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Caracterização Bioquímica e Molecular de Enzimas Trombolíticas Obtidas de Isolados Microbianos dos Açores

Universidade dos Açores
Departamento de Biologia
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Dissertação para obtenção do grau de Mestre em Ciências Biomédicas, sob orientação do Professor Doutor Nelson José de Oliveira Simões e Doutor Duarte Nuno Tiago Toubarro.

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Abstract

Bacillus sp. present a great diversity and a high productivity of protease. The group of Biotechnology Department of the Universidade dos Açores has a microbial isolates bank which includes about 1600 isolates of Bacillus sp. Some of the biggest potential thrombolytic enzymes are isolated from Bacillus sp. (streptokinase and natokinase). In this work we tested 79 isolates, previous tested to find a thrombolytic enzyme.

The 79 isolates were tested for proteolytic activity in agar casein plate. Then the fibrinolytic activity and the thrombolytic activity of selected isolates were tested. Was determinate the group species of the bacterial isolates with higher activity and were selected 1 isolate from each group. The optimal pH and the optimal temperature of the extracellular protease produced by 11 isolates from Bacillus sp. were determinate also.

A hemolytic test was made to the enzymes with a higher thrombolytic activity. After we check for fibrinolytic, thrombolytic and hemolytic activity were selected 2 isolates. These 2 isolates were tested for aPTT and PT activity, for plasminogen activator activity, fibrinogenolytic activity and euglobulin lysis time. 1 bacterial enzyme was selected for biochemical characterization and purification.

From the 79 isolates with high proteolytic activity 27 were chosen by their high activity in pH 7.5 and 37˚C. From these 27 bacterial isolates 11 were spotted with fibrinolytic activity and thrombolytic activity (just 6% of the initial 79 isolates). 4 of these enzymes (S97B, S88A, S178C and 99D) belongs to *Bacillus cereus* group, 4 belongs to *Bacillus mycoides* group (S101C, S115C, S26A and S62A) and 3 belongs to *Bacillus subtilis* group (S157E, S122C and S150C). Were selected 2 isolates from the *Bacillus mycoides* group (S115C and S101C) because they didn't presented hemolytic activity. The isolate S115C didn't interfere with aPPT test neither with PT test, otherwise S101C increase the aPPT, interfering with the normal coagulation time. Was also evidence that S101C is a plasminogen activator (t-PA) and S115C is not. Moreover S115C presented more than 10x fibrinolytic activity then S101C, thus enzyme S115C was selected for biochemical characterization and purification. Biochemical characterization

showed that S115C was a high inhibited by Benzamidine, STI, Chymostatin and PTCK indicating that S115C is a chymotrypsin-like serine protease. Suc-Ala-Ala-Pro-Phe-pNa was a specific substrate which indicates chymotrypsin activity. The activity of these enzyme was highly enhanced by Mn and slightly enhanced by Na and Ca^2. This enzyme was inhibited by metal ions Mg^{2+}, Cu^+ and $Ni2^+$.

The zymogram of the purified enzyme revel a digestion band higher than 135 kDa and another band at 75kDa. SDS-page of purified fraction revel 2 bands one at 75 kDa and another at 140kDa. The SDS-page bands were cut and sent to analysis of mass spectrometry (Ms/Ms).

I Introduction

The blood coagulation cascade is initiated when subendothelial tissue factor is exposed/expressed to the blood flow following either the damage or activation of the endothelium. This may occur as a consequence of the perforation of the vessel wall or activation of endothelium by chemicals, cytokines, or inflammatory processes (Camera, M. et al, 1999).

When it occurs in the heart, clots may cause blockage of blood flow to the muscle tissue (myocardium), cutting the supplemental oxygen to tissue and causing cell death. With increasing age of the organism, the production of these enzymes begins to decrease, making it more susceptible to blood clotting. This mechanism can lead to myocardial infarction or cerebral, and other pathological conditions. Since endothelial cells exist all over the body, such as arteries, veins and lymphatic system, the deficit in the production of thrombolytic enzymes can lead to the development of thrombotic conditions virtually anywhere in the body.. Thrombotic diseases typically include cerebral hemorrhage, myocardial infarction and cerebral, and angina pectoris but also includes other diseases caused by blood vessels with low flexibility in cases of senile dementia or patients with diabetes. (Kotb, 2012).

1. Blood Coagulation

The coagulation process is currently a matter very well studied and continues to be the subject of research and new discoveries.

Several proteins involved in the clotting process and inhibitors have been discovered and new interactions between these system components. Increased knowledge on the coagulation process enabled the development of new diagnostic coagulation tests, and the discovery of new anti-thrombotic and hemostatic drugs (Bombeli and Spahn, 2004).

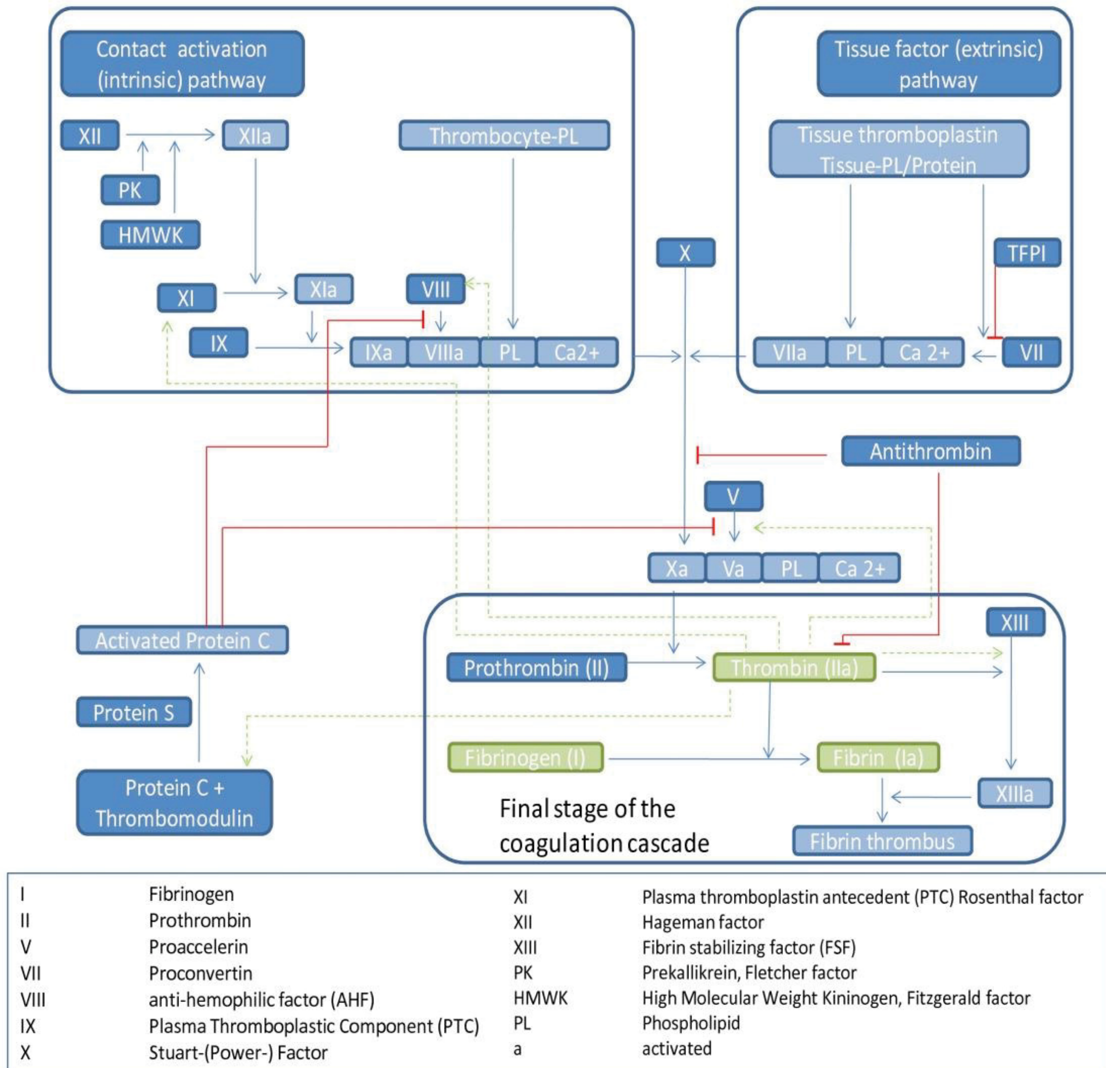
The coagulation process under normal physiological conditions is initiated through the extrinsic pathway, which is dependent on a tissue factor, also called thromboplastin or factor III (Butenas et al., 2000).

In the initiation phase of coagulation, damaged tissue releases the tissue factor (TF), which in turn binds to, factor VIIa, already present in blood and forms the factor VIIa–tissue factor complex (*extrinsic factor Xase*), which activates the zymogens factor IX to factor IXa and factor X to factor Xa The limited amounts of the serine protease factor Xa produced generate picomolar concentrations of thrombin (factor IIa), which partially activates platelets and cleaves the pro-cofactors factor V and factor VIII generating the active cofactors factor Va and factor VIIIa, respectively (Bombeli and Spahn, 2004).

Factor VIIIa forms the *intrinsic factor Xase* complex with the serine protease, factor IXa, and activates factor X. Factor Xa forms the *prothrombinase* complex with the cofactor, factor Va, which is the primary activator of prothrombin. The produced thrombin amplifies its own generation by activating factor XI and completing activation of platelets and factors V and VIII (Pieters, J. et al, 1989). Thrombin also cleaves fibrinogen and factor XIII to form the insoluble crosslinked fibrin clot. The procoagulant processes are attenuated by a variety of inhibitors, which inactivate either serine proteases or cofactors (Bombeli and Spahn, 2004). Except for the first two steps in the intrinsic pathway, calcium ions are required for promotion or acceleration of all the blood-clotting reactions (Jesty and Beltrami, 2005).

The activation of the TF: VII by factor Xa is the main positive feedback mechanism of coagulation process. When the TF is available in the plasma, this binds with inactive factor VII to form VIIa form. Most of the available TF binds to the inactive form of FVII, giving rise to the TF: VII, this is because the levels of FVIIa medium are low in plasma, about 0.5% of the total FVII, resulting in a small proportion of the complex TF: VIIa formed, but sufficient to activate FX and the feedback process occurs which leads to the conversion of TF:VII to TF: VIIa. The FVIII activation by thrombin is another positive feedback step. The regulatory factor VIII is a cofactor for factor IXa, but this circulates the blood in the inactive form. And the FIXa does not act in the coagulation cascade while the FVIII is in the inactive form (Jesty and Beltrami, 2005). Furthermore thrombin activates factor V cofactor to accelerate the activation of FII (prothrombin) at FXa and FXIa to still FXI leading to increase FIXa (Bombeli and Spahn, 2004). The following

stage is called propagation and is characterized by keeping the production of thrombin, in order to ensure sufficient blood clot formation, by converting fibrinogen into fibrin.



I	Fibrinogen	XI	Plasma thromboplastin antecedent (PTC) Rosenthal factor
II	Prothrombin	XII	Hageman factor
V	Proaccelerin	XIII	Fibrin stabilizing factor (FSF)
VII	Proconvertin	PK	Prekallikrein, Fletcher factor
VIII	anti-hemophilic factor (AHF)	HMWK	High Molecular Weight Kininogen, Fitzgerald factor
IX	Plasma Thromboplastic Component (PTC)	PL	Phospholipid
X	Stuart-(Power-) Factor	a	activated

Figure 1 Coagulation cascade: the conversion of fibrinogen into fibrin is triggered by thrombin and calcium. (Jockenhoevel, et al.,2011)

There is the phase of clot stabilization, where the thrombin reaches higher levels and are sufficient to activate FXIII (one transglutaminase) establishing covalent bonds between the soluble fibrin monomers to yield an insoluble mesh stable fibrin. In this phase the inhibitor by thrombin activatable fibrinolysis (TAFI) protects the clot fibrinolysis attack (Bombeli and Spahn, 2004).

At the end of the coagulation cascade, factor XIIIa creates covalent bonds between fibrin molecules, which precipitate and form a clot, which fibers are more resistant to the activity proteolytic and mechanical disruption. The presence of Factor XIII is highly important because the fibrin fibers need to be stabilized to form a strong structure capable of preventing loss of blood circulatory system (Standeven et al., 2007).

Fibrin is organized by two chains of fibrinogen, crosslinked, α and γ and non-crosslinked chain, β (McDonagh, et al., 1972). Factor XIIIa is capable of generating structures containing various combinations of crosslinks between γ and α chains of fibrin, giving a high resistance to fibrinolysis. It is not yet fully known the effect of different cross-linking the fibrin clot formation, however it is known that increasing the rigidity of blood clots is attributed to crosslinks between α chains (Collet et al., 2005).

2. Fibrinolysis

Fibrinolysis is a process that prevents blood clots from growing and becoming problematic. The main enzyme in this process is plasmin, a proteolytic enzyme whose sole role is to dissolve fibrin. Plasmin is produced in an inactive form, plasminogen, in the liver. Plasminogen cannot cleave fibrin. Instead, it is incorporated into the clot when it is formed and then activated into plasmin later. Plasminogen is activated to plasmin by tissue plasminogen activator (t-PA). The tissue plasminogen activator (t-PA), which is released by the endothelium, in the presence of fibrin acts as a cofactor and catalyze the conversion of glu-plasminogen (GPG) to glu-plasmin (GPN). t-PA activates plasminogen at a very low rate. (Dobrovolsky and Titaeva, 2002).

Plasmin digests fibrin fibers and some other protein coagulants such as fibrinogen, Factor V, Factor VIII, prothrombin, and Factor XII. Therefore, whenever plasmin is formed, it can cause lysis of a clot by destroying many of the clotting factors, thereby sometimes even causing hypocoagulability of the blood (Muszbek, *et al*., 2011).

This process begins digestion of fibrin (FN) by catalyzing cleavage after specific arginine and lysine residues in α, β, and γ chains of fibrin. Fibrin is modified to FN' a form that has in its structure carboxyl terminal lysine residues, which promote the binding of tissue-type plasminogen activator (t-PA) and GPg to fibrin. These reactions are catalyzed by GPn and LPn. LPg is approximately 20-fold better than GPg as a substrate for tPA-catalyzed formation of plasmin (LPn). Thus, modification of FN to FN′ represents positive feedback in the fibrinolytic cascade, somewhat similar to that represented by thrombin-catalyzed activation of factors V and VIII in the coagulation cascade by thrombin (Nesheim, 2003).In addition, thrombomodulin of endothelial cells is important in the regulation of the balance between the two systems, it is able to convert thrombin anticoagulant enzyme, directing it to the activation of protein C and can also convert thrombin for an enzyme antifibrinolytic, directing it to the activation of TAFI (Nesheim, 2003).

3. Pathologies related to fibrinolysis

The high expression of these activators, leads to the appearance of abnormal bleeding, but they are very rare conditions (Booth et al., 1983). Excessive production activator plasminogen activator (u-PA) within the alpha-granules of platelets, is related to Quebec syndrome, an extremely rare autosomal dominant hemorrhagic disease (Diamandis et al, 2009; Veljkovic et al, 2009). Several studies have associated high levels of PAI -1 with venous or arterial thrombosis and the risk of septic shock and multiple organ failure (Sanchez Miralles et al, 2002; Williams, 1989). The change of plasminogen activators also appears to be linked to the onset of diseases. There are sporadic reports on t-PA deficient patients with thrombosis (Brandt, 2002). Moreover, the high expression of these activators, leads to the appearance of abnormal bleeding, but they are very rare conditions (Booth et al., 1983).

The plasminogen deficiency in both the heterozygous and homozygous form, is related to the appearance of thrombosis in the presence of other genetic defects, such as mutation of factor V Leiden (Demarmels Biasiutti et al., 1998). Other pathologies related to a severe deficiency in plasminogen can lead to the development of Woody conjunctivitis, a rare ocular disease characterized by the

formation of fibrin rich pseudomembranes essentially the tarsal conjunctiva (Seregard and Schuster, 2003).

4. Thrombolytic Therapy

In general, there are three thrombolytic therapy options for patients, by administration of anticoagulants and antiplatelet agents, and finally through the use of fibrinolytic enzymes.

Anticoagulants act by blocking one or more stages of the coagulation cascade that culminates in the formation of fibrin. Some drugs used can also act by inhibiting synthesis of coagulation factors while others increase the anticoagulant activity which occurs naturally in blood and prevent the formation of platelet stopper (Kotb, 2012). Heparin is a example of an anticoagulant, whose main purpose lies in its inhibition of thrombin and factor IIa and Xa in the coagulation cascade. In addition has a short half-life, it is associated with bleeding, osteoporosis, alopecia, thrombocytopenia and hypersensitivity (Fitzmaurice et al., 2002). Warfarin is an anticoagulant that inhibits coagulation interfering with the incorporation of vitamin K dependent coagulation factors Vitamin K, including factors II, VII, IX and X. (Kotb, 2012).

Antiplatelet agents are used to prevent clot formation or prevent it from becoming larger. Antiplatelet drugs such as dipyridamole, clopidogrel and ticlopidine act through inhibition of platelet activating factor and collagen. Aspirin is the most widely used antiplatelet drug and its mode of action is through inhibition of platelet aggregation. (Patrono, et al., 2002).

4.1. Enzyme Therapy

Different enzymes purified from various microbial, animal and plant sources have been used in varied ranges of physiological, medicinal, and industrial applications. Among them, fibrinolytic enzyme is one which has a significant medical applications for the treatment of cardiovascular diseases

(CVDs) caused by intravascular thrombosis. CVDs are the leading cause of death throughout the world (Mender et al, 2011).

Enzymes are the bio-catalysts playing an important role in all stages of metabolism and biochemical reactions. Unlike heparin and warfarin, fibrinolytic enzymes promote lysis of pre-existing thrombus. Nowadays the biggest producer of fibrinolytic enzymes are Bacillus spp (Annex Figure 1). Depending on the mode of action of these enzymes, they may be classified into two types, plasminogen activators (PA) and enzymes plasmin type which degrade directly fibrin, which in turn leads to rapid and complete destruction of thrombus (Kotb, 2013).

The group of plasminogen activator enzymes include streptokinase, Urokinase (Duffy, 2002), and tissue plasminogen activator (t-PA) genetically modified (Lijnen and Collen, 2004). Urokinase is the best Known human plasminogen activator. This enzyme is capable of catalyzing the conversion of the inactive zymogen plasminogen to the active proteinase plasmin, Urokinase is extracted from urine (Andreasen, et al., 1997). Urokinase is too expensive and after intravenous administration there is a significant risk of hemorrhagic (Yang, et al., 2012).

Streptococcus hemolyticus and Staphylococcus aureus produce staphylokinase and streptokinase respectively, where both are used in thrombolysis (Collen and Lijnen, 1994). Streptokinase can potentiate the body's own fibrinolytic pathways by converting plasminogen to plasmin. The streptokinase is an effective thrombolytic agent of bacterial (streptococci). This enzyme enhances the fibrinolytic pathway by converting plasminogen to plasmin.

t-PA produced by recombinant DNA technology, have the same properties of the molecule that activates the endogenous fibrinolytic system, and therefore do not develop allergic responses and is considered more specific for the lysing of the clot (Mine et al., 2005)

There is a large set of fibrinolytic enzymes were discovered in particular of the genus Bacillus sp isolated from traditional fermented foods (Peng et al., 2005). Natokinase is an enzyme that is extracted from Natto, a fermented soy

bean. It functions include directly degrading a fibrin (the main component of thrombi), activating pro-Urokinase (precursor for Urokinase that is a thrombolytic enzyme in the body) and increasing the amount of tissue plasminogen activator (t-PA) that produces a thrombolytic enzyme, plasmin. In addition, recent research has revealed that Nattokinase has a function of degrading plasminogen activator inhibitor, PAI-1 and reducing the euglobulin lysis time, and therefore, it has a function of improving the thrombolytic activity. Furthermore, the reduction of blood pressure has also been confirmed (Urano et al., 2001). An additional study indicates that nattokinase can also be purified from TKU015 culture supernatant of strain Pseudomonas sp. isolated from soil which has high activity on fibrin (Wang et al., 2009).

According to Suzuki et al. (2003), a supplementary diet with natto is related to the shortening of the euglobulin lysis time, which is an indicator of the total intrinsic plasma fibrinolytic activity. Simultaneously, this enzyme does not prolong the clotting time, indicating that it may be used as a safe food supplement, has no unwanted side effects.

More recently was discovered in B. amyloliquefaciens subtilisin DC-4 of Chinese fermented soybean designated Douchi (Peng et al., 2003), this enzyme can activate t-PA and it can dissolve fibrin directly (Yang, et al. 2012). Unlike Urokinase and streptokinase has no side effect and it has no toxic. Douchi like Natokinase has a convenient oral administration, and stability in the gastrointestinal tract (Sumi et al., 1990).

These enzymes also have some negative aspects such as the low specificity of active centers, which leads to adverse effects including gastrointestinal bleeding (Turpie et al., 2002); systemic fibrinogenolysis accompanied by bleeding problems is also often found as well as neural complications (Caramelli et al., 1992), intracranial hemorrhages (Kase et al., 1992).

The limited efficacy and undesired side effects of these thrombolytic agents pose problems for their clinical application. Much research is made in order to overcome these problems by seeking increasing the thrombolytic activity of the target or for improving targeting of these proteins on the clots. Furthermore both streptokinase and urokinase dependent and act by the activation of plasminogen to plasmin (indirect action), making it important to direct the further research for the discovery of novel agents that are able to act directly on fibrin (Caramelli et al., 1992).

4.2. Proteases

The fibrinolytic enzymes are mostly proteases. These catalyze hydrolysis reactions on proteins and act specifically in the interior peptide bonds. Most living cells produce different types of proteases, but most of it is produced by microorganisms.

Proteases are subdivided into two groups depending on their mode of action, are called exopeptidases or endopeptidases. The exopeptidases cleave peptide bonds near the amino or carboxy terminus of the substrate, whereas endopeptidases cleave peptide bonds distant end group of the substrate. Based on the functional group present at the active site of the proteases, they can be classified into four families: the serine proteases, aspartic proteases, cysteine proteases and metalloproteases. Fibrinolytic proteases are for the most serine or metalloproteases and have great importance in the food, pharmaceutical and residual maintenance industry (Kotb, 2012).

More than one third of all the known proteolytic enzymes belong to the group of serine proteases, distributed in 40 families. Serine proteases are characterized by the presence of a catalytic triad composed of the residues of the amino acids aspartic acid, serine and histidine. They are present in eukaryotes, prokaryotes, in archaea and viruses, and participate in diverse physiological processes, including proteases of the digestive system (trypsin and chymotrypsin), immune system (complement factors B, C and D), enzymes

involved in coagulation (factors VIIa, IXa, Xa and XIIa), fibrinolytic enzymes (urokinase, tissue plasminogen activator and plasmin) and reproductive system (acrosin).

The trypsin-like proteases are the most abundant group of serine proteases. For a long time just two groups of known serine proteases, the trypsin-like enzymes (clan PA) exhibit a double b-barrel fold, whereas the subtilisin-like enzymes (clan SB) have a parallel b-sheet structure. The peptidases of clan SC, which were discovered considerably later (Rawlings, et al., 1991), display an a,b-hydrolase fold with the same catalytic triad as found with the classic serine peptidases, chymotrypsin and subtilisin (serine, histidine and aspartic acid), but with an opposite handedness. However, in recently identified clans (SE, SF, SH, SJ, SK, SP, SR) the members of the catalytic triad and thus the mechanism of action changed to some extent. (Polgar, 2005).

II Materials and Methods

1. Bacterial growth and enzyme extract preparation

The group of Biotechnology Department of the Universidade dos Açores have 1600 isolates of Bacillus spp. This isolates were collected from Azorean soils of São Miguel Island. 79 bacterial isolates, previously tested for protease producers, were cultivate at 37˚C at 200rpm during 24 hrs in nutrient broth (0.5% Peptone, 0.3% beef extract and yeast extract, 0.5% NaCl, pH 7). After growth, bacteria was separated by centrifugation at 10000g for 10 min and supernatant was filtrated using 0.2 um membrane (Milipore) and then was concentrated using through tangential Concentration System (Minimate ™ TFF Capsule) with a 5K cut-off membrane.

2. Proteolytic activity

Bacillus isolates (79 isolates) were screened for their ability to hydrolyzed casein in 2% (w/v) agar plates containing 0.1% (w/v) of skin dry milk dissolved in 2% (w/v) agar for bacterial use, 2 ml of Tris HCI, pH 7.5, 25M buffer for buffering the medium. In the casein plate was made wells to pipette the bacterial supernatant.

Inoculations were made of the 79 isolates in test tubes with 5 ml of nutrient broth and the supernatant was collected. 40 µL of supernatant from each isolate were pipetted into the wells in casein plate and was incubated for 24 hours at 37˚C.

3. Fibrinolytic activity

3.1. Agarose plate assay

Fibrinolytic protease activity was estimated according to the method described by Astrup and Mullertz, 1952. Using a fibrin plate containing a 56,25 mg of fibrinogen from human plasma (Sigma) dissolved in 7,5ml of Tris-HCl(25mM), pH 7.5 with 75mM NaCl, were added to this solution 7,5ml of 1.2% agarose and 100 µl of thrombin (Sigma) (100U/ml). The mixture was

homogenized and poured into the plate, reaching a thickness of 6-7mm, the plate was incubated for 30 min at 37˚C. Wells were made with 0.5 cm of radius.

Serial dilutions of enzyme were made (15 μg, 7.5 μg, 3.95 μg and 1.8 μg in Tris-HCl pH7.5 solution) and serial dilutions of Urokinase (Sigma)(5 μg, 2.5 μg, 1.25 μg, 0.6 μg) 20 μL of each were pipetted into the well, then the plate was incubated at 37˚C for 1, 2, 4, 6, 8, 12 hours. Plate scans were made for presence of digestion halos around each well where the sample was applied, the diameters of the digestion halos were measured.

3.2. 96-well plate assay

The method was developed for cost reasons to test the enzymatic activity on fibrin and plasminogen activation it was used a 96 well plate method. This method required less than 4% of human fibrin and human thrombin necessary compared with the previous plate method.

In witch well of the 96-well plate was pipetted 50 μL of a fibrinogen solution (2mg of fibrinogen and 50 μl of Tris HCl pH 7.5 25mM with75mM NaCl) and 20 μl of diluted thrombin (0.02 μL of thrombin (100U/ml) and 19.98μl of distillated water). The plate was incubated for 30 min at 37 ˚C and then 20μg of samples were pipetted into witch well. Absorbance was read at 30 min intervals during 4 hours at a wavelength of 405nm, the decrease of absorbance meant the digestion of the fibrin clot.

4. Plasminogen activation

Plasminogen activation assay was performed in a 96-well plate assay and agarose plate assay as described above in Fibrinolytic activity (3) with minor modifications. 5 μg plasminogen was mixed with the samples, for testing the enzyme was added 15 μg of enzyme and 5 μg of plasminogen to an Eppendorf tube. Two controls were made one with 5 μg of plasminogen diluted in buffer Tris-HCl (100mM), pH 7.5 and another with 5 μg of Urokinase diluted in buffer Tris-HCl (100mM), pH 7.5.

Samples containing plasminogen were incubated at 37˚C for 5 min before being applied in the wells. The other samples containing just enzyme were

loaded directly into the wells, 30 μL of each mix of enzyme and plasminogen were pipetted into the respective wells of the fibrin plate and incubated at 37˚C. The digestion was followed by plate scan was made every hour during 6 hours in fibrin plate assay or absorbance readings at 405nm in 96-well plate assay.

5. Fibrinogenolytic assay

5.1 Evaluation of Fibrin clot formation

To investigate the fibrinogenolytic activity of the enzyme was used the 96 well plate method (3.2). 20 μg of sample was added to 50 μL of a fibrinogen solution (2mg of fibrinogen and 50 μL of Tris HCl pH 7.5 25mM with75mM NaCl) and a pre incubation of 10 min at 37˚C was made before adding 20 μL of thrombin solution (0.02 μL of thrombin (100U/ml) and 19,98 μl of distillated water). In positive control was used 20 μg of Urokinase and a negative control with Tris-HCl buffer were made to compare the fibrinogenolytic activity Absorbance was read at 15 min intervals during 5 hrs at a wavelength of 405nm.

5.2 SDS-page analysis of fibrinogen chains digestion

The fibrinogenolytic activity was also assayed according to Salazar et al. (2007), with some modifications. The method consists of pre-incubation of enzyme with fibrinogen to obtain a profile of degradation of fibrinogen chains (α, β and γ) through an SDS-page electrophoretic separation. For this 20μg of enzyme was added to 100 μL (0.08μg/μL) of fibrinogen from human plasma (Sigma) dissolved in Tris-HCl (25mM) pH 7.5 with 75mM NaCl and incubated at 37˚C. Aliquots of 20 μL were taken from the mixture at different times (0, 10, 40 and 1440 min). For a control was prepared a mix of 10 μg Urokinase (Sigma) and 50 μL of fibrinogen from human plasma (Sigma) dissolved in Tris-HCl (25mM) pH 7.5 with 75mM NaCl and incubated at 37˚C. Aliquots of 20 μL were taken from the mixture at different times (0, 20, and 1440 min). To the aliquots were added 5μL of sample buffer containing β-mecaptoethanol. Samples were incubated for 15 min at 95˚C and analyzed by a SDS-page. The separation of the proteins on the gel was visualized with Coomassie Blue staining.

6. Bacterial species groups identification

In order to obtain the different restriction profiles, proceeded to the extraction of DNA and PCR amplification of the 16S rRNA gene. In order to obtain bacterial pellet to collect DNA, 5 µL of the bacterial culture was centrifuged at 5000g for 5 min, the supernatant was discarded and the pellet was washed with 500 µl of H_2O milli-Q and stored at -80˚C, during 15 min. Then the samples were placed on 95˚C for 10 min to lysis the cell. Finally the cell suspension was centrifuged at 11000g for 5 min and the supernatant was stored at -80°C until needed.

PCR reaction mixture was used 18.8 µl H2O milli-Q, 2.5 µL of buffer Tris-HCl, pH 8.3 with MgCl2, 0,5 µL of dNTP, 10mM, 1µL of forward primer, 1 µL of reverse primer, 0,2µL Taq DNA polymerase and finally 0,5 µL of DNA.

The PCR conditions for amplification of the 16S rRNA gene were: initial denaturation at 95˚C for three min followed by denaturation at 95 °C for 30 seconds, annealing at 50˚C for 30 seconds, extension for 1 min at 72˚C by 35 cycles and final extension at 5 min 72˚C. Finally the reaction was cooled at 4 °C. Then, the amplified fragments were analyzed using an electrophoretic run on 1.2% agarose gel. A molecular weight marker (BIORON GmbH) of 100 Kb was used.

After confirmation of 16S amplification the samples were digested with restriction enzyme AluI (AG'CT). For the reaction mix 5 µL of amplified product was joined with 5U of restriction enzyme in 20 µL total volume. Then incubated for 1 hr at 37ºC. Digested fragment was separated into 1.2% of agarose gel, a 100 Kb molecular weight marker (BIORON GmbH) was used and the bands were stained with ethidium bromide.

7. 16S Amplification and Sequencing

A 16S amplification was made using a primer 8f 5'AGA GTT TGA TCC TGG CTC AG3' and 1492r 5' CCG TTA CCT TGT TAC GAC TT3'. For PCR

reaction mixture was used 18.8 µl H2O milli-Q, 2.5 µL of buffer Tris-HCl, pH 8.3 with MgCl2, 0,5 µL of dNTP, 10mM, 1µL of forward primer, 1 µL of reverse primer, 0,2µL Taq DNA polymerase and finally 0,5 µL of DNA. The conditions for amplification of the 16S gene were: initial denaturation at 95˚C for three min followed by denaturation at 95 °C for 30 seconds, annealing at 50˚C for 30 seconds, extension for 1 min at 72˚C by 35 cycles and final extension at 5 min 72˚C. Finally the reaction was cooled at 4 °C.

The amplified DNA was purified with a PCR purification kit (Wizard SV Gel and Clean Up System, Promega) and then the sample was sent to sequencing DNA.

8. SDS-page

For this electrophoretic analysis we used a vertical electrophoresis system Mini protean II Cell (Bio-Rad). There was a separation gel at 12% by the addition of 4 ml of acrylamide 30%, 2.5 ml buffer 0.375 M Tris-HCl, pH 8, and 0.1 ml of 10% SDS, made up to volume with MilliQ water up to a total of 10 ml.

To polymerized the gel was added 75 µL of 10% APS and 30 µL TEMED. After its polymerization, butanol was removed and washed with MilliQ water. Once prepared the acrylamide gel concentration of 7% final concentration, was poured into the running gel and the comb is placed to create the wells during polymerization. The samples were dissolved in sample buffer with β-mecaptoethanol and incubated at 95˚C for 15 min. The electrophoretic run occurred for 90 minutes at constant 24 Ampere 150 volts. The separation of the proteins on the gel was visualized with Coomassie Blue staining.

9. Thrombolytic Activity

To determine the in vitro thrombolytic activity, was used a total blood clot provided by Centro Médico Dr Forjaz Sampaio, collected with a serum-Z-gel tubes. The blood clot was cut into small squares, with approximately 0.3 cm of length wise. Then the fragments were placed in 96-well-plate and washed with

0.1 M phosphate buffer solution pH 7 and 0.8% NaCl, to remove red blood cells detached from the clot. To a negative control was added 60 µL of 0.1M phosphate buffered saline. To check the thrombolytic activity of the enzyme a 60 µL mix was made with enzyme and 0.1M phosphate buffered saline.Then the plate was incubated for 37˚C for 4 hours. The microplate was scanned each 15 min to record the fragment size in each.

10. Euglobulin lysis time

This assay was based on the method described by Smith et al, 2003. Consist in preparation of a euglobulin fraction from human blood plasma which is analyzed over time by reading absorbance at 405 nm. With tis assay we study the effect/action of the enzyme on the euglobulin lysis time. The euglobulin fraction was prepared by adding 400 µL of citrated human plasma to 3.6 ml of 0.25% acetic acid (v/v), placed on ice for 30 min and centrifuged at 2000g at 20˚C for 15 min.

The supernatant was discarded and the tubes were inverted on paper swab for 3 min. The precipitate was resuspended by addition of 400 µL 0.1 M sodium borate solution, pH 9.0. lysis assay were prepared in a pre-warmed 37˚C 96-well plate 150 µL of Euglobulin , 5 µL of thrombin solution, 5 µL of 0.1M $CaCl_2$ and 30 µL 0.1 M Tris-HCl. The enzymatic activity was tested replacing 0.1 M Tris-HCl for the enzyme (10µg) and the enzyme was incubated for 15 min with euglobulin before adding thrombin and $CaCl_2$. The control with Urokinase (Sigma) was made with 5µL of Urokinase (100/ml) dissolved in Tris-HCl 0.1M pH 7.5. The absorbance was measured kinetic spectrophotometer reader every 15 min during 500 min.

11. Hemolytic assay

For detection of hemolysis were used Columbia CNA Agar with 5% of Sheep Blood Medium from Biomerieux. Wells were made to the plate and 20µg of enzyme diluted in 0.1 M phosphate buffer solution pH 7 and 0.8% NaCl were pipetted into each well. Scans were made during 1 hour intervals to see formation of a hemolytic disc surrounding the well. A positive control was made with 30µL

of H_2O Mili-Q and a negative control was made with 40 µL of PBS buffer with 0.8% of NaCl. The CAN plate was incubated for 6 hrs at 37˚C.

12. Purification of the Fibrinolytic Enzyme

All chromatograhies were performed on a AKTA-FPLC system at 7˚C. The enzyme fractionation was done using 4 liters of 18 hrs bacterial culture and the supernatant was then separated by centrifugation at 8000g, 10min, filtrated with a 0.2 um membrane and concentrated with tangential Millipore Concentration System (Minimate ™ TFF Capsule) with a 5K membrane.

Bacterial supernatant was used to fractionate in successive chromatographies. The first one was an anionic exchange chromatography at pH 8.8, using a Capto Q column. The column was equilibrated with 50mM of Tris-HCl pH8.8 (Buffer A) protein was eluted with 5 steps of 0%, 15%, 30% 60% and 100% 50mM Tris-HCl, pH 8.8 with 1M of NaCl (Buffer B). Eluted proteins automatically collected using a chromatography system collector. The proteolytic activity was determinate with a 96-well-plate fibrin assay.

Active fractions were pooled, concentrated and applied in a HitrapQ column equilibrated with 50mM Tris-HCl, pH 8.8 (Buffer A). Proteins were eluted with 50 mM Tris-HCl, pH 8.8 with 1M NaCl (Buffer B) in a gradient (0%; 5%; 5% to 30%; 30% to 50% and 50% to 100%). After testing each fraction of the Hitrap Q the active fraction was applied in an anionic exchanger column Mono Q, equilibrated with 50mM Tris-HCl, pH 8.8. The protein was eluted by Tris-HCl pH8.8 with 1M of NaCl, The different fraction were tested in a 96-well plate and the fraction with the best fibrin digestion was used.

13. Zymogram of Purified Enzyme

The enzyme fraction with fibrinolytic activity detected was separated on SDS-PAGE supplemented with gelatin. The zymogram was performed on gel with 12% acrylamide / bisacrylamide in 1.5 M Tris-HCl buffer solution (pH 8.8) copolymerized with gelatin to 0.10%. Sample (20 µL) were loaded on the gel with 15 µL of the purified enzyme, and 5 µL sample buffer. After the electrophoretic

run on a Mini Protean Cell II (Bio-Rad) system, the gel was washed 2 times for 30 minutes with 2.5% Triton X-100 and removed with 3 washes in distilled water for 10 minutes.

After that the gel was incubated for 3 hrs in buffer 50 mM Tris-HCl, pH 7.5, at 37˚C.The areas in which it occurred proteolytic activity were detected by Coomassie blue staining..

14. SDS- page of purified fraction

After the chromatography purification, the soluble purified protein concentration was determinate using a NanoDrop spectrophotometer (Thermoscientific). The purified protein was precipitated with acetone and tricloroacetic acid (TCA).

The purified fraction was added 5µL of sample buffer with β-mecaptoethanol, the mix of Sample and Buffer was incubated at 95˚C for 15 min. The electrophoretic run occurred for 90 minutes at a constant 24 Ampere 150 volts. The separation of the proteins on the gel was visualized after a Coomassie Blue staining.

15. Mass Spectrometric analysis of the Enzyme (Ms/Ms)

After separation of the protein by SDS-PAGE gel, the band corresponding to the pure fraction was cut from the gel and placed in Eppendorf tubes and covered with milli-Q water. Samples were sent to the Group of Mass Spectrometry at the Institute of Chemical and Biological Technology (ITQB, Oeiras) for analysis.

The monoisotopic mass of the analyzed proteins were obtained using a MALDI-TOF-MS model Voyager-DE STR (Applied Biosystems). The external calibration masses was performed using a mixture of peptides patterns PepMix1 (Laserbio Labs). The masses obtained were used to search the NCBI database (www.ncbi.nlm.nih.gov) using the public version of the Mascot software (www.matrixscience.com).

16. Biochemical Characterization of the Enzyme

16.1 Effect of pH and Temperature

To determinate the optimal pH were used 0.1M of Tris-HCl buffer at pH 6.5, 7.5 and 8.5. For each pH range was added to an eppendorf, 20 μL of buffer, 30 μL of enzyme fraction, and 50 μl of 2% azocasein. In a negative control the sample was replaced by buffer. The effect of temperature on the enzymatic activity was also determined by azocasein. The assay was performed at different temperatures, 28°C, 37°C and 48°C. For each temperature an Eppendorf prepared with 20 μL of buffer, 30 μL of enzyme fraction, and 50 μl of 2% azocasein. A negative control for each temperature, where only buffer was added and the solution was made substrate.

16.2 Effect of Ions and Solvents and on enzymatic activity

To determine the effect of various solvents and ion on the enzymatic activity was carried a standard azocasein test with 5 μL 0.1M of ions, 30 μL of enzyme fraction, 15 μL of buffer solution and 50 μL of 2% azocasein (w/v). In this case a positive control was made without sample and solvents. Was made a negative control containing 0.1M Tris-HCl buffer. Ions used were the $HgCl_2$, $MgCl_2$, $CuSO_4$, NiCl2, $MnCl_2$, KCl, NaCl, and CaCl2. To check for enzyme stability with solvents were used Urea, SDS, β-Mercaptoethanol, Chaps, Triton, Tween and DTT.

16.3 Substrate specificity

Proteolytic activity was measured using specific chromogenic substrates. MetSuc-Ala-Ala-Pro-Met-pNa, Suc-Ala-Ala-Pro-Phe-pNa and Suc-Gli-Gli-Phe-pNa to analyses chymotrypsin- like activity; Bz-Phe-Val-Arg-pNa and Bz-Pro-Phe-Arg-pNa to analyse thrombin- like activity; Bz-Gli-Gli-Arg-pNa for Urokinase- like activity; Z-D-Arg-Gli-Arg-pNa for factor Xa like activity; and the D-Ile-Pro-Arg-pNa for plasminogen activator like activity. Enzyme was mixed with

witch chromogenic substrato dissolved in 0.5 M Tris-HCl pH 7.5. The formation of p-nitroaniline was monitored at 405 nm by BioRad reader.

16.4 Effect of Inhibitors on enzymatic activity

The effect of various protease inhibitors on enzyme activity was tested with specific substrate. Was added to 5µL of specific substrate, 15 µL of buffer, 15 µL of enzyme fraction, and 5 µL of inhibitor. The mixture was pre-incubated at room temperature for 5 min and then was added to 5 µL of Specific substrate. The inhibitors used were benzamidine (serine protease inhibitor), STI (soybean trypsin inhibitor), E64, phenanthroline (metallopeptidases inhibator), PMSF (srine protease inhibator), phosphoramidon, cysteine, EDTA (mataloprotease inhibator), leupeptin, chymostatin (Chymotrypsin-like inhibator), TPCK and antithrombin. A control was made without inhibitor. The activity was monitored at 405nm after 1 hour.

17. In Vitro anticoagulation assay

This test was used to evaluate the Activated Partial Thromboplastin (APPT) and Thrombin time (TT) values. This test was carried out in three different situations automatically using a coagulometer with approved methodology for coagulation tests in laboratory. The test was performed at Centro Médico Forjaz Sampaio, in which the movement of magnetic beads allows to calculate the plasma clotting time. It was very important to detect quantitative and qualitative coagulation abnormalities induced by enzyme in plasma constituents.

The assay was performed according manufacturer's instruction and laboratory standards. Were used commercial reagents Cefalin and $CaCl_2$ to induced the human plasma clot. The coagulometer was preheated for 3 min, in the first situation was added to 100 µL of plasma to a cuvette with a metal bead and the coagulation was induced by 10µL of 0.2M $CaCl_2$, when the metal beads stooped the coagulation time was measured in seconds. To compare the time of

clot formation with a standard situation was added 80 µL of plasma and 20 µL of enzyme solution (5µg/µL) to the cuvette with the magnetic bead. Finally added 50 µl of 0.02 M calcium chloride to the mixture in the cuvette and the coagulation time was measured through the magnetic bead movement. After the coagulation the cuvette were incubated at 37˚C and scans were made during 3 hours.

III Results

1. Proteolytic activity of the Bacterial Isolates

The purpose of this research was determining bacterial isolates producing proteases in larger amount. From the 79 isolates tested were chosen the isolates with the largest digestion halo (Figure 1. and Annex Figure 2). From this result were selected 27 isolates with higher proteolytic activity (Figure 1): S115C (1.62 cm), S122C (1,38 cm), S127E (1.17cm), S144B (1.09 cm), S62A (1.06 cm), S25E (1.05 cm), S101C (1.03 cm), S25B (1.01 cm), S150C (1 cm), S157E (1 cm), S99A (0.96 cm), S99D (0.88 cm), S61B (0.78 cm), S140E (0.75 cm), S52D (0.75 cm), S109A (0.75cm), S117E (0.71 cm) S97B (0.7 cm), S125B (0.68 cm), S26A (0.65 cm), S88A (0.62 cm), S144D (0.6 cm), S62D (0.59cm), S178B (0.58 cm), S54D (0.57cm), S137B (0.5 cm), S148A (0.5 cm)

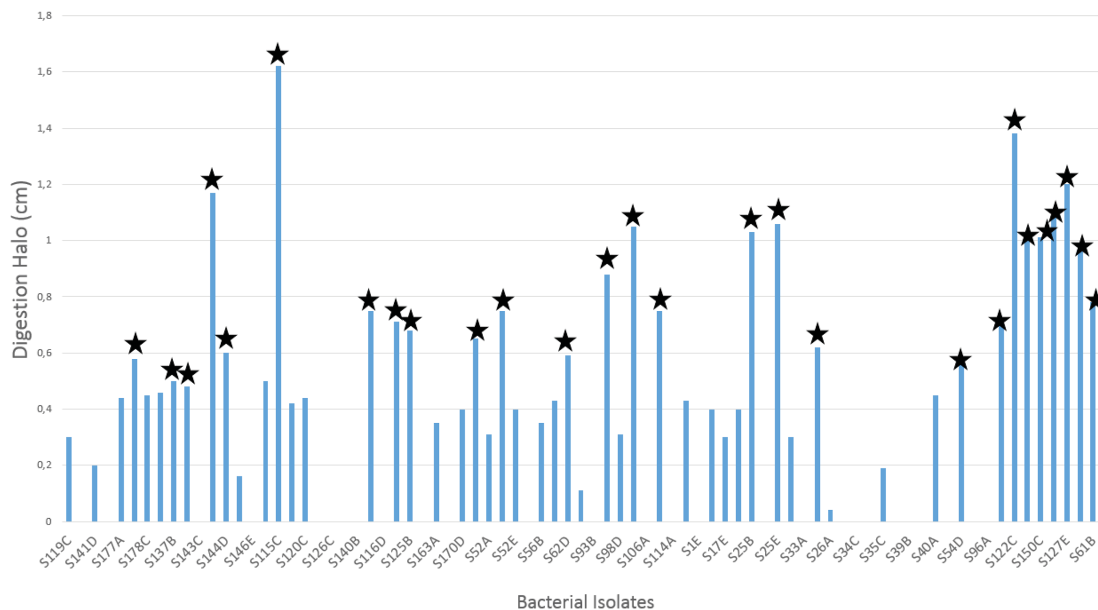


Figure 1: Agar casein plate assay. Casein digestion halos in centimeters (cm) from a total of 79 bacterial isolate. The plates were incubated 24hrs at 37 ºC.

2. Screening of fibrinolytic activity

The fibrinolytic activity was detected by plate method with the appearance of digestion around halos. In total 14 showed fibrinolytic activity but

we selected the 11 with more activity. Therefore the radius of halos in cm was measured in each sample and compared with Urokinase as a positive control. Were select 14 isolates with higher fibrinolytic activity (≥1.5 cm) with: S122C (2.28 cm), S150C (2.31), S157E (2.24), S178C (1.9 cm), S115C (1.5 cm), S62A (2.12 cm), S99D (2.54 cm), S101C (1.91 cm), S88A (2.33 cm), S26A (2.28 cm) and S97B (2.01 cm) (Figure 2, Annex: Figure 2).

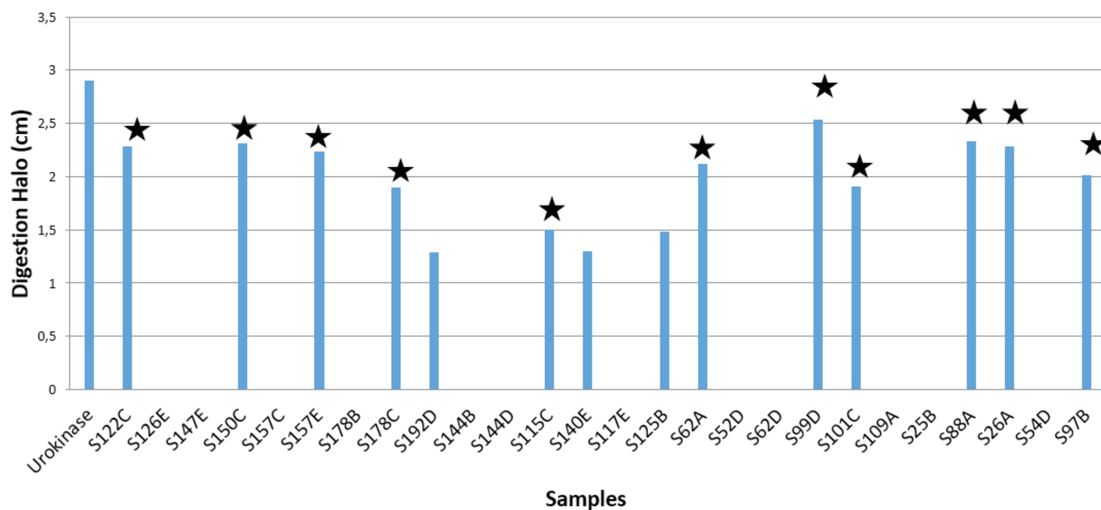


Figure 2: Fibrinolytic activity of the 27 samples. Fibrin digestion halos in centimeters (cm) in agarose fibrin plate. After incubation for 16 hrs at 37˚C.

3. Thrombolytic Activity in vitro

The enzymes that present higher fibrinolytic activity were tested for thrombolytic activity. The clot digestion was measured by reduction of clot size ant the release of hematocytes in the well (the increment of color in the well).

It is observed that in first hour of incubation the samples S122C, S157E, S97B and S150C present high digestion. After 3 hours we can see that S115C, is starting to digest the clot by the shape of the clot and the color of the well (Figure 3). Looking at the rest of the isolate we can not see a significant diference in the blood clots shape.

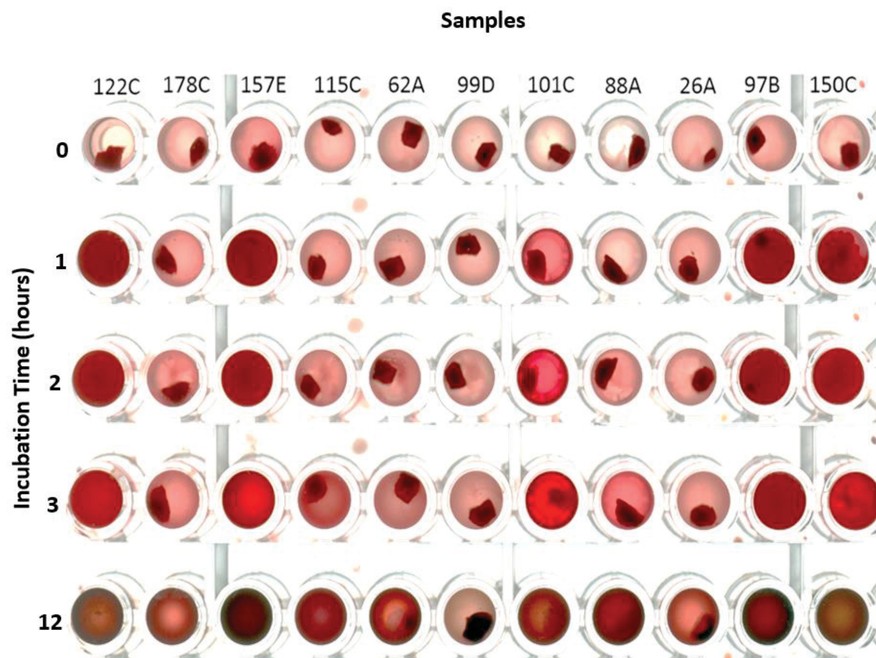


Figure 3: 96-well Microplate with enzymatic digestion of blood clots throughout the incubation period at 37˚C.

4. Genetic identification of isolates based on PCR-ARDRA profile

In order to identify the groups of bacillus the 16S rRNA of all bacterial strains used in this work was amplified and a restriction enzyme profile was obtained (Wu et al., 2006).

It was possible to amplify the 16S gene from all isolates yielding a band of 1500 bp, which corresponds to the size of the expected fragment. Were detected 3 different restriction profiles (Figure 4.A) one presenting fragments of 600 bp, 220 bp and 180 bp, another one with 550 bp, 220 bp and 180 bp fragments and another group with fragments of 450 bp, 280 bp and 230 bp.

According to Wu et al., 2006 the first group correspond to Bacillus group I witch include 3 fragments of 600 bp, 220 bp and 180 bp. Bacillus Group II has a similar profile to group I, except the largest fragment is 550 bp. The restriction profile of Group VII contain a fragment of 450 bp, a fragment with 280 bp and a fragment with 230 bp (Figure 4.B)

The ARDRA profile (Figure 4, B) genetic Bacillus group I, corresponds to the species of *B. cereus, B. anthracis* or *B. thuringiensis*, in Bacillus group II we can find *B. mycoides* and *B. weihenstephanesis*. The genetic profile of group VII corresponds to species of bacteria like *B. subtilis*, *B. pumilus*, B. *amyloliquefaciens* or *B. atrophaeus*.

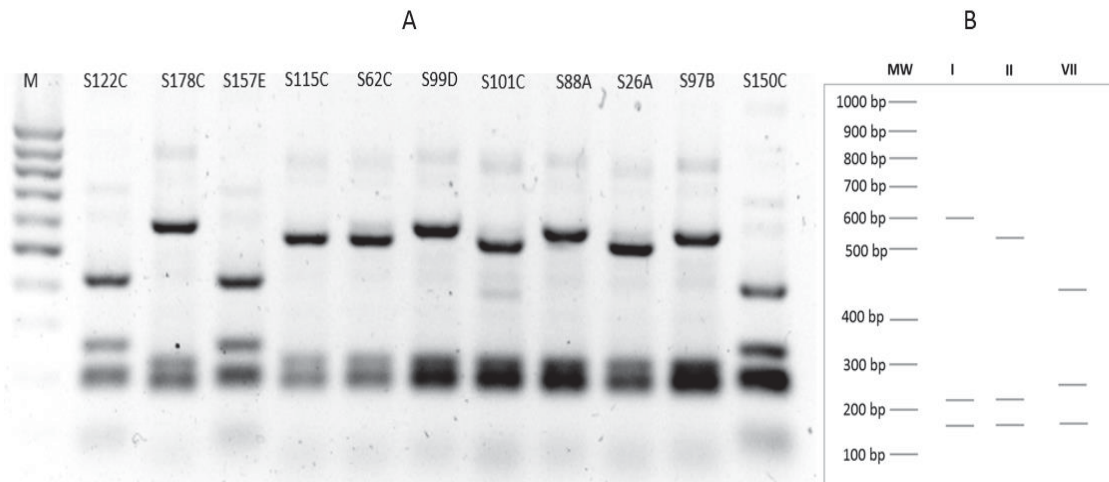


Figure 4: Electrophoresis gel with different restriction profile ARDRA by AluI. A) Pattern of different isolates digested with AluI. B) Three different 16S rRNA restriction profiles by AluI.

Table 1: The three different genetics groups of 11 isolate

Group	Isolate	Bacillus sp.
I	S178C, S99D, S88A, S97B	*B. cereus* and *B. anthracis*
II	S115C, S101C, S62C, S26A	*B. mycoides* and *B weihenstephanesis*
VII	S157E, S122C, S150C	*B. subtilis, B. pumilus, B. atrophaeu* and *B.amyloliquefaciens*

.

5. Optimal Temperature and pH

Of the three Bacillus groups studied so far we want to select one isolated from each group. The selection of these isolates had as parameters the fibrinolytic activity and its activity in the physiological conditions of the human organism, pH 7.5 and temperature 37˚C (Figure 6). pH and temperature assays were very important to help choose the right isolate with fibrinolytic activity in human physiological conditions. The optimal pH for the most isolates was pH 7.5 (Figure 5).

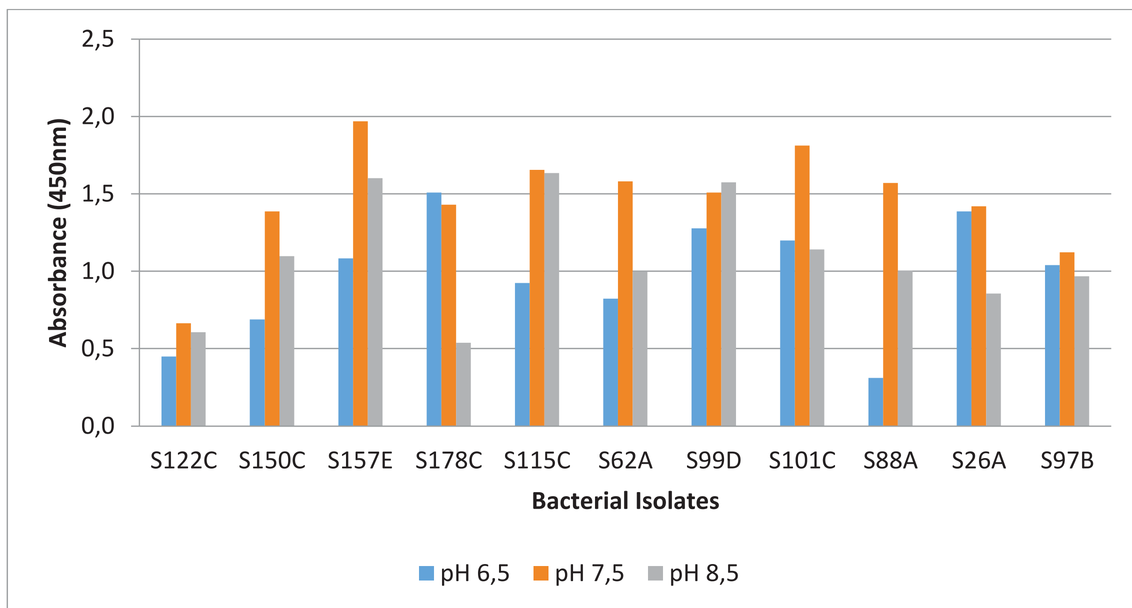


Figure 5: Effect of pH on the enzymatic activity with generalist substrate azocasein.

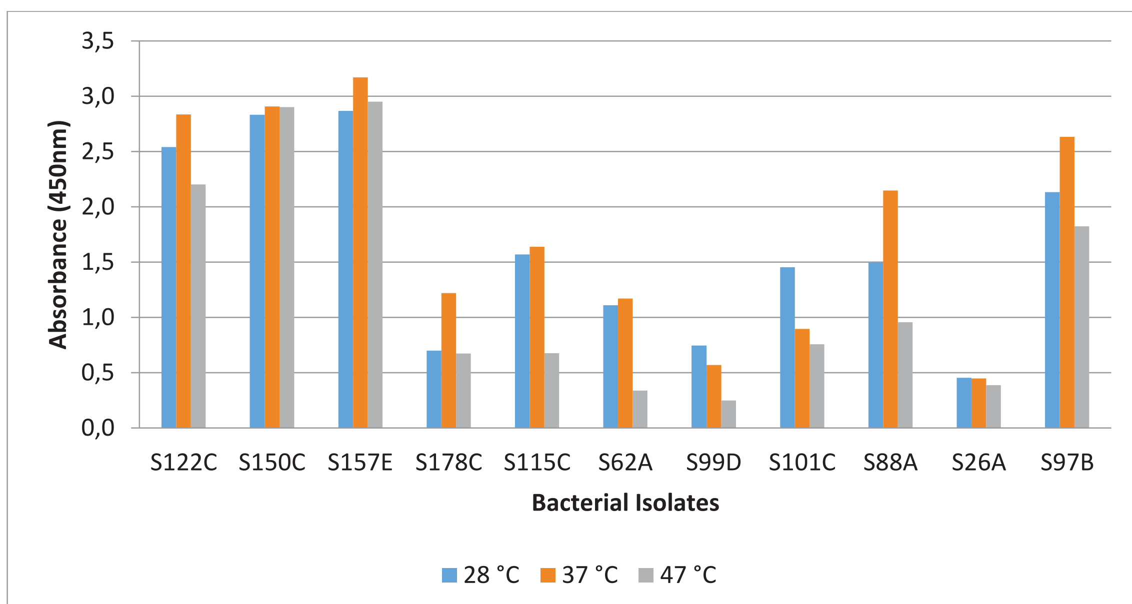


Figure 6: Efect of temperature on the enzymatic activity with generalist substrate azocasein.

The bacterial isolates with a better activity at a pH7.5 were S122C, S150C, S157E, S115C, S62A S101C, S88A and S97B (Figure 5). In terms of temperature, the isolates with a better activity at 37˚C were S122C, S157E, S178C, S115C, S62A S101C, S88A and S97B. S99D AND S101C had an optimal temperature 28˚C (Figure 6).

6. Hemolytic activity

For the hemolytic activity (Figure 7, Annex: Figure 3), was used a Columbia CNA Agar plate with Sheep Blood Medium and the activity was detected by a presence of a clear halo around the wells. The positive control used was MilliQ H2O, which causes lysis of hemocytes through osmotic movement of water into the interior of cells, the diameter was about 4 cm. As for the most of the samples the hemolytic halo was between 0.5 cm and 2.1 cm.

It was found that two enzymes, S115C and S101C had small smudge, indicating that this two enzymes does not cause red blood cells lysis. Curiously this 2 enzymes correspond to the same bacillus group (*B. Mycoides*). Moreover previous tests showed that S115C and S101C had fibrinolytic activity, becoming the best choices for the further investigation.

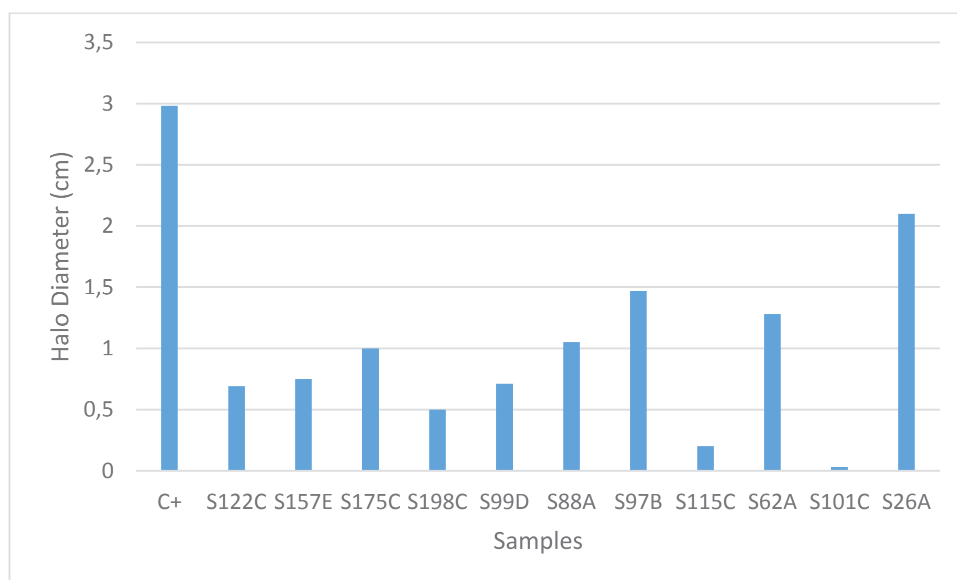


Figure 7: Hemolytic assay: hemolytic halos in centimeter (cm) after a incubation of 4 hour at 37˚C. the positive control (C+) was distillated water.

7. Influence of S115C and S101C on clotting time and plasma clot lysis

The assay of the activated partial thromboplastin time (aPTT) and Prothrombin time (PT) allows to investigate the enzyme effect on the clotting time and the action on the plasma clot. No significance differences in PT were showed between control and respective cuvettes containing enzyme S115C and S101C.

In aPTT test the objective was to evaluate the effect of enzyme on the coagulation time, and both enzymes were applied at the start of the test, acting during the process of coagulation. It was found that the cuvettes containing enzyme S101C presented an accentuated increase in activated partial thromboplastin time, 55 seconds, S101C caused alteration in the intrinsic and common pathway of coagulation. Thus S115C didn’t show a significant variation clotting time was 35 seconds.

In the PT assay both enzymes didn’t show significates differences, prothrombin time of S115C and S101C were, respectively, 13 seconds and 13,2 seconds didn’t disturbed the extrinsic pathway of coagulation.

S115C values are inside the reference values for healty humans. The reference range of the aPTT is 30-40 seconds and the reference range of the PT is 11-14 seconds.

The cuvettes with the clot and the enzyme were incubated at different times at 37˚C, the plasma clots showed some changes in their shape and size during the incubation period, reaching smaller after 3 hours of incubation. The biggest reduction on the plasma clots was seen in the cuvette with S115C (Figure 8).

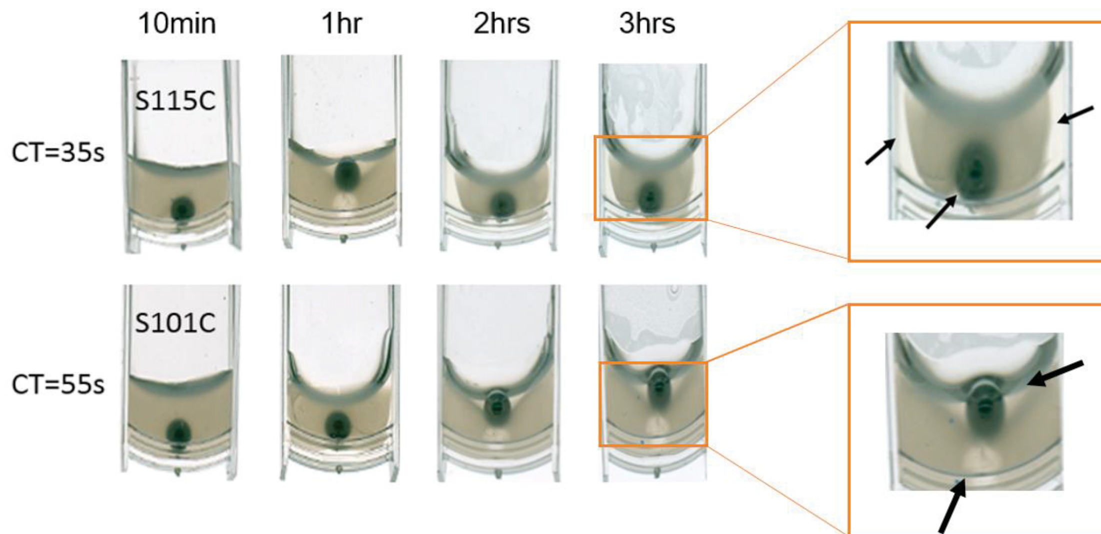


Figure 8: Clotting time and plasma clot lysis during the incubation period (10min, 1hr, 2hrs and 3 hrs). S115C and S101C were applied after clotting enzyme; CT-clotting time. (→) plasmatic clot digestion

8. Plasminogen activation of S101C and S115C

To compare the plasminogen activation we created two different situations. the first one the well was supplemented with plasminogen the other one the well didn't have plasminogen, it was found that the well with plasminogen the enzyme digestion was enhance with the presence of S101C while S115C didn't show significant difference as expected. This result indicates that S101C is a great plasminogen activator, increasing meaningfully the fibrinolytic activity (Figure 9). Fibrinolytic enzyme activity of S101C and S115C was detected by a fibrin 96 well plate method. S115C showed more lytic activity, thus S101C is a great plasminogen activator.

At this point of the work we selected enzyme S115C for its fibrinolytic, hemolytic and thrombolytic activity this enzyme didn't altered the normal and healthy coagulation pathway as confirmed with aPTT, PT test and euglobulin lysis time. S115C was named BmK.

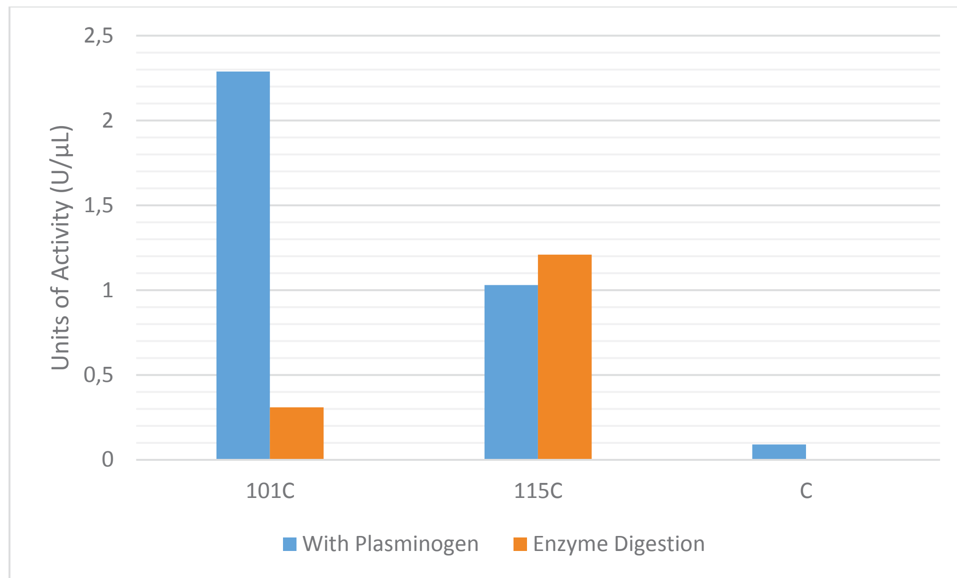


Figure 9: Plasminogen activator and fibrinolytic activity of S101C and S115C after 2 hrs incubation at 37˚C. Control (c) just with plasminogen.

9. In vitro Thrombolytic Activity of BmK

We tested BmK's ability to degrade the clot through the examination of clot fragments size, a photo was taken to the clots in 30min intervals (Figure 10). BmK digest the whole blood clot in 90 min while the negative control didn't digest the blood clot as espected.

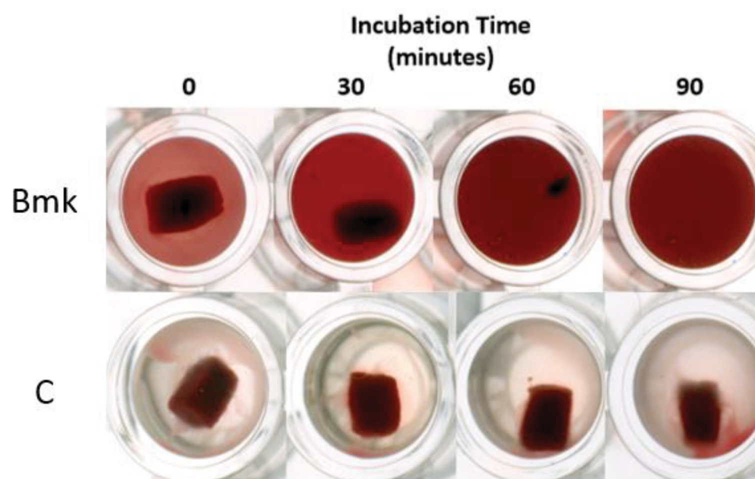


Figure 10: 96-well Microplate with enzymatic digestion of blood clots throughout the incubation period. BmK (30μg); C - negative control with 0.1 M phosphate buffered saline solution.

10. Enzyme Purification

During the fractionation process, it was found that the enzyme of interest has an alkaline isoelectric point, pH was above 8. In the first chromatography, CaptoQ. The fractions were collected and concentrated in peaks, each peak was tested with a 96-well plate method with fibrin. Was identified one peak with a higher enzymatic activity in exclusion, this peak was named CQP1 and the second peak eluted also showed activity.

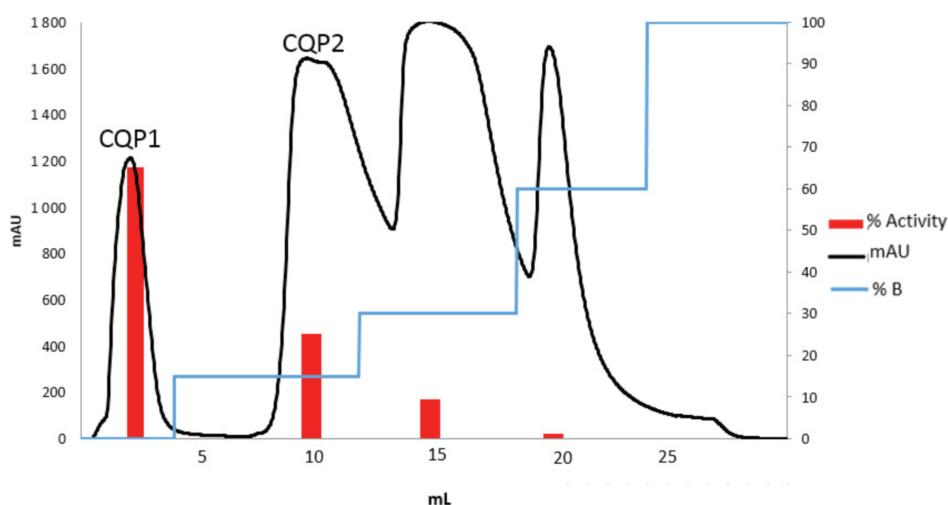


Figure 11: Anion-exchange chromatography with Capto Q column. mAU - Absorbance; % B- salt gradient; % activity of Fibrinolytic activity.

The peak with higher activity obtained in capto Q (CQP1) was injected in a anionic exchange chromatography using a HitrapQ column (Figure 12). In this chromatography separation was obtained two clear peaks of protein. The first peak (HQP1) eluted in the exclusion showed low activity, in the last peak (HQP2) showed 65% of fibrinolytic activity (Figure 12). The fraction corresponding to the peak containing the protein of interest, at pH 8.8, is positively charged and was retained in the stationary phase with a moderate ionic strength and left the column when the eluent gradient increased.

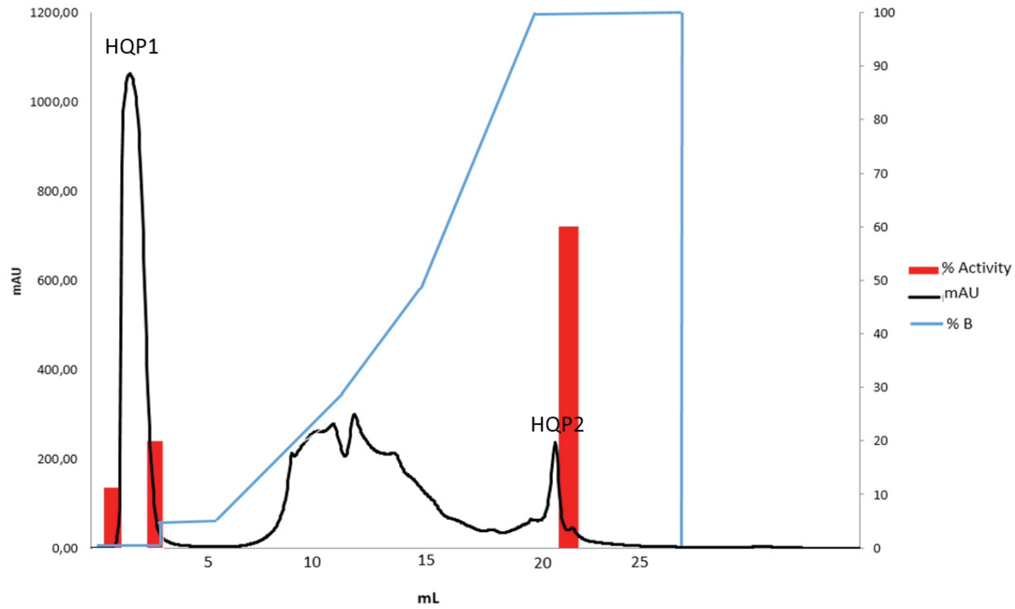


Figure 12: Anion-exchange chromatography HitrapQ column. mAU - Absorbance; % B- salt gradient; % activity of Fibrinolytic activity.

The HQP2 from HitrapQ was injected in a MonoQ anionic exchange column. After testing all the fractions was selected the #16 and #17 that belong to the last peak (MQP6). #16 and #17 had, respectively 58% and 42% of fibrinolytic activity (Figure 13). #16 and #17 were pooled and used for SDS-page and Zymogram.

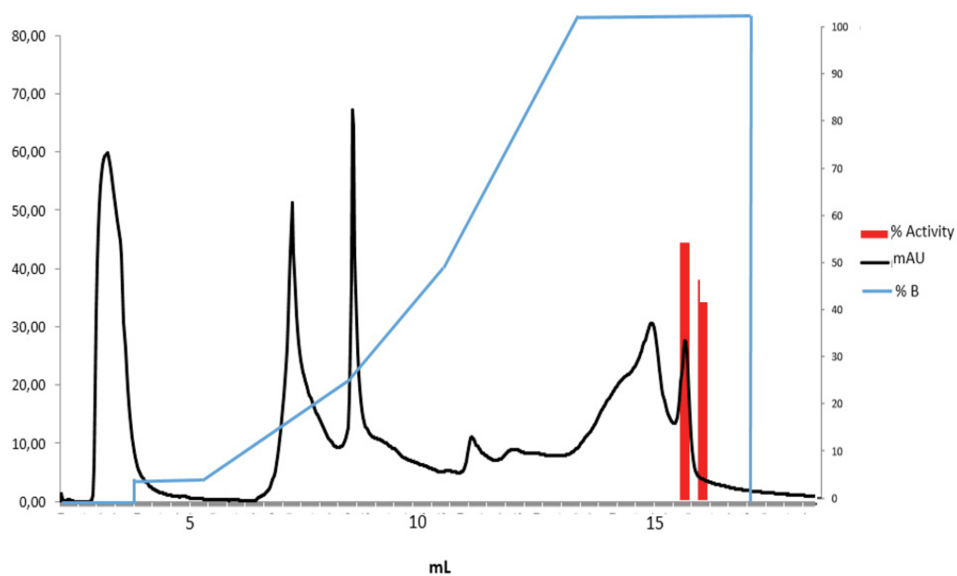


Figure 13: Anion-exchange chromatograpgy MonoQ column. mAU - Absorbance; % B-salt gradient; % activity of Fibrinolytic activity.

Table 2: Purification table of BmK

Sample	mL	mg/mL	[mg]	Total act. (U)	Specific act. (U/mg)	Purification Factor
Bmk	1,5	57,6	83,4	483	6	
CQP1	0,8	6,03	4,824	201	42	7
HQP2	0,7	0,585	0,409	258	631	109
MQP6	1	0,08	0,08	279	3488	698

11. Zymogram and SDS-page of Purified Enzyme.

The zymogram of chromatography fraction of BmK enzyme revel a digestion band higher than 135 kDa and another band at 75kDa. SDS-page of fraction revel 3 bands one at 37 kDa, one at 75 kDa and another higher than 180kDa (Figure 14). The band with 75kDa and the band with 200 kDa in the SDS-page were the ones with activity according to the zymogram (Figure 14.A) these two bands were cut and sent to Protein Mass Spectrometry Analysis (Ms/Ms).

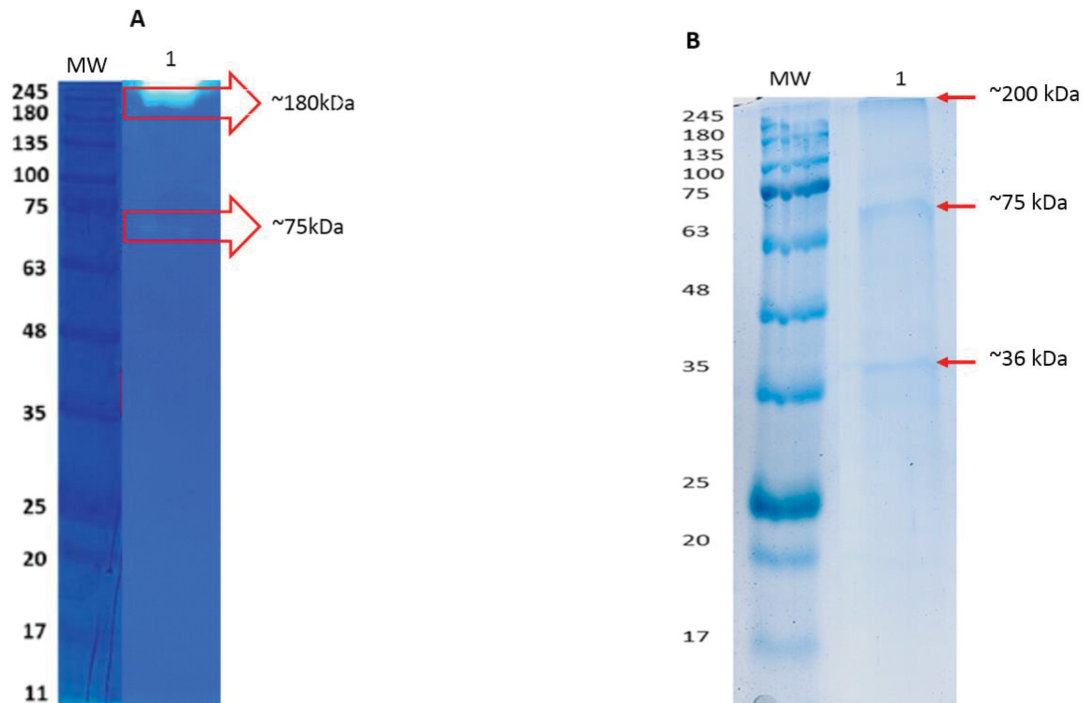


Figure 14: A Zymography of the chromatography BmK Enzyme (1) **B** SDS-page of the chromatography fraction. MW- molecular weight; 1-Chromatography fraction of BmK Enzyme.

12. Euglobulin lysis time S115C.

The influence of BmK enzyme on the coagulation time or the lysis time. We had the same test running in different situations. The first one as positive control the test was performed under normal conditions without the addition of enzyme, the second situation was applied the enzyme S115C fractionated and in the third situation Urokinase was added to the clot. To see if the enzyme Bmk interfere with intrinsic coagulation pathway was made a pre incubation of 10 min with enzyme and euglobulin before adding thrombin e calcium.

In the first situation we can see that in 350 minutes the Euglobulin clot started to decrease, as expected according to the a normal references for euglobulin clot lysis time, at 500 minutes the clot disappeared completely. In the situation designated by BmK the fractionate S115C enzyme was present and the absorbance increase, which is a sign of clot formation, however after 15 min started digesting slowly the clot and at 255 min the digestion of the clot was complete, which indicates the euglobulin clot lysis. These results

confirm once again the fibrinolytic activity of this enzyme, which acts upon the fibrin fibers reducing the time of the euglobulin clot lysis (Figure 15).

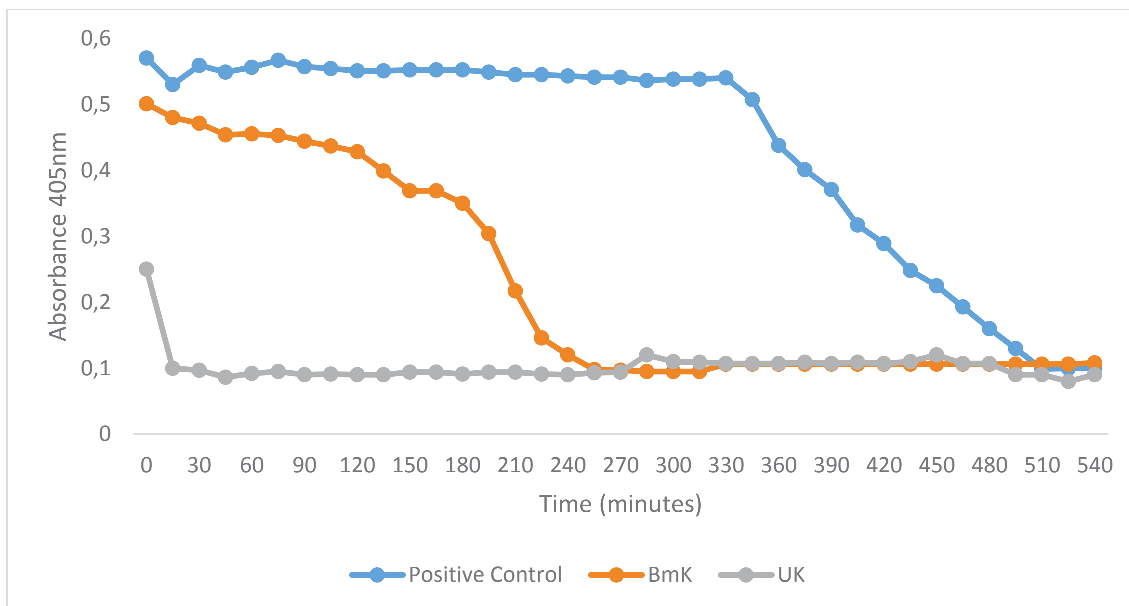


Figure 15: The euglobulin lysis time in different situations. Normal situation (Positive control); BmK enzyme fraction application at start of test (BmK); Urokinase control (UK). Incubation during 550 minutes at 37˚C.

In the situation were Urokinase UK was added it was found that there wasn't a big increase in absorbance, although in 15 min the absorbance was at 0.1 nm, this fact indicates that the clot was already dissolved.

13. Biochemical characterization of S115C

13.1 Effect of Ions and Solvents on enzyme activity

In the assay azocasein to different ions (Figure 16), it was found that $MnCl_2$, NaCl and $CaCl_2$ ions contribute to the increase of enzyme activity, however the optimal enzyme cofactors is Manganese, since this reaches an activity increase of around 95%, while that Calcium and Sodium contributes only a slight increase in activity. All other ions cause an inhibition of the activity, $NiCl_2$ inhibits the enzymatic activity in 37%, and $CuSO_4$ inhibit the activity of around 31%, MgCl and KCl inhibits less than 15%, HgCl2 does not interfere with the normal enzyme activity.

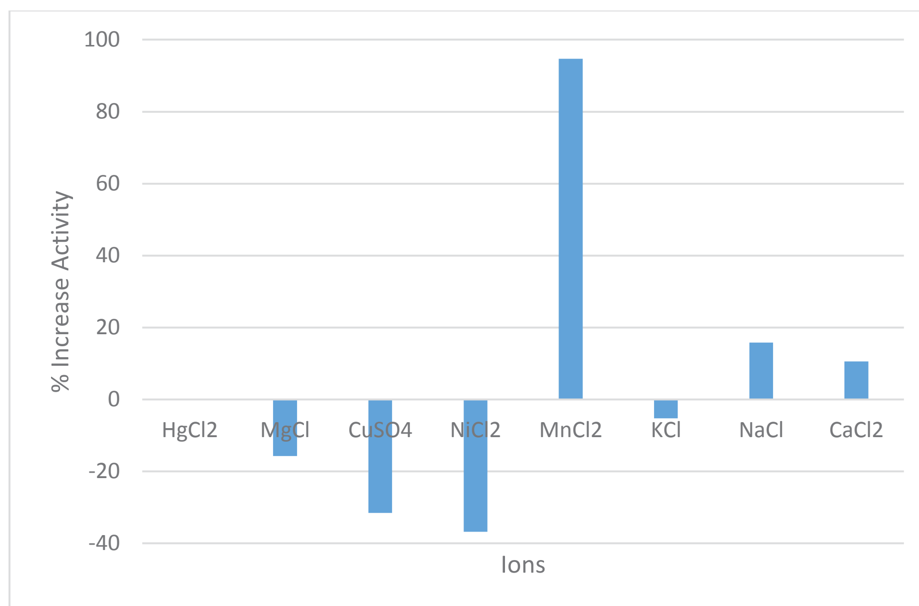


Figure 16: Effect of ions in enzyme activity with the substrate general azocasein. Incubation for 3 hour at 37˚C.

With regard to solvents, SDS was the only solvent that didn't interfere with the activity, however β-mercaptoethanol inhibits completely the enzyme activity. Urea, Chaps Triton and DTT showed a strong inhibitory effect (>60%) (Figure 17). SDS is an ionic detergent the enzyme is not stable in presence of nonionic solvents and denaturant agents.

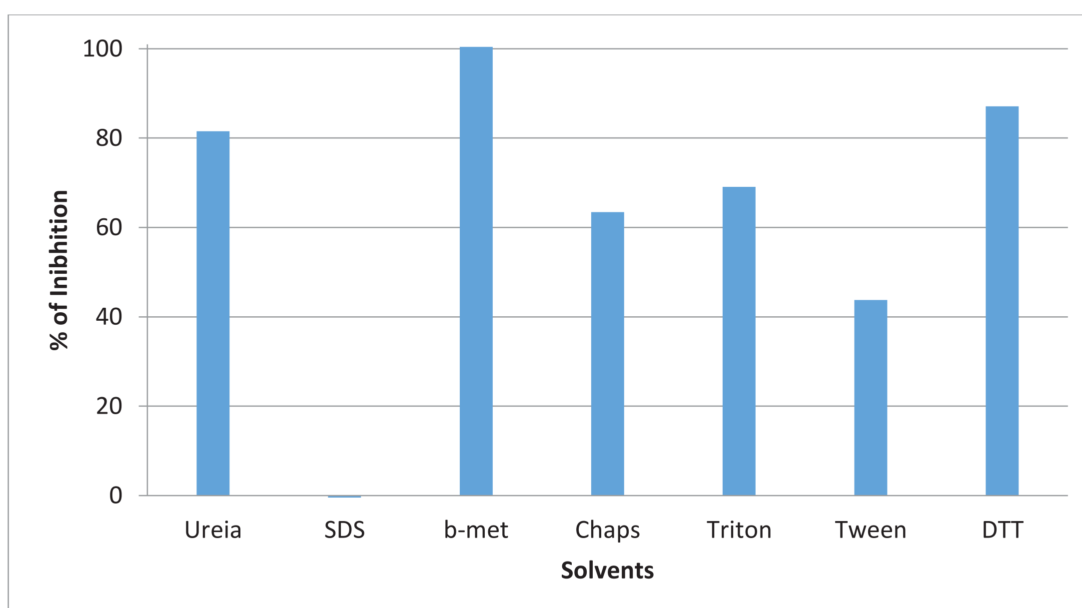


Figure 17: Effect of solvents in enzyme activity with substrate general azocasein. Incubation for 3 hrs at 37˚C.

13.2 Substrate specificity

Different synthetic chromogenic substrates were used to screen the enzyme specificity. The enzyme has shown greater specificity for the Suc-Ala-Ala-Pro- Phe-pNA substrate whose absorbance value increase (Figure 18). The substrate is specific for chymotrypsin, confirming again that enzyme in question belongs to the family of serine proteases of the chymotrypsin.

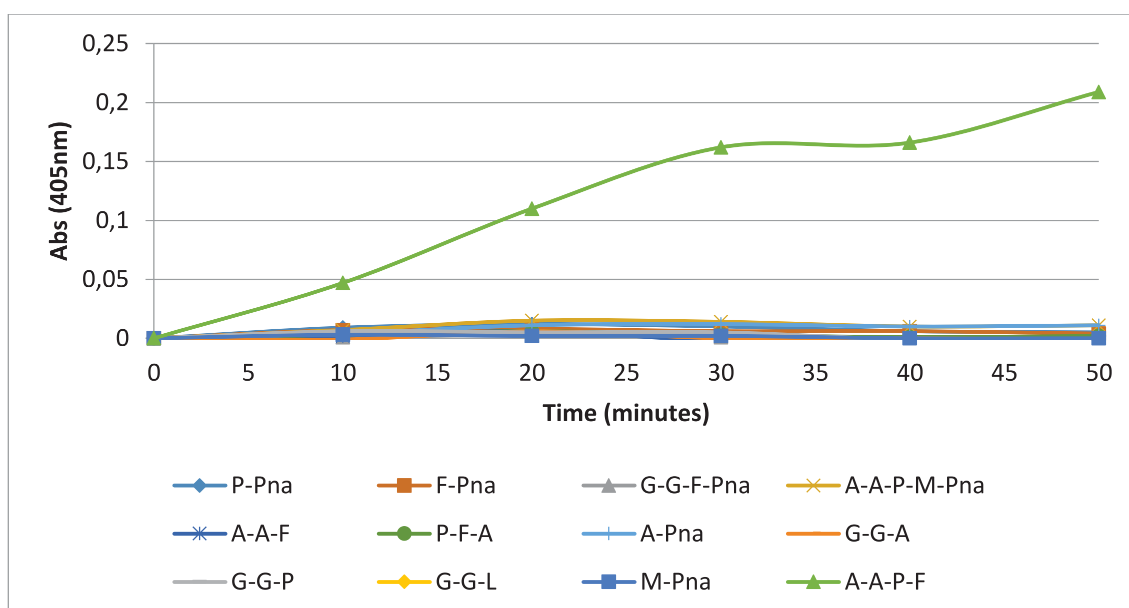


Figure 18: Enzyme activity with different specific substrates. Incubation for 1 hr at 37˚C.

13.3 Effect of Inhibitors in enzyme activity

Benzamidine, STI, Chymostatin and TPCK were the inhibitors with highest percentage of inhibition of the enzyme BmK 100%, 100%, 93% and 82%, respectively (Figure 19). Benzamidine and STI are inhibitors of serine proteases and trypsin-like enzymes, TPCK is a serine protease inhibitor. The Chymostatin is a strong inhibitor of many proteases, particularly chymotrypsin, serine proteases of the chymotrypsin-like. Since the inhibitor is PMSF large spectrum of action on serine proteases. The metalloprotease inhibitor EDTA showed a inhibitory effect on the enzyme (70%), serine protease inhibitors like PMSF also affect the activity (68%) (Figure 19). The percentage of inhibition is low, below 20%, with antitrombin, since antithrombin is used as a inhibitor of thrombin, plasmin, trypsin and factors IXa, Xa and XIa clotting cascade.

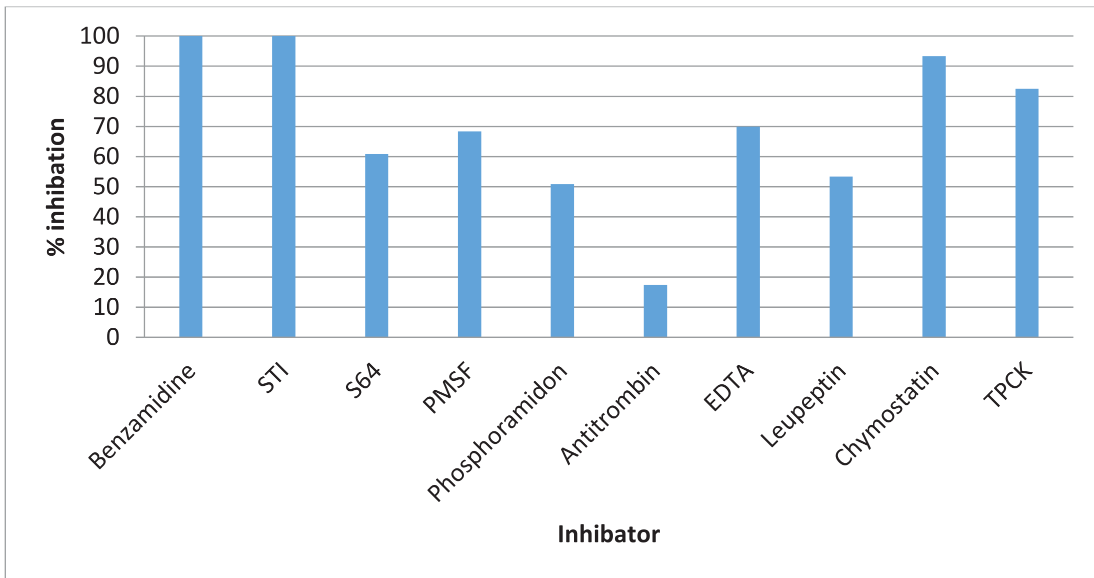


Figure 19: Effect of Inhibitors in enzyme activity with substrate general azocasein. Incubation for 3 hrs at 37˚C.

14. Fibrinolytic activity of BmK

BmK showed a transparent area surrounding the well on the fibrin plate, this area is the lytic area where the fibrin had been degraded into soluble peptides (Figure 20). Was also observed that BmK digest fibrin at a dose dependent ratio and comparing with UK digest fibrin less than 3 times.

Figure 20: BmK enzyme fraction in different concentrations (15μg, 7.5μg, 3.75μg, 1.8μg). Urokinase in different concentrations (5μg, 2.5μg, 1.25μg, 0.62μg). Incubation for 4hrs at 37˚C.

To calculate the activity constant according to Wang et al., 2012, relates to the halo radius of enzymatic digestion with the diffusion time in the fibrin plate (R/√t) for both enzymes, allowing to estimate the activity units of the enzyme of interest from commercial Urokinase of known activity (Table 3). The activity constant obtained for the BmK and the enzyme Urokinase, was 2.77 and 3.31 respectively. The constant value of BmK was 1.25 less then UK value.

Table 3: Calculation of the activity constant for different incubation times and estimating the activity units per ug through known Urokinase

Time (hrs)	BmK	Uk
1	2,81	3,53
2	2,31	2,89
3	2,73	3,24
4	2,81	3,38
Mean	2,77	3,31

.

To investigate the fibrin digestion pattern we made a SDS-page using fibrin that was already digest by 15 µg and 5 µg of BmK. A positive control was made using fibrin digest by 5 µg of Urokinase. This pattern correspond to a incubation of 3 hours. Fibrin has the same structure than fibrinogen the only difference is that fibrin is insoluble. We can see that with Urokinase fibrin were degraded in various bands resulting one high molecular weight band at approximately 40 kDa and various other ranging between 15 and 35 kDa. BmK35 and BmK5 showed a similar digestion pattern, the difference is that with a concentration of 35µg the bands are darker suggesting an enhanced digestion of fibrin (Figure 21).

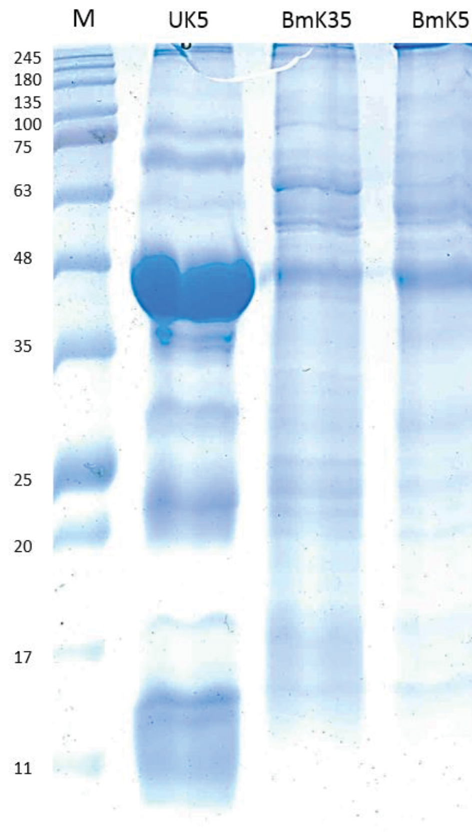


Figure 21: Fibrinolytic SDS-page. M (Molecular Weight) Mix of 5 µg of Urokinase and fibrin (UK5); Mix 35µg of BmK enzyme fraction and fibrin (BmK35). Mix 5µg of BmK enzyme fraction and fibrin (BmK5).

15. Plasminogen activation of BmK

The fibrinolytic activity on the BmK enzyme was compared in the presence and absence of plasminogen. In the presence of plasminogen the lytic area of the Bacillus enzyme was not enhanced (Figure 22). As expected Urokinase that was used as a positive control, its halo degradation was enhance with plasminogen as compared with the well without plasminogen, indication plasminogen activation.

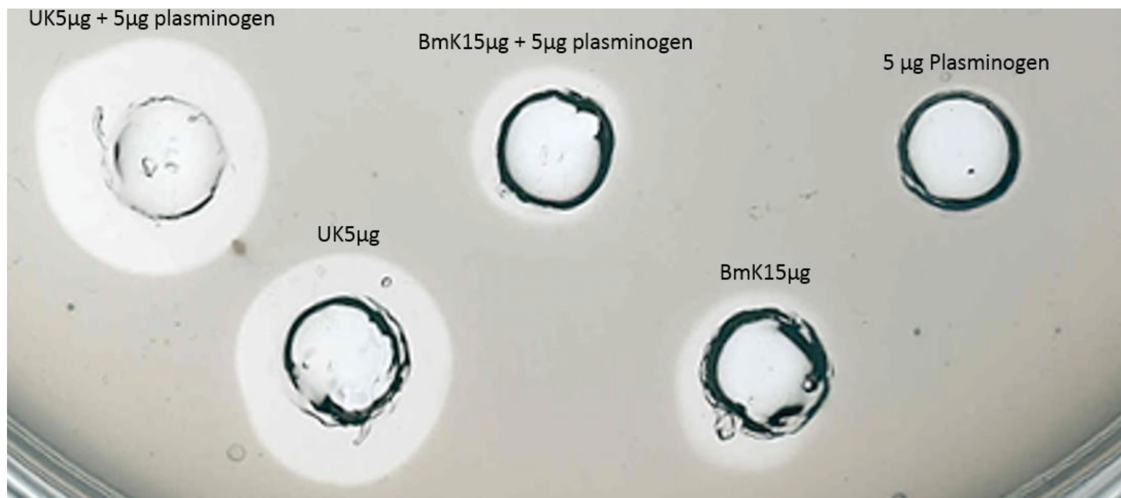


Figure 22: Plasminogen activation assay. Urokinase (UK5µg); BmK enzyme fraction (BmK15µg); Plasminogen (5µg). Mix Urokinase and plasminogen (Uk5µg+5µg plasminogen; Mix Bmk fraction with plasminogen (Bmk15µg+5µgpalsminigen).

16. Fibrinogenolytic activity of BmK

To investigate the interaction between Bmk and fibrinogen was made a separation by SDS-PAGE gel which was charged with a mix of fibrinogen and BmK and this mixture was incubated at different times. A control was made mixing fibrinogen with Urokinase and was incubated in different times (Figure 23).

Fibrinogen consists of α chain of 64 kDa, 57 kDa β chain and the γ chains having 48 kDa molecular weight.

As can be seen, soon after mixing the enzyme with the fibrinogen the degradation of fibrinogen didn't happened. We found that the pattern of fibrinogen, α, β and γ chains, is the same even with 24 hours of incubation. This shows that Bmk has a high specify for fibrin and not for fibrinogen. With the control with Urokinase (UK) we can see at 0 min an instant degradation of α, β and γ chains.

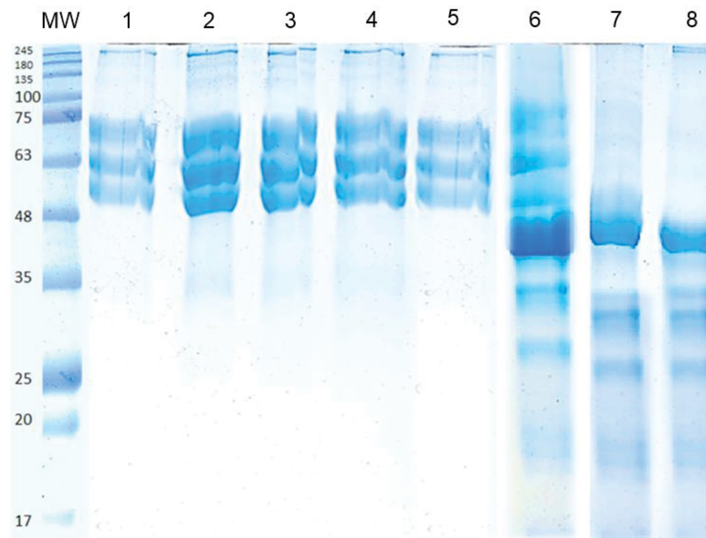


Figure 23: Fibrinogenolytic SDS-PAGE. Fg-fibrinogen. And mix of enzyme and fibrinogen with different incubation periods. MW-Molecular Weight; 1-Fibrinogen; 2 Mix fibrinogen and enzyme 0 min; 3- Mix fibrinogen and enzyme 10 min; 4- Mix fibrinogen and enzyme 40 min; 5- Mix fibrinogen and enzyme 24hrs; 6- Mix fibrinogen and Urokinase 0 min; 7- Mix fibrinogen and Urokinase; 40 minutes; 8- Mix fibrinogen and Urikinase 24hrs.

The UK degradation profile was completely set after a 20 min incubation, disappearing the other two α and β chains of fibrinogen. The degradation profile has diverse bands resulting from the degradation of various fibrinogen chains. One molecular band that after 24 hour still intact was a 48kDa band (γ).

IV Discussion

From the 79 isolates with proteolytic activity 27 were selected with better activity in physiological conditions, pH 7.5 and 37˚C. These 27 bacterial isolates were tested for fibrin activity and 11 show fibrinolytic activity. From this 11 only 5 bacterial isolates presented thrombolytic activity.

After obtained the ARDRA restriction profiles (amplified ribosomal DNA restriction analysis) of 16S rRNA with the enzyme AluI to 11 bacterial isolates. It was possible to distinguish three different genetic groups based on the restriction band profile obtained. The isolates S178C, S99D, S88A and S97B belong to group I, with a characteristic profile of *Bacillus cereus* species belonging to the group containing the species *B. anthraci*, *B. thuringiensis* and *B. cereus*. As for Group II which corresponds to the species *B. mycoides* and *B. weihenstephanesi* belong isolated S115C, S101C, S62C and S26A. For the genetic group VII belong to isolates S122C, S150C and S157E witch correspond to the species *B. subtilis*, *B. pumilus, B. and B. amyloliquefaciens*. Several strains of the species B. subtilis and *B. amyloliquefaciens* are described in other studies as producers of fibrinolytic enzymes.

Previous studies shown that fibrin is a very difficult to digest, only 6% of the isolates studied in this work were capable of digestion a blood clot. It is noted that the three dimensional conformation and the disposition of the fibers of fibrin into a blood clot is different and more complex than the configuration shown an agarose plate comprising only insoluble fibrin. At the end of the clotting cascade which culminates in the activation of thrombin which converts fibrinogen to insoluble fibrin is the activation of other important factors. From the three fibrinogen chains (α, γ, β), only the α and γ chains, undergo cross-linking by factor XIIIa. During clot formation in the early stages of polymerization, cross-linking occurs within the protofibrils, between the various chains, resulting in dimers γ training and the multiple connections between α chains, resulting in formation of α polymers (Collet et al., 2005; Chen and Doolittle, 1969; McKee et al., 1970).

From the fibrinolytic enzymes selected enzymes only 2 didn't presented hemolytic activity. In previous studies physiological abnormal activities has been have been identified, some of them include immunomodulatory and hypocholesterolemic actions, and antitumor, anti-inflammatory, anti-allergic, anticoagulation, and antithrombin activity as well as fibrinogenolysis stimulation (Lu et al., 2010; Wang et al., 1995). After hemolytic assay were chosen two *Bacillus mycoides*, S115C and S101C. This enzymes that curiously belongs to the same Bacillus group specie, didn't cause red bool cells lysis proving to be capable of digest blood clots without interfere with blood function.

A thrombolytic test was made to investigate the ability of S115C and S101C to degrade blood clots with all its constituents present and the tests is used to evaluate the influence of the enzyme in clot digestion. After analyzing the results obtained in the test, it was found that S115C enzyme was able to completely degrade a blood clot after 90 minutes, contrasting with S101C which didn't digest the clot, blood clot stayed intact.

The analysis of the results obtained in the plasminogen activation assay, it is concluded that S115C enzyme is capable of degrading the fibrin directly without activation of plasminogen, otherwise S101C like Urokinase is a plasminogen activator (Veljkovic, DK. *et al*. 2009). S115C enzyme which digest directly the fibrin fibers similar to Nattokinase (Kotb, 2013).

Activated partial thromboplastin time (aPTT) allows to determinate the blood plasma coagulation time. With this assay we evaluate the effect of S115C and S101C on clotting time and subsequently their action on the plasma clot. After analyzing the results, it was found that the S115C enzyme did not affect the normal clotting time, and later caused a slight deterioration of plasma clot. With these results it was concluded that S115C enzyme does not interfere with the coagulation process unlike S101C that elongated significantly the normal clotting time.

In this work we had always in consideration finding a enzyme that didn't interfere with the human biological function.

S115C was chosen not just for its high specify for fibrin digestion but also its no specify for fibrinogen. The fibrinogenolytic activity of the S115C enzyme (Bmk) was determined, and the purpose was to investigate whether the enzyme is capable of degrading the fibrinogen chains α, β and γ (McKee et al, 1970). A positive control with Urokinase was made to compare the digestion patterns.

The enzyme BmK didn't degraded fibrinogen α, β and γ chains, even after a 24 hour incubation. So BmK enzyme is able to digest easily fibrin but don't digest fibrinogen which is in its soluble state, however its insoluble fibrin fibers degradation capacity is high. With the positive control UK the digestion of α and β chains was instant, in 0 min we have a digestion pattern.

The fact that BmK didn't showed fibrinogenolytic activity is beneficial to future enzyme applications, because the intravenous application of the enzyme will not interfere with fibrinogen concentration in the blood. Patients don't have the risk of bleeding problems (Karlsson, et al., 2002).

It was also possible to evaluate the effect of the enzyme in the Euglobulin Lysis Time. In this case, what is being assessed is the time that elapses from the clot formed until lysis. The enzyme was applied before euglobulin clot induction. In this situation Bmk was mixed with euglobulin and a pre incubation was made for 5 min, next was added calcium and thrombin, it was found that the plasma clot actually occur. It was concluded that the enzyme hasn't degraded fibrin as it was being formed by thrombin and calcium and the euglobulin clot formed. Euglobulin clot started to disappeared around 380 min thus euglobulin clot with BmK started to been digest at 30 min

It is noted that in Euglobulin Lysis Time method, unlike APTT method, the amount of involved fibrin or is produced is in very minor amounts, allowing for greater sensitivity to the assay as the proportion of enzyme and substrate are more balanced. These results confirm once again the fibrinolytic activity of this enzyme, which acts reducing the time of the euglobulin clot lysis.

For the characterization of S115C enzyme (BmK) were studied the effect of the various ions / solvents and inhibitors on enzyme activity and was determinate the specific substrates of the enzyme. The optimum operating

temperature of BmK enzyme is approximately 37°C which is the physiological temperature of the human body. The enzyme exhibits maximum activity in neutral pH, with an optimum pH of 7.5, very close to the pH of human blood, indicating that this enzyme will offered a good activity if administered by intravenous injection. For the purposes of the ions on the enzyme activity, it was found that MnCl2, NaCl and $CaCl_2$ ions contribute to increase the activity, however the optimal enzyme cofactors is manganese, since this achieves a greater increase in activity while sodium and calcium only accounts for a slight increase in activity.

It was found that Benzamidine, STI, Chymostatin and TPCK were the ones highest percentage of inhibition of the enzyme BmK 100%, 100%, 93% and 82%, respectively. Benzamidine and STI are inhibitors of serine proteases and trypsin-like enzymes, TPCK is a serine protease inhibitor. The Chymostatin is a strong inhibitor of many proteases, particularly serine proteases of the chymotrypsin-like. Chymostatin and PMSF were those who had higher percent inhibition of the BmK enzyme. The Chymostatin is a strong inhibitor of many proteases, particularly chymotrypsin, serine proteases of the chymotrypsin-like, chymases and lysosomal cysteine proteases such as captesinas B, H and L. The results are quite informative to BmK enzyme, indicating that it belongs to the family of serine proteases of the chymotrypsin (Barret et al., 2013).

For the test on the specific substrates, it was found that the enzyme has degraded only one substrate, with greater specificity with the Suc-Ala-Ala-Pro-Phe-pNA substrate, these substrate is specific for chymotrypsin and cathepsin G, confirming again that BmK enzyme in question belongs to the family of serine proteases of the chymotrypsin. (Barrett et al., 2013) Serine proteases are characterized by the presence of a catalytic triad composed of the residues of the amino acids aspartic acid (electrophile), serine (nucleophile) and histidine (base). The serine acts as a very reactive residue (nucleophile) forming a covalent bond with the substrate. The optimum catalytic activity of these enzymes is set at pH values between 7 and 9 (Perona, et al., 1995).

Through the fibrin plate assay it was possible to estimate the fibrinolytic activity of the BK enzyme from commercial Urokinase. Both enzymes were

applied to the fibrin plate, BmK was applied with a concentration of 15µg and Uk was applied with 5µ. The calculation of the activity constant was calculated and Urokinase activity is higher than BmK, as expected. Urokinase activity constant was 3.31 and BmK constant was 3.31 less 1.25 units than UK.

With Urokinase we can see a faster digestion of fibrin by activation of plasminogen, in the other hand it's known that is a very expensive enzyme and have some clinical disadvantages, after intravenous administration there is a significant risk of bleeding and allergic reaction (Yang, et al., 2012). Bmk showed its capacity of digest directly fibrin unlike the biggest part of commercial fibrinolytic enzymes that are plasminogen activators (t-PA). Also this bacillus sp. enzyme didn't interfere with normal coagulation pathway and blood functionality as confirmed with aPPT and PT test, hemolytic test and fibrinogenolytic test.

V Future Works

The biggest part of thrombolytic agents such as Urokinadse, steptokinase, genetically tissue-type plasminogen activators (t-AP), have also been widely applied in thrombolytic treatments, but they seem not to be affective because of many undesirable side effects. To be specific, the patient may be vulnerable to resistance to reperfusion, occurrence of acute coronary reclusion, allergic reaction and bleeding complication (Bode, et al., 1996). Furthermore both streptokinase and urokinase dependent and act by the activation of plasminogen to plasmin (indirect action), making it important to direct the further research for the discovery of novel agents that are able to act directly on fibrin (Caramelli et al., 1992). The limited efficacy and undesired side effects of these thrombolytic agents pose problems for their clinical application. Much research is made in order to overcome these problems by seeking increasing the thrombolytic activity of the target or for improving targeting of these proteins on the clots.

Therefore, the findings for new save and inexpensive fibrinolytic enzyme is very essential. BmK don't interfere with normal coagulation pathway and blood functionality as confirmed with aPPT and PT test, hemolytic assay and fibrinogenolytic activity assay. This chymotrypsin-like protease demonstrated a

good activity in physiological conditions and its ability of digest directly fibrin without activation plasminogen. Future works in vivo with BmK would be an important step towards a new potential enzyme with pharmacological applications.

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VII Annex

Microorganism	Food	Name of enzyme	References
B. natto, NK	*Natto*, Japan	Nattokinase	Fujita et al. (1993)
B. amyloliquefaciens DC-4	*Douchi*, China	Subtilisin DFE	Peng et al. (2003)
Bacillus sp. CK	*Chungkook-jang*, Korea	CK	Kim et al. (1996a, b)
Bacillus sp. DJ-4	*Doen-jang*, Korea	Subtilisin DJ-4	Kim and Choi (2000)
Bacillus sp. DJ-2	*Doen-jang*, Korea	bpDJ-2	Choi et al. (2005)
Bacillus sp. KA38	*Jeot-gal*, Korea	*Jeot-gal* enzyme	Kim et al. (1997)
B. subtilis QK02	Fermented soybean	QK-1 and QK-2	Ko et al. (2004)
Bacillus firmus NA-1	*Natto*	–	Seo and Lee (2004)
B. subtilis IMR-NK1	*Natto*	–	Chang et al. (2000)
Bacillus sp.	*Tofuyo*, Japan	SMCE	Fujita et al. (1993)
Katsuwonus pelamis	Skipjack, Japan	Katsuwokinase	Sumi et al. (1995)
Bacillus sp.	*Kimchi*, Korea	Bacillus protease	Noh et al. (1999)
Armillaria mella	*Armillaria mella*	Neutral metalloprotease	Kim and Kim (1999)
Bacillus sp. KDO-13	Soybean paste, Korea	–	Lee et al. (2001)

Figure 1: Bacilli from traditional food (Kotb, 2012).

Microorganism	References
Bacilli	
B. subtilis BK-17	Jeong et al. (2001)
B. subtilis A1	Jeong et al. (2004)
B. subtilis 168	Kho et al. (2005)
Actinomyces thermovulgaris	Egorov et al. (1976)
Streptomyces	
S. megasporus SD5	Chitte and Dey (2000, 2002)
S. spheroids M8-2	Egorov et al. (1985)
Streptomyces sp. Y405	Wang et al. (1999a)
Fungi	
A. ochraceus 513	Batomunkueva and Egorov (2001)
Cochliobolus lunatus	Abdel-Fattah and Ismail (1984)
F. oxysporum	Tao et al. (1997, 1998)
Fusarium pallidoroseum	El-Aassar (1995)
P. chrysogenum H9	El-Aassar et al. (1990)
Pleurotus ostreatus	Choi and Shin (1998)
R. chinensis 12	Xiao-Lan et al. (2005)
Algae	
C. intricatum	Matsubara et al. (1998)
C. latum	Matsubara et al. (1999)
C. divaricatum	Matsubara et al. (2000)

Figure 2: Sources of microbial enzymes (Kotb, 2012).

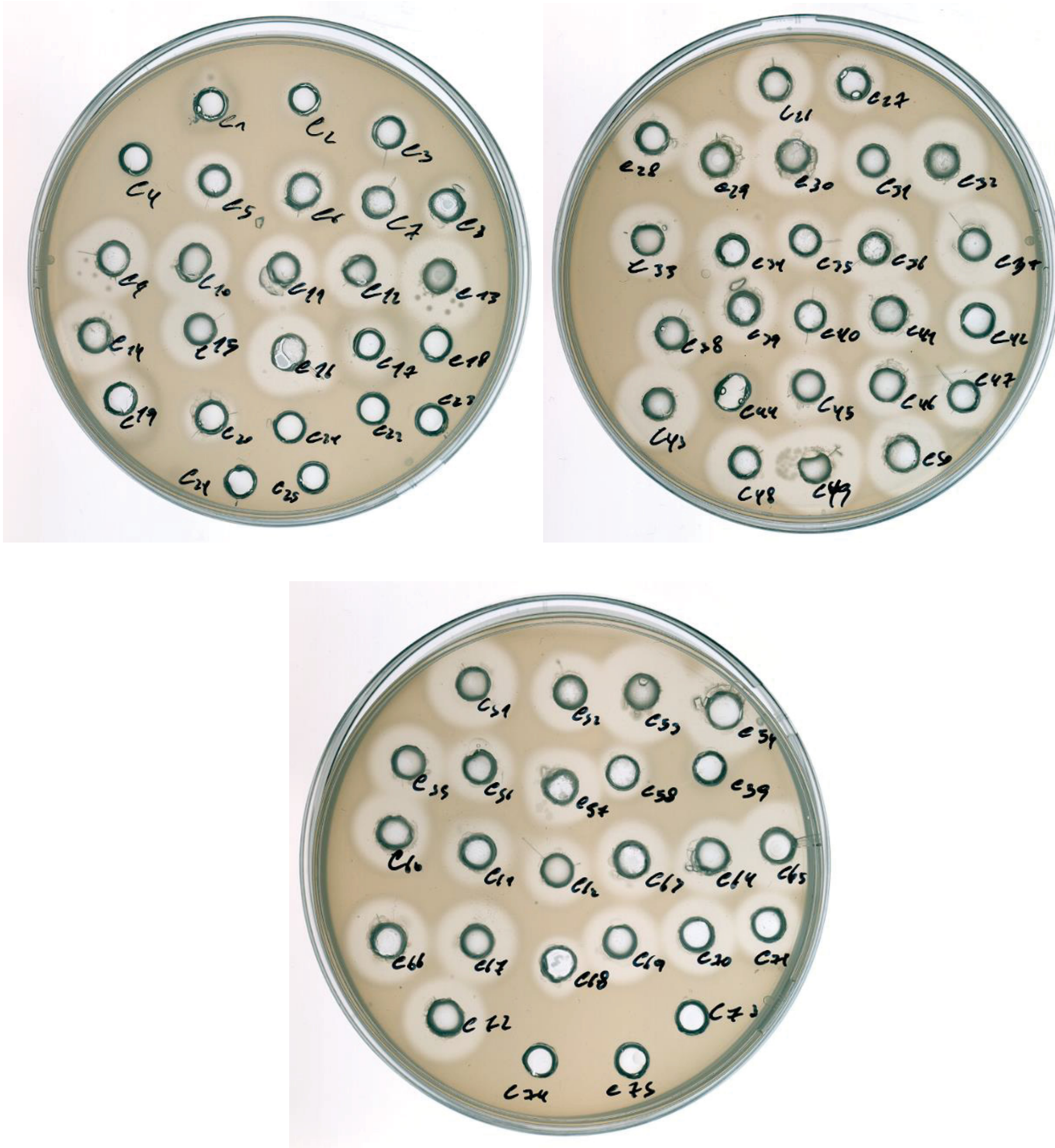

Figure 3: Agar Casein Plates. plates were incubated 24hrs at 37 ºC.

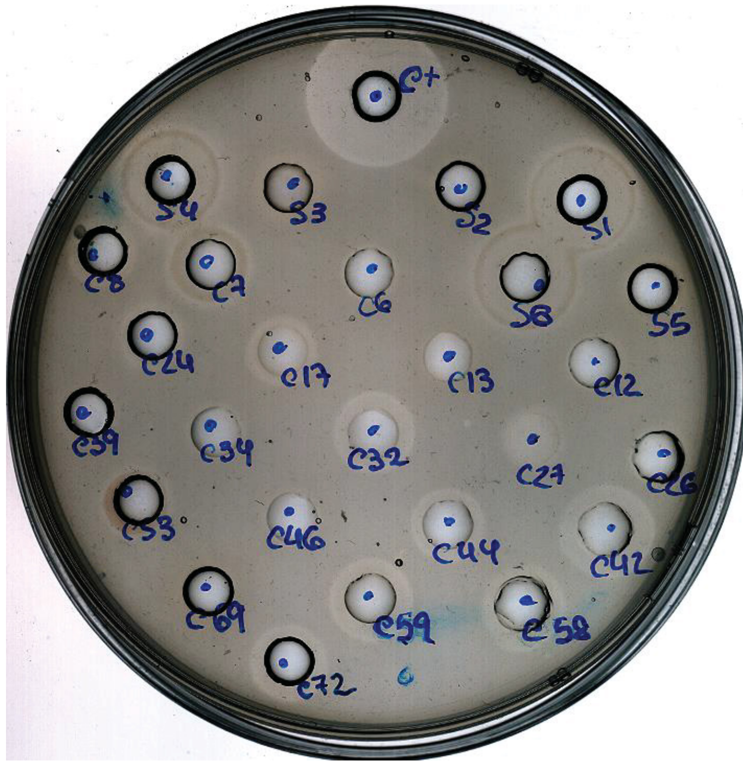

Figure 4: Agarose Fibrin Plate. The plates were incubated 16 hrs at 37 ºC

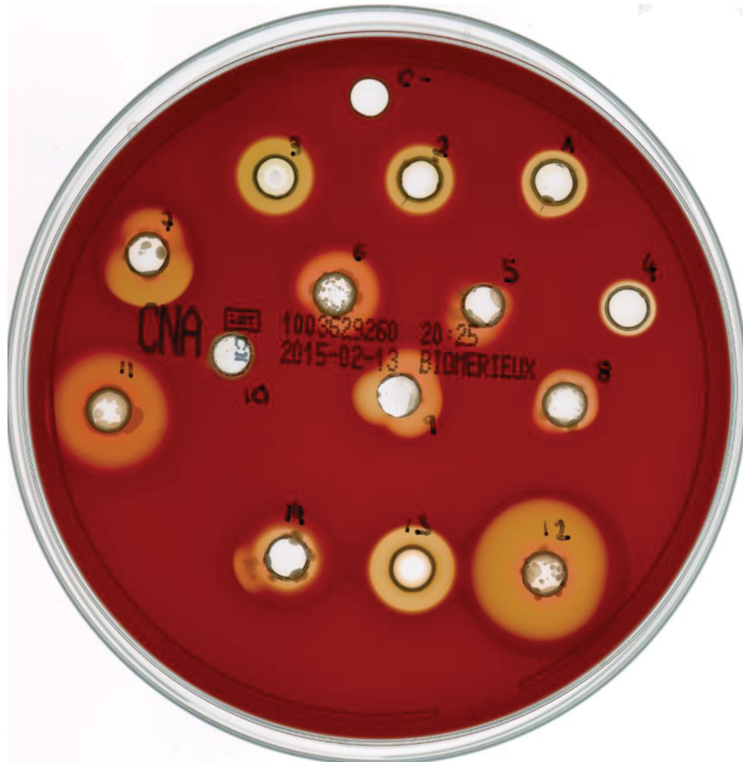

Figure 5: Hemolytic assay plate (CAN plate with sheep blood). Incubation for 6 hrs at 37˚C.