75 column	7.0	20	5910	N/A
Flammulina velutipes	Protein precipitation: ethanol	CM-celullose DEAE Sephadex A-50 Sephadex G-75 Superdex 75	6.0	18.52	144.12	N/A
Fomitella fraxinea	80% Ammonium sulfate precipitation	DEAE-sepharose FF Superdex 200	7.0	34.6	62.30	N/A

Fusarium sp	ammonium sulfate, First at 40%, and then at 60%	Sephadex G-25 MonoQ Superdex 75	7.4	158.5	76.111	8.1
Lyophyllum shimeji	Protein precipitation: ethanol	anion exchange Mono Q 5/5 Superdex 200 100/300	7.0 - 8.0	80.9	469.3	N/A
Murcor subtilissimus	N/A	DEAE-Sephadex A50	N/A	N/A	N/A	N/A
Paecilomyces tenuipes	Protein precipitation: ethanol	CM-cellulose DEAE-Sepharose CL-6B Sephadex G-75 POROS 20 HQ ion exchange	4.0–7.0	550.70	1431.81	N/A
Perenniporia fraxinea	Protein precipitation: ethanol	CM-cellulose DEAE-Sepharose CL-6B Sephadex G-75 Superdex 75	2.0 – 10.0	1915	900	N/A
Pleurotus eryngii	80% Ammonium sulfate precipitation	DEAE-Sepharose Sephacryl S-300 HR	2.0 – 10.0	29.3	52.8	N/A
Pleurotus ostreatus	80% Ammonium sulfate precipitation	Sephadex G-50 phenyl Sepharose Sephadex G-150 anion exchange Mono Q 5/5	7.5 to 8.0	52	2062	N/A
Rhizopus chinensis	70% Ammonium sulfate precipitation	Octyl-Sepharose FF DEAE-Sepharose FF Sephacryl S-100	7.8 – 10.4	893.1	2,143.4	8.5±0.1
Tricholoma saponaceum	N/A	DEAE-cellulose anion exchange Mono S	6.0 – 10.0	45.9	43.4	N/A
Xylaria curta	60% Ammonium sulfate precipitation	Q-sepharose anion exchange Sephacryl S-300	4.0 – 9.0	9.19	36.67	N/A

Sixteen different genera were observed in the fungi used in the selected articles (Armillaria mellea, Cordycep militaris, Cordycep militaris korea, Cordycep sinensis, Flammulina velutipes, Fomitella fraxinea, Fusarium sp, Lyophyllum shimeji, Murcor subtilissimus, Paecilomyces tenuipes, Perenniporia fraxinea, Pleurotus eryngii, Pleurotus ostreatus, Rhizopus chinensis, Tricholoma saponaceum, Xylaria curta).

3.3. pH, Temperature, Activator and Inhibitor of Enzyme

A summary of the pH, Temperature, Activator and Inhibitor of Enzyme from various literature is presented in Table 3. Six genera showed optimal activity. Enzymes are produced at different pH ranges. Armillaria mellea (2.0 – 10.0), Cordycep militaris (5.0 - 10.0), Cordycep militaris Korea (3.0 – 11.0), Cordycep sinensis (2.0 – 12.0), Flammulina velutipes (2.0 – 10.0), Fomitella fraxinea (FFP-1: 7.0 – 9.0, FFP-2: 4.0 – 11.0). At the same time, Murcor subtilissimus data is not displayed.

Table 3 pH, Temperature, Activator and Inhibitor of Enzyme

Species	pH	Temp (◦ C)	Activator	Inhibitor
Armillaria mellea	2.0 – 10.0	33	Ca^{2+}, Mg^{2+}	Cu^{2+}, Co^{2+}, EDTA, substrate S-2586 for chymotrypsin
Cordycep militaris	5.0 - 10.0	37	Ca^{2+}, Cu^{2+}	Fe^{3+}, PMSF, Aprotinin, Pepstatin, SBTI
Cordycep militaris korea	3.0 - 11.0	30 and 40	Cu^{2+}, and Ba^{2+}	Aprotinin, EDTA, and EGTA
Cordycep sinensis	2.0 – 12.0	40	$MgCl_2$, $MnCl_2$, $CdCl_2$, $BaCl_2$, $CoCl_2$, $CaCl_2$, $NiCl_2$, $HgCl_2$ and $CuSO_4$	Aprotinin, Benzamidine hydrochloride, E-64, Na2-EDTA, EGTA and PMSF
Flammulina velutipes	2.0 – 10.0	20 - 30	Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Mg^{2+}	PMSF, TLCK, TPCK, aprotinin, EDTA, EGTA, peptasin A
Fomitella fraxinea	- FFP-1: 7.0 – 9.0 - FFP-2: 4.0 – 11.0	- FFP1: below 30 - FFP-2: below 40	- FFP1: N/A - FFP-2: Co2+, Zn2+	- FFP1: PMSF, Aprotonin - FFP-2: Cu^{2+}, Ni^{2+}, Hg^{2+}, EDTA, 1, 10-phenanthroline
Fusarium sp	8.5	45	$MgCl_2$, $ZnCl_2$, $CoCl_2$, $CaCl_2$, and $CuSO_4$	EDTA and PMSF
Lyophyllum shimeji	8.0	37	$CuCl_2$, $NaCl_2$, $MnCl_2$, $CoCl_2$, $ZnCl_2$, $MgCl_2$, and $CaCl_2$	Cu^{2+} and Co^{2+}, PMSF and TPCK
Murcor subtilissimus	N/A	N/A	N/A	N/A
Paecilomyces tenuipes	5.0	35	$CaCl_2$, $CoCl_2$, $ZnCl_2$, $MgCl_2$, and $MnCl_2$	EDTA, EGTA, PMSF, TLCK, TPCK, and Aprotinin
Perenniporia fraxinea	6.0	35–40	Mn^{2+}, Mg^{2+} and Ca^{2+}	PMSF, APMSF, TLCK, TPCK, EDTA, EGTA, Aprotinin, and Pepstatin A
Pleurotus eryngii	5.0	40	N/A	PMSF, TLCK, TPCK, EDTA, DFP, SSI, Pepstatin A, and Phenantrolin
Pleurotus ostreatus	8.0	4	Zn^{2+} or Co^{2+}	PMSF, TPCK, Leupeptin, Pepstatin, Iodoacetic acid, p-chloromercuribenzoate and E-64
Rhizopus chinensis	10.5	45	N/A	N/A
Tricholoma saponaceum	7.5	55	Mg^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}	Cu^{2+}, Hg^{2+}
Xylaria curta	8.0	35	Cu^{2+}, Mn^{2+}	Fe^{2+}, Zn^{2+}

3.4. Storage and Stability of Enzyme

A summary of the Storage and Stability of Enzyme from various literature is presented in Table 4. Each enzyme has different characteristics in its storage. This is due to differences in their respective activities. Enzymes will be stable if stored according to their characteristics.

Table 4 Storage and Stability of Enzyme

Species	Storage	Stability
Armillaria mellea	N/A	The enzyme was very stable in a pH range of 5.0–8.0 at 37 °C for one hour, but above pH 8.0, enzyme stability degenerated abruptly.
Cordycep militaris	Purified fibrinolytic enzyme (0.151 mg/mL) was stored at −20 °C and −70 °C	Highly stable even after repeated cycles of freeze−thawing. One week at four °C in the presence of 15% glycerol Liquid form: retained 56% and 76% of its activity when stored at −20 °C and −80 °C for 14 months. Lyophilized form: highly stable at four °C, −20 °C, and −80 °C for the same period and retained 62%, 83%, and 101% of its activity, respectively.
Cordycep militaris korea	Optimum temperature for enzyme activity was determined by measurement of enzyme activity in 50 mM Tris–HCl buffer (pH 7.0) over a temperature range of 20–70◦ C	To observe the thermal stability of the enzymes, the enzyme activity was measured after incubation of the enzyme at various temperatures (20–70 C) in 50 mM Tris–HCl buffer (pH 7.0) for 1 h
Cordycep sinensis	Thus, CSP is a robust enzyme, which is highly stable at temperatures below 50°C.	Temperature stability analysis indicated that CSP maintained 100% of its proteolytic activity when exposed to temperatures of 10, 20, 30 and 40 °C for 2 h
Flammulina velutipes	N/A	pH range of 5.0 to 8.0 at 37C for 1 h, but above pH 8.0 protease stability degenerated abruptly
Fomitella fraxinea	N/A	FFP1: The enzyme was quite stable in the pH range of 7.0-9.0 at 25C for 1h. FFP2: was active in the pH range of 5.0-9.0 and exhibited maximum activity at pH 5.0. The enzyme was very stable over a broad pH range of 4.0-11.0
Fusarium sp	N/A	Fu-P was stable over a pH range of 6–9 for 4 h at 4°C (date not shown). The optimum temperature of the enzyme was 45°C and the enzyme activity was stable below 37°C
Lyophyllum shimeji	Stored in a 70◦ C deep freezer	To observe the thermal stability of the enzymes, the enzyme activity was measured after incubation of the enzyme at various temperatures (40◦ C, 50◦ C, and 60◦ C) in 20 mM Tris HCl buffer (pH 8.0) for 1 h
Murcor subtilissimus	N/A	N/A
Paecilomyces tenuipes	N/A	The enzyme was relatively stable below 40◦ C. The enzyme was relatively stable between 20 and 35◦ C, and the enzyme activity decreased slowly with time
Perenniporia fraxinea	N/A	The optimum temperature of the enzyme activity was determined by the measurement of residual activity after incubation 0.1 ml fibrinolytic enzyme at different temperatures (20–80 C) for 1 h
Pleurotus eryngii	N/A	The enzyme was very stable at pH 4.0–6.0 with an optimum pH 5.0 at 40◦ C
Pleurotus ostreatus	N/A	Thermal stability of purified protease was determined after incubation of the preparation at different temperatures for 15 min followed by the assay. Fibrinolytic protease was stable up to 50 C and the remaining activity was 80% at 60◦ C

Rhizopus chinensis	The enzyme was very stable in the range of pH 6.8–8.8 at 37°C for 24 h, but became unstable out of this range	The pH stability of the enzyme was investigated in the range of pH 2.8–11.8 by measuring the residual activity after incubation at each pH for 24 h
Tricholoma saponaceum	The enzyme was stable up to 30°C and the maximum fibrinolytic activity was at 55°C	The thermal stability of the purified enzyme has been measured at pH 7 using the fibrin plate method with the temperature range from 20°C to 80°C. Above 30°C, it loses stability slowly and at 80°C, it does not show any fibrinolytic activity.
Xylaria curta	The optimum temperature for xylarinase activity was 35°C. The enzyme was relatively stable till 40°C. However, above 40°C the activity of the enzyme was sharply reduced	Stable in the pH range of 7.0–8.0

4. Discussion

In the pharmaceutical industry, the role of fibrinolytic enzymes is very important, so the purity in purpose must be very good. The process usually uses multiple chromatography sequentially, such as gel filtration, ion exchange, and affinity chromatography. Pre-purification techniques are also carried out, such as salt deposition, centrifugation and filtration[24], as seen in Table 3, out of 16 articles presenting fibrinolytic. The purified enzyme activity uses a combination step of various types of column chromatography according to the desired molecule.

Several studies have used ammonium sulphate in protein pre-purification. Precipitation using ammonium sulphate salts is generally better than other organic solvents, causing hydrophobic interactions between protein molecules where protein solubility becomes low and it will agglomerate, making it easier to separate[25] Furthermore, other studies using ethanol. Precipitation using ethanol is one of the methods used to remove SDS and other impurities in protein samples to avoid losing protein during purification[26].

Enzyme activity is strongly influenced by temperature and pH. In addition, an optimal temperature is also needed so that activity can be determined. The optimal pH for enzyme activity can be determined by measuring the remaining enzyme activity from the process carried out with various stages. The optimal enzyme pH, i.e. the range of pH at which the enzymes show the greatest fibrinolytic activity, varies from pH 2 and 11, indicating a variety of enzymes that can defend themselves in acidic or alkaline conditions. However, most of the enzymes studied showed optimal pH in the range of 5.0 (12.5%), 6.0 (6.25%), 7.5 (6.25%), 8.0 (18.75%) and 10.5 (6.25%).

The optimum pH and temperature information is crucial for developing basic research and applications. It is the basis for developing valid alternatives for treating thrombosis, so it is very important for the work where the production and characterization of fibrinolytic enzymes, parameters with optimal pH, temperature, and stability, are determined. Inhibitors and activators can modulate the rate of enzymatic reactions and play an essential role in regulating enzyme activity. Inhibitors can act on enzymes by modifying the structural characteristics that determine the mechanism of action and consequently inhibiting enzymatic activity. Based on the research described, several compounds were able to inhibit the movement of the enzymes studied, e.g. serine protease inhibitors (Fe3+, PMSF, Aprotinin, Pepstatin, SBTI)[27], Metalloprotease (Cu2+, Co2+, EDTA, substrate S-2586 for chymotrypsin)[8].

5. Conclusion

Almost all articles present complete purification and characterization, indicating pH 2-10 and 20-45°C as conditions for enzyme activity. However, various sources have a fairly wide temperature range. So that the optimal temperature must

adjust to the characteristics of each fungus regarding purification and characterization. Even though more research has been conducted, there must be an important part in the development and application of this enzyme; it is necessary to pay attention to its further development and complex characterization, exploring the physicochemical properties of enzymes.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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