Purification and Biochemical Characterization of a Novel Fibrinolytic Enzyme from edible mushroom *Lentinula edodes*

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KEYWORDS

Lentinula edodes; fibrinolytic enzyme; production; fermentation

ABSTRACT:

The crude fibrinolytic enzyme was purified to full homogeneity using DEAE sepharose chromatography and gel filtration on Sephadex-G200. An over all of 69.5% fold purification with 3.5 recovery was obtained. The apparent molecular mass of the purified enzyme was estimated to be 44 kDa. The optimum pH and temperature of the purified enzyme (LEF) were 6 and 35˚C, respectively. LEF was completely inhibited by Hg^{2+} and partially inhibited by Cu^{2+}, Ni^{2+} and Al^{3+}. Its activity strongly enhanced by Zn^{2+}, Mg^{2+}, Ca^{2+}, K^{+} and Fe^{2+} in descending order. EDTA and EGTA inhibited the enzyme activity suggesting that it is a metalloprotease. LEF was also inhibited by the serine inhibitors PMSF and aprotinin. The pure enzyme showed strong specificity to fibrin as substrate *in vitro*. The amidolytic activity toward synthetic substrates showed high specificity to the synthetic peptide N-Succinyl-Ala-Ala-Pro-Phe-pNA suggesting that it is a chymotrypsin-like protease. Additionally, it was discovered that the enzyme decreased fibrinogen (FIB) and prothrombin activity (PA) levels while prolonging activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT). These investigations suggest that the enzyme has a lot of promise for use as a natural remedy to cure and prevent thrombotic illnesses.

1. Introduction

Fibrin is the primary protein component of a blood clot, which is formed from fibrinogen by thrombin [1]. Fibrin causes thrombosis, leading to myocardial infarction and other cardiovascular diseases in the blood vessels. The insoluble fibrin fiber is hydrolyzed by plasmin, which is generated from plasminogen by plasminogen activators, such as tissue plasminogen activator, vascular plasminogen activator, blood plasminogen activator, urokinase, Hageman factor, and streptokinase-plasminogen complex [2]. The hydrolysis of fibrin is known as fibrinolysis. Fibrin clot formation and fibrinolysis are normally well balanced in the biological system. However, in the unbalanced state, the clots are not lysed, and therefore thrombosis occurs [3]. The fibrinolytic agents available today for clinical use are mostly plasminogen activators such as a tissue-type plasminogen activator (tPA), a urokinase-type plasminogen activator, and the bacterial plasminogen activator streptokinase. Despite their widespread use, all these agents have undesired side effects, exhibit low specificity for fibrin, and are also relatively expensive. Therefore, the searches for other fibrinolytic enzymes from various sources are being continued. Over the last decade, potent fibrinolytic enzymes have been discovered from a variety of sources, such as earthworms [4, 5], snake venoms [6, 7], insects [8], food-

grade microorganisms [9-12], marine creatures [13], herbal medicines [3], and fermented food products like Japanese natto [14, 15, 13], Korean chungkook-jang [16], and Chinese douchi [1].

In recent years, mushrooms have become an attractive source of various physiologically active compounds [17, 18]. They are commonly used as food and food flavoring substances and also in traditional oriental medicines. Their extracts have been reported to exert hematological, antiviral, antitumorigenic, hypotensive, and hepatoprotective effects [2, 19]. They constitute an important source of thrombolytic agents. Many fibrinolytic enzymes were identified in the fruiting bodies of different medicinal mushrooms, such as *Armillaria mellea* metalloprotease [20, 21] *Grifola frondosa* aminopeptidase [22] and *Pleurotus ostreatus* metalloprotease [23, 24], Indeed, the presence of fibrinolytic enzymes in the fruiting bodies of some mushrooms has been identified, although the presence of these enzymes in their mycelia is not clear. Furthermore, protease genes from several bacteria, fungi, and viruses have been cloned and sequenced with the prime aims of overproduction of the enzyme, delineation of the role of the enzyme in pathogenicity, and alteration in enzyme properties to suit its commercial application. The ability and functionality of *L. edodes* in the formation of polysaccharides are currently the main subjects of the majority of study on the organism. A fibrinolytic enzyme from *L. edodes* had not been reported in the literature prior to our current findings. The productivity of the liquid fermentation of the fibrinolytic enzyme from *L. edodes* was much increased in this initial investigation by optimizing the growth conditions. Purifying the fibrinolytic enzyme from *L. edodes* fermentation and examining its characteristics, including its thrombolytic and anticoagulant effects, are the goals of this work.

2. Methods

a. Organism and culture conditions

The white-rot fungus *L. edodes* Que'let NRRL0633 was kindly provided by king Abdulaziz University, Saudi Arabia. *L. edodes* strain was stored on PDA slants. The mycelia were transferred from the slants to the plate medium. Aseptic inoculation of the mycelia was performed in 250 mL shake flasks, each containing 50 mL of fermentation media composed of 2.9% fructose, 3.6% soybean cake, 0.25% KH_2PO_4, and 0.2% $MgSO_4$. The fermentation process was conducted at a temperature of 24°C and a speed of 160 r/min for 6 days. After that, the resulting mixture underwent centrifugation at a rate of 10,000 r/min for 10 min at 4°C. The resulting supernatant was then considered the crude enzyme extract. Prasad et al. [25].

b. Purification of fibrinolytic enzyme from L. edodes

The culture filtrate (1.5 liters) fractionated by precipitation with $(NH_4)_2SO_4$ between up to 80% saturation at 4°C. The precipitate dissolved in least amount of 20 mM Tris-HCl buffer (pH 7.4), and desalted by dialysis over night against the same buffer [29]. The previous partially purified enzyme extract was applied to DEAE-sepharose column (2.5 × 10 cm) (Pharmacia) equilibrated with 20 mM Tris-HCl buffer (pH 7.4). Proteins were eluted with linear gradient of NaCl from 0.0-0.7 M. The flow rate was 1.0 mL min^{-1}. Fractions of 3 mL were collected; protein content and fibrinolytic enzyme activity were assayed in each fraction. Fractions containing fibrinolytic activity from DEAE-sepharose column were pooled, concentrated by lyophilization, redissolved in 10 mM sodium-phosphate buffer pH 6.8 and applied to Sephadex G-200 column (Pharmacia). Elution was carried out by the same buffer. Fractions collected and assayed for fibrinolytic activity and protein content.

c. Fibrinolytic enzyme assay (LEF)

Fibrinolytic enzyme activity in the culture filtrate was measured spectrophotometrically as described by Datta et al. [26]. Ten µg of human fibrinogen solution (prepared in 10 mM Tris-HCl buffer pH 7.4 containing 0.15 M NaCl) was added to human thrombin (0.1 NIH unit), and allowed to stand for 1 h at room temperature. The formed clots (fibrin) were mixed with 1 ml of filtrate (as a source of crude fibrinolytic enzyme) and incubated at 37°C for 1 h. The amount of solubilized peptides was measured per one ml reaction mixture using procedure of Bradford [27]. One unit of enzyme activity was expressed as the amount of enzyme releasing 1µmol of soluble peptides per min.

d. Proteolytic enzyme assay

Proteolytic activity was determined according to Shen et al. [28] by measuring the release of acid-soluble material from azocasein (Sigma) by absorbance at 366 nm. One unit of protease activity was defined as the amount required producing enough acid-soluble material from azocasein to yield an absorbance of 0.1 at 366 nm, after 1 h of incubation at 37°C.

e. Molecular Weight Determination

The molecular weight of the enzyme was determined by SDS-PAGE according to the method described by Laemmil [30] after electrophoresis, the gels were stained with silver nitrate. The molecular mass markers used were low molecular weight standards (Sigma).

f. Effects of Temperature and pH on the Activity of LEF

For 6 h, the blood fiber plate was incubated with the purified enzyme (100 U mL^{-1}) at temperatures ranging from 20°C to 80°C. Regarding thermos-stability, The usual fibrin plate method was then used to quantify the remaining fibrinolytic activity. The purified enzyme's ideal pH was found at 37°C in 20 mM of several buffers (pH 2.0–10.0). In order to assess pH stability, it was incubated in a variety of pH buffers for 2, 4, 6, and 24 h at 37°C. The remaining fibrinolytic activity was then measured.

g. Effects of Metal Ions, Protease Inhibitors, on LEF

The effect of various metal ions (K^+, Cu^{2+}, Na^+, Mg^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, and Al^{2+}) at a concentration of 5 mM on enzyme activity was investigated. The metal ions and enzymes were incubated together at 37°C for 12 h, followed by measurement of residual enzyme activity.

The effects of different protease inhibitors (ethylene diamine tetraacetic acid (EDTA), phenylmethyl sulfonyl fluoride (PMSF), aprotinine, soybean trypsin inhibitor (SBTI), N-α-tosyl-L-phenyl alanine chloromethyl ketone (TPCK), and pepstatin) on the enzyme activity were studied. These inhibitors were mixed with an equal volume of LEF and incubated at concentrations ranging from 1.0 mM to 10 mM at 4°C for 12 h.

The effects of some other reagents on the enzyme activity were also studied. It was incubated with various additives (20 mmol L^{-1} cysteine, 5 mmol L^{-1} reduced glutathione, 5 mmol/L oxidized glutathione, 0.5% β-mercaptoethanol, 1% peptone, 1% gelatin, and 1% bovine serum albumin), organic solvents (10% acetone, 10% glycerol), and denaturants (urea and SDS) at 4°C for 12 h. The remaining enzyme activity was measured accordingly.

In vitro Analysis of Anticoagulant Activity of L. edodes

The blood of six healthy volunteers was centrifuged twice at 3000 r/min for 20 min at 4°C to create platelet-poor plasma (PPP), and the plasma in the supernatant was extracted for usage. For 3 min, the reactants of the various groups (Table I) were incubated at 37°C. Five coagulation indices, including prothrombin

time (PT), fibrinogen content (FIB), activated partial thrombin time (APTT), thrombin time (TT), and prothrombin activity (PA), were measured in vitro using an automatic thrombin analyzer.

Table I. The experimental method for the analysis of anticoagulant activity.

Group	Composition
A	500 µL PPP
B	500 µL PPP, 500 µL normal saline
C	500 µL PPP, 500 µL heparin sodium (30 U mL^{-1})
D	500 µL PPP, 500 µL CFE (10 U mL^{-1})
E	500 µL PPP, 500 µL CFE (30 U mL^{-1})

A; Blank control, B; Negative control, C; Positive control, D; *L. edodes* low dose; E; *L. edodes* high dose.

Results and Discussion

3.1 Enzyme Purification and Molecular Mass Determination

The partially purified enzyme obtained by fractional precipitation with ammonium sulphate recorded 60.8% recovery with 3.5 fold purification. Three separate peaks of fibrinolytic activities were observed by using DEAE-sepharose (Figure 1). The composite sample containing the three active peaks was further applied onto Sephadex G-200 chromatography where one main peak appeared showing high fibrinolytic activity (Figure 2). An overall of 3.5 fold purification with 69.1% recovery were attained (Table 1). The apparent molecular mass from the relative mobility by the marker proteins on SDS-PAGE was estimated to be 44 kDa (Figure 3). The enzyme was signaled as (LEF). Similar or relatively similar molecular masses were observed by Paik et al. [33] who purified fibrinolytic protease from *Bacillus subtilis* KCK-7 with an apparent molecular mass of 44 kDa. Kim et al. [29] purified and characterized a fibrinolytic enzyme from mycelia of the mushroom *Perenniporia fraxinea* with apparent molecular mass of 42 kDa. Higher molecular masses than LEF were detected by Kim et al. [16] who purified a fibrinolytic enzyme from the medicinal mushroom *Cordyceps militaris* with molecular mass of 52 kDa by SDS-PAGE. However Lower molecular masse than LEF were detected in *P. ostreatus* by Shen *et al*. [28] who purified fibrinolytic enzyme from mycelia (32 kDa) and fruiting bodies (19 kDa) of *P. ostreatus* using combination of chromatographies.

3.2. Effect of pH values

The optimum pH for the LEF was observed at pH 6.0 (Figure 4). This enzyme was highly active at pH range of 5.0–8.0 at 37°C for 1 h but below or above this range, the enzyme activity declined rapidly. Similarly, the optimum pH of *P. ostreatus* purified fibrinolytic enzyme was 6.5. The enzyme was stable in pH range of 6.0-7.0 but above 7.0 the enzyme stability decreased drastically [28]. Maximum activity of purified fibrinolytic enzyme was detected at pH 6.0 in the mushroom *Cordyceps militaris* [34]. The optimum pH of LEF is also similar to those of FFP2 from *Prenniporea fraxinea* [29, 35]. PoMEP from fruiting body of *P. ostreatus* [22], AMMP from *Armillaria mellea* [35], FP1 and FP2 of *P. sajor-caju* [36]. The fibrinolytic enzymes from *B. subtilis* DC-33 showed stability over a wide range of pH 5–12 with maximum activity at pH 8.0 [5].

3.3. Effect of temperature

The optimum temperature of LEF was found to be 35˚C but when the enzyme was exposed to temperature over 37°C the fibrinolytic activity degenerated abruptly (Figure 5). As the temperature increased above 45°C the enzyme rendered inactive. The fibrinolytic enzyme in

Table 1. Purification scheme of fibrinolytic enzyme in *L. edodes*

Purification step		Total protein (µg mL^{-1})	Fibrinolytic activity (U mL^{-1})	Specific activity (U µg^{-1})	Purification fold	Recovery (%)
Crude filtrate		9.1	24.1	3.2	1	100
$(NH_4)_2SO_4$		5.1	44.3	5.9	2.3	60.8
DEAE-sepharose	**LF1**	0.20	36.7	158.4	49.5	1.2
	LF2	0.26	24.8	91.8	28.7	1.8
	LF3	0.42	16.1	38.8	12.1	4.2
Sephadex G-200		0.18	48.7	22.4	69.5	3.5

Cordyceps militaris was active between 20-40°C with an optimum activity at 37°C. The activity decreased rapidly at temperature greater than 40˚C [16]. The optimum temperature of fibrinolytic enzyme purified from the mushroom *Perenniporia fraxinea* was found to be 35°C. The enzyme become less active above 45°C and completely inhibited above 55°C [29]. Lower temperature than that of LEF was detected in other mushrooms by Cui et al. [34] who found that the optimum temperature of fibrinolytic enzyme purified from *Cordyceps militaris* mycelial filtrate was 25°C. Lee et al. [35] observed that the optimum temperature of fibrinolytic enzyme purified from the mushroom *Armillaria mellea* was 33°C.

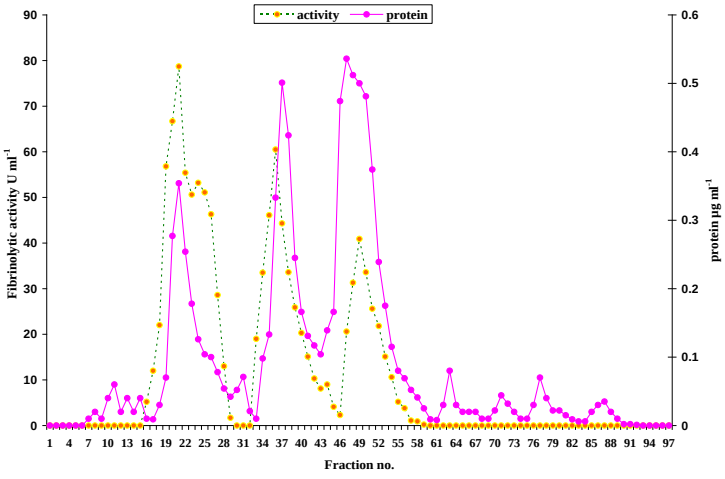


Figure 1. Typical elution profile for the behaviour of *LEF* enzyme on DEAE-sepharose.

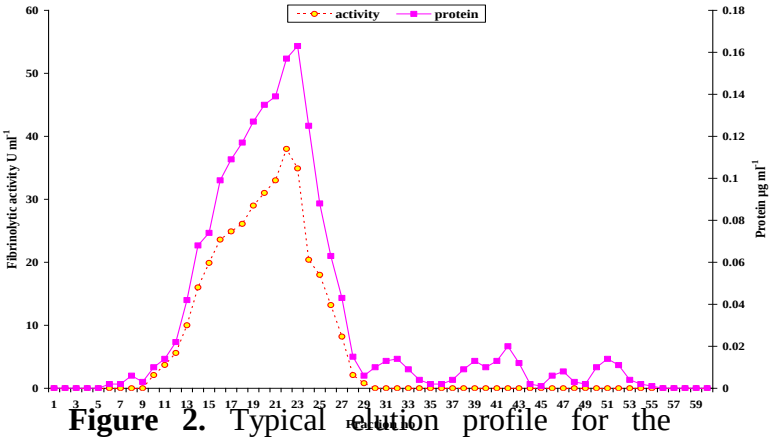
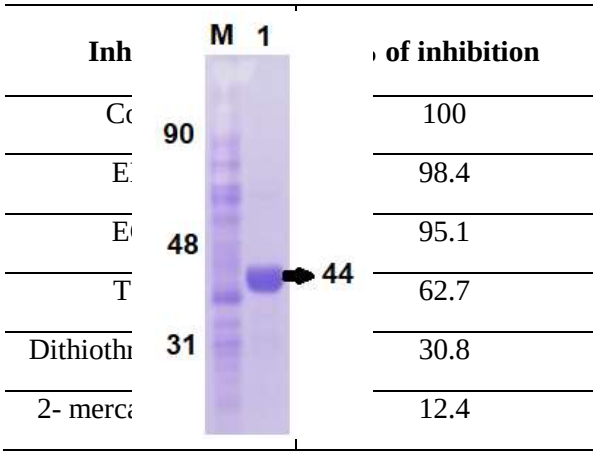


Figure 2. Typical elution profile for the behaviour of *LEF* enzyme on Sephadex G-200.

3.4. Effect of different protease inhibitors

Table 2 showed that EDTA and EGTA strongly inhibited LEF activity indicated that it's a metalloprotease. TPCK, DTT and mercaptoethanol exerted low effect on the fibrinolytic activity. LEF was inhibited by the serine protease inhibitors PMSF and aprotinin. The cystiene protease inhibitors TLCK and the aspartic protease inhibitor pepstatin A exerted low effect on the enzyme activity. Wang et al. [5] reported that the fibrinolytic activity in *B. subtilis* DC33 completely inhibited by PMSF, DTT and pepstatin A which are well known inhibitors of serine proteases. Kim et al. [29] indicated that the fibrinolytic activity of enzyme purified from the mushroom *Perenniporia fraxinea* was inhibited by EDTA and EGTA suggesting that it's a metalloprotease.



Inh	of inhibition
C	100
E	98.4
E	95.1
T	62.7
Dithiothr	30.8
2- merc	12.4

Figure 3. SDS-PAGE for purified LEF. lane (M) protein standard- KDa; bovine serum albumin, 65; ovoalbumin 45; lactate dehydrogenase, 35 and lane (1) purified LEF.

3.5. Effect of different metal ions on fibrinolytic enzyme activity

The effect of metal ions and protease inhibitors on LEF was investigated. The enzyme activity was completely inhibited by Hg^{2+} and partially inhibited by Ca^{2+}, Ni^{2+} and Al^{3+}. The fibrinolytic activity was strongly enhanced by Zn^{2+}, Mg^{2+}, Ca^{2+}, K^{+} and Fe^{2+} in decreasing order and moderetly stimulated by Co^{2+}, Mn^{2+}, Fe^{3+} and Ba^{2+} (Table 3). It could be concluded that the LEF was enhanced by Zn^{2+} up to 140% appeared strongly as serine-metalloprotease requiring Zn^{2+} for its catalytic activity. This observation was also recorded in other microbial fibrinolytic enzymes. It has been reported that some fibrinolytic enzyme from various sources are serine and or metalloprotease requiring Zn^{2+}, Ca^{2+} or Mg^{2+} [22, 35]. The fibrinolytic enzyme from *Rhizopus chinensis* 12 and *Streptomyces* sp. Y405 are both serine and metalloproteases [37] However, the fibrinolytic enzyme from *P. ostreatus* [23], *P. sajor-caju* [48] are Zn^{2+} requiring metalloprotease.

3.6. Substrate specificity, amidolytic, Km and Vmax determination

The enzyme showed highest specificity to the synthetic N-Succinyl-Ala-Ala-Pro-Phe-pNA (for subtilisin or chymotrypsin). High degree of specificity was shown also toward N-benzoyl-Phe-Val-Arg-pNA (for trypsin or thrombin). The enzyme LEF was highly specific to fibrin, fibrinogen and gelatin but not specific to azocasien, elastine or albumin (Table 4). The Km and Vmax of the enzyme for N-Succinyl-Ala-Ala-Pro-Phe-pNA were determined to be 0.46 mM and 32.1 U mL^{-1}, respectively. Similarly, when the fibrinolytic activity of *B. subtilis* DC-33 using fibrin as substrate (was taken as 100) the relative activity of the enzyme to fibrinogen, casein and serum albumin were 132, 18 and 12, respectively. It hydrolysed N-Succinyl-Ala-Ala-Pro-Phe-pNA effectively with Km of 046 mM, Kcal/Km of 1.88×10^{5} $S^{-1}M^{-1}$, respectively. The enzyme also degraded D-Phe-Pipecolyl-Arg-pNA, aspartic substrate for thrombin with Km of 43.9 mM, Kcal/Km of 4.62 S^{-1} M^{-1} [5]. The fibrinolytic enzymes from *Rhizopus chinesis* showed high specificity for N-Succinyl-Ala-Ala-Pro-Phe-pNA and the Km value was 0.17 mM [37]. The fibrinolytic enzyme from mushroom *Fomitella fraxinea* showed a broad specificity for synthetic substrates and the km and Vmax values for N-Succinyl-Ala-Ala-Pro-Phe-pNA were 0.23 mM and 39.68 U ml^{-1} [35]. The most sensitive substrates for fibrinolytic enzymes isolated from *B. lipoliquefacience* DC-4 was N-Succinyl-Ala-Ala-Pro-Phe-pNA for subtilisin or chymotrypsin [38].

Table 2. Effect of different protease inhibitors on LEF activity at 1.0 mM

Table 3. Effect of different metal ions on LEF activity from *L. edodes* at 1.0 mM

In Vitro Analysis of Anticoagulation Activity of LEF

The coagulation test and the inhibitory impact of LEF on blood clot formation in vitro are evaluated as part of the anticoagulant activity evaluation procedure. A vital clinical

diagnostic tool frequently used to detect fibrinolysis or coagulation disorders in vitro is the coagulation screen, which consists of activated partial thromboplastin time (APTT), prothrombin time (PT), prothrombin activity (PA), thrombin time (TT), and fibrinogen (FIB) assays [45]. The activity of the clotting cascade's intrinsic and extrinsic routes is measured by PT and APTT, respectively. PA is more able to capture coagulation factor activity. The time needed for fibrinogen to transform into fibrin is denoted by TT, while the amount of fibrinogen found in a sample is indicated by FIB.

An automatic coagulation analyzer was used to determine the effect of LEF on coagulation screening indices after it had been incubated with the plasma of healthy volunteers for three minutes at 37°C. Figure 6 presents the findings. The findings showed that both the high- and low-dose groups of LEF displayed significantly higher levels of APTT, PT, and TT and concurrently lower levels of FIB and PA as compared to the blank group and negative control group. Furthermore, the effect seen in the positive control group outperformed that of the low-dose LEF group, although the high-dose LEF group showed a more noticeable effect than the positive control group.

Metal ions	% of inhibition
Control	100
Mg^{2+}	206.3
Co^{2+}	77.9
Cu^{2+}	50.9
Ca^{2+}	122.7
Mn^{2+}	67.2
Hg^{2+}	0.0
Zn^{2+}	155
Fe^{2+}	99.4
Fe^{3+}	57.1
Ni^{2+}	42.9
Ba^{2+}	45.5
K^{+}	93.3
Al^{3+}	37.4

LEF impeded the cascade of coagulation in both the intrinsic and extrinsic mechanisms of coagulation, as evidenced by the rise in APTT and PT levels (Figure 6A,B). It was hypothesized that LEF might block coagulation factors XII, XI, V, and VII in both the endogenous and exogenous coagulation pathways, prevent coagulation factor X from activating to Xa, prevent prothrombin from converting to thrombin, and lower PA levels (Figure 6C). LEF may hydrolyze thrombin in the interim. Consequently, a decrease in thrombin causes fibrinogen to convert into fibrin over a longer period of time, which is why TT levels improved (Figure 6D).

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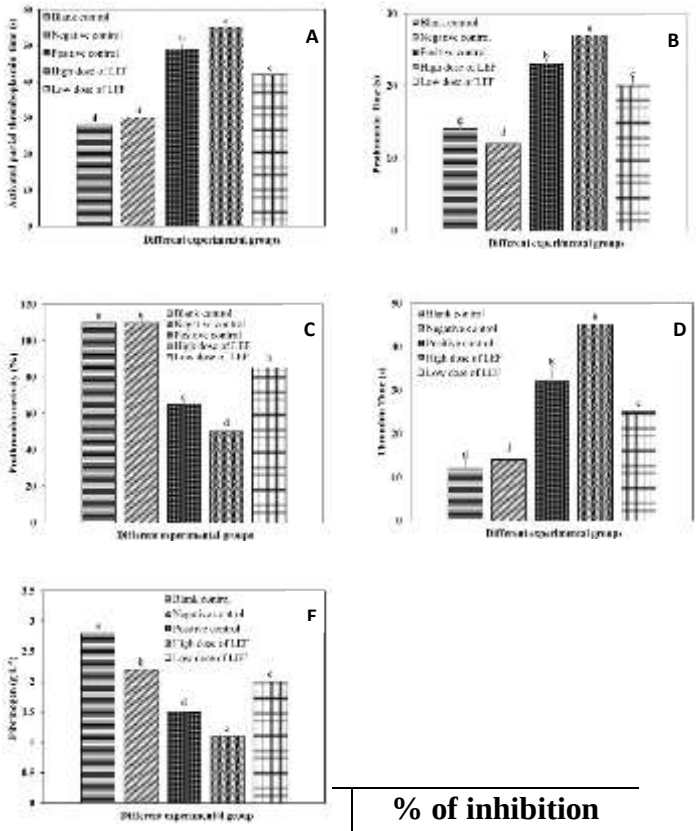
However, as Figure 6E illustrates, LEF may lower the amount of FIB. This outcome further supported the experimental findings that showed LEF has the ability to hydrolyze fibrinogen and damage its structural integrity, preventing it from converting to fibrin and lowering the amount of FIB. According to the coagulation index testing results, LEF may be used as a strong plasma anticoagulant or fibrinolytic drug to successfully avoid thrombosis. Anticoagulant administration, however, necessitates a careful balance between under- and over-treatment.

The fibrinolytic enzyme from *Pleurotus ferulae* considerably extended the levels of APTT and PT in vitro, according to Choi et al. [45]. Yang et al. [46] found that the fibrinolytic protease might raise plasma levels of PT and APTT. *Petasites japonicus*'s fibrinolytic enzyme [47] markedly raised the APTT level but had no discernible effect on PT.

Table 4. Effect of natural and synthetic substrates on LEF activity at 1.0 mM



	% of inhibition
N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide	100
N-benzyol-Phe-Val-Arg-p-nitroanilide	35.9
Gly-Phe-p-nitroanilide	53.8
Phe-Ala-p-nitroanilide	66.7
Gly-p-nitroanilide	82.7

Figure 6. Effects of LEF on coagulation index in vitro. The levels of (**A**) APTT, (**B**) PT, (**C**) PA, (**D**) TT, and (**E**) FIB in different experimental groups. Different letters indicate significant differences (p .05).

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest

The author declares no competing interests.

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