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Research paper

Fibrin(ogen)olytic and antiplatelet activities of a subtilisin-like protease from *Solanum tuberosum* (*St*SBTc-3)

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ABSTRACT

Plant serine proteases have been widely used in food science and technology as well as in medicine. In this sense, several plant serine proteases have been proposed as potential anti-coagulants and anti-platelet agents. Previously, we have reported the purification and identification of a plant serine protease from *Solanum tuberosum* leaves. This potato enzyme, named as *St*SBTc-3, has a molecular weight of 72 kDa and it was characterized as a subtilisin like protease. In this work we determine and characterize the biochemical and medicinal properties of *St*SBTc-3. Results obtained show that, like the reported to other plant serine proteases, *St*SBTc-3 is able to degrade all chains of human fibrinogen and to produces fibrin clot lysis in a dose dependent manner. The enzyme efficiently hydrolyzes β subunit followed by partially hydrolyzed α and γ subunits of human fibrinogen. Assays performed to determine *St*SBTc-3 substrate specificity using oxidized insulin β-chain as substrate, show seven cleavage sites: Asn3-Gln4; Cys7-Gly8; Glu13-Ala14; Leu15-Tyr16; Tyr16-Leu17; Arg22-Gly23 and Phe25-Tyr26, all of them were previously reported for other serine proteases with fibrinogenolytic activity. The maximum *St*SBTc-3 fibrinogenolytic activity was determined at pH 8.0 and at 37 C. Additionally, we demonstrate that *St*SBTc-3 is able to inhibit platelet aggregation and is unable to exert cytotoxic activity on human erythrocytes *in vitro* at all concentrations assayed. These results suggest that *St*SBTc-3 could be evaluated as a new agent to be used in the treatment of thromboembolic disorders such as strokes, pulmonary embolism and deep vein thrombosis.

1. Introduction

Proteolytic enzymes (peptidases), usually known as proteases, are important enzymes which play a significant role in various biochemical mechanisms to maintain metabolic processes of all organisms [1]. These enzymes, belonging to group 3 (hydrolases) and subgroup 4 (hydrolases of peptide bonds) (E.C.3.4.), carried out the hydrolysis of peptide bonds [2,3]. The enzyme subclass of proteases (EC 3.4) is in turn divided into seven catalytic classes according to their catalytic mechanism. Includes proteases named as serine, cysteine, aspartic, glutamic, metallo, and threonine, with a seventh group including peptidases with unidentified mode of action [3].

Several proteases, including serine proteases, interfere with haemostasis and act either as procoagulants or as anticoagulants [4–6]. According to their action mechanism, thrombolytic agents are classified in two types: one is plasminogen activator, such as tissue type plasminogen activator (t-PA) and urokinase; the other is the plasmin-like protein, e.g. nattokinase [7] and lumbrokinase [8] which can directly degrade the fibrin of blood clots, thereby dissolving the thrombi rapidly and completely [9]. However, these enzymes are expensive and patients may suffer from undesirable side effects such as gastrointestinal bleeding, allergic reaction, and

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resistance to repercussion [10,11]. Therefore, antithrombotic, anticoagulant and thrombolytic reagents from various sources have been investigated [12].

Plant serine proteases have been widely used in food science and technology as well as in medicine; however, few plant serine proteases have found a place in industry [13]. Like their counterparts in mammalians, snakes and microorganisms, plant serine proteases have been proposed as potential anti-coagulants and anti-platelet agents. In this sense, Siritapetawee et al. [14] have proposed the potential use of AMP48 (a serine protease from *Artocarpus heterophyllus*) as antithrombotic for treatment thromboembolic disorders such as strokes, pulmonary embolism and deep vein thrombosis. Additionally, Fonseca et al. [15] have purified and characterized a serine protease with fibrinogenolytic activity from *Euphorbia milii* latex. The potential use in medicinal treatment, for example for thrombosis has been suggested for a serine protease with fibrinogenolytic activity and high stability from *Euphorbia cf.* latex named as EuP-82 [1]. Choi et al. [16] have reported the isolation and characterization of a new bifunctional fibrinolytic serine protease from the green algae *Codium fragile* named as Codiase, with thrombolytic, anticoagulant, and antiplatelet activities. Also, the purification of a chymotrypsin-like fibrinolytic serine protease from *Petasites japonicus*, has been reported by Kim et al. [17].

Previously, we have reported the purification, identification and biochemical characterization of a potato subtilisin-like protease, named as *St*SBTc-3 [18]. In this study we report the ability of *St*SBTc-3 to degrade human fibrinogen and fibrin *in vitro*. Additionally, results obtained demonstrate that this potato protease inhibits platelet aggregation induced by collagen. All these results and the fact that *St*SBTc-3 is not cytotoxic to human erythrocytes *in vitro*, suggest a potential industrial and therapeutic applications for this protease.

2. Experimental section

2.1. Purification and identification of StSBTc-3

*St*SBTc-3 was purified from intercellular washing fluid of potato leaves following the method previously described by Fernandez et al. [18]. Briefly intercellular washing fluid of potato leaves was obtained by vacuum infiltration-centrifugation procedure with infiltration buffer (50 mM HCl-Tris (pH 8), 3.5% (w/v) NaCl, 0.1% (v/v) 2-mercaptoethanol and 20 15 μL/15 ml of Tween20). Then, a two-step chromatographic procedure was employed to purify *St*SBTc-3 from the fluid. The first step of purification was an Ion-exchange chromatography on Mono-Q HR 5/5. The column was equilibrated in 50 mM sodium acetate (pH 5.2) and eluted with a linear gradient of 0–500 mM NaCl at 1 mL min^{-1} flow rate. In the second step, the fractions with specific activity were concentrated and loaded into a Superose 12 10/300 size exclusion column (GE Healthcare GE, 17-5173-01) and eluted with 0.05 M sodium acetate buffer (pH 5.2), 0.3 M NaCl using a flow rate of 0.5 mL min^{-1}. The peak at 75 kDa correspond to *St*SBTc-3 as reported previously in our lab by Fernandez et al. [18]. *St*SBTc-3 purity and identity was confirmed digesting the *St*SBTc-3 purified fraction [18] by trypsin and following by MALDI-TOF-mass spectrometry and subsequently MS/MS analysis. The resulting spectra was used to search for matching proteins in the NCBI database (http://www.ncbi.nlm.nih.gov) using the Mascot search program (http://www.matrixscience.com, Matrix Science, London, England) and the potato genome database Solgenomics (solgenomics.net). As described by Fernndez et al. [18], MALDI-TOF mass spectra and the sequences of the three tryptic peptides were obtained. BLASTp and tBLASTn searches against the potato genome database show 100% of identity with a subtilisin-like protease from *Solanum tuberosum* (Transcript and protein ID: PGSC0003DMT400027148 and PGSC0003DMP400018521 respectively).

2.2. Protein concentration determination

Protein concentration was measured by the bicinchoninic acid method (BCA) [19], using bovine serum albumin for the standard calibration curve. Briefly, 25 μl of protein solution was mixed with 200 μl of BCA reagent. The mixture was incubated at 37 C for 30 min and then measured in a microplate Reader ELx800 (BioTek Instruments) at a wavelength of 540 nm.

2.3. Thrombin clotting time (TCT)

TCT was measured as described by Ignjatovic [20] using an IL ACL 200 Coagulation Analyzer, Instrumentation Laboratory (USA) with the TCT assay kit provided by Wiener Labs (Argentina) following the manufactures instruction. 100 μl of poor-platelet plasma were incubated with different amounts of *St*SBTc-3 (from 0 μM to 1 μM), during 2 h at 37 C.

2.4. Determination of StSBTc-3 fibrinogenolytic activity

Fibrinogenolytic activity of *St*SBTc-3 was tested as described by Siritapetawee et al. [1] using a modified method of Satake et al. [21]. The reaction mixture was composed of 40 μg of human fibrinogen and 2 μM of *St*SBTc-3 in a final volume of 100 μl. The reaction mixture was incubated at 37 C for 2 h. The reaction was stopped by adding 40 μl of 10% (w/v) trichloroacetic acid (TCA). The digested peptides were collected by spinning the mixture at 12.000 × g for 20 min. The peptide concentration of the supernatant was measured using the BCA assay (Subsection 2.2). Control assay was performed using the reaction mixture without enzyme. One unit of enzyme activity was determined as the amount of enzyme required to produce an increase in absorbance of 0.01 at 540 nm min^{-1} (37 C).

2.5. Specificity of fibrinogenolytic activity of StSBTc-3

Subunits of human fibrinogen digested by *St*SBTc-3 were analyzed by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS) using a method from Rajesh et al. [22] with slight modifications. Fresh human plasma from healthy informed volunteers was used to obtain fibrinogen enriched fraction as described by Zamora González [23]. 40 μg of fibrinogen enriched fraction were incubated with several amounts of *St*SBTc-3 (from 0 μM to 3.2 μM) at 37 C during 2 h in a final volume of 50 μl. The reaction was stopped by adding 20 μl of denaturing buffer containing: 1 M urea, 4% (w/v) SDS and 4% 2-Mercaptoethanol and heated at 95 C during 5 min. All proteins were analyzed by SDS-PAGE using 15% (p/v) acrylamide gel [24]. Gels were stained with colloidal Coomasie Brillant Blue G250 [25]. Densitometric analysis of the SDS-PAGE gel bands were performed using Image J software [26]. The same methodology was used to perform the time-dependant assays using 3 μM of StSBTc-3 varying the incubation times from 0 min to 300 min.

2.6. Specificity studies of StSBTc-3 proteolytic activity

Oxidized β-chain (5 mg/ml) was incubated with purified *St*SBTc-3 in 0.1 M formic (pH 3.1). After 1, 3 and 24 h at 37 C, the reaction mixtures were centrifuged and the peptide fragments were separated by reverse-phase HPLC using Vydac C18 column, HPLC model Providence, (SHIMADZU) software LCSolution. The chromatography was carried out at room temperature and the column was

equilibrated with 0.1% Trifluoroacetic acid (TFA). The peptides were eluted with a linear gradient of acetonitrile (0–80%) in 0.1% TFA at a flow rate of 1.5 mL min^{-1}. Amino acid composition and *N*-terminal amino acid sequencing were performed to characterize the isolated peptides. *N*-terminal amino acid sequences were determined by mass spectrometry using a MALDI-TOF-TOF spectrometer, Ultraflex II (Bruker).

2.7. Effects of pH and temperature on fibrinogenolytic activity

The optimal pH was examined varying the pH of the reaction mixture between 3 and 12. The buffers used were 25 mM sodium acetate (pH 3–5), 25 mM sodium phosphate (pH 6–7), 25 mM Tris-HCl (pH 8–9), and 25 mM glycine-NaOH (pH 10–12). Human fibrinogen substrate was dissolved in the respective buffers. The optimal pH of *St*SBTc-3 on fibrinogenolytic activity was assayed following the method described in Section 2.4. The optimal temperature was determined in the temperature range of 10 C–95 C and the fibrinogenolytic activity of *St*SBTc-3 was assayed following the method described in 2.4.

The pH stability was determined by pre-incubating *St*SBTc-3 with respective pH buffers (pH range from 3 to 12) at 37 C for 24 h. After incubation, the activity of the treated enzyme was determined by the method described in 2.4 at optymum conditions. The temperature stability was determined by pre-incubating *St*SBTc-3 at several temperatures (range from 10 C to 95 C) for 24 h. Then the activity of treated enzyme was assayed following the method described in 2.4. at optymum conditions.

All determinations described above were done in triplicate. The residual activity of the previously treated sample was evaluated according to the standard condition (Section 2.4) taken as 100%.

2.8. Fibrinolytic activity by microplate clot lysis assay

The decrease in clot turbidity of fibrin polymer by *St*SBTc-3 was examined in a 96-well plate in triplicate using an absorbance microplate Reader ELx800 (BioTek Instruments) at a wavelength of 405 nm. The reaction mixture was prepared as described by Choi et al. [16] with different amounts from 0.5 μM to 3 μM of *St*SBTc-3 in a final volume of 100 μl.

2.9. Haemolysis assay

Haemolysis assays were performed as described by Muñoz et al. [27]. Fresh human red blood cells (hRBC) were rinsed in PBS and centrifuged for 10 min at 800 × g three times and resuspended in PBS to a final erythrocyte concentration of 4% v/v. The hRBC suspension (100 μl) was added to a 96-well microtiter plate and incubated with different concentrations 0.5 μM; 1 μM; 2 μM; 3 μM of *St*SBTc-3 in PBS buffer. Controls of zero and 100% haemolysis consisted of hRBC suspended in PBS and 1% Triton X-100, respectively. The suspensions containing hRBC were incubated with agitation for 1 h at 37 C. The samples were centrifuged at 800 × g for 10 min, and the release of hemoglobin was monitored by measuring the absorbance of the supernatant at a wavelength of 550 nm in a microplate Reader ELx800 (BioTek Instruments).

2.10. Preparation of washed human platelets

Rich-platelet plasma (RPP) was obtained from fresh human plasma from healthy informed volunteers according to Marx et al. [28]. Platelets were separated by centrifugation of RPP at 800 × g for 15 min and then washed twice with 3.8 mM pH 6.5 Hepes buffer containing: 137 mM NaCl; 2.7 mM KCl; 1 mM $MgCl_2$; 5.6 mM glucose; 0.35% bovine serum albumin and 0.4 mM EDTA. The washed platelets were resuspended in 3.8 mM pH 7.4 Hepes buffer. The platelets were counted by Coulter Counter (Coulter Electronics, USA) and adjusted to a concentration of 3.10 platelets ml^{-1}.

2.11. Determination of human platelet aggregation

Platelet aggregation was measured using an Aggregometer 700 (Chrono Log Corp, USA) as previously described by Rho et al. [12]. Briefly, washed platelets (3.10 platelets ml^{-1}) were incubated at 37 C in the with or without 0.2 μM of *St*SBTc-3 for 3 min in the presence of 1 mM $CaCl_2$, then platelet aggregation was induced by addition of collagen 5 mg/ml. The resulting aggregation, measured as the change in light transmission, was recorded for 3 min.

2.12. Statistical analysis

All data are the mean ± standard deviation of at least three experiments. Statistical significance of the results was evaluated using ANOVA, and Bonferroni or Dunnet *t*-test as post-test. All P values were two-sided. All statistical analyses were performed in GraphPad Prism 6 for Windows Version 6.01, September 21, 2012.

3. Results and discussion

3.1. Fibrin(ogen)olytic activity

In order to elucidate the ability of *St*SBTc-3 to degrade human fibrinogen *in vitro*, assays were performed according to the described in Subsection 2.5. Several amounts of *St*SBTc-3 were incubated for 2 h with human fibrinogen and the degradation products were analyzed by SDS-PAGE 15% [24]. Human fibrinogen is composed of two sets of three polypeptide chains termed as α, β and γ subunits. The approximate molecular weights of α, β and γ chains are 63, 56 and 47 kDa respectively. The three chains are joined by disulfide bonds within the *N*-terminal of *E*-domain [29,30]. As illustrated in Fig. 1, all α, β and γ chains of fibrinogen were susceptible to cleavage by *St*SBTc-3 at all concentrations assayed in a dose dependent manner. This result is consistent with previous results reported for plant serine proteases with fibrinogenolytic activity, which are able to degrade fibrinogen in a dose dependent manner [14,1]. Differences in the fibrinogenolytic cleavage specificity between *St*SBTc-3 and other plant serine proteases with fibrinogenolytic activity [31,14] were observed in time dependent assays (Fig. 2) Results showed in Fig. 2 demonstrate that *St*SBTc-3 completely hydrolyze β subunit of human fibrinogen and partially hydrolyze α and γ subunit. This result is in disagree with the data reported by Siritapetawee [14] to a serine protease with human fibrino(geno)lytic activities from *Artocarpus heterophyllus* latex named as AMP48. Authors demonstrate that this protease digest α chain of fibrinogen first and γ subunit thereafter [14]. Like with AMP48 and unlike *St*SBTc-3, Choi et al. [16] reported the ability of a serine protease with fibrinogenobrinolytic activity from the marine green algae *Codium fragile*, named as Codiase, to target α-chain of fibrinogen first followed by γ- and β-chains. Preferences in the digestion of fibrinogen chains of AMP48; Codiase and *St*SBTc-3 make a difference between these plant proteases and proteases with fibrinogenolytic activity from other sources. α-fibrinogenase from different sources including snake venom [32], *Lampetra japonica* [33], Codium species [34,35], *Paecilomyces tenuipes* [36], and *Perenniporia fraxinea* [37] preferentially hydrolyze α-subunit. However, the γ-chain does not appear to be a second specific target. On the other hand, plasmin, a plasma serine protease which plays an important role in the dissolution of blood clots, completely hydrolyzes all subunits of fibrinogen in the same manner [14].

To corroborate the *St*SBTc-3 *in vitro* effect on human fibrinogen

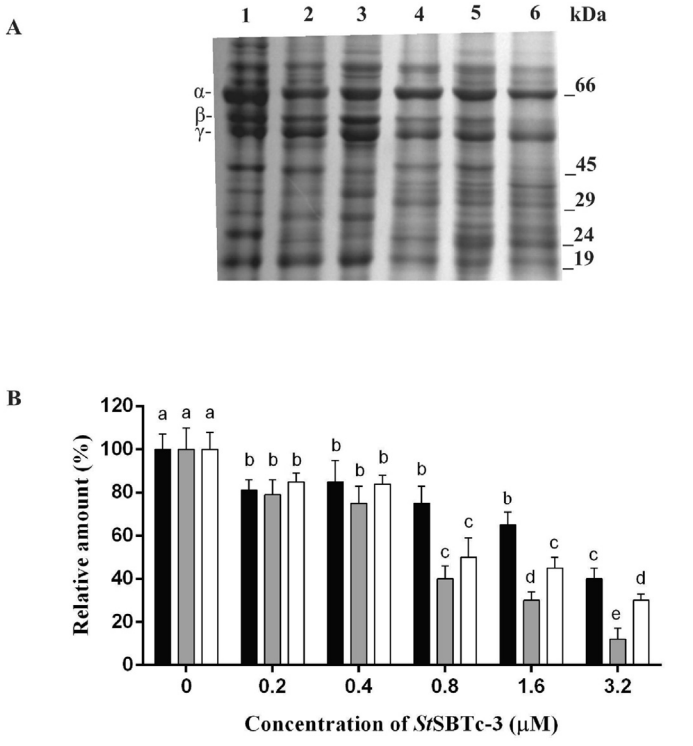

Fig. 1. Fibrinogenolytic activity of *St*SBTc-3 analyzed by dose-dependent. Hydrolysis patterns of StSBTc-3 on fibrinogen enriched fraction from human plasma are shown on SDS-PAGE 15%. (**A**) Fibrinogen enriched fraction of human plasma (40 μg) was incubated with 0 μM (lane 1), 0.2 μM (lane 2), 0.4 μM (lane 3), 0.8 μM (lane 4), 1.6 μM (lane 5) and 3.2 μM (lane 6) of *St*SBTc-3 for 2 h at 37 C. (**B**) Densitometric analysis of fibrinogen chains corresponding to SDS-PAGE 15% bands: α chain (black bars); β chain (grey bars); γ chain (white bars). Graphics and values represent the media of three independent experiments (Media ± SD). Different letters indicate significant differences with $p < 0.001$ and $p < 0.01$ for one-way ANOVA and Tukey tests respectively.

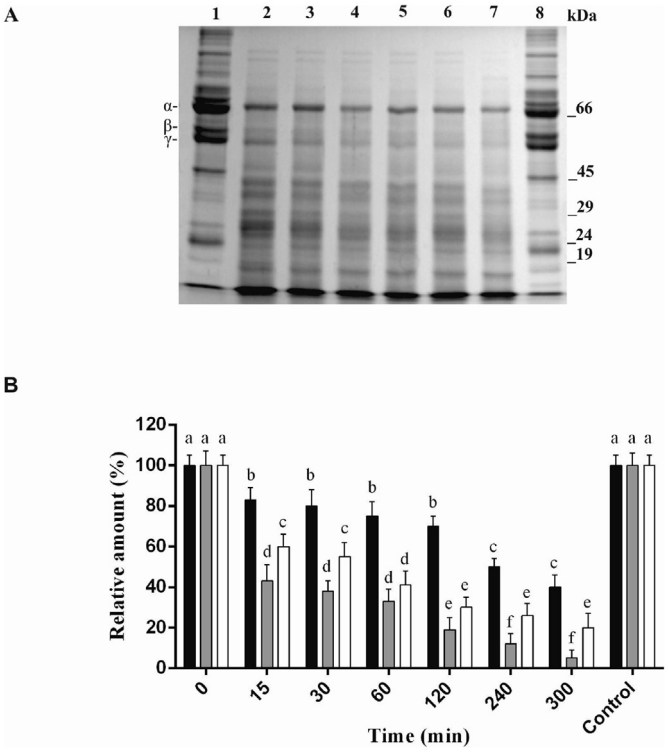

Fig. 2. Fibrinogenolytic activity of *St*SBTc-3 by time-dependent. Hydrolysis patterns of *St*SBTc-3 on fibrinogen enriched fraction from human plasma are shown on SDS-PAGE 15%. (**A**) Fibrinogen enriched fraction of human plasma (40 μg) was incubated with *St*SBTc-3 (3 μM) for 0 min (lane 1); 15 min (lane 2); 30 min (lane 3); 60 min (lane 4); 120 min (lane 5); 240 min (lane 6) and 300 min (lane 7); and 300 min without *St*SBTc-3 (lane 8) at 37 C. (**B**) Densitometric analysis of fibrinogen chains corresponding to SDS- PAGE 15% bands: α chain (black bars); β chain (grey bars); γ chain (white bars). Graphics and values represent the media of three independent experiments (Media ± SD).Different letters indicate significant differences with $p < 0.001$ and $p < 0.01$ for one-way ANOVA and Tukey tests respectively.

degradation, thrombin clotting time assays (TCT) were performed. TCT assay is a coagulation test to evaluate the conversion of fibrinogen to fibrin. Some factors that made TCT longer are lower doses of fibrinogen and defects on its quality [20]. As shown in Table 1, *St*SBTc-3 increases thrombin time in dose dependent manner. Data obtained show that *St*SBTc-3 is able to increase TCT by more than 40% (1 μM of *St*SBTc-3). This amounts were in the same magnitude order than the amounts necessary to produce human fibrinogen degradation reported for other plant serine proteases [14,1].

The effect of *St*SBTc-3 on fibrin clot lysis was examined using an *in vitro* fibrin polymer lysis assay [16]. Fibrin clot was first generated from fibrinogen by thrombin and clot lysis was then initiated by addition of *St*SBTc-3 as described in Section 2.8. Subsequent dissolution of fibrin polymer was measured with a turbidity assay. As shown in Fig. 3, *St*SBTc-3 was able to induce clot lysis compared

Table 1
Effect of *St*SBTc-3 on thrombin clotting time (TCT). Poor-platelet plasma was incubated with different concentrations 0 μM; 0.08 μM; 0.16 μM; 0.22 μM; 0.30 μM; 0.38 μM and 1 μM of *St*SBTc-3 for 1 h at 37 C. Values represent the media of three independent experiments (Media ± SD).

*St*SBTc-3 (μM)	TCT (s)
0.00	16.2 ± 0.1
0.08	17.5 ± 0.1
0.16	18.4 ± 0.1
0.22	19.4 ± 0.1
0.30	20.1 ± 0.2
0.38	20.5 ± 0.1
1.00	23.3 ± 0.3

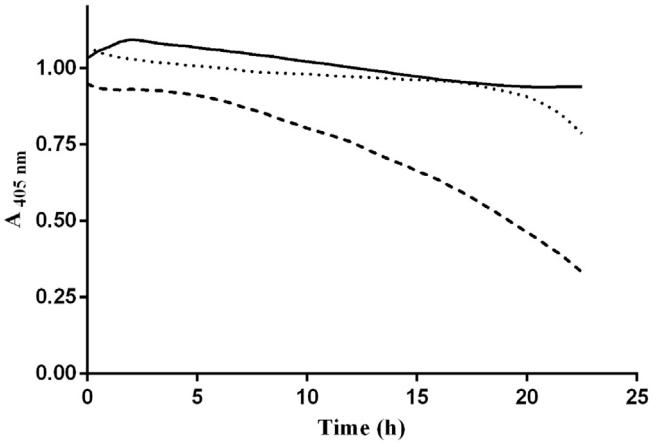

Fig. 3. Lysis of fibrin clot by *St*SBTc-3. Fibrin clot lysis was monitored by turbidity assay. Fibrin clot was prepared as described in experimental section by combining thrombin and fibrinogen and lysis was initiated by adding different concentrations of *St*SBTc-3: 1 μM (full line); 2 μM (dot line); 3 μM (segmented line). The decrease in turbidity was measured at 405 nm. Values represent the media of three independent experiments.

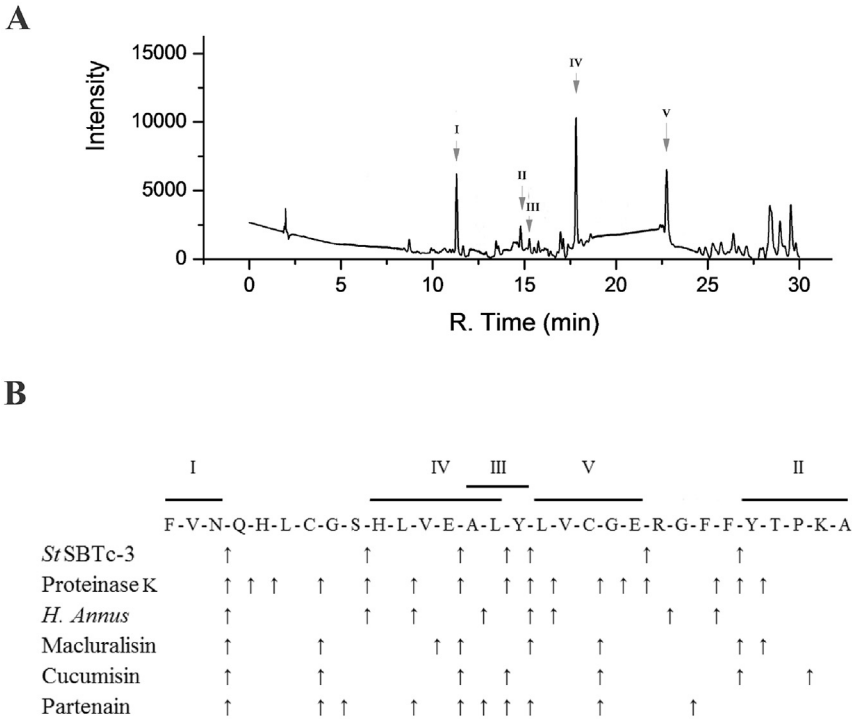

Fig. 4. Hydrolysis of bovine insulin β-chain by *St*SBTC-3. A. h.p.l.c. of the soluble peptide fragments of the B-chain of insulin produced by *St*SBTc-3. A sample of 100 μl of the trifluoroacetic acid-soluble degradation products of insulin B-chain was subjected to h.p.l.c. as described in the Experimental section. This is a typical profile of the separated products. The peptides were eluted with a linear gradient of acetonitrile (0–80%) in 1% (w/v) trifluoroacetic acid. The corresponding absorbance peaks from several h.p.l.c. separations were pooled, and the peptides were identified in subsequent analyses. B. The arrows indicate the peptide bonds cleaved in oxidized insulin β-chain by *St*SBTc-3 and other seine proteases: proteinase K [43]; *Helianthus annuus* proteinase [41]; Macluralisin [41]; Cucumisin [44]; Partenain [45].

with untreated control. *St*SBTc-3 concentration necessary to produce 50% of clot lysis after 15 h of incubation was the same, approximately, than the amount reported to Codiase [38]. Similar amounts able to produce fibrin degradation products were reported to AMP48 [14].

3.2. Digestion of oxidized insulin β-chain by StSBTc-3

The hydrolytic specificity of *St*SBTc-3 on oxidized insulin β-chain was analyzed as a probable explanation of the differences found between this enzyme and other plant serine proteases in the specificity to degrade fibrinogen. The proteinase was incubated with this substrate and the insulin peptide fragments were separated by RP-HPLC and identified by *N*-terminal amino acid sequencing. The same insulin cleavage pattern was obtained after 1, 3 and 24 h incubation, indicating that all possible peptide bonds

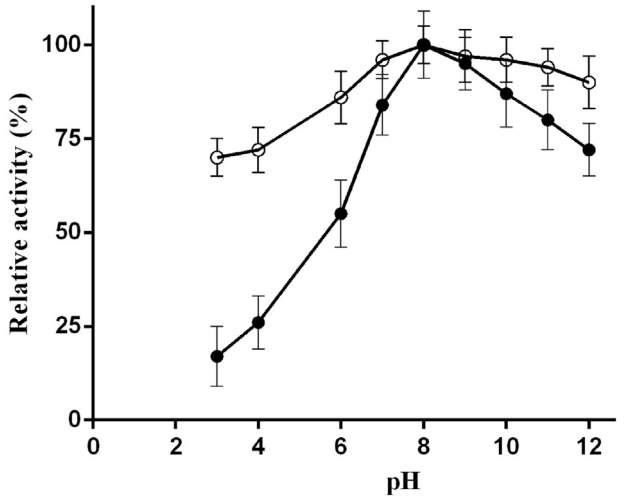

Fig. 5. Effect of pH on the fibrinogenolytic activity of *St*SBTc-3. The pH optimum (black dots) and stability (white dots) of the fibrinogenolytic activity of *St*SBTc-3 were determined in the pH range from 3 to 12. The activity was evaluated with the method described in experimental section (2.4). Values represent the media of three independent experiments (Media ± SD).

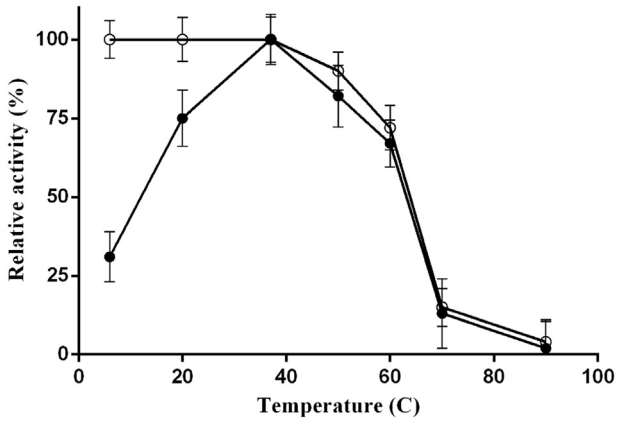

Fig. 6. Effects of temperature on the fibrinogenolytic activity of *St*SBTc-3. The temperature optimum and stability of fibrinogenolytic activity of *St*SBTc-3 were determined in the temperature range from 5 C to 90 C. The activity was evaluated with the method described in experimental section (2.4). Values represent the media of three independent experiments (Media ± SD).

Table 2
Effect of *St*SBTc-3 on human red blood cells. Fresh human red blood cells (hRBC) were incubated with different concentration of *St*SBTc-3 dissolved in PBS. Controls zero and 100% haemolysis consisted of hRBC suspended in PBS and 1% (w/w) TRITON X-100, respectively. The release of hemoglobin was monitored by measuring the absorbance of the supernatant at 550 nm. Values represent the media of three independent experiments (Media ± SD).

Treatment	Concentration (μM)	Haemolysis of hRBC (%)
PBS buffer	–	1.9 ± 0.2
TRITON X-100 buffer	–	100
*St*SBTc-3	0.5	2.1 ± 0.3
*St*SBTc-3	1	1.8 ± 0.4
*St*SBTc-3	2	2.2 ± 0.4
*St*SBTc-3	3	2.1 ± 0.4

were already cleaved after 1 h.

Seven cleavage sites were identified for the action of the *St*SBTc-3: Asn3-Gln4; Cys7-Gly8; Glu13-Ala14; Leu15-Tyr16; Tyr16-Leu17; Arg22-Gly23 and Phe25-Tyr26 (Fig. 4). As shown in Fig. 4, *St*SBTc-3 preferentially attacked the peptide bonds after the hydrophobic amino acid residues Leu, Tyr, Phe, which agrees well with the specificity pattern of subtilisin [39,18]. In addition, the bonds between residues Glu13-Ala14 and Cys7-Gly8 formed by glutamic or cysteic residues were cleaved. A comparable cleavage pattern was observed in the course of insulin β-chain hydrolysis with plant serine proteases from *Cucumis melo*, *Trichosanthes cucumeroides*, *Parthenium agentatum*, *Maclura pomifera* (named as Macluralisin). As reported for a serine protease isolated from *Helianthus annuus*, a fungal proteinase A1 from *Trichosanthes cucumeroides* and human plasmin [40,41], *St*SBTc-3 hydrolyzes the amino acid bond between Arg22-Gly23 (Fig. 4). However, *St*SBTc-3 is unable to cleave the peptide bound between Lys29-Ala30 previously reported to human plasmin [42]. Future assays focused on find *St*SBTc-3 cleavage sites on human fibrinogen will be performed in order to relate these results with *St*SBTc-3 fibrinogen subunits cleavage specificity.

3.3. Effect of pH and temperature on the fibrinogenolytic activity of StSBTc-3

Effects of pH and temperature (Figs. 5 and 6) on fibrinolytic activity and stability of *St*SBTc-3 were similar with data reported for other plant; bacterial and green algae serine proteases with fibrinogenolytic activity [1,14,16,38,40,46]. *St*BTc-3 was found to be active at pH values ranging from 7 to 11 and temperatures from 20 C to 60 C. The optimal pH for the fibrinogenolytic activity was 8 (Fig. 5) and the optimal temperature was 37 C (Fig. 6). Fibrinogenolytic activity of *St*SBTc-3 was found to be stable at pH values ranging from 3 to 12 (manteining 75–100% of it relative activity) and at temperatures from 10 C to 60 C (manteining 75–100% of it relative activity). These results are consistent with results previously reported to *St*BTc-3 DEVDase activity [47].

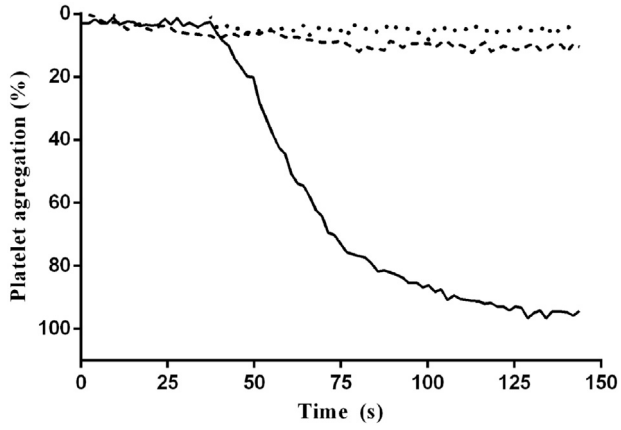

Fig. 7. Antiplatelet activity of *St*SBTc-3. Platelet aggregation was tested according to described in experimental section. Washed human platelets were incubated with 0.3 μM of *St*SBTc-3 (dot line); collagen 5 mg/ml (full line) and collagen 5 mg/ml with 0.2 μM *St*SBTc-3 (segmented line). Values represent the media of three independent experiments (Media ± SD).

3.4. In vitro StSBTc-3 haemolytic activity

The cytotoxic activity of *St*SBTc-3 was tested on human erythrocytes *in vitro*. *St*SBTc-3 did not show haemolytic activity against human erythrocytes at all concentrations assayed (Table 2). This result is in accordance with the results reported by Majumdar et al. [38] for a fibrinolytic serine protease from *Brevibacillus brevis* strain FF02B with a high therapeutic potential, named as Brevithrombolase.

3.5. Effect of StSBTc-3 on platelet aggregation in vitro

Platelet aggregation is a complex phenomenon that involves several intracellular biochemical pathways. Platelets readily aggregate in response to a variety of endogenous signals and secrete various compounds that cause further aggregation; they can initiate thrombus formation and precipitate thromboembolism, leading to ischemic diseases [48,49]. Antiplatelet activity has been reported for several plant proteases and plant extracts [16,50,51]. In order to elucidate if *St*SBTc-3 has antiplatelet activity, platelet aggregation assays mediated by collagen were performed (Fig. 7). Results obtained here demonstrate that *St*SBTc-3 inhibits platelet aggregation induced by collagen at a lower dose (0.2 μM) than the *St*SBTc-3 amount necessary to produce both, fibrin clot and fibrinogen lysis *in vitro*.

4. Conclusions

The results obtained in this work show that *St*SBTc-3 has fibrinogenolytic activity in a dose dependent manner. Additionally, we demonstrate that contrary to plasmin, α-fibirnogenases and other plant serin proteases, *St*SBT3 completely hydrolyzes the β subunit of human fibrinogen but partially hydrolyzes α and γ subunit. *St*SBTc-3 concentrations necessary to produce fibrinogen and fibrin clot lysis were similar to those previously reported for other plant serine proteases with fibrin(ogen)olytic activity *in vitro*. *St*SBTc-3 specificity assay using oxidized insulin β-chain as substrate shows seven cleavage sites, all of them were previously reported for other serine proteases with fibrinogenolytic activity. Biochemical analyses determine that fibrinolytic activity of *St*SBTc-3 is active in a wider range of pH from 7 to 11 and temperatures from 20 C to 60 C. This enzyme is stable in a broad range of pH and temperatures. Data here obtained show that *St*SBTc-3 is a potential bifunctional agent for thrombolytic therapy because also inhibits platelet aggregation induced by collagen. It is interesting that *St*SBTc-3 seems to inhibit platelet aggregation at lower dose and at shorter incubation times than the required for the fibrino(geno)lytic activity. However, future assays will be necessary to determine *St*SBTc-3 antiplatelet mechanism. Additionally, haemolysis assays demonstrate that *St*SBTc-3 is not cytotoxic to human erythrocytes at all concentrations assayed. All these results indicate that *St*SBTc-3 is a serin protease with high potential industrial and therapeutic applications in thrombolytic therapies.

Author contributions

A.P. performed the assays, drew the figures and analyzed the data. M.E.F., F.M., and M.B.F help to perform biochemistry assays. D.N.G. help to perform platelet aggregation assays. A.P. help to perform TCT assays. M.G.G. designed the research and the manuscript, and analyzed the data. M.G.G. and A.P. wrote the manuscript. G.R.D and G.G. revised the manuscript and checked English language.

Conflicts of interest

The authors declare no conflict of interest.

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