Purification and biological characterization of 62 kDa enzymes from *Synadenium carinatum* latex

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Abstract

Background: Plant Proteases have been described in the literature as being able to interfere in biological processes and behaving as antitumor, antiinflammatory, anthelmintic and antifungal agents. The aim of the present work was the purification and characterization of *Synadenium carinatum* 62 kDa enzyme and evaluation of in antimicrobial and antiparasitic effects.

Results: A 62 kDa serine protease with fibrinolytic activity was purified from *S. carinatum* latex by the combination of ion exchange and affinity chromatography. The 62 kDa enzyme showed high proteolytic activity on bovine fibrinogen at optimal pH 7,0 and 37 °C. The 62 kDa enzyme of *S. carinatum* degraded the fibrinogen α-chain and the β-chain, while the fibrinogen γ-chain remained unchanged. In the presence of protease inhibitors as benzamidine; leupeptin; PMSF; EDTA and phenanthroline the fibrinogenolytic activity of the *S. carinatum* 62 kDa enzyme was inhibited in the presence of the PMSF, suggesting that it is a member of the serine-proteases family. Interestingly, in the presence of EDTA, the enzyme had increased enzymatic activity. The fibrinogenolytic activity of the enzyme was inhibited in the presence of Cu^{2+} and Zn^{2+}. In biological assays, the *S. carinatum* 62 kDa enzyme showed non-cytotoxic on peritoneal macrophages, murine bone-marrow-derived macrophages and fibroblasts. In addition, the enzyme showed antiparasitic activity on *Toxoplasma gondii* infection *in vitro* in high concentration.

Conclusions: In that sense, this study sheds perspectives for future pharmacological applications of *S. carinatum* enzymes.

Keywords: *Synadenium carinatum*, proteases, fibrinogenolytic activity, *Toxoplasma gondii*.

Backgroud

Traditional medicine has been used by the majority of the world population for thousands of years. The World Health Organization (WHO) reported that an estimated 80% of the population in developing countries has traditionally used medicinal plants for their primary health care [1]. Several studies on the use and effects of plants bioactive molecules have been conducted throughout the world with a marked increasing in the areas of the cell biology, biotechnology, biochemistry and pharmaceutics [2]. The benefic effects of plants have been the main source for research in the treatment of diseases and injuries, such as tumors, inflammation, fungi, parasites and others microorganism infection [3, 4, 5].

Euphorbiaceae is a complex and heterogeneous family of angiosperms and contains approximately 8.000 species that have been identified worldwide [6, 7]. This family is able to secrete latex and the characterization of its components allowed the identification of a wide variety of molecules, such as terpenoids, alkaloids, proteases, lectin and many other particles [8].

Synadenium carinatum is a Euphorbiaceae plant commonly found in Brazil. It is known as "Janaúba" or "Leitosinha", whose latex is traditionally used for injuries, immunomodulatory effects in *Toxoplasma gondii* infection [9] and immunostimulatory effects in the immunization protocol against *Neospora caninum* [10]. Plant latex is a viscous fluid and colloidal in nature, which contains proteins, alkaloids, tannins, terpens, starch, sugars, oils, resins, gums and enzymes. Studies with *S. grantii*, another specie of *Synadenium* gender, demonstrated the presence of proteases with nematicidal action [11] and antiproliferative activity in human tumor cells [12]. Plant proteases have been described in the literature as being able to interfere in biological processes and behaving as antitumor, anti-inflammatory, anthelmintic and antifungal agents [13, 14, 15].

Considering the essential role of plants proteases, the aim of the present work was the purification and characterization of *S. carinatum* 62 kDa enzyme and evaluation of the antimicrobial and antiparasitic effects.

Results

Purification of the *Synadenium carinatum* latex enzyme

Purification of the *S. carinatum* enzyme was performed in two steps. First the *S. carinatum* latex (17.70 mg) was purified by ion exchange chromatography on DEAE Sephacel column producing four major protein peaks (DS1– DS4) (Fig. 1A) and SDS-PAGE profile. When analyzed under non-reducing conditions, demonstrated several

peptide components, with relative molecular masses (*Mr*) ranging from 12 to 120 kDa (Fig. 1B). After this, the peak DS2 was fractioned on a D-galactose agarose, resulting in galactose non-binding fraction (flow-flue) with a single band of 62 kDa (Fig. 1C).

Fibrinogenolytic activity

The fibrinogenolytic activity of *S. carinatum* fractions was demonstrated in Fig. 2A and B. The peak DS2 and galactose non-binding fraction showed higher proteolytic activity on fibrinogen when compared with the other peaks obtained after chromatography in DEAE-Sephacel column and D-galactose agarose respectively. Concerning the concentration and time, the *S. carinatum* 62 kDa enzyme completely degraded bovine fibrinogen Aα and Bβ-chains and apparently not degraded the γ-chain at a concentration of 8 μg and with 30 min of reaction (Fig. 2C and D).

Stability tests showed that the intervals of optimum temperature and pH for the fibrinogenolytic activity were 25–56 ^{o}C (Fig. 3A) and 7.0–10.0 (Fig. 3B), respectively, evidenced by degradation products of the bovine fibrinogen.

Concerning the effect of inhibitors, the proteolytic activity was reduced after pre-incubation with PMSF e and was potentiated in the presence of EDTA (Fig. 4A). When the effect of different ions in the fibrinogenolytic activity was evaluated, we observed that Cu^{2+} and Zn^{2+} ions completely blocked the effect of the *S. carinatum* enzyme and consequently it not degraded bovine fibrinogen (Fig. 4B).

Biological activity

Cell viability assay

Cytotoxic effects from different concentrations of *S. carinatum* 62 kDa enzyme was tested on peritoneal macrophages, murine bone-marrow-derived macrophages and fibroblasts. After incubating 18h, *S. carinatum* enzyme did not change the viability of these cells (Fig. 5).

Antimicrobial activity

The antimicrobial activity of the *S. carinatum* 62 kDa enzyme was also examined and the results were measured by the zones of bacterial growth inhibition around each of the disks, comparing with positive controls. *S. carinatum* enzyme not presented antimicrobial activity against *S. aureus*, *E. coli* and *P. aeruginosa* (data not shown).

Antiparasitic Activity

Effect of the *S. carinatum* 62 kDa enzyme on *T. gondii* infection and replication in HFF cells was verified and shown in Fig. 6. The pretreatment of *T. gondii* tachyzoites with *S. carinatum* before infection of HFF cells showed a dose-response inhibitory curve that reached up to 51% of inhibition and showed an IC_{50} of 254,09 µg/mL for the infection index (Fig. 6A). Concerning the inhibition of intracellular parasite replication, *S. carinatum* enzyme showed a dose-dependent inhibition, however, although the highest concentration was able to inhibit 48% on *T. gondii* replication, it was not possible to calculate the IC_{50} (Fig. 6B).

Discussion

Plants latex are composed of a variety of bioactive chemical resources, which the most common components are proteases, that present in large quantities in their fractions (serum and rubber particles) [16, 17]. The discoveries of these natural proteases may lead to the identification of model compounds for the development of novel therapeutic agents for thromboses and tumors, as has been demonstrated in studies [13, 18]. In this sense, we evaluated the activity of a latex enzyme of *S. carinatum*, with 62 kDa concerning its biological and enzymatic characteristics.

In the purification process by ion exchange with DEAE-Sephacel of the crude extract, the peaks were evident in the first fractions of the chromatography (1st-40th fraction), as well as the class III peroxidase of *Marsdenia megalantha* latex [18]. Separation of lectin from peak 2 was performed by galactose affinity chromatography. Lectin extraction is successful when performed by D-galactose as shown in studies that had this protein as interest [9].

The protein profile of *S. carinatum* latex revealed by SDS-PAGE a 62 kDa enzyme. Previous studies have reported that proteases of other latex from the Euphorbiaceae family, such as *Euphorbia heterophylla* and *Euphorbia mauritanica*, also exhibited similar molecular weights to 77.2 and 73 kDa, respectively [19, 20].

The degradation of the fibrinogen α and β chains by the *S. carinatum* 62 kDa enzyme was observed at concentrations of 8 and 16 µg of crude protein. The same profile is expressed by the proteases found in *Maclura spinosa,* at similar doses of 7.5 and 10 µg was able to cleave the fibrinogen subunits [21]. There was a reduction of fibrinogen activity after 30 minutes with the *S. carinatum* 62 kDa enzyme, similarly to *Euphorbia milii* latex protease [22].

The effect of pH on the proteolytic activity of *S. carinatum* 62 kDa enzyme on the fibrinogen substrate was evaluated, it was shown wide range of activity between pH 7,0 and 10,0. Similar results were found in other species of Euphorbiaceae. Siritapetawee *et al.* [23] found stability at a wide pH range, between 4,0 and 12,0 for a protease purified from *Euphorbia cf. lactea* latex. Torres *et al.* [24] observed the degradability capacity of a peptidase from *Vasconcellea quercifolia* latex with activity above 80% between pH 6,0 and 9,5.

The evaluation of the effect of temperature on the activity of fibrinogen proteolysis of the *S. carinatum* 62 kDa enzyme showed high activities between 25 °C and 56 °C and impaired activity above 70 °C. Bindhu *et al.* [25] reported optimal temperature between 37 °C and 50 °C for the cysteine proteases of *Calotropis gigantea* latex, and above that temperature, the enzymatic activity decreased markedly. Patel *et al.* [26] observed optimal *Euphorbia hirta* latex protease activity at 50 ºC on fibrinogen substrate.

The *S. carinatum* 62 kDa enzyme had its fibrinogenolytic activity reduced after preincubation with PMSF, irreversible inhibitor of the serine proteases and was potentiated in the presence of EDTA (metalloprotease inhibitor). These preliminary results suggest that the enzyme is a serine protease since it was inhibited by an irreversible inhibitor of serine proteases and the increase in its activity is explained by the elimination of trace amounts of heavy metal ions from the solution by EDTA. Raskovic *et al*. [27] demonstrated that the activity of the collagenolytic serine protease of *Ficus carica* latex was also reduced by PMSF and moderately enhanced by EDTA. The proteolytic activity of the *S. carinatum* enzyme was strongly inhibited by Cu^{2+} and Zn^{2+}. Previous studies have reported that a protease of *Euphorbia cotinifolia* was significantly inhibited by Zn^{2+} and Cu^{2+} ions [28].

Regarding to the biological activities, the *S. carinatum* 62 kDa enzyme showed no antimicrobial activity against *P. aeruginosa*, *S. aureus* and *E. coli*. Recent studies have shown that the methanolic extract of *Ficus carica* latex also did not present antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*, except *Proteus mirabilis* [29].

The *S. carinatum* 62 kDa enzyme did not present cytotoxic effects for the BMDM, HFF and peritoneal macrophage cells by the MTT assays, even when administered in high concentration (100 μg/mL). Latex of *Jatropha neopauciflora*, from the Euphorbiaceae family, presented the same non-cytotoxic profile when tested in two different cell lines [30].

Finally, the *S. carinatum* 62 kDa enzyme also showed antiparasitic activity in *T. gondii in vitro* infection. The infection of the parante tachyzoites with the 62 kDa enzyme prior to the infection in HeLa cells was able to control the infection, as demonstrated by the dose-dependent inhibition curves, with values of IC_{50} 254,09 μg/mL. A similar effect was observed in regards to the dose-dependent inhibition of intracellular replication of the parasite. These findings indicate that this *S. carinatum* 62 kDa enzyme was effective when tested directly against the parasite, with a greater reduction in infection rate than in the parasite replication. A recent study reported that the latex crude extract of *Euphorbia tirucalli* had anti-Toxoplasmosis activity against tachyzoites, with IC_{50} values of 135,4 μg/mL for the infection rate and IC_{50} 137,1 μg/mL for the replication rate [31].

Conclusion

In conclusion, the present investigation describes the biological and enzymatic characterization of a 62 kDa enzyme from *S. carinatum* latex. It demonstrated fibrinogenolytic activity to fibrinogen α and β chains, with optimum pH between 7,0 and 10,0 and temperatures range variates at 37 °C. The enzymatic activity of the *S. carinatum* 62 kDa enzyme in the presence of different protease inhibitors showed significant reduction under effect of PMSF, suggesting that the it is a member of the serine-proteases family. However, in the presence of EDTA, the enzyme had increased enzymatic activity. The *S. carinatum* 62 kDa enzyme was not cytotoxic to HFF, BMDM and peritoneal macrophage cells and demonstrated antiparasitic activity by *T. gondii*. These observations correlate with previous findings indicating that Euphorbiaceae species has medicinal properties and should be better explored.

Methods

Preparation of crude extract

S. carinatum latex was collected of this plant, which were grown under natural conditions in the University Campus localized in Uberlândia, Minas Gerais, Brazil in September of 2017. A voucher specimen (HUFU 848354) was identified and deposited at the Herbarium of the Federal University of Uberlândia.

Crude extracts from *S. carinatum* were obtained from small incisions in the distal branches of plants and by mixing 15 mL aliquots with water at a ratio of 1:10. These extracts were then incubated 4 °C for 24h and stored −80 °C for 2 months. Afterwards, a

rubber-like material was removed and the suspension was centrifuged at 12,000 g for 20 min at 4 °C.

Purification of *S. carinatum* enzymes

A crude extract (CE) of *S. carinatum* was separated by chromatography in a DEAE-Sephacel column (1.7 × 35 cm). The proteins were then eluted with a convex concentration gradient (50 mM − 1 M) of the ammonium bicarbonate buffer (pH 7.8). All peaks were monitored by measuring absorbance at 280 nm on a spectrophotometer BioSpec-Mini (Shimadzu Biotech, Japan) at a flow rate of 20 mL/h and fractions of 3.0 mL/tube were collected. The two peak was pooled, lyophilized and purified in immobilized D-galactose-agarose (Pearce, Rockford, IL, USA) to remove the contaminating proteins with affinity to galactose. Briefly, the column was balanced with 0.9% NaCl and the galactose non-binding proteins (fraction of interest) were removed with same buffer. After purification, the fraction was concentrated and dialyzed (against water) by diafiltration in centricon Vivaspin 6 (GE HealthCare) tube with exclusion limit of <50 kDa, stored at −20 °C and resuspended in phosphate buffer (PBS, pH 7.8) for use. Protein concentrations were determined by the Bradford method [32], using bovine serum albumin as standard.

Electrophoretic analysis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described by Laemmli [33] using 12% (w/v) gels. Electrophoresis was carried out at 26 mA/gel in Tris-glycine buffer (pH 8.3) containing 0.01% of SDS. The molecular mass standard proteins used were BenchaMarckTM Protein Ladder and Novex Sharp Pre-Stained Protein Standard. Gels were stained with Coomassie blue R-250, 0.2% (w/v).

Enzymatic Activities

Fibrinogenolytic Activity

The fibrinogenolytic activity of the *S. carinatum* enzymes was determined in SDS-PAGE as described previously [34], with modifications. Briefly, 10 µL of bovine fibrinogen (stock solution at 3 mg/mL, Sigma-Aldrich) was incubated with different concentrations of the enzyme (4, 8 and 16 µg) at 37 °C for 30 min. After incubation, the reaction was stopped by adding SDS sample buffer. The hydrolysis profile was followed by SDS-PAGE at 12% gel.

To study the effect of time on fibrinogenolytic activity, 8 µg of enzyme was added to fibrinogen and incubated at different time intervals (5, 15 and 30 min). The reaction was stopped and the enzymatic activity determined as above described.

Effect of Temperature and pH on fibrinogenolytic Activity

The effect of temperature on the fibrinogenolytic activity was verified by preheating for 30 min of *S. carinatum* 62 kDa enzyme (8 µg) at temperatures ranging from 25 °C to 100 °C, following incubation with bovine fibrinogen as above described. The reactions were stopped by adding SDS sample buffer and the hydrolysis profile was visualized by SDS-PAGE.

To study the effect of pH, 8 µg of enzyme 62 kDa was reconstituted at varying pH values, using the specified buffers in the respective pH range of 4–10. For pH 4-5: 0.2 M sodium acetate buffer; pH 7: PBS; and pH 9-10: 0.05 M Tris buffer containing 0,15 M NaCl and incubated with bovine fibrinogen for 30 min. After incubation, the reactions were stopped as above described.

Effect of Inhibitors and Ions on fibrinogenolytic Activity

The stability of the *S. carinatum* enzyme was evaluated on the basis of its proteolytic activity on fibrinogen in the presence of different protease inhibitors as PMSF and benzamidine (serine proteases, Sigma-Aldrich); leupeptin (cysteine proteases, Sigma-Aldrich) and EDTA and Phenanthroline (metalloproteases, Sigma-Aldrich), and bivalent ions (Ba^{2+}, Ca^{2+}, Cu^{2+},Mg^{2+}, MN^{2+} and Zn^{2+}), all reagents at concentration of 5 mM. Aliquots of 8 µg of enzyme and 5 µL of inhibitors or ions were preincubated for 30 min at 37 ºC, fibrinogenolytic activity was assayed as described above.

Biological activity

Cell cytotoxicity assay

Cytotoxicity of *S. carinatum* 62 kDa enzyme was assessed by determining cellular viability using MTT assay as previously described [35]. Murine peritoneal macrophages (Mø) and murine bone-marrow-derived macrophages from Balb/c mice (BMDM), and Human Foreskin Fibroblasts (HFF) were cultured separately in 96-well plates (1×10^5 cells/well) in triplicate, in the presence of *S. carinatum* 62 kDa enzyme in different concentrations (10, 30, 100 and 300 µg/mL). As controls, cells were incubated with complete RPMI medium. After 24h of incubation at 37 °C and 5% CO_2, cells were washed

and pulsed with 10 μL of thiazolyl blue at 5 mg/mL in 90 μL of complete RPMI medium 4h prior to the end of the culture. Formazan particles were solubilized in 10% sodium dodecyl sulfate (SDS) and 50% *N*,*N*-dimethyl formamide (Sigma-Aldrich). The optical density was read after 30 min at 570 nm in a plate reader (Titertek Multiskan Plus, Flow Laboratories, McLean, VA, USA). Results were expressed as percentage of cell viability in relation to the controls.

Antiparasitic activity

The antiparasitic activity of the *S. carinatum* 62 kDa enzyme was verified *in vitro* in the *T. gondii* infection following the protocol previously described [36]. HFF cells were cultured on 13-mm round glass coverslips into 24-well plates (1×10^5 cells/well/200 μL) for 24h at 37 °C and 5% CO_2. *T. gondii* tachyzoites (RH strain) were obtained from previously infected HFF cells, washed in RPMI medium and pretreated for 1h at 37 °C and 5% CO_2 with *S. carinatum* 62 kDa enzyme in different concentrations (1, 3, 10, 30, 100 and 300 μg/mL) or with medium alone (control). Next, parasites were washed and incubated with HFF cell monolayers on coverslips at 2:1 (parasite: host cell) rate of infection (2×10^5 tachyzoites/well/200 μL) for 24h at 37 °C and 5% CO_2. Cells were washed with 0.9% NaCl to remove non-adherent parasites, fixed in 10% buffered formalin for 2h and stained with 1% toluidine blue for 5s. Coverslips were mounted on glass slides and cells were examined under light microscope with regards to *T. gondii* infection index (percentage of infected cells per 100 examined cells) and parasite intracellular replication (mean number of parasites per cell in 100 infected cells).

Results were expressed as percentages of inhibition of infection as well as of parasite intracellular replication for each treatment in relation to controls. The median inhibitory concentration (IC_{50}) of *S. carinatum* 62 kDa enzyme was calculated by extrapolation of the corresponding dose-curve response on a log linear plot employing the portions of the curve that transected the 50% response point [37]. All experiments were performed in triplicate of each concentration, analyzed in 3 independent observations.

Antimicrobial effect of S. carinatum enzymes

The antimicrobial activity of *S. carinatum* 62 kDa enzyme was assessed by disk diffusion susceptibility. The first step of this assessment was to apply a bacterial inoculum of approximately 2×10^8 CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Bacteria specimens tested included *Staphylococcus aureus* (ATCC

25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Paper filter disks (0.5 mm diameter) were prepared with *S. carinatum* 62 kDa enzyme at 15.0, 10.0 and 5.0 µg/per disk unit, and then placed on the inoculated agar surface. Sterile water disks were used as negative controls for all bacteria and as positive controls were used Oxacillin 1 µg for *S. aureus*, Ampicillin 10 µg for *E. coli* and ceftazidime 30 µg for *P. aeruginosa*. The plates were incubated for 16–24h at 37 °C prior to determining results by measuring the growth inhibition zones (millimeters) around each of the disks. This experiment was performed with triplicate disk of each concentration and 3 independent observations.

Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, USA). The one-way ANOVA test followed by Dunett's post-test was applied for cell cytotoxicity and anti-toxoplasmosis activity.

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Figure legend

Fig. 1 Purification and identification of the 62 kDa enzyme from *S. carinatum.* **A** Chromatography profile of the partial protease purification on DEAE-Sephacel column showed 4 peaks (DS1 to DS4). **B** SDS-polyacrylamide gel at 12%. Fractions of *S. carinatum* were analyzed in non-reducing conditions. Mr: molecular size markers; lane 1, DS1; lane 2, DS2; lane 3, DS3 and lane 4, DS4. **C** Electrophoretic profile of the DS2 peak after D-galactose purification demonstrated a single band of 62 kDa (lane1).

Fig. 2 Proteolysis of bovine fibrinogen by the *S. carinatum* 62 kDa enzyme. **A** Fibrinogen was incubated or not with fractions of *S. carinatum* obtained after DEAE chromatography at 37 °C for 30 min and then analyzed on SDS-PAGE (12%). Lane 1, bovine fibrinogen chains, (α, β, and γ) are shown on the left; lane 2, DS1; lane 3, DS2; lane 4, DS3 and lane 5, DS4. **B** Bovine fibrinogen pre-incubated with galactose non-binding or binding proteins at 37 °C for 30 min. Lane 1, bovine fibrinogen chains (α, β, and γ); lane 2, galactose non-binding proteins – 62 kDa enzyme of *S. carinatum*; lane 3, galactose binding proteins. **C** Effect of different concentrations of *S. carinatum* 62 kDa enzyme on the Fibrinogenolytic activity. Lane 1-3 fibrinogen incubated with 4 μg, 8μg and 16 μg, of *S. carinatum* 62 kDa enzyme respectively. **D** Time-dependent. Lanes 1-3: fibrinogen incubated with enzyme for 5; 15 and 30 minutes, respectively.

Fig. 3 The pH and temperature effects on fibrinogenolytic activity of the *S. carinatum* 62 kDa enzyme. **A** Temperature-dependent fibrinolytic activity. *S. carinatum* 62 kDa enzyme samples (8 μg) were incubated at different temperatures for 30 min. Lane 1, bovine fibrinogen chains (α, β, and γ); lane 2-8, 25º; 37º; 56º, 60º, 70º, 80º and 90 ºC respectively. **B** Effect of different ranges of pH (4 to 10) on the proteolysis activity of the *S. carinatum* 62 kDa enzyme. (*****) optimal temperature and pH for enzymatic activity.

Fig. 4 Effect of inhibitors and ions on fibrinogenolytic activity of the *S. carinatum* 62 kDa

enzyme. **A** *S. carinatum* 62 kDa enzyme preincubated with different Inhibitors for 30 min, at 37 ^{o}C. Lane 1 bovine fibrinogen chains (α, β, and γ); lane 2, *S. carinatum* 62 kDa enzyme without inhibitors; lane 3, benzamidine; lane 4, leupeptin; lane 5, PMSF; lane 6, EDTA and lane 7, phenanthroline. **B** *S. carinatum* 62 kDa enzyme preincubated with different ions (5 mM) for 30 min and added to bovine fibrinogen for 30 min at 37 °C. Lane 1 bovine fibrinogen chains (α, β, and γ); lane 2, *S. carinatum* enzyme without ions. Lanes 3-8, 62 kDa enzyme preincubation with Cu^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Ca^{2+} and Zn^{2+} respectively.

Fig. 5 Cytotoxic effects of *S. carinatum* 62 kDa enzyme against cell lines. Percentage of cell viability in BMDM, Mø and HFF demonstrating the non-cytotoxic effects of *S. carinatum* enzyme even at higher concentrations.

Fig. 6 Effect of pretreatment of *T. gondii* tachyzoites with *S. carinatum* 62 kDa enzyme in different concentrations (1, 3, 10, 30, 100 and 300 µg/mL) or with medium alone (control): **A** on *T. gondii* infection and **B** intracellular replication in HFF cells**.** Results are expressed as mean and standard deviation of the percentages of inhibition of infection and intracellular replication related to controls. Dotted lines show the inhibitory concentration of 50%.

Fig. 1

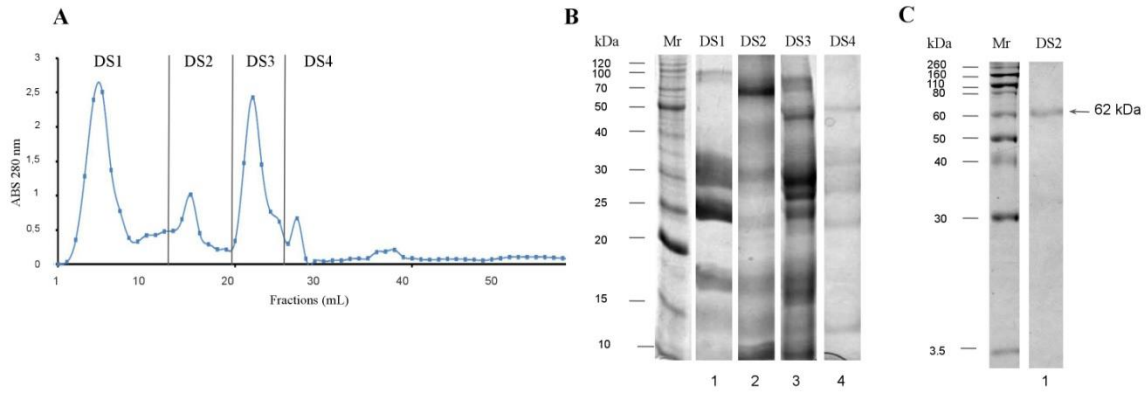

A
DS1
DS2
DS3
DS4
ABS 280 nm
Fractions (mL)
B
kDa
Mr
DS1
DS2
DS3
DS4
C
kDa
Mr
DS2
62 kDa

Fig. 2

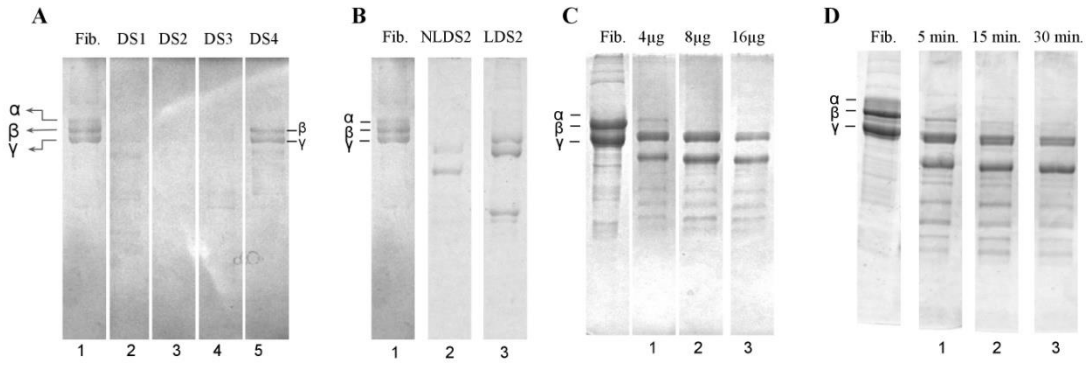

A
Fib.
DS1
DS2
DS3
DS4
α
β
γ
B
Fib.
NLDS2
LDS2
C
Fib.
4µg
8µg
16µg
D
Fib.
5 min.
15 min.
30 min.

Fig. 3

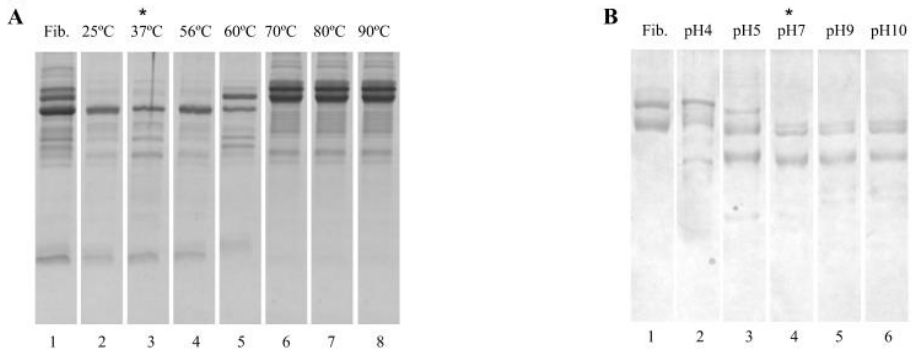

A
Fib.
25ºC
*
37ºC
56ºC
60ºC
70ºC
80ºC
90ºC
1
2
3
4
5
6
7
8
B
Fib.
pH4
pH5
*
pH7
pH9
pH10
1
2
3
4
5
6

Fig. 4

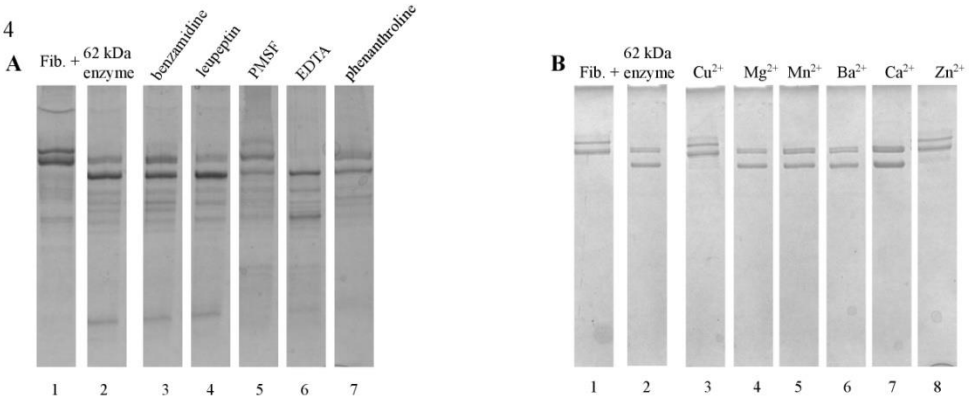

A
Fib. +
62 kDa enzyme
benzamidine
leupeptin
PMSF
EDTA
phenanthroline
1
2
3
4
5
6
7
B
Fib. +
62 kDa enzyme
Cu2+
Mg2+
Mn2+
Ba2+
Ca2+
Zn2+
1
2
3
4
5
6
7
8

Fig. 5

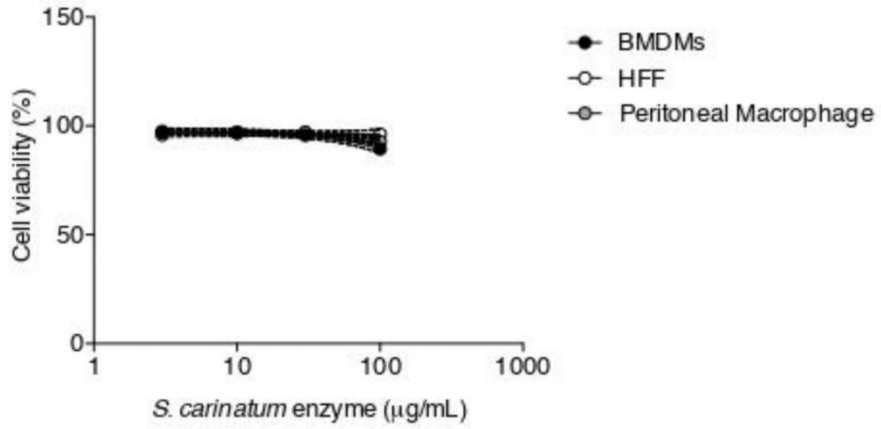
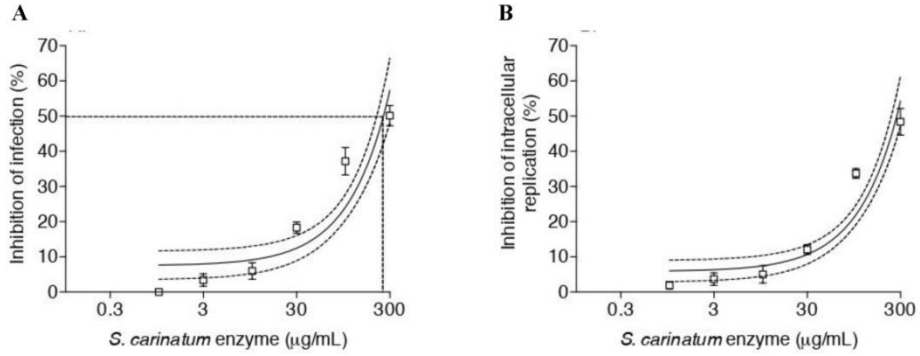

150
100
50
0
Cell viability (%)
1
10
100
1000
S. carinatum enzyme (μg/mL)
BMDMs
HFF
Peritoneal Macrophage

Fig. 6


A
70
60
50
40
30
20
10
0
Inhibition of infection (%)
0.3
3
30
300
S. carinatum enzyme (μg/mL)
B
70
60
50
40
30
20
10
0
Inhibition of intracellular replication (%)
0.3
3
30
300
S. carinatum enzyme (μg/mL)