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Evaluation of partial thromboplastin time, thrombin time and prothrombin time over treated plasma using a fibrinolytic protease

Avaliação do tempo parcial de tromboplastina, tempo de trombina e tempo de protrombina sobre o plasma tratado usando uma protease fibrinolítica

Evaluación del tiempo de tromboplastina parcial, tiempo de trombina y tiempo de protrombina en plasma tratado con proteasa fibrinolítica

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Abstract

Fibrinolytic proteases are enzymes that degrade the fibrin, a main component of the blood clot. Thus, the present study aimed to understand the evaluation of aPTT, TT and PT on plasma treated with a fibrinolytic protease produced by *Mucor subtillissimus*. To assess the anticoagulant effect, the protease was used in different concentrations of 0.5-2.5mg/mL. The tests showed that the enzyme promoted a significant prolonged time over the PT clotting time as concentration increased. In the aPTT assay, fibrinolytic protease practically did not prolong the clotting time, even with increased enzyme concentration. In TT it was verified that at all times of contact with thrombin, be it 5-30 minutes, there was no interference on the action of thrombin or fibrinogen on the formation of thrombi. Since only the prothrombin time among all those studied was altered, it is suggested that the fibrinolytic protease under study affects the extrinsic pathway of coagulation. Thus, it was possible to observe that the serine protease promoted a significant prolonged time on the clotting time of the PT as the enzyme concentration increased, but the serine protease did not cause any type of change in TT and aPTT.

Keywords: Thrombosis; Fibrinolytic agents; Coagulation; Plasma.

Resumo

As proteases fibrinolíticas são enzimas que degradam a fibrina, principal componente do coágulo sanguíneo. Assim, o presente estudo teve como objetivo compreender a avaliação do TTPa, TT e TP em plasma tratado com uma protease fibrinolítica produzida por *Mucor subtillissimus*. Para avaliar o efeito anticoagulante, a protease foi utilizada em diferentes concentrações de 0,5-2,5mg/mL. Os testes mostraram que a enzima promoveu um tempo prolongado significativo ao longo do tempo de coagulação do TP com o aumento da concentração. No ensaio de TTPa, a protease fibrinolítica praticamente não prolongou o tempo de coagulação, mesmo com o aumento da concentração da enzima. No TT verificou-se que em todos os momentos de contato com a trombina, seja de 5 a 30 minutos, não houve interferência na ação da trombina ou do fibrinogênio na formação de trombos. Como apenas o tempo de protrombina entre todos os estudados foi alterado, sugere-se que a protease fibrinolítica em estudo afeta a via extrínseca da coagulação. Assim, foi possível observar que a serina protease promoveu um prolongamento significativo do tempo de coagulação do PT conforme a concentração da enzima aumentou, mas a serina protease não causou nenhum tipo de alteração no TT e no TTPa.

Palavras-chave: Trombose; Agente fibrinolítico; Coagulação; Plasma.

Resumen

Las proteasas fibrinolíticas son enzimas que degradan la fibrina, el principal componente de los coágulos sanguíneos. Así, el presente estudio tuvo como objetivo comprender la evaluación de aPTT, TT y TP en plasma tratado con una proteasa fibrinolítica producida por *Mucor subtillissimus*. Para evaluar el efecto anticoagulante, se utilizó la proteasa a diferentes concentraciones de 0,5-2,5 mg/ml. Las pruebas mostraron que la enzima promovió un tiempo prolongado significativo durante el tiempo de coagulación de PT con una concentración creciente. En el ensayo APTT, la proteasa fibrinolítica prácticamente no prolongó el tiempo de coagulación, incluso con el aumento de la concentración de enzima. En TT, se encontró que en todo momento de contacto con trombina, ya sea de 5 a 30 minutos, no hubo interferencia en la acción de trombina o fibrinógeno en la formación de trombos. Como solo se alteró el tiempo de protrombina entre todos los estudiados, se sugiere que la proteasa fibrinolítica en estudio afecta la vía extrínseca de la coagulación. Así, fue posible observar que la serina proteasa promovió una prolongación significativa del tiempo de coagulación de PT a medida que aumentaba la concentración de enzima, pero la serina proteasa no provocó ningún tipo de cambio en TT y APTT.

Palabras clave: Trombosis; Fibrinolíticos; Coagulación; Plasma.

1. Introduction

Cardiovascular diseases are the leading cause of death in the world. It is estimated that 17.7 million people died from cardiovascular disease in 2015, representing 31% of all deaths globally. Of these deaths, it is estimated that 7.4 million occur due to cardiovascular diseases and 6.7 million due to strokes. More than three quarters of deaths from cardiovascular disease (CVDs) occur in low- and middle-income countries (Hu et al., 2019). Among the various types of CVDs, a thrombosis is among the most common causes of death today and may be responsible for the increase in the number of deaths (SBC, 2020). In Brazil, according to the Brazilian Society of Cardiology until September 2020, more than 320 thousand people lost their lives due to heart problems (Kotb et al., 2012). For people with cardiovascular disease or high cardiovascular risk (due to the presence of one or more risk factors such as hypertension, diabetes, hyperlipidemia or already known disease) early diagnosis and treatment is essential, through counseling or appropriate management services medicines (Hu et al., 2019). The formation of a clot in a blood vessel (intravascular thrombosis) is a major cause of CVDs (Kotb et al., 2012).

The main protein component of blood clots, a fibrin, is formed from fibrinogen via proteolysis by thrombin and can be hydrolyzed by plasmin to prevent thrombosis in blood vessels. In an unbalanced situation due to some disorders, the clots are not hydrolyzed and, therefore, thrombosis occurs (Lopez-Sendon et al., 1995). In general, there are four therapy options for thrombosis: anticoagulants, antiplatelet agents, mechanical and surgical treatments, and fibrinolytic enzymes. Anticoagulants and antiplatelet drugs are used to treat CVDs for a long time. However, they are not able to dissolve an existing thrombus. Fibrinolytic enzymes, on the other hand, have become more substantial in the treatment of cardiovascular diseases since they can lyse the fibrin clot within blood vessels (Kumar, S.S & Sabu, A., 2019).

Fibrinolytic enzymes produced by microorganisms have the potential to inhibit blood clotting and are capable of degrading fibrin. In recent years, microbial fibrinolytic enzymes have been described in the treatment and prevention of

cardiovascular diseases. Among therapeutic applications, such enzymes can act as anticoagulants, thrombolytics and anti-inflammatory drugs (Chang et al., 2012). Some potential microorganisms, such as bacteria of the genus *Bacillus* and *Xanthomonas*, cyanobacteria, fungi and Streptomyces have been described as sources of fibrinolytic agents (Hu et al., 2019; Vijayaraghavan et al., 2019; Chang et al., 2012; Banerjee et al., 2013; Medeiros et al., 2013; Nascimento et al., 2015; Chandramohan et al., 2019). These proteases have an important role because they are enzymes of industrial interest responsible for about 60% of the total enzyme market in the world and 40% of the total worldwide sale of enzymes (Ravikumar et al., 2012). Fungi have proven to be a good source of these enzymes, with the possibility of large-scale production, facilitating the production and purification of this bioproduct. In recent years, special attention has been paid to the production, assessment of acute toxicity, genotoxicity and cytotoxicity in different cell lines of fibrinolytic enzymes that have a high potential to degrade the fibrin clot using the genus Mucor, especially the species Mucor subtilissimus, in addition to assess its fibrinogenolytic and hemolytic capacity (Nascimento et al., 2016; Nascimento et al., 2017; Silva et al., 2019).

The use of coagulometric methods to evaluate the anticoagulant activity of proteases, such as that produced by *Mucor subtillissimus* UCP 1262, are based on the formation of the fibrin clot, which can be visualized in the tube, in manual techniques, or detected photometrically, through the devices called coagulometers. The coagulometric methods are Prothrombin Time (PT), Activated Partial Thromboplastin Time (aPTT), Thrombin Time (TT), circulating anticoagulant research, fibrinogen measurement and factor measurement (Zago et al., 2013). The Prothrombin Time is a test performed to evaluate the factors involved in the extrinsic and common pathways of the coagulation cascade, factors: VII, V, X, prothrombin and fibrinogen. The test may be prolonged in the deficiencies of one or more of the factors above, as well as in the presence of an inhibitor of any of these factors (Kasvi, 2019; Fleury, 2020). Partial activated thromboplastin time corresponds to the time taken to coagulate the recalcified plasma in the presence of cephaline. It aims to evaluate the intrinsic and common pathways of the coagulation cascade (prekallikrein, high molecular weight kininogen, factors XII, XI, IX, VIII, X, V, prothrombin and fibrinogen). aPTT is used to detect deficiencies or inhibitors of coagulation factors of the intrinsic or common pathway, in addition to being used for monitoring anticoagulation with heparin (Kasvi, 2019; Fleury, 2020). Thrombin time, on the other hand, assesses the rate of conversion of fibrinogen to fibrin in the last step of the common final pathway, its greatest utility is in the evaluation of conditions in which there is a reduction in this conversion, such as in the presence of heparin, fibrin degradation products paraproteins or even in the presence of dysfibrinogenemia (Zago et al., 2013).

Thus, the present study aimed to understand the evaluation of partial thromboplastin time, thrombin time and prothrombin time on plasma treated with a fibrinolytic protease produced by *Mucor subtillissimus* UCP 1262, since research intervention in this area can help in development of safer thrombolytic agents for therapeutic use.

2. Methodology

2.1 Obtaining fibrinolytic protease

The microorganism used to produce the enzyme was the filamentous fungus *Mucor subtilissimus* UCP 1262 (SISGEN AA30B0B) isolated from the Caatinga and obtained from the Collection of the Catholic University of Pernambuco (UNICAP). The fungus was kept in Czapek medium at a temperature of 30ºC. The preparation of the inoculum, the production of the fibrinolytic enzyme by solid state fermentation and the enzymatic extraction was performed as described by Nascimento et al, (2015). The fibrinolytic protease extracted as mentioned above was initially precipitated with 40-60% ammonium sulfate and then purified by DEAE-Sephadex A50 ion exchange chromatography as described by Nascimento et al. (2017). The methodological support was based on the work of Nascimento et al. (2015, 2016, 2017).

2.2 Determination of clotting times

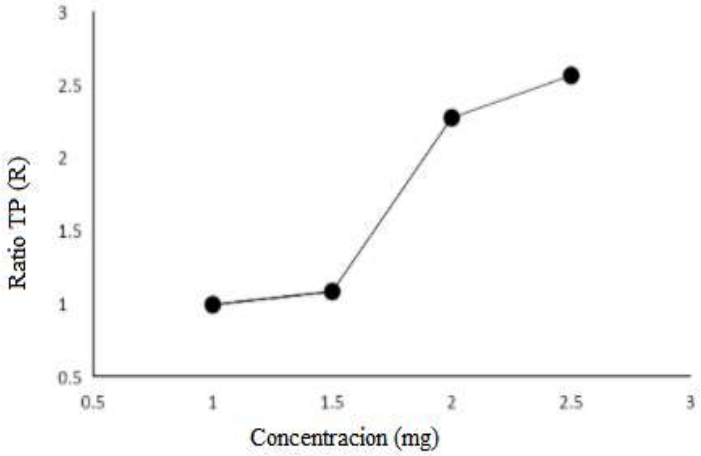
The times of prothrombin (PT) and partially activated thromboplastin (aPTT) were performed in a semi-automatic coagulometer BFT II. A standard plasma was used for the tests provided by the manufacturer. The controls of the PT and TT assays were performed with 20µL and 50 µL of saline solution (NaCl, 0.9%) and 100µL of plasma, incubated for 60 seconds, with subsequent addition of 50µL of the RecombiPlastin 2G reagent. The control of the aPTT assay was performed with 20µL and 50µL of saline solution (NaCl, 0.9%), 100µL of plasma and 50µL of the reagent for aPTT (aPTT-SP (liquid)), incubated for 180 seconds and subsequent addition of 50 µL of 0.025M calcium chloride. To evaluate the anticoagulant effect, purified fibrinolytic protease was used in different concentrations of 0.5mg/mL, 1mg/mL, 1.5mg/mL, 2mg/mL and 2.5mg/mL. The tests were performed in triplicate and the results were expressed as a function of R (ratio of sample coagulation time to control coagulation time).

For the evaluation of thrombin time (TT), the formation of thrombi in vitro was used according to the methodology of Wang et al. (2011) with some modifications. First, 50 µL of fibrinolytic protease (1 mg/mL) was placed in contact with 100 µL of thrombin (20 U/mL) for 5, 10 and 30 min, shortly after this period, each time the solution was transferred to eppendorfs (2 mL) containing a mixture of 400 µL of 150 mM Tris-HCl-NaCl pH 7.75, 100 µL of 245 mM phosphate buffer pH 7.0 and 0.72% bovine fibrinogen, being placed in a water bath at 37 ºC until observation of fibrin clot formation.

3. Results and Discussion

The tests showed that the fibrinolytic protease produced by *Mucor subtilissimus* UCP 1262 promoted a significant prolonged time over the PT clotting time as the enzyme concentration increased (Figure 1). A similar curve was obtained graphically by the serine protease extracted from the leaves of *Leucas indica*, lunatrombase, which at a concentration of 500 nM significantly increased TP (Gogoi et al., 2018). Similar results were observed by Sun et al. (2015), where a significant prolonged increase in TP was also observed in a manner dependent on the concentration of a fibrinolytic protease (AfeE) produced by *Streptomyces* sp. CC5, as well as the protease-CFR15 produced by *Bacillus amyloliquefaciens* MCC2606, which also showed an increase in PT, both in relation to dose and time, compared to the control (Devaraj et al., 2018). PT measures the factors involved in the extrinsic and common routes (FVII, X, II and Fibrinogen), being independent of the intrinsic route. The test depends on the level of vitamin K dependent factors (II, VII and X), being used to control patients using oral anticoagulants (Zago et al., 2013).

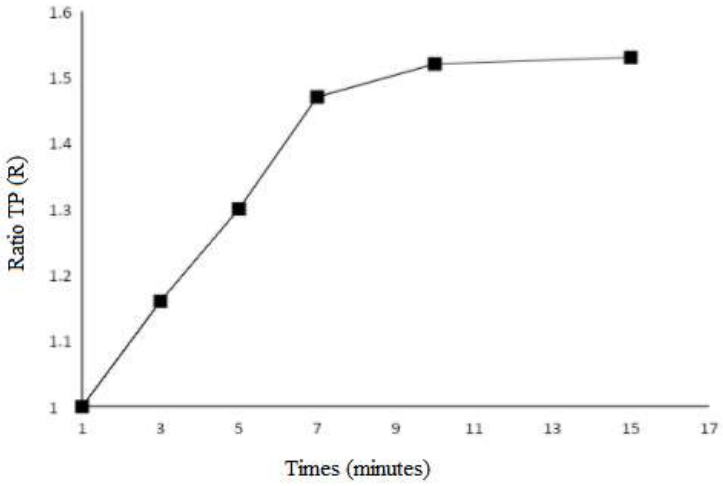
Figure 1. Prothrombin time (PT) as a function of fibrinolytic protease concentration (mg). R = Relationship between the enzyme clotting time and the control clotting time.



Source: Authors.

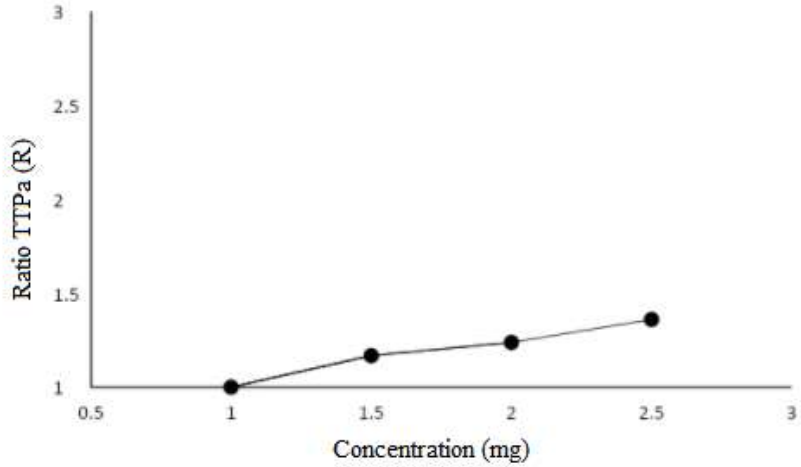
A concentration of 1 mg/mL was used to verify the monitoring of fibrinolytic protease activity on PT in blood plasma over time (Figure 2). In this PT test, it can be observed that the fibrinolytic protease prolonged the clotting time up to the first 7 minutes, after which there was a stabilization of time, with no more effect of the enzyme in relation to time. In the aPTT assay, fibrinolytic protease practically did not prolong the clotting time, even with the increase in the enzyme concentration, as can be seen in Figure 3. Similar results were observed by Gogoi et al. (2018), where the serine protease extracted from Leucas indica leaves did not affect the TTPa, which shows that the fibrinolytic protease produced by *Mucor subtilissimus* UCP 1262 does not have any effects on the intrinsic and common pathways of blood coagulation.

Figure 2. Prothrombin time (PT) of fibrinolytic protease as a function of time (minutes). R = Relationship between the enzyme clotting time and the control clotting time.



Source: Authors.

Figure 3. Activated partial thromboplastin time (aPTT). R = Relationship between the sample's clotting time and the control clotting time.



Source: Authors.

At thrombin time (TT) it was evaluated whether the fibrinolytic protease produced by *Mucor subtilissimus* UCP 1262 would alter the time required for clot formation after the addition of thrombin. Thus, it was found that at all times of contact with thrombin, be it 5, 10, or 30 minutes, there was no interference on the action of thrombin or fibrinogen on the formation of thrombi, always it was necessary on average 10 min at 37ºC for complete fibrin formation as described by Wang et al., (2011).

The prothrombin time was the only one that showed an alteration, a prolongation of its time, it is suggested that the fibrinolytic protease under study affects the extrinsic pathway of coagulation. Our results indicate that the fibrinolytic protease produced by *Mucor subtilissimus* UCP 1262 can block the activation of the coagulation cascade by inhibiting the interaction with factor VII. It is known that this factor is secreted by hepatocytes in its zymogen form and is known as the hyaluronic acid binding protein 2 gene (HABP2), a circulating plasma serine protease that has several effects on hemostasis, vascular biology, inflammation, and cancer, and that, once activated, it can be inhibited by several circulating protease inhibitors (Byskov et al., 2020) and as demonstrated in our studies by the fibrinolytic protease produced by *Mucor subtilissimus* UCP 1262.

The hydrolytic action of the plasmin-like protease is already known, that is, acting directly on fibrin (Nascimento et al., 2017). However, some of its behaviors differ in terms of plasmin under physiological conditions, since the fibrinolytic enzyme studied degrades specifically fibrin, and not the other blood components like fibrinogen, factor V and factor VIII that are degraded by plasmin. Thus, the fibrinolytic enzyme does not alter the thrombin time (TT) in which it is influenced by the concentration of fibrinogen and the presence of inhibitors of fibrin formation (Byskob et al., 2020). Although fibrinolytic protease also has a fibrinogenolytic property as demonstrated in the studies by Silva et al. (2019), this property cannot be considered as an anticoagulant action, since the degradation of fibrinogen by fibrinolytic protease produced by *Mucor subtilissimus* UCP 1262 was only observed after 90 min of incubation with the enzyme and even so only degrading the γ chain, which is suggested by Park et al., (2013) an advantage for removing fibrin clots *in vivo*.

4. Conclusion

Thus, it was possible to observe that the serine protease obtained by *Mucor subtilissimus* UCP 1262 promoted a significant prolonged time on the TP clotting time as the enzyme concentration increased, but the serine protease did not cause

any types of changes in TT and TTPa. It is suggested, therefore, that the enzyme under analysis affects the extrinsic pathway of the coagulation cascade.

Future studies will be carried out using the fibrinolytic enzyme *in vivo*, that is, the protease will be injected into animals and later their blood will be collected to evaluate the hematological behavior after the fibrinolytic enzyme.

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