Applied Microbiology and Biotechnology (2018) 102:3145–3158
https://doi.org/10.1007/s00253-018-8821-z

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Heterologous expression of rTsHyal-1: the first recombinant hyaluronidase of scorpion venom produced in *Pichia pastoris* system

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Received: 17 November 2017 / Revised: 18 January 2018 / Accepted: 20 January 2018 / Published online: 17 February 2018

Abstract

In general, hyaluronidases have a broad potential application on medicine and esthetics fields. Hyaluronidases from animal venoms cleave hyaluronan present in the extracellular matrix, acting as spreading factors of toxins into the tissues of the victim. However, the in-depth characterization of hyaluronidase from animal venoms has been neglected due to its instability and low concentration in the venom, which hamper its isolation. Thus, heterologous expression of hyaluronidase acts as a biotechnological tool in the obtainment of enough amounts of the enzyme for structural and functional studies. Therefore, this study produced a recombinant hyaluronidase from *Tityus serrulatus* scorpion venom, designated as rTsHyal-1, in the *Pichia pastoris* system. Thus, a gene for TsHyal-1 (gb|KF623285.1) was synthesized and cloned into the pPICZαA vector (GenScript Corporation) for heterologous expression in *P. pastoris*. rTsHyal-1 was expressed in laboratorial scale in a buffered minimal medium containing methanol (BMM) for 96 h with daily addition of methanol. Expression of rTsHyal-1 resulted in a total protein yield of 0.266 mg/mL. rTsHyal-1 partially purified through cation exchange chromatography presented a specific activity of 1097 TRU/mg, against 838 TRU/mg for the final expressed material, representing a 1.31-fold purification. rTsHyal-1 has molecular mass of 49.5 kDa, and treatment with PNGase F and analysis by mass spectrometry (MALDI-TOF) indicated a potential N-glycosylation of 4.5 kDa. Additionally, de novo sequencing of rTsHyal-1, performed in MALDI-TOF and Q Exactive Orbitrap MS, resulted in 46.8% of protein sequence coverage. rTsHyal-1 presents the highest substrate specificity to hyaluronan followed by chondroitin-6-sulfate, chondroitin-4-sulfate, and dermatan sulfate and showed an optimum activity at pH 6.0 and 40 °C. These results validate the biotechnological process for the heterologous expression of rTsHyal-1. This is the first recombinant hyaluronidase from scorpion venoms expressed in the *P. pastoris* system with preserved enzyme activity.

Keywords *Tityus serrulatus* · Scorpion venom · Hyaluronidase · Heterologous expression · *Pichia pastoris*

Introduction

Scorpion venoms are an opalescent dispersion mixture containing several enzymes, low molecular mass components, neurotoxins, and cytolytic and antimicrobial peptides, among others, with great therapeutic and biotechnological potentials. These components can act in synergy, resulting in an extreme and rapid immobilization and/or death of the prey (Kuhn-Nentwig 2003; Calvete 2017).

The distinct signs and symptoms observed after scorpion envenoming are consequence of the great complexity of the venoms and may evolve from a local pain to cardiorespiratory failure and death (Pucca et al. 2015). Neurotoxins are the main responsible for the envenoming syndrome and the most studied toxin class in scorpion venoms; however, the diffusion of these toxins through the prey's tissues is increased due to hyaluronidases present in this venom (Bordon et al. 2015; Cologna et al. 2009). Venom hyaluronidases are enzymes that hydrolyze preferentially hyaluronan, which is the major glycosaminoglycan present in the extracellular matrix (Bordon

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et al. 2015; Amorim et al. 2017). These enzymes hydrolyze the glycoside linkages β1➔4 between the residues N-acetyl-β-D-glucosamine and D-glucuronate from hyaluronan, producing tetrasaccharides and hexasaccharides (Bordon et al. 2015). Therefore, these enzymes are known as "spreading factors" in the envenoming by increasing the venom diffusion in the victim's tissues (Pukrittayakamee et al. 1988).

In general, hyaluronidases present several therapeutic applications and have already been used to increase the diffusion of active substances (such as drugs), in esthetic medicine and in the treatment of hyaluronan-induced diseases (such as some types of cancers) (Wohlrab et al. 2014). Concerning scorpion hyaluronidases, the first enzyme was reported in the venom of Indian scorpion *Heterometrus scaber* in 1975 (Nair and Kurup 1975). Meanwhile, it took 14 years to isolate the first scorpion venom hyaluronidase, which was isolated from *Heterometrus fulvipes* specie and had its structural and functional features characterized (Ramanaiah et al. 1990).

Until this moment, a "scorpion hyaluronidase" search in data banks retrieves 21 deposits in UniProt (just four revised deposits) and 30 in NCBI databases. Although omics approaches have assisted the identification of new hyaluronidases, these enzymes are present in low proportion in scorpion venoms, beyond being extremely unstable (Pessini et al. 2001; Bordon et al. 2015). These factors hamper their isolation from venoms, as well as their in-depth functional and structural characterization (Tan et al. 2016; Wiezel et al. 2015). Thus, these issues result in few reports of hyaluronidases that were actually purified from the venoms and could have their structure and function investigated (Ramanaiah et al. 1990; Horta et al. 2014; Pessini et al. 2001; Morey et al. 2006; Batista et al. 2007; Feng et al. 2008).

Scorpion hyaluronidases are glycoproteins and show molecular masses ranging from 43 to 50 to 82 kDa (Pessini et al. 2001; Horta et al. 2014; Ramanaiah et al. 1990). Their optimum activity is achieved between pH 4–6 and 30–37 °C, and these enzymes considerably lose their activity above 40 °C and with extreme pH (Ramanaiah et al. 1990; Pessini et al. 2001; Morey et al. 2006; Feng et al. 2008). Enzyme activity of hyaluronidase can be inhibited by heparin, dithiothreitol (DTT), flavonoids, and some cations, such as Cu^{2+} and Fe^{3+} (Ramanaiah et al. 1990; Morey et al. 2006; Feng et al. 2008). In addition, the enzyme activity seems to be influenced by variations in the animal's diet and habitat (Venancio et al. 2013; Pucca et al. 2014; Rodríguez-Ravelo et al. 2013). Concerning their structure, to date no hyaluronidase X-ray crystallographic structure from scorpion was obtained and there are only molecular models for TsHyal-1 and TsHyal-2 from *T. serrulatus* (Horta et al. 2014) and BmHYI from *M. martensii* scorpions (Xia et al. 2014). The catalytic site is conserved among all venom hyaluronidases, and the number of putative N-glycosylation sites varies from three in TsHyal-2 to five in BmHYI and TsHyal-1 (Horta et al. 2014; Xia et al. 2014).

Despite the diversity of potential applications presented by hyaluronidases, the main difficulty in performing applied research with these molecules is due to limitations in obtaining the native toxin. Thus, heterologous expression of proteins can overcome these obstacles, leading to production of sufficient amounts of toxins for their comprehensive characterization. Therefore, production of a recombinant hyaluronidase may enable the biotechnological and therapeutic applications of this scorpion venom enzyme.

In this way, we produced a recombinant hyaluronidase in the *Pichia pastoris* system, since this expression system is capable of performing post-translational modifications. In addition, in this system the expressed proteins are released into the culture medium, which facilitates the purification procedures. Moreover, the recombinant enzyme was enzymatically and structurally characterized in order to validate the heterologous expression process. It is worth mentioning that this is the first recombinant hyaluronidase from scorpion produced in a *P. pastoris* system.

Material and methods

Construction of a recombinant expression vector

The cDNA sequence encoding TsHyal-1 hyaluronidase from *T. serrulatus* (gb|KF623285.1), described by Horta et al. (2014), was used for the design of the gene. The gene for TsHyal-1 was synthesized and cloned into the pPICZαA vector by GenScript Corporation (USA) (http://www.genscript.com/gene_synthesis.html), for heterologous expression in the *P. pastoris* system.

For the gene design, the TsHyal-1 sequence was inserted between the *Xho*I and *Sal*I cloning sites (Fig. 1). In order to allow the expression of the recombinant protein with the native N-terminal, the kexin 2 (Kex2) cleavage site was inserted downstream the *Xho*I cloning site in the gene synthesis. The synthetic gene was designed to avoid the expression of 6xHistidine-tag in the C-terminal of the recombinant protein. For that, a stop codon (TAG) was added between the TsHyal-1 sequence and the *Sal*I cloning site. The entire sequence of the gb|KF623285.1 deposit was cloned in the synthetic gene, and codon optimization for the TsHyal-1 sequence was not performed.

Transformation of *Pichia pastoris* and recombinant clone selection

The recombinant plasmid pPICZαA-TsHyal (2 μg) containing the TsHyal-1 coding sequence was linearized with the restriction enzyme *PmeI* (New England Biolabs, UK). This linearized plasmid was transformed into *P. pastoris* (KM71H strain) cells by electroporation in the equipment GenePulser II

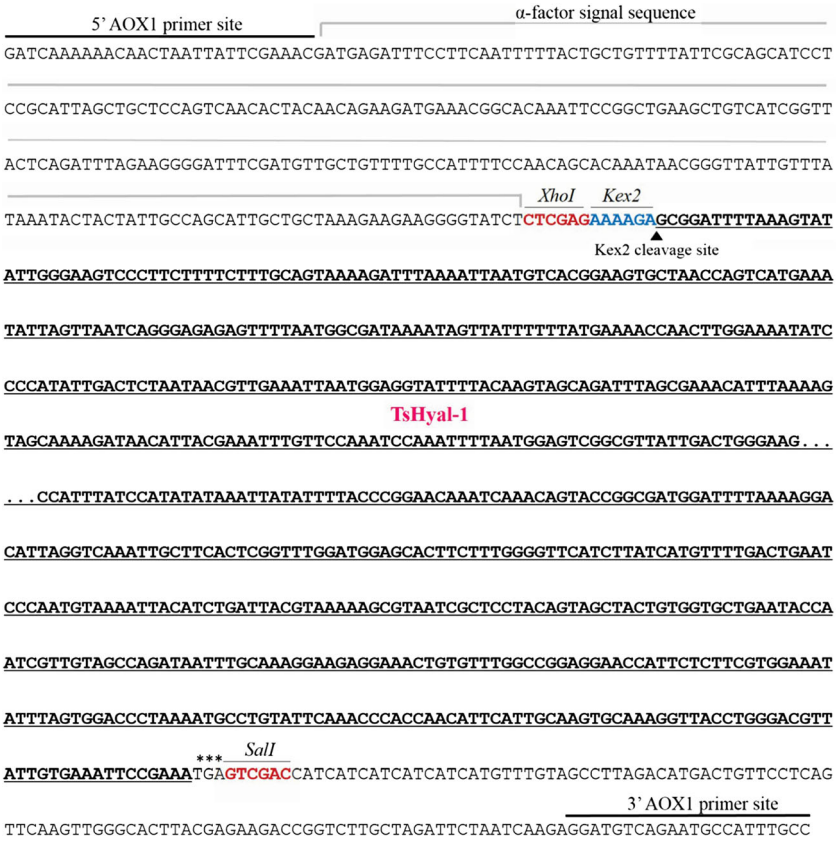

Fig. 1 Gene design of the recombinant plasmid pPICZαA containing the insert for the TsHyal-1 sequence. TsHyal-1 cDNA was synthesized aside the α-factor signal peptide. The recombinant plasmid has a cloning site for *Xho*I (in red) and Kex2 enzyme cleavage site (in blue). A stop codon (TGA) was added between the TsHyal-1 sequence and the *Sal*I cloning site (in red), in order to avoid the expression of 6xHistidine-tag in the protein C-terminal. Insert region encoding TsHyal-1 (KF623285.1) is shown in bold and underlined

(Bio-Rad Laboratories, USA), at 1500 V, 25 mF, and 200 Ω. An additional transformation was performed with a plasmid without insert as a negative control of expression. After electroporation, cells were plated in yeast extract peptone dextrose medium agar (YPDS, 1% yeast extract, 2% peptone, 2% dextrose, 2% agar) containing 100 μg/mL of Zeocin and incubated at 30 °C for 3 days. For the selection of transformants, the colonies were submitted to a polymerase chain reaction (PCR) followed by a product visualization on 1% agarose gel to confirm if the insert was incorporated in the yeast genomic DNA. For this reaction, a specific forward primer for the 5′ region of the TsHyal-1 sequence (5′-CTCGAGAAAAGAGC GGATTTTAAAG-3′) and a reverse primer for the 3′ region of the plasmid AOX gene (5′-GCAAATGGCATTCTGACATC C-3′) were used.

Screening of the optimum conditions for protein expression

To determine the best expression condition for the recombinant protein, we tested different media, pHs, and induction times for the heterologous expression. The expressions were carried out as described by the EasySelect™ *Pichia* Expression manual (Invitrogen, USA), with modifications. The positive colonies containing the TsHyal-1 coding sequence were selected and individually inoculated into a 24-well deep-well plate (Whatman, USA) containing 3 mL of buffered complex medium containing glycerol (BMGY, 1% yeast extract, 2% peptone, 1.34% Yeast Nitrogen Base with ammonium sulfate and amino acids (YNB), 4×10^{-5} biotin, 1% glycerol, 100 mM potassium phosphate pH 6.0) per well to generate biomass. As negative control, a clone containing the plasmid pPICZαA without insert of TsHyal-1, was inoculated under the same described conditions. The plate was incubated at 30 °C under constant stirring of 190 rpm. After 48 h, the cultures were centrifuged at 1500×*g* and the supernatant was discarded. The cells were resuspended in 2 mL of buffered complex medium containing methanol (BMMY, 1% yeast extract, 2% peptone, 1.34% YNB, 4×10^{-5} biotin, 0.5% methanol, 100 mM potassium phosphate, pH 6.0) for the induction of expression. The cultures were incubated at 26 °C under constant stirring of 190 rpm, and 100% methanol was added to each culture to a final concentration of 0.75% every 24 h to maintain the induction. We collected aliquots of 100 μL after 144 h of induction, and the supernatants were analyzed by 13.5% SDS-PAGE and monitored for hyaluronidase activity.

The clone with the highest hyaluronidase activity was subjected to a new screening to determine the ideal pH and medium composition to obtain the most active recombinant hyaluronidase. Therefore, the selected colony was inoculated under the same conditions described previously for biomass

generation. Then, after 48 h of biomass generation, the cells were centrifuged and resuspended in the following conditions: (a) buffered complex medium containing methanol at different pHs (BMMY, pH 4.0, 6.0, and 7.0); (b) buffered minimal medium containing methanol at different pHs (BMM, 1.34% YNB, 4×10^{-5} biotin, 0.5% methanol, 100 mM potassium phosphate, pH 4.0, 6.0, or 7.0); and (c) unbuffered minimal medium containing methanol (MM, 1.34% YNB, 4×10^{-5} biotin, 0.5% methanol). Aliquots of 100 μL were collected after 96, 120, and 144 h of induction, and supernatants were analyzed on 13.5% SDS-PAGE and evaluated for hyaluronidase activity. The conditions that allowed the expression of rTsHyal-1 with higher activity and lower number of contaminants were used for the expression of this protein in laboratory scale.

Laboratory-scale expression of rTsHyal-1

The chosen colony from plate screening was pre-inoculated in 10 mL of BMGY medium and incubated at 30 °C under constant stirring of 190 rpm. After 16 h, the culture was inoculated into 500 mL of BMGY medium and incubated at 30 °C under constant stirring of 190 rpm until reaching the absorbance between 0.2 and 0.6 at 600 nm. Thereafter, the culture was centrifuged at 1500×*g*, the supernatant was discarded, and the cells were resuspended in 100 mL of BMM medium pH 7.0 and incubated at 26 °C under constant stirring of 190 rpm. For the maintenance of the induction, methanol replacement was performed at the final concentration of 0.75% every 24 h. Aliquots of 1 mL for each day of induction were collected for protein quantification, evaluation of hyaluronidase activity, and SDS-PAGE. After 96 h of induction, the expression culture medium was centrifuged at 3000×*g*, for 10 min, and the supernatant was separated, filtered through a 0.2-μm pore membrane, and stored at 4 °C. This material was used for the purification of the recombinant protein.

Purification of rTsHyal-1

A dialysis bag (Fisherbrand® regenerated cellulose dialysis tubing, nominal MWCO 6000–8000, Fisher Scientific, Pittsburgh, PA, USA) was filled with the filtered supernatant of the expression culture medium (100 mL) and placed in a bath (4 L) of cold ultrapure water (18.2 MΩ·cm, Milli-Q water, Millipore, Bedford, MA, USA). At least four replacements of the bath water at 4 °C for 24 h were performed to completely remove all of the salt and components with molecular mass less than 6 kDa.

After dialysis, the medium was filtered through a 0.22-μm membrane and applied on a C10/40 column (1 × 40 cm, GE Healthcare, Uppsala, Sweden) packed with CM-cellulose-52 cation exchange microgranular resin (GE Healthcare) and connected to a fast protein liquid chromatography (FPLC) ÄKTApurifier UPC10 system (GE Healthcare). The column was equilibrated with 50 mM sodium acetate buffer pH 6.0 (buffer A). The sample (30 mL) was applied on the column, and absorbance was monitored automatically at 280 nm. The elution was performed with a step concentration gradient to 100% of 1 M sodium acetate pH 6.0 (buffer B), at a flow rate of 0.5 mL/min, collecting 1 mL per tube. The obtained fractions were concentrated on a centrifugal filter unit (Amicon® Ultra-15 Centrifugal Filter Ultracel® 3K, regenerated cellulose 3000 NMWL, Millipore) and tested for hyaluronidase activity. The fractions were stored at 4 °C for subsequent assays. Quantification was performed by absorbance method 280/205 nm (Scopes 1974).

SDS-PAGE, zymography, and densitometry

Zymography was performed according to Cevallos et al. (1992), based on the electrophoresis system described by Laemmli et al. (1970). In this method, hyaluronic acid (0.4 mg/mL) was incorporated into the gel matrix during polymerization of SDS-PAGE (13.5% or 10%). Electrophoresis was performed in the following conditions: 120 V, 40 mA, and 10 W for 1 h and 10 min. Gels were stained with PlusOne Coomassie Blue PhastGel® R-350 (GE Healthcare) or silver nitrate, depending on the amount of protein applied. The molecular mass of rTsHyal-1 was estimated under non-reducing conditions. For densitometry analysis, we used the DenGo densitometer (Qualiterm, Brazil) for gel electrophoresis and the accompanying program.

Hyaluronidase activity

Hyaluronidase activity was quantitatively determined by the turbidimetric assay as described by Pukrittayakamee et al. (1988) and adapted to a 96-well microplate. For this assay, we used an acetate buffer (200 mM sodium acetate and 150 mM NaCl pH 6.0), 10 μg hyaluronan (0.5 mg/mL in water), and the expression medium or fraction/enzyme to the final volume of 200 μL. The reactional mixture was incubated for 30 min at 37 °C, and the reaction was stopped with the addition of 100 μL of 5% cetyltrimethylammonium bromide (CTAB) and 4% NaOH. Then, the absorbance of the mixture was read at 400 nm in a microplate reader (Sunrise Basic, Tecan, Switzerland). Hyaluronidase activity was expressed as the percentage of hydrolyzed hyaluronan, considering the absorbance of the tube in which no hyaluronan was added as 100% of hydrolysis. The absorbance of the tube containing 10 μg of hyaluronan, without enzyme, was considered as 0% of hydrolysis. Turbidity-reducing units (TRU) are expressed as the amount of enzyme required to hydrolyze 50% (5 μg) of hyaluronan, and the specific activity is turbidity-reducing units per milligram of enzyme. This assay was also performed to determine the conditions for maximum activity, such as pH

(3.0 to 9.0) and incubation temperatures (0 to 100 °C), as well as specificity on 0.5 mg/mL of different substrates such as chondroitin-4-sulfate (chondroitin sulfate A), dermatan sulfate (chondroitin sulfate B), and chondroitin-6-sulfate (chondroitin sulfate C) (Sigma, USA).

Mass spectrometry analysis

For peptide mass fingerprinting (PMF), rTsHyal-1 was extracted from SDS-PAGE and subjected to reduction, alkylation, and enzymatic digestion. For that, 5 μg of the recombinant protein was reduced with 2 μL of 100 mM DTT and 6 μL of 50 mM NH_4HCO_3 pH 7.8 and incubated at 58 °C for 1 h. The sample was then alkylated with 2 μL of 500 mM iodoacetamide (IAA) at room temperature in the dark for 1 h. After this step, the recombinant protein was digested with trypsin (Thermo Scientific, USA) in 50 mM NH_4HCO_3 in a ratio of 1/30 (trypsin/protein) at 37 °C for 4 h. After digestion, the reaction was stopped with addition of formic acid to a final concentration of 1% and subjected to desalting on a 10-μL pipette tip containing C18 reversed-phase media (ZipTip® C18 tips, Millipore) with elution in a solution of acetonitrile/water/formic acid (49.8/50/0.2). The sample was spotted with 1 μL of 2,5-dihydroxybenzoic acid matrix (DHB 10 mg/mL in 0.2% formic acid and 50% acetonitrile). The tryptic digests were analyzed by MALDI-TOF Ultraflex II (Bruker Daltonics, USA) operated in reflected positive mode, equipped with Smart Beam Laser. The analyzer was previously calibrated with Peptide Standard II from Bruker Daltonics. The PMF was analyzed using the programs FlexAnalysis 3.0, BioTools 3.2, and Sequence Editor.

In addition, the digestion was also analyzed by a ULPC nanoACQUITY (Waters, UK) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, USA). The chromatographic system is equipped with a 100 μm × 5 cm monolithic PepSwift capillary column (Thermo Scientific, USA). Elution of peptides was performed in a gradient from 3 to 50% of solution B (A: water/0.1% formic acid; B: acetonitrile) in 80 min at a flow rate of 0.7 mL/min. The raw data were analyzed by Pmi-Byonic software based on the amino acid sequence of TsHyal-1 from the database.

For determination of glycosylation, approximately 2 μg of rTsHyal-1 was digested by PNGase F (Roche Molecular Biochemicals, Germany) in two steps. First, the protein was incubated with 5 U of PNGase F in 50 mM NH_4HCO_3 buffer at 37 °C for 8 h. Then, another 3 U of the enzyme was added to the sample which was incubated at 37 °C for 16 h. After this period, the sample was desalted by ZipTip® C18 tips (Millipore) with elution in acetonitrile/water/formic acid solution (49.8/50/0.2). The sample was spotted with 1 μL of the 2,5-dihydroxybenzoic acid matrix (DHB 10 mg/mL in 0.2% formic acid and 50% acetonitrile) and analyzed by MALDI-TOF Ultraflex II (Bruker Daltonics, USA) as described above.

Results

Heterologous expression of rTsHyal-1

After the transformation of *P. pastoris*, we performed a screening of all positive colonies for the insert using PCR. The enzyme activity was monitored during all the process. For this, all colonies were tested for hyaluronidase activity and evaluated by SDS-PAGE. All positive colonies showed efficiency in expression and secretion of soluble active rTsHyal-1 after 144 h of induction. Then, the colony that secreted the most active rTsHyal-1 was submitted to a screening of optimal expression conditions. The higher hyaluronidase activity (838 UTR/mg) was obtained with 96 h of induction in BMM culture medium at pH 7.0. No rTsHyal-1 expression was observed in pH 4.0 and MM medium (Fig. 2a). Interestingly, a lower number of contaminants of high molecular mass were observed using BMM culture medium at pH 6.0 and pH 7.0 (Fig. 2b).

Amino acid supplementation (such as peptone and yeast extracts) in the culture medium can influence the heterologous expression by competing for protease substrates and inhibiting protease induction caused by nitrogen restriction (Sreekrishna et al. 1997; Macauley-Patrick et al. 2005). The addition of peptone in the culture medium (BMMY) influenced the expression of rTsHyal-1, which led to production of a greater number of contaminants (mainly of high molecular mass). Furthermore, expression in BMMY medium may have resulted in the co-expression of a protease with rTsHyal-1 that could result in recombinant protein degradation. This scenario might explain the lower hyaluronidase activity of the BMMY expression medium, when compared to the medium without supplementation (BMM). The use of yeast extracts and peptones in complex media can provide several batch-to-batch variations in the expression process. Therefore, it is desirable to eliminate any complex components from the medium, in order to make it easier to standardize the production procedure and to validate the medium and process itself (Macauley-Patrick et al. 2005).

Temperature reduction during the induction step could also improve the yield of recombinant protein (Macauley-Patrick et al. 2005; Gao et al. 2015). Therefore, rTsHyal-1 expression was performed at 26 °C. The expression in higher temperatures, such as 30 °C, may affect the stability of the recombinant protein, alter its folding and solubility, and lead to protease production (Macauley-Patrick et al. 2005). These described optimal expression conditions were utilized to express rTsHyal-1 in laboratory scale.

During the laboratory-scale expression, aliquots of 100 μL were collected each 24 h of induction for protein quantification, determination of hyaluronidase activity, and analysis on SDS-PAGE (Fig. 3). The increase of protein concentration (Fig. 3a, b) and hyaluronidase activity (Fig. 3c, d) are directly proportional to time. Expression was ended after 96 h of

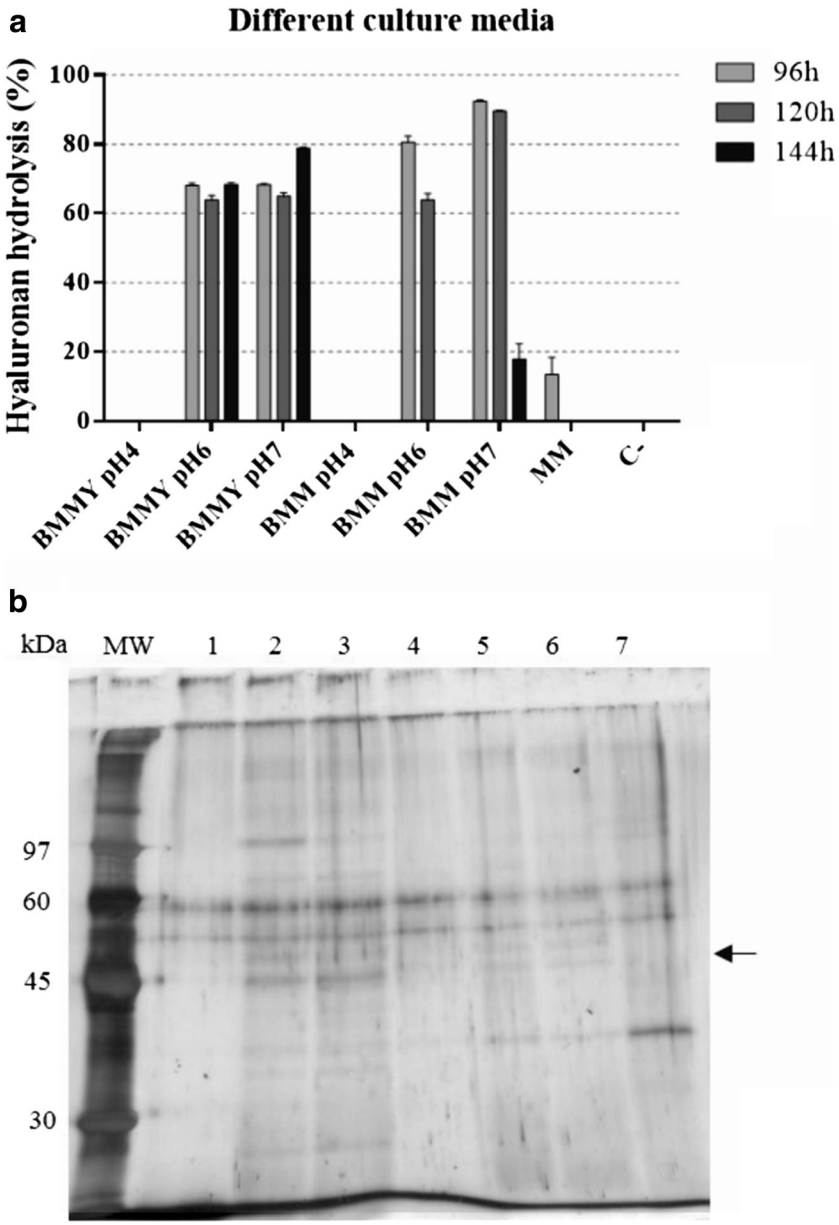

Fig. 2 Screening of optimum conditions for protein expression. **a** Hyaluronidase activity determined turbidimetrically (10 μL of each supernatant of the expression culture medium). **b** SDS-PAGE (10%) of supernatants of the culture media after 144 h of induction. MW: molecular weight marker; 1: BMMY pH 4.0; 2: BMMY pH 6.0; 3: BMMY pH 7.0; 4: BMM pH 4.0; 5: BMM pH 6.0; 6: BMM pH 7.0; 7: MM. rTsHyal-1 is indicated by the arrow. C−: negative control (BMMY culture medium pH 6.0 without insert)

induction, and the material was quantified by Scopes' method. A total of 25.27 mg of protein (0.266 mg/mL) was produced in laboratory scale. This material was used in the following assays as well as for rTsHyal-1 purification.

The dialyzed medium was concentrated threefold and then subjected to SDS-PAGE and zymography. It is possible to notice in Fig. 4 that the band near 45 kDa represents rTsHyal-1, which is confirmed by the presence of the active band in zymography. In addition, rTsHyal-1 seems to be aggregated in the supernatant since it is possible to observe the presence of the band with hyaluronidase activity in the upper part of the gel. Additionally, the zymogram also presents active bands of approximately 60 kDa and above 97 kDa (Fig. 4). These bands could represent rTsHyal-1 with a different glycosylation profile, such as hyperglycosylation.

Purification of rTsHyal-1

rTsHyal-1 was purified from the culture supernatant by cation exchange chromatography (Fig. 5a) resulting in two main fractions, designated as C1 and C2. Hyaluronidase activity was detected only in fraction C2, presenting 80% of hyaluronan hydrolysis. The homogeneity of this concentrated fraction was evaluated in a 13.5% SDS-PAGE (Fig. 5b). It was possible to notice an electrophoretic band around 45 kDa and its corresponding band in the zymogram, which proves that fraction C2 corresponds to the active hyaluronidase. Thus, active rTsHyal-1 (1097 TRU/mg) could be obtained by a one-step chromatographic procedure. The recovery and yield of the purification procedure are presented in Table 1.

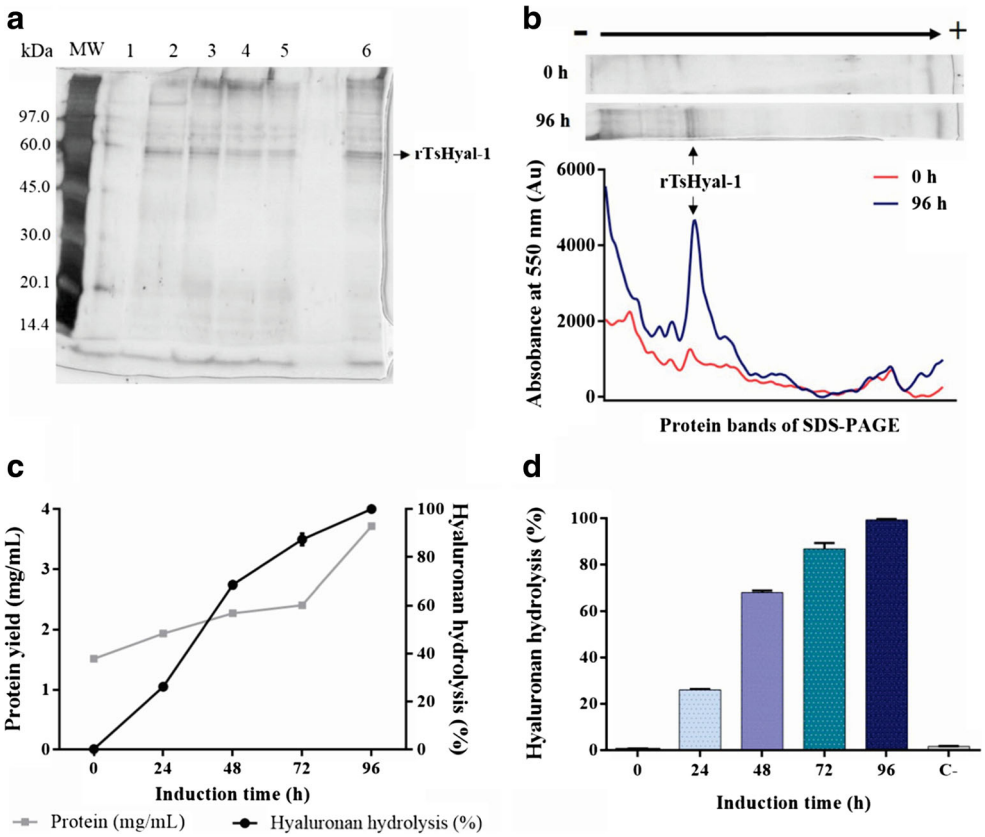

Fig. 3 Time analyses of rTsHyal-1 expression in BMM medium pH 7.0 (until 96 h of induction). **a** 13.5% SDS-PAGE stained with silver; rTsHyal-1 is indicated with the arrow. MW: molecular weight marker; 1: 0 h; 2: 24 h; 3: 48 h; 4: 72 h; 5: 96 h; 6: supernatant after 96 h of induction, dialyzed, filtered, and three times concentrated. **b** Densitometry of culture supernatant proteins in SDS-PAGE comparing 0 and 96 h of induction. Absorbance at 550 nm and the graphic created by DenGo software. **c** Protein yield and hyaluronidase activity according to induction time. **d** Hyaluronidase activity during expression (h). C−: BMM culture medium pH 6.0 without insert

Purification of rTsHyal-1 showed low yield, probably due to the low expression of hyaluronidase in the culture medium. The protocol uses a weak cation exchange resin and under the conditions described above presented the best results regarding rTsHyal-1 purification, since it is possible to observe that the protein interacted with the resin and eluted with approximately 250 mM of sodium acetate, pH 6.0. At this pH, rTsHyal-1 carries a net positive charge, since its theoretical isoelectric point (pI) is 8.7, calculated with the ProtParam program (http://www.expasy.ch/tools/protparam.html). Thus, at pH below its pI, the enzyme was positively charged and could interact with the negatively charged carboxymethyl groups of the resin. In addition, no hyaluronidase activity was detected in the unretained components fraction. Moreover, hyaluronidase activity assay was performed at similar concentrations of sodium acetate, which warrants the choice of such buffer for fractionation, leading to the optimum conditions for the enzyme activity.

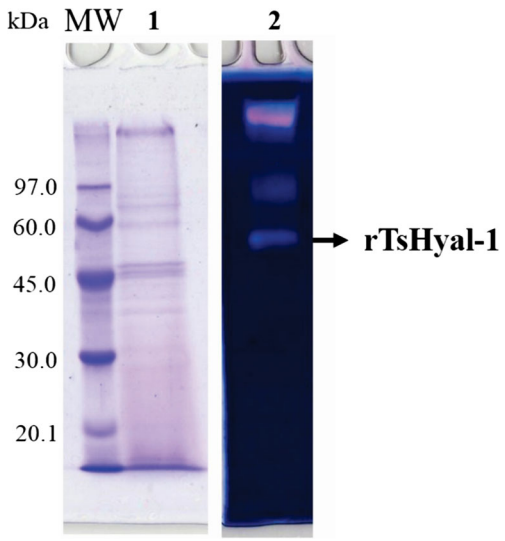

Fig. 4 SDS-PAGE (10%) containing hyaluronan. The gel was stained with PlusOne Coomassie Blue PhastGel® R-350 (MW and lane 1) and with Stains-all for evaluation of the hyaluronidase activity (lane 2). Lanes 1 and 2: three times concentrated expression culture supernatant; MW: molecular weight marker

Characterization of enzyme activity of rTsHyal-1

In order to verify the optimum temperature for the activity of rTsHyal-1, the enzyme was incubated with the substrate hyaluronan at different temperatures (Fig. 6a). rTsHyal-1 presented optimum temperature for activity around 40 °C, and it was still active for up to 6 months when stored in the purification buffer at − 20 and 4 °C. The optimum pH for the catalytic activity of rTsHyal-1 on hyaluronan was also evaluated, and rTsHyal-1 presented optimum pH for enzyme activity at 6.0 (Fig. 6b). To evaluate the specificity of rTsHyal-1 against different substrates, the turbidimetric assay was performed

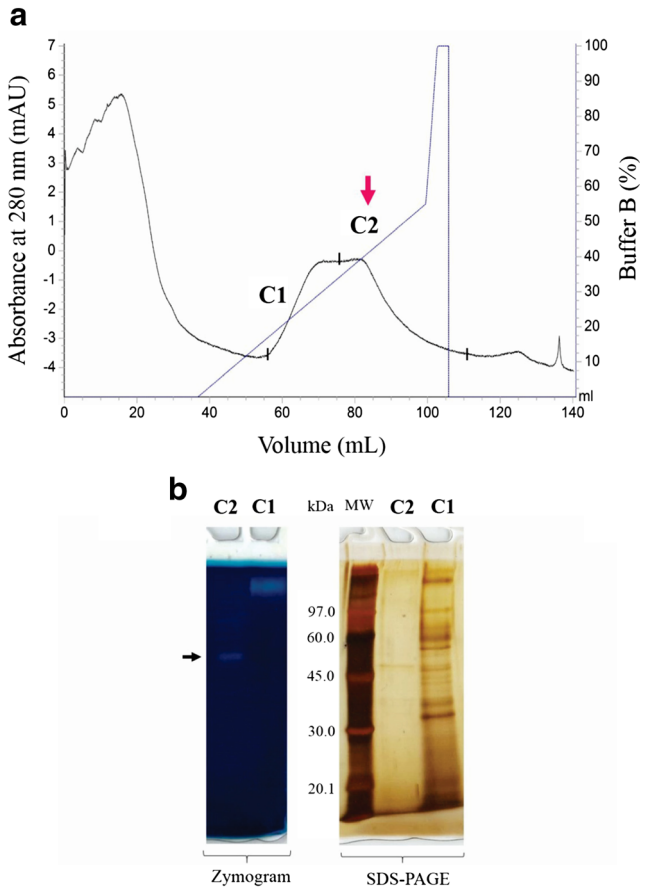

Fig. 5 Chromatographic profile of the expression culture supernatant. **a** Cation exchange chromatography was performed on a C10/40 column (1 × 40 cm, GE Healthcare, UK) packed with CM-cellulose-52 microgranular resin (GE Healthcare, UK) equilibrated with 50 mM sodium acetate buffer pH 6.0 (buffer A). The column was connected to the FPLC system, and the absorbance was monitored automatically at 280 nm. Fractions were eluted with a concentration gradient from 50 mM to 1 M sodium acetate pH 6.0 (buffer B), at a flow rate of 0.5 mL/min, collecting 1 mL per tube. **b** SDS-PAGE profile (13.5%) of C1 and C2 fractions and their respective zymogram. MW: molecular weight marker. The arrow indicates the fraction with hyaluronidase activity

with chondroitin-4-sulfate (chondroitin sulfate A), dermatan sulfate (chondroitin sulfate B), chondroitin-6-sulfate (chondroitin sulfate C), and hyaluronan. rTsHyal-1 showed higher activity on hyaluronan substrate, followed by the chondroitin sulfates C, A, and B (Fig. 6c). Although rTsHyal-1 has partially degraded chondroitin sulfates C, A, and B, the higher hydrolysis specificity was with hyaluronan. This is the first time that the substrate specificity for *T. serrulatus* hyaluronidase has been evaluated.

Mass spectrometry

C2 fraction extracted from SDS-PAGE was subjected to digestion with trypsin, and the PMF was obtained by MALDI-TOF. Analysis of the PMF in the Sequence Editor and BioTools softwares shows that the tryptic digests identified had a 26.5% coverage of the TsHyal-1 sequence (Fig. 7a).

In order to obtain a greater coverage of the rTsHyal-1 sequencing, we used nanoACQUITY UPLC coupled to a Q Exactive Orbitrap high-resolution mass spectrometer and the software Pmi-Byonic. A 28.6% coverage of rTsHyal-1 sequence was obtained (Fig. 7b). In this analysis, 47 spectra provided 14 different peptides related to the rTsHyal-1 sequence. Among them, only two peptides (143–158 and 339–354) were the same as those obtained in the previous assay, corresponding to 8.3% of the enzyme sequence. Therefore, considering the peptides obtained in these two assays, the coverage of the rTsHyal-1 sequence was 46.8%.

Glycosylation determination

rTsHyal-1 extracted from the gel was deglycosylated with PNGase F. This enzyme is capable of cleaving between N-acetylglucosamine binding and N-glycosylation asparagine residue. PNGase F cleaves all types of N-glycosylations, except those bound to fucose (Morelle and Michalski 2007). The experimental mass of 49.5 kDa was estimated by electrophoresis for the rTsHyal-1 glycosylated form. According to the Sequence Editor software, the oxidized TsHyal-1 presents a theoretical average mass of 44.606 kDa. The difference between the mass estimated by electrophoresis and the theoretical mass of the protein may indicate a possible glycosylation with a theoretical molecular mass around 4900 Da. This fact corroborates to the analysis of the deglycosylated protein on MALDI-TOF, in which an ion of 4515.8 Da was observed after treatment with PNGase F (Fig. 8). rTsHyal-1 shows five putative N-glycosylation sites (Fig. 7a). Additionally, the *P. pastoris* expression system is capable of promoting post-

Table 1 Total protein recovery and hyaluronidase activity of rTsHyal-1 after fractionation of the supernatant of the expression BMM pH 7.0 culture medium on a CMC-52 column

Purification step	Protein		Enzyme activity			
	Total (mg)	Recovery (%)	Total (TRU)	Yield (%)	Specific activity (TRU/mg)[a]	Purification factor
Culture medium (15 mL)	4.0	100.0	3352.0	100.0	838	1
C2 CMC-52 (1 mL)	0.1	2.5	109.7	3.3	1097	1.3

[a] Turbidity-reducing units (TRU): amount of protein required to hydrolyze 50% (5 μg) of hyaluronan after 30 min of incubation at 37 °C

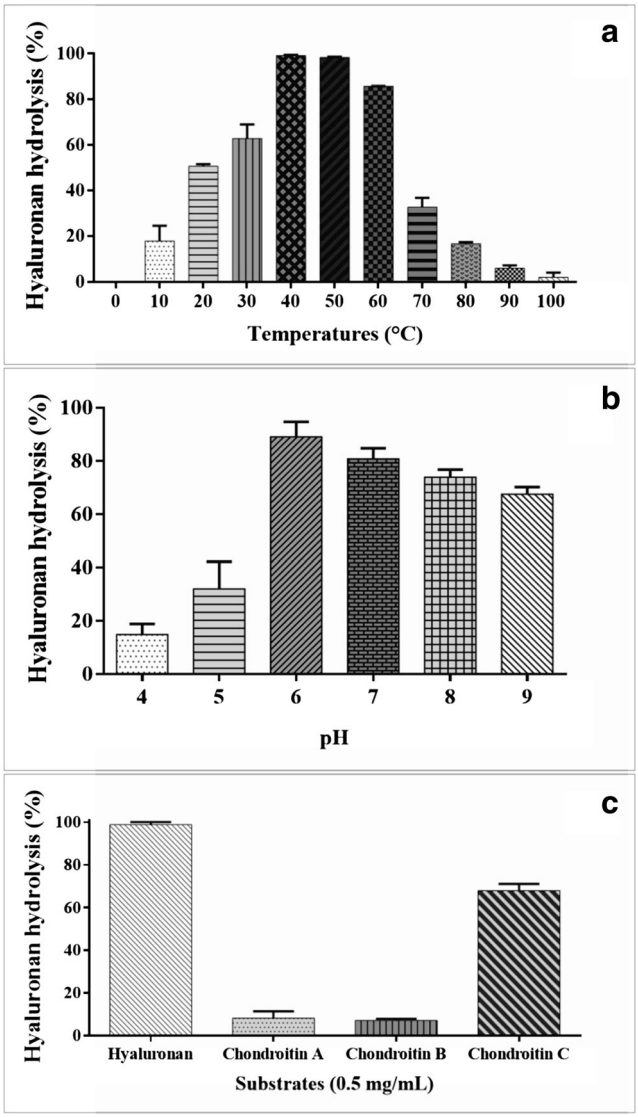

Fig. 6 Characterization of rTsHyal-1 enzyme activity. **a** Temperature. rTsHyal-1 (approximately 3 μg) was incubated with hyaluronan (0.5 mg/mL) in 200 mM sodium acetate buffer, pH 6.0, containing 150 mM NaCl at different temperatures for 30 min. **b** pH. Turbidimetric activity was performed in 200 mM sodium acetate buffer containing 150 mM NaCl at different pHs. Recombinant hyaluronidase (approximately 3 μg) was incubated with 20 μL of hyaluronan (0.5 mg/mL) at 37 °C for 30 min. **c** Substrates. rTsHyal-1 (approximately 3 μg) was incubated for 30 min at 37 °C with 20 μL of 0.5 mg/mL hyaluronan or chondroitin sulfates A, B, or C in 200 mM sodium acetate buffer at pH 6.0 containing 150 mM NaCl. The values correspond to the mean ± SEM (standard error of the mean, $n = 3$)

translational modifications performed by higher eukaryotic beings, such as glycosylations (Cereghino and Cregg 2000).

Discussion

The choice of TsHyal-1 from the *T. serrulatus* venom was based on the three-dimensional model analysis performed by Horta et al. (2014) in which the sequence/structure of hyaluronidase isoforms TsHyal-1 and TsHyal-2 are compared. Both sequences present some conserved sites for the enzyme activity; however, TsHyal-2 has a variation in the amino acid position 219, in which histidine was replaced by a tyrosine, and this mutation leads to a slight structural difference in the active site cleft of TsHyal-2 when compared to TsHyal-1. Thus, this variation could cause differences in the substrate specificity between these two isoforms (Horta et al. 2014). In addition, TsHyal-1 presents five possible N-glycosylation sites, whereas TsHyal-2 has only three sites.

The first recombinant hyaluronidase reported was from *Streptococcus pneumoniae*, and it was produced in the *Escherichia coli* system in 1994 (Berry et al. 1994). This expression showed a yield of 1 mg/L, but the recombinant hyaluronidase was cleaved by endogenous proteases into fragments of 89 and 94 kDa. The next year, in 1995, Lu et al. performed the heterologous expression of the first recombinant animal venom hyaluronidase from wasp *Dolichovespula maculata*, also in *E. coli* (Lu et al. 1995). However, the enzyme lost its activity due to refolding and underwent proteolysis during expression. The presence of different electrophoretic bands in SDS-PAGE (Fig. 2b) could be due to the presence of cleaved hyaluronidase.

In general, hyaluronidases from different organisms have already been expressed in several systems, such as bacteria (Gmachl and Kreil 1993; Skov et al. 2006; Berry et al. 1994; Lu et al. 1995; King et al. 1996; Bakke et al. 2011; Ferrer et al. 2013), yeast (Reitinger et al. 2008; Jin et al. 2014; Chen et al. 2016; Kang et al. 2016), plants (Jung et al. 2010; Li et al. 2014), insect cells (Clement et al. 2012; Soldatova et al. 2007; Hofinger et al. 2007; Ng et al. 2005; Soldatova et al. 1998), and mammalian cells (Frost et al. 1997). However, to the best of our knowledge, up to now only two venom hyaluronidases have been expressed in a *P. pastoris* system, one related to bee venom (Reitinger et al. 2008) and the other one to leech saliva (Jin et al. 2014; Kang et al. 2016). Therefore, the present study reports for the first time the heterologous expression of a hyaluronidase from scorpion in a yeast system.

There are several heterologous expression systems available for recombinant protein production. The simplest is the expression system in *E. coli*. Although there are new strategies for targeting protein to the periplasmic space or to the culture medium which facilitates purification and folding of the recombinant protein and avoid the expression in inclusion bodies in the bacteria, this system is not capable of promoting post-translational modifications (Hofinger et al. 2007; Wingfield 2015; Mergulhão et al. 2005). These post-translational modifications, especially glycosylation, may be crucial for enzyme activity, for example, of hyaluronidases (Li et al. 2002; Goto et al. 2014). On the other hand, yeasts exhibit rapid growth and ability to make various post-translational modifications performed by higher eukaryotic cells, such as proteolytic processing, folding, disulfide bridging, and

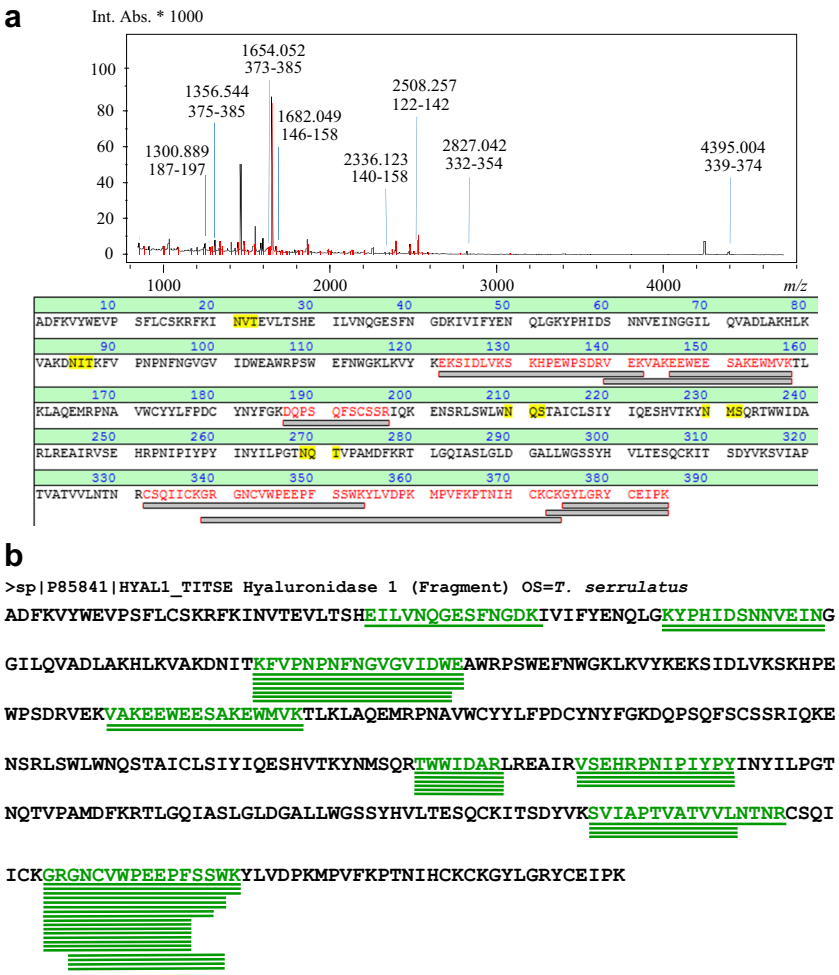

Fig. 7 Mass spectrometry analysis of rTsHyal-1. **a** rTsHyal-1 PMF after trypsin digestion. The PMF was obtained by MALDI-TOF and analyzed by the softwares Sequence Editor and BioTools. Sequencing showed 26.5% coverage of TsHyal-1 (fragments in gray) sequence. Possible glycosylation sites are highlighted in yellow. **b** Peptides obtained by Q Exactive mass spectrometer and analyzed by Pmi-Byonic software (28.6% coverage of rTsHyal-1)

glycosylation. In addition, the yeast expression system is able to secrete the recombinant protein into the culture medium, facilitating its production and purification. *P. pastoris* is easier to genetically manipulate and culture than mammalian cells and can be grown to high cell densities, which can generate less costs for the process (Cregg et al. 2000; Cereghino and Cregg 2000; Brondyk 2009; Daly and Hearn 2005).

As described above, *P. pastoris* is able to do several post-translational modifications that are performed by higher eukaryotes, especially N- and O-glycosylations (Macauley-Patrick et al. 2005). As shown in the SDS-PAGE and zymogram (Fig. 4), the medium culture presents several active bands above 45 kDa, besides a band around 60 kDa. The recombinant protein may become hyperglycosylated even if its native form is not, since *P. pastoris* can add glycosylations on different N-glycosylation sites present in the native protein (Macauley-Patrick et al. 2005). This fact was observed with the recombinant glucosamylase from *Aspergillus awamori* which presented 20 kDa more than native protein corresponding to glycosylations (Heimo et al. 1997).

Studies regarding hyaluronidase expression in *P. pastoris* reports variations on yields of the recombinant protein. The recombinant hyaluronidase from bee, Hya, showed a yield of 0.195 mg/mL (Reitinger et al. 2008), while H6LHyal from leech presented 0.42 mg/mL (Jin et al. 2014). In the attempt to purify rTsHyal-1, several chromatographic approaches were tested, among them anion exchange, affinity, gel filtration, and reversed phase. Treatment with denaturants (i.e., 8 M urea) was also tested for the culture medium after the end of the expression, but it was not possible to undo the protein aggregate for purification. As previously described, the best result was obtained using a CMC-52 column, since the recombinant protein interacted with the resin and was eluted with the gradient of buffer and hyaluronidase activity was not detected in the unretained components fraction. Several studies reported difficulties in purification of recombinant hyaluronidase, among them the loss of enzyme activity after the refolding step (Justo Jacomini et al. 2014; Hofinger et al. 2007; Lu et al. 1995), proteolysis (Berry et al. 1994; Soldatova et al. 1998), and the irreversible binding to the chromatographic resin

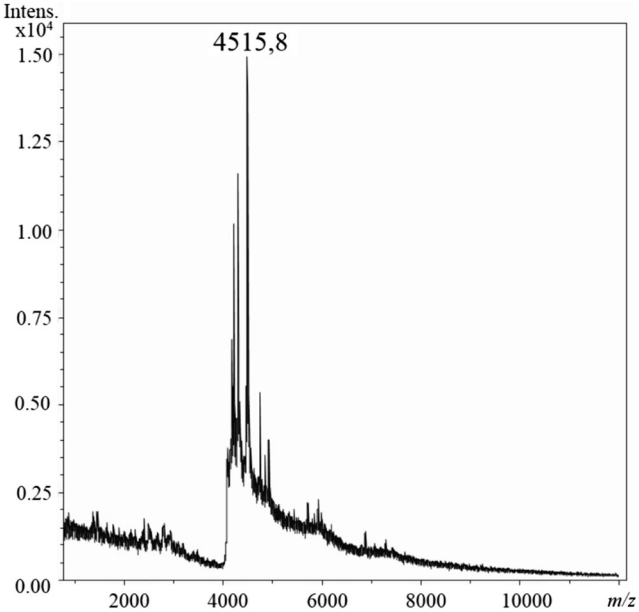

Fig. 8 Glycosylation analysis of rTsHyal-1. Mass spectrum of deglycosylated rTsHyal-1 treated with PNGase F obtained by MALDI-TOF. The sample was analyzed in the 2,5-dihydroxybenzoic acid matrix operated in reflected positive mode, and the analyzer was previously calibrated with Peptide Standard from Bruker Daltonics. The mass spectrum was analyzed using FlexAnalysis 3.0 software

(Soldatova et al. 1998). As previously reported, the purification method chosen in this study did not result in a high yield of rTsHyal-1; however, it was possible to partially isolate the active enzyme with the one-step procedure.

Concerning the optimum conditions for rTsHyal-1 activity, the maximum activity was detected when incubated with hyaluronan at 40 °C and pH 6.0. Pessini et al. (2001) evaluated these parameters for the native hyaluronidase isolated from *T. serrulatus* venom, and the enzyme presented similar values, with the optimum temperature in 40 °C, but also highly active from 30 to 50 °C. Native hyaluronidase from *T. serrulatus* is active in a pH range of 4.0 to 8.5, while rTsHyal-1 showed high hyaluronidase activity (> 60%) between pH 6.0 and 9.0. Among the native forms of scorpion hyaluronidase, generally the optimum pH for activity ranges from 4.0 to 6.0 (Ramanaiah et al. 1990; Morey et al. 2006; Feng et al. 2008).

Frost et al. (1997) classified hyaluronidases based on their optimum pH for activity: acid-active enzymes (active between pH 3.0–4.0) or neutral-active (active between pH 5.0 and 6.0). Both the native hyaluronidase (described by Pessini et al. 2001) and recombinant TsHyal-1 from *T. serrulatus* showed optimum activity at pH 6.0; thus, hyaluronidases from this scorpion venom belong to the neutral-active class. In the case of scorpion's accidents, the sting reaches the epidermis in which the pH can range from 5.0 to 7.4 in a healthy tissue (Ohman and Vahlquist 1998; Lambers et al. 2006). In this pH range, *T. serrulatus* hyaluronidase presents high activity, which may aid in the diffusion of the venom components in the victim's tissue, acting as a spreading factor (Bordon et al. 2012).

Hyaluronidases from scorpion venoms are classified as EC 3.2.1.35. This subclass is found in mammalian spermatozoa, lysosomes, venom of hymenoptera, and venom of snakes and comprises endo-β-N-acetyl-D-hexosaminidases capable of hydrolyzing hyaluronan in the β1➔4 position among residues of N-acetyl-β-D-glucosamine and D-glucoronate. These enzymes are hyaluronoglycosaminidases and have hydrolytic and transglycosydase activities, and may degrade hyaluronan, chondroitin sulfate (mainly chondroitin sulfate A, and chondroitin sulfate C), and, in a lesser extent, dermatan sulfate (chondroitin sulfate B or beta-heparin) (Bordon et al. 2015). Therefore, as expected, rTsHyal-1 exhibited the hydrolysis profile expected for its class EC 3.2.1.35. This is the first time that the substrate specificity for *T. serrulatus* hyaluronidase has been evaluated.

Mass spectrometry analysis validated the production of rTsHyal-1 by confirming 46.8% of the amino acid sequence. The rTsHyal-1 exhibits five possible N-glycosylation sites, and during the ionization process, glycopeptides can suppress the ionization of non-glycosylated peptide leading to a low detection of the tryptic digests (Mechref 2012). In addition, the detection of tryptic peptides containing glycosylation depends on the type of sugar present. In particular, oligomanose, the main N-glycosylation performed by *P. pastoris*, is not cleaved so easily compared to other N-glycans (Zaia 2004). Skov et al. (2006) carried out a similar analysis and obtained only 64% coverage with the PMF of the recombinant hyaluronidase of vespula *Vespula vulgaris*. Therefore, these factors could explain why it was not possible to obtain a complete coverage of the rTsHyal-1 sequence.

The presence of a glycan in rTsHyal-1 was confirmed by the deglycosylation and mass spectrometry analyses, which provided a mass of 4515.8 Da related to the glycosylation. Similar studies corroborate with this finding, as an example of a recombinant hyaluronidase SFHYA1 from *Synanceja* fish that presented an increase of 4.5 kDa in its molecular mass, which was supposed to be a glycosylation (Ng et al. 2005). The recombinant human Hyal-1 showed a difference of approximately 4.9 kDa related to N-glycosylation after the treatment with PNGase F. Moreover, Hyal-1 lost 40% of its activity, which may indicate that this glycosylation plays an important role in its enzyme activity (Hofinger et al. 2007).

These results describe a successful production of an active rTsHyal-1 in a yeast heterologous system which allowed its structural and enzyme characterization, being a pioneer study in the production of the first recombinant hyaluronidase of scorpion in a *P. pastoris* system. The determination of the best heterologous expression conditions of *T. serrulatus* hyaluronidase in *P. pastoris* will allow the in-depth structural and functional studies of this enzyme. Hyaluronidases present several therapeutic and biotechnological applications in various fields

(Wohlrab et al. 2014). This study may generate prospects for expanding the therapeutic and biotechnological application potential of these enzymes through the possibility of the recombinant production of this active enzyme in a heterologous system.

Funding information This study received financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, São Paulo Research Foundation, scholarship to FGA, n. 2011/12317-3 and 2013/26083-0; grant to ECA, n. 2012/14996-8), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, The National Council for Scientific and Technological Development, grants n. 402508/2012-2, n. 303689/2013-7, and n. 449960/2014-5), and the Support Nucleus for Research on Animal Toxins (NAP-TOXAN-USP, grant n. 12-125432.1.3).

Compliance with ethical standards

Ethical statement approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest The authors declare that they have no conflict of interest.

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