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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

A hemolytic peptide from the mycophilic fungus *Sepedonium chrysospermum* (Bull.) Fr.

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Abstract The hemolytic activity of an extract of the mycoparasite *Sepedonium chrysospermum* (teleomorph *Hypomyces chrysospermus*) was detected and characterized. Extraction of the fungal biomass by methanol yielded a fraction in which the hemolytic activity against human red blood cells corresponded to a peptide with a molecular mass of 7,653.72 Da and an isoelectric point of approximately 5.8. The peptide was temperature resistant, and the hemolysis was only partially inhibited, even after a 30-min pre-incubation at 100°C. Its hemolytic activity was unaffected by treatment with proteolytic enzymes such as trypsin. Among the divalent cations assayed, Hg^{2+} was the strongest inhibitor of hemolysis. The reducing agent, dithiothreitol, and the membrane lipid, cholesterol, demonstrated concentration-dependent inhibitory activities. Finally, hemolytic activity triggered by the peptide was analyzed by scanning electron microscopy, and a pore-forming activity was detected.

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Introduction

Mycophilic fungi of the anamorphic genus *Sepedonium* (teleomorphs in *Hypomyces*, *Hypocreales*, Ascomycota) infect and parasitize the fruit bodies (or sporomata) of boleticolous species, forming conidia in the final stage of the infection process (Gams et al. 2004; Neuhof et al. 2007). The preferred hosts are genera within *Boletales*, such as *Boletus*, which include the most popular and expensive edible wild-grown mushrooms, *Boletus edulis* and relative group. Other genera parasitized by *Sepedonium* are *Xerocomus* and *Paxillus*. Infections lead to total necrosis and the formation of large quantities of yellow aleurioconidia (Sahr et al. 1999; Põldmaa et al. 2011). Mycoparasitic fungi are a diverse and prolific source of bioactive compounds with great value for biotechnological and biopharmaceutical applications (Mukherjee et al. 2011). Metabolites previously isolated from *Sepedonium chrysospermum* (Bull.) Fr. include the polyketide sepedonin and its derivatives (Wright et al. 1970; Nagao et al. 2006), the antifungal compound chrysodin (Closse and Hauser 1973), the family of chrysospermins, 19-membered peptaibols with antibacterial and antifungal activities (Dornberger et al. 1995), the cyclic pentapeptide chrysosporide (Mitova et al. 2006), and the peptaibol antibiotic, chrysaibol (Mitova et al. 2008).

In this paper, we study a peptide derived from *S. chrysospermum* cultures with hemolytic activity. Several reports describe hemolysins as important virulence factors in

the pathogenic processes of many clinical microorganisms, causing hemorrhagic septicemia and diarrhea (Honda et al. 1976; Raimondi et al. 2000; Han et al. 2002). Hemolysins appear to be associated with the toxicity of *Aspergillus fumigatus* Fresen. (Sakaguchi et al. 1975; Ebina et al. 1984; Rementeria et al. 2005) and with sporadic intoxications following human and animal ingestions of large quantities of fresh *Pleurotus ostreatus* (Jacq.) P. Kumm. (Al-Deen et al. 1987; Žužek et al. 2006). Hemolysins were isolated from the microfungi *Aspergillus niger* Tiegh. (Donohue et al. 2006), *Stachybotrys chartarum* (Ehrenb.) S. Hughes (Vesper et al. 2001), *Penicillium chrysogenum* Thom (Donohue et al. 2005), and *Trichophyton mentagrophytes* (C.P. Robin) Sabour. (Schaufuss et al. 2007). Whether hemolysin plays a common role in different organisms remains to be elucidated (Ngai and Ng 2006).

To our knowledge, this is the first report of a hemolytic peptide produced by a mycoparasitic fungus. The aim of this study was to characterize the hemolytic agent of *S. chrysospermum* and its effect on human red blood cells.

Materials and methods

Strain collection and culture conditions

S. chrysospermum was isolated from fresh basidiomata of the edible mushroom *B. edulis* Bull., collected in Liguria, Italy. For isolation, the conidia were aseptically removed from the infected host and were transferred to modified Sabouraud Dextrose Agar plates (1.5% agar, 2% glucose, and 1% peptone); the strain was grown at 24°C in dark conditions. Identification was made following classic mycological methods using specific literature (Rogerson and Samuels 1989; Sahr et al. 1999; Põldmaa 2000) and online keys (Põldmaa et al. 2011). Subcultures of the isolate were deposited at the Mycotheca Universitatis Taurinensis in Turin, Italy (MUT 4752).

S. chrysospermum extract

The strain was inoculated in flasks containing 25 ml of Sabouraud broth (2% glucose and 1% peptone) and was grown under static conditions in the dark for 12 days at 24°C. The cultures were centrifuged (4,500×*g*, 30 min, 4°C), and fungal biomasses (mycelia and conidia) were washed three times in phosphate-buffered saline (PBS; 100 mM sodium phosphate buffer pH 7.2 and 150 mM NaCl) and were then weighed and stored at −20°C until extraction. The cells were resuspended in methanol or PBS using a ratio (*w*/*v*) of 1:3 for wet weight/solvent and were sonicated for 5 cycles (2 min on and 2 min off) in a sonicating water bath. After centrifugation (4,500×*g*, 15 min, 4°C), PBS supernatants were directly collected and stored at −20°C; methanol supernatants were taken to dryness under a stream of air, and the dried pellets were then stored until use.

Protein determination

Protein concentration was estimated by the bicinchoninic acid assay (Smith et al. 1985) using a BCA Protein Assay Kit (Thermo Scientific Pierce) and bovine serum albumin as the standard.

Hemolytic activity assay

This assay has been described previously (Andreeva et al. 2006; Singh and Kaur 2008). Human erythrocytes (RBCs) were purified from freshly drawn blood obtained from the Blood Transfusion Center, G. Gaslini (Genoa, Italy). The blood was centrifuged (500×*g*, 10 min, 4°C), and the serum and the upper layer of white blood cells were removed. Erythrocytes were washed three times with PBS and brought to a final concentration of 4% *v*/*v* in PBS, approximately 4.76×10^8 cells/ml. Blood suspensions (0.1 ml, in micro-centrifuge tubes) were combined with 0–100 μl of the dried pellets redissolved in methanol (0–40 μg/ml); the final volumes were adjusted to 0.7 ml by adding PBS. After a 30-min incubation period at 37°C, unlysed erythrocytes were pelleted by centrifugation (500×*g*, 2 min). The absorbances of the supernatants were measured at 540 nm. Supernatants from erythrocytes treated with 0.1% *v*/*v* Triton X-100 and 15% *v*/*v* methanol were used as complete and spontaneous lysis controls, respectively. One hemolytic unit (HU) was defined as the concentration of hemolysin required to achieve 50% lysis of human RBCs.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Protein species were visualized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to the Laemmli method (Laemmli 1970). The dried pellets were resuspended in PBS and SDS-PAGE sample buffer (125 mM Tris-HCl pH 6.6, 20% *v*/*v* glycerol, 2% *w*/*v* SDS, and 10% *v*/*v* β-mercaptoethanol). After heating (97°C, 3 min), samples were loaded on 16% (*w*/*v*) polyacrylamide gels in SDS-PAGE running buffer (25 mM Tris-base, 192 mM glycine, and 0.1% *w*/*v* SDS). Protein electrophoresis was conducted at 120 V. Protein bands were visualized by silver staining according to the Vorum protocol (Vorum et al. 1999).

Two-dimensional gel electrophoresis

The dried pellets were resuspended in rehydration buffer containing 7 M urea, 2 M thiourea, 2% *w*/*v* CHAPS,

10 mM dithiothreitol (DTT), 2% *v*/*v* carrier ampholytes pH 3-10 (GE Healthcare) and 40 mM Tris-HCl. The samples were loaded onto linear IPG strips (7 cm, pH 3–10) (GE Healthcare) and were incubated overnight at room temperature. Isoelectric focusing was then performed on a Multiphor II apparatus (GE Healthcare). Focused strips were equilibrated for 20 min in a solution of 6 M urea, 26 mM DTT, 4% *w*/*v* SDS, 30% *v*/*v* glycerol, and 0.1 M Tris-HCl pH 6.8, followed by 6 M urea, 380 mM iodoacetamide, 4% *w*/*v* SDS, 30% *v*/*v* glycerol, and 0.1 M Tris-HCl pH 6.8 for 20 min. The strips were then directly applied to 16% SDS-polyacrylamide gels and separated at 120 V. After silver staining, gels were recorded on an Image Scanner (Amersham Biosciences).

Electrospray ionization mass spectrometry

The dried pellets were resuspended in an 85:15 mixture of 0.1% *v*/*v* formic acid in water (eluent A)/0.1% *v*/*v* formic acid in acetonitrile (eluent B) and were analyzed on an Agilent 1100 MSD system, consisting of a capillary chromatograph equipped with a diode array detector coupled to an ion trap mass spectrometer equipped with an orthogonal geometry electrospray source. High-pressure liquid chromatography (HPLC) separation was performed on an Agilent Zorbax SB C18 column (250×1 mm; particle size, 5 μm) at a flow rate of 12 μl/min. The gradient was as follows: from 0 to 10 min, eluent B was held at 15%; eluent B was then brought linearly to 70% in 35 min; after that, eluent B was then brought linearly to 100% in 5 min. Chromatograms were monitored at 220 and 260 nm, and mass spectrometry (MS) spectra were acquired in the positive ion mode in an *m*/*z* range including, time by time, the expected multi-charged ions.

Erythrocyte gel overlay

To determine if the electrophoretic band was the active hemolysin, dried pellets were resuspended in a small volume of PBS, and aliquots were electrophoresed on 8% PAGE using a non-denaturing, non-reducing buffer system; the samples were heated to 97°C for 3 min before loading. After electrophoresis, according to a previous protocol (Don et al. 2004), the gel was divided into two parts. One part was washed three times with PBS and was then overlaid with a solution of 2% *w*/*v* human erythrocytes suspended in 0.8% *w*/*v* agarose (37°C), 3.0 mm thick; the erythrocyte–agarose layer solidified on top of the polyacrylamide gel at room temperature. After incubation at 4°C for 12 h, the overlaid gel was examined for zones of hemolysis. The other part of the gel was silver-stained.

Effect of temperature on hemolytic activity

To determine the effects of temperature on hemolytic activity, 0–100 μl of dried pellets resuspended in methanol (0–40 μg/ml) was mixed with PBS (600 μl total volume) and was pre-incubated for 30 min at different temperatures. Pre-washed RBCs (100 μl, 4% *w*/*v*) were then added. After incubation at 37°C for 30 min, the OD_{540nm} of the supernatant was measured.

Effect of a reducing agent and trypsin on hemolytic activity

A reducing agent (DTT) and trypsin were tested for their capacities for inhibiting hemolytic activity. Aliquots of extract (300 μl, 75 μg protein total) were incubated for 30 min at 37°C with different concentrations of DTT or trypsin; the total volume was brought up to 1 ml with PBS, pH 7.2. Then, 100 μl of the pre-incubated materials, corresponding to 1.5 HU (10.71 μg/ml), was mixed with 100 μl of a 4% *w*/*v* RBC suspension and 500 μl of PBS (pH 7.2); after incubation at 37°C for 30 min, the samples were assayed for hemolytic activity (Chung et al. 2001).

Effect of different carbohydrates, metal ions, and cholesterol on hemolytic activity

Hemolysis inhibition tests were performed as follows. Seventy-microliter solutions of different carbohydrates (200 mM) in PBS, 20-μl solutions of different divalent cations (350 mM) in water, or 1–10 μl of cholesterol in different concentrations of ethanol solutions was added to 30 μl of extract with 1.5 HU; the volumes were brought up to 600 μl with PBS, pH 7.2. The mixtures were allowed to stand for 30 min at room temperature and were then mixed with 0.1 ml of a 4% *w*/*v* suspension of human erythrocytes. After incubation at 37°C for 30 min, the remaining hemolytic activity was measured. In controls for all of the above solvents without hemolytic agent added, no hemolysis occurred.

Scanning electron microscopy

Human erythrocytes (0.1 ml of a 4% *w*/*v* suspension) were treated with 30 μl of extract with 2 HU; final volumes were brought up to 0.7 ml with PBS. After incubation at room temperature for 1 min and 15 min, samples were fixed in 3% *v*/*v* glutaraldehyde in 0.1 M PBS, pH 7.2. They were then post-fixed in 1% aqueous osmium tetroxide in 25 mM phosphate buffer, dehydrated in a graded ethanol series and transferred onto glass cover slips. Each preparation was air-dried and sputtered with gold. The specimens were viewed and photographed with an S360 SEM (Leica-Cambridge) operating at 20 kV.

Statistical analysis

The results are presented as the mean values ± standard deviations (SD) (n=3). Two-tailed Student's t tests were employed for comparisons between control and experimental conditions, and p values of less than 0.05 were considered significant. Experimental data were analyzed using Minitab 15 Statistical Software (Minitab Inc.).

Results

Isolation and characterization of a hemolytic peptide from *S. chrysospermum* extract

S. chrysospermum was cultured for 12 days in Sabouraud broth as described in "Materials and methods." Two extracts were obtained: one in aqueous solution (PBS) and the other in methanol. Both samples were analyzed for protein content and were characterized by SDS-PAGE. The methanol extract had a lower protein content compared to the PBS extract; the protein recoveries were approximately 0.6 mg/g fungal mass for the methanol extract and 3 mg/g for the saline extract. When subjected to SDS-PAGE under reducing conditions, the PBS extract exhibited numerous protein bands after silver staining, while in the methanol extract, a band with a low molecular mass (about 7 kDa) was predominant (Fig. 1a). The homogeneity of the peptide was confirmed by means of 2-DE gel analysis. Only one spot was visualized, with an isoelectric point of approximately 5.8 (Fig. 1b). A very weak signal corresponding to a molecular weight of 14 kDa could also be seen by SDS-PAGE; this band is probably due to dimerization, as it disappeared in the bidimensional electrophoresis, where denaturating and reducing conditions were stronger.

To investigate whether this low molecular weight peptide was indeed responsible for the hemolysis, the extract was subjected to native gel separation, followed by in-gel visualization of hemolytic activity. After electrophoresis, a single band was detected by silver staining in the native gel; its hemolytic activity was visualized on an erythrocyte/agarose overlay gel as a translucent zone that appeared after overnight incubation (Fig. 1c). For a deeper structural study of the peptide, an electrospray ionization (ESI) mass spectrometry analysis was then performed. HPLC/MS-TIC (total ion current) chromatograms for the extract (data not shown) allowed us to identify a well-resolved peak with a retention time of 43.7 min, corresponding to a 70% organic phase in the eluent, which was consistent with the hydrophobicity of the isolated peptide. The multi-charged spectrum was deconvoluted, yielding a neutral mass of 7,653.72 Da (Fig. 1d), supporting the data from the SDS-PAGE separation. Attempts to structurally characterize this molecule were

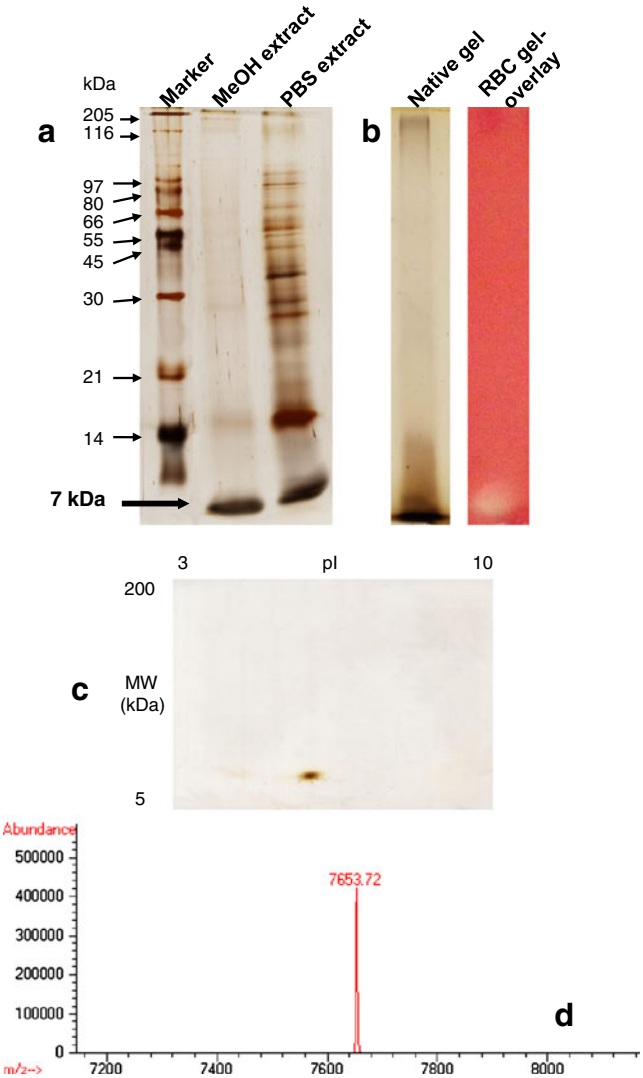

Fig. 1 *S. chrysospermum* hemolytic peptide isolation. **a** Methanol and PBS extracts separated on 16% SDS-PAGE. **b** Methanol extract separated on native polyacrylamide gel and visualized by silver staining (*left lane*) or by hemolytic spots on erythrocyte-agarose overlay after overnight incubation (*right lane*). **c** Methanol extract separated on two-dimensional SDS-PAGE. **d** Deconvoluted ESI showing the neutrally charged mass spectrum of the hemolytic peptide. Gels represent triplicate runs. The *arrow* shows the hemolytic peptide

conducted using proteolytic enzymes, such as trypsin and chymotrypsin. Unfortunately, the digests did not yield peptides that were suitable for sequencing.

Effect of temperature on the hemolytic peptide

Figure 2 shows a dose–response curve of the hemolytic activity of the *S. chrysospermum* peptide assayed at five different pre-incubation temperatures. The curve has a steep and sigmoidal shape, which suggests that lysis was accompanied by some co-operative effect between toxin molecules, which was more evident when the peptide

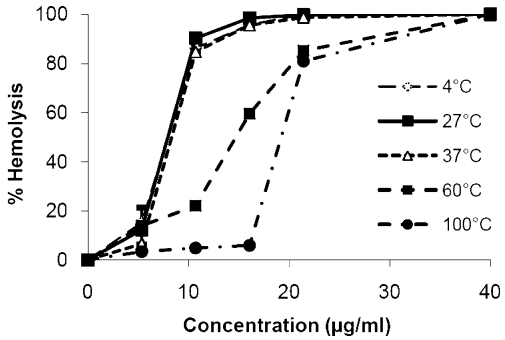

Fig. 2 Activity of *S. chrysospermum* hemolysin, assayed at various concentrations, after pre-incubation at different temperatures for 30 min. Results represent means±SD (n=3). Some *error bars* are within the *symbols*

concentration was increased. This co-operation might consist of aggregation or oligomerization of the toxin, leading to more stable and efficient structures that interact with erythrocyte membranes and that consequently induce the hemolytic event. The curves for the 60°C and 100°C pre-incubations (Fig. 2) show a marked rightward shift in the amount of hemolysin necessary to induce 50% hemolysis in the standard assay conditions (37°C). This pattern indicates that the hemolytic potency was reduced when the pre-incubation temperature was increased. However, at a concentration of 3 HU/ml, more than 80% activity was retained, even after pre-incubation for 30 min at 100°C. If the concentration of hemolysin exceeded a certain threshold, the peptide acquired a resistance to thermal denaturation.

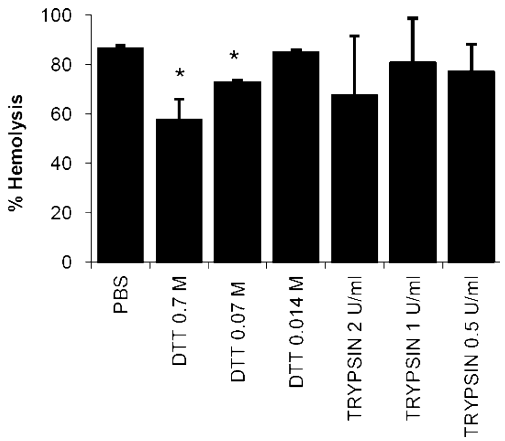

Fig. 3 Effects of DTT and trypsin on hemolytic activity. Aliquots were incubated for 30 min at 37°C with the indicated chemicals and concentrations. Then, a 1:10 dilution of the pre-incubated material (1.5 HU) was assayed for hemolytic activity. Results represent means±SD (n=3). *p<0.05 indicates a statistically significant difference when compared to incubation in buffer only (PBS). A two-tailed Student's t test was employed for comparison

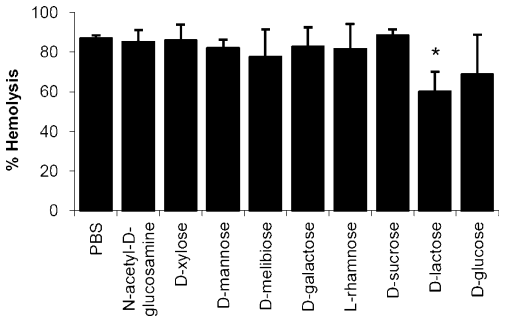

Fig. 4 Effects of exposure of *S. chrysospermum* extract to various carbohydrates (20 mM) for 30 min. Results represent means±SD (n=3). *p<0.05 indicates a statistically significant difference when compared to incubation in buffer only (PBS). A two-tailed Student's t test was employed for comparison

Effect of a reducing agent and trypsin on hemolytic activity

Figure 3 describes the hemolytic activity of the peptide in the presence of a proteolytic enzyme (trypsin) or a reducing agent (DTT). Trypsin was not effective in diminishing the hemolytic activity; as has been previously shown in mass spectroscopy analysis, it was not able to efficiently hydrolyze the peptide. DTT reduced hemolysis by about 30% and 14%, respectively, when assayed at concentrations of 0.7 and 0.07 M (Fig. 3). No effect was detected when DTT was tested at a lower concentration (0.014 M). The effectiveness of DTT in lowering the hemolytic activity is consistent with its capacity to reduce disulfide bonds and then alter protein tertiary structures.

Effect of sugar, cholesterol, and divalent cations on hemolytic activity

Figure 4 shows the influence of sugars on hemolytic activity. It is well known that erythrocyte membranes are characterized by the presence of many glycoproteins and glycolipids. The interactions between the peptide and

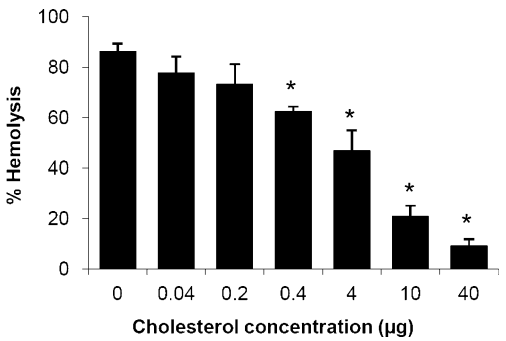

Fig. 5 Effects of different concentrations (0–10^{-4} M) of cholesterol on hemolytic activity. Results represent means±SD (n=3). *p<0.05 indicates a statistically significant difference when compared to incubation in buffer only (PBS). A two-tailed Student's t test was employed for comparison

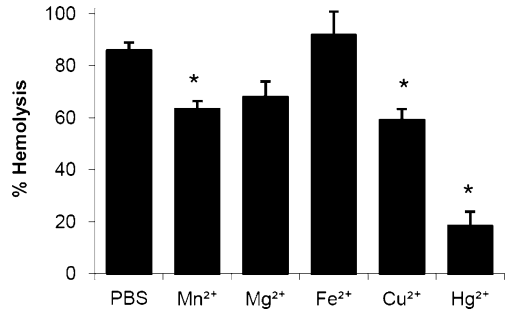

Fig. 6 Effects of bivalent metal ions (10 mM) on hemolytic activity of *S. chrysospermum* hemolysin. Results represent means±SD (n=3). *p<0.05 indicates a statistically significant difference when compared to incubation in buffer only (PBS). A two-tailed Student's t test was employed for comparison

erythrocyte membranes could involve the presence of specific sugar residues. Among the various carbohydrates assayed, only D-lactose partially reduced the hemolytic activity by approximately 30% (Fig. 4). Other sugars, such as *N*-acetyl-D-glucosamine, D-glucose, D-melibiose, D-xylose, D-mannose, D-sucrose, L-rhamnose, and D-galactose, tested at 20 mM, showed no significant inhibitions of hemolytic activity (p>0.05 when compared to PBS).

Cholesterol is an essential component of eukaryotic membranes; it plays a pivotal role in membrane organization and dynamics. Thus, the interaction of membrane peptides with cholesterol represents an important determinant. Our data (Fig. 5) show that cholesterol inhibited *S. chrysospermum* hemolysin in a concentration-dependent manner. A final lipid concentration of approximately 10^{-6} M (0.4 μg) reduced hemolysis by only 24%, but concentrations above 3×10^{-5} M (10 μg) strongly inhibited hemolytic activity (77%).

Erythrocyte membranes could be stabilized by the presence of divalent cations; this effect could lead to a partial inhibition of hemolytic activity. In Fig. 6, the effects of bivalent cations (10 mM) on hemolytic activity are shown. Hg^{2+} exhibited the strongest inhibition (68%), whereas Cu^{2+} (27%) and Mn^{2+} (23%) were considerably less effective. Little or no inhibitory effects were detected when other ions (Fe^{2+} and Mg^{2+}) were tested.

S. chrysospermum hemolysin is a pore-forming peptide

To gain morphological insights on the mechanism of hemolysis, human erythrocytes were incubated with *S. chrysospermum* extract, and the resulting effects on cell structure were observed by SEM. Untreated human erythrocytes (Fig. 7a) and erythrocytes treated with *S. chrysospermum* extract for 1 min (Fig. 7b) maintained their biconcave discoid shape, with a relatively smooth surface; erythrocytes treated with *S. chrysospermum* extract for 15 min displayed ghost-like remnants with pores (Fig. 7c), many cells lacked integrity, and membrane debris was visible (Fig. 7d). Lysis likely arose from osmotic leakage caused by the formation of pores in the cell membrane, followed by severe membrane damage. Thus, we hypothesize that *S. chrysospermum* hemolysin is inserted in the lipid bilayers of erythrocyte membranes because of its hydrophobicity, inducing the destabilization of the structure and the subsequent formation of pores.

Discussion

In this paper, for the first time, a hemolytic peptide derived from a mycophilic microfungus was isolated and characterized. Features of the peptide, such as its accumulation on the fungal surface, its poor solubility, and its tendency to form aggregates in aqueous solutions, made the purification by chromatography a difficult task. In this report, we describe a rapid and selective protocol that entailed solubilization of the fungal mass in methanol and subsequent extraction by sonication of the cell-bound hemolytic peptide. This adopted procedure resembles one that was used to purify *Staphylococcus cohnii* hemolysins (Rözalska and Szewczyk 2008); it is very different from the traditional methods used to purify hemolysins from basidiomycetes and ascomycetes, such as *Schizophyllum commune* Fr. (Han et al. 2010), *Pleurotus eryngii* (DC.) Quél (Ngai and

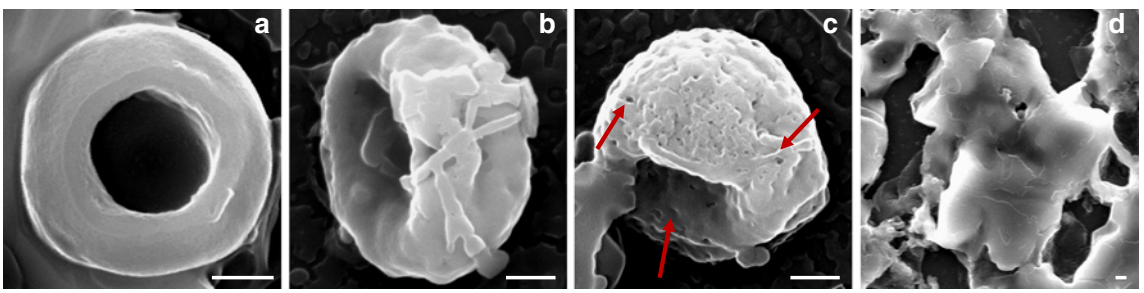

Fig. 7 Scanning electron micrographs showing the effect of *S. chrysospermum* hemolysin (2 HU) on human erythrocytes after incubation for 15 min. **a** Control untreated erythrocytes. **b** Erythrocytes treated with *S. chrysospermum* extract for 1 min. **c** Erythrocytes treated with *S. chrysospermum* extract for 15 min showing numerous pores in the membranes. **d** Completely destroyed erythrocytes. *Arrows* highlight hemolysin-induced pores. The *bar* on each micrograph represents 1 μm

Ng 2006), *P. ostreatus* (Berne et al. 2002), *Agrocybe aegerita* (V. Brig.) Singer (Berne et al. 2002), *A. niger* (Donohue et al. 2006), and *S. chartarum* (Vesper et al. 2001). The molecular weight and an interesting thermostability make this peptide similar to hydrophobins, which are unique hydrophobic protein constituents of fungal cell walls (Neuhof et al. 2007; Wösten 2001; Linder et al. 2005). However, this peptide is also structurally similar to other microfungal hemolysins: it is an aggregating peptide with an acidic p*I* and a low molecular weight, like *S. chartarum* (Vesper et al. 2001) and *P. chrysogenum* (Donohue et al. 2005) hemolysins.

In contrast to the thermolability of *S. commune*, *P. ostreatus*, *P. eryngii*, and *A. aegerita* hemolysins, *S. chrysospermum* hemolysin was stable in high-temperature treatments. The sigmoidal dose–response curve, seen in Fig. 2, suggests a co-operative effect between toxin molecules. Once the toxin reaches the lipid bilayer, interaction with the hydrophobic matrix may occur, and hemolysis could take place rapidly. Unlike hemolysins in *S. commune* and *P. eryngii*, which would be inactivated by cooking before consumption, *S. chrysospermum* hemolysin, likely associated with *S. chrysospermum*-infected basidiomycetes, could retain its activity. Thus, the potential toxicity of *Boletales* infected with *S. chrysospermum* should be investigated in more detail. The best-known thermostable hemolysin is *Vibrio parahemolyticus* (Sakurai et al. 1973), but there are other heat-stable hemolytic proteins, such as those that were isolated from *Ancylostoma caninum* hookworms (Don et al. 2004) and *Albizia leebeck* seeds (Lam and Ng 2011).

The isolated peptide shares several features with other hemolysins: a low isoelectric point has been also observed in *A. aegerita* hemolysin (Berne et al. 2009); additionally, susceptibility to Hg^{2+} and Cu^{2+} is similar to that reported for *P. ostreatus*, *P. eryngii*, *S. commune*, and *Vibrio fluvialis* hemolysins (Han et al. 2002). Divalent cations could inhibit hemolysis by stabilizing the erythrocyte membrane; alternatively, they could combine with the toxin, thus preventing the binding of the peptide to the membrane (Malovrh et al. 1999). Inhibition of hemolysis by carbohydrates has been reported for other hemolytic toxins, which is suggestive of a carbohydrate protection mechanism for erythrocyte membranes (Chung et al. 2001). The binding of cholesterol and the capacity to form pores in host cell membranes are major characteristics of hemolytic peptides (Raghuraman and Chattopadhyay 2005). Inhibition of hemolysis by cholesterol confirms the affinity of the peptide for lipophilic molecules, suggesting that the peptide binds to the RBC membrane in a lipid-mediated manner.

Resistance to degradation by trypsin may be due to the absence of a cutting site; alternatively, it may be that the presence of a strong tertiary structure, stabilized by cross-linked bonds, prevents peptide denaturation. This observation may also reflect the presence of unusual amino acids, which has already been hypothesized for bacterial amyloid proteins (Jordal et al. 2009).

Each pore induced from *S. chrysospermum* hemolytic peptides seems to be at the center of a ring-like depression in the erythrocyte membrane. The pores are approximately 50 nm in diameter; this value is close to the pore size reported for *A. caninum* hemolysin (Don et al. 2004) and is larger than those reported for *P. ostreatus* and *P. eryngii* hemolysins (Berne et al. 2009).

In summary, in this study, we described a simple purification strategy that led to the identification of a pore-forming peptide with hemolytic activity on human erythrocytes in vitro. The peptide exhibited remarkable features, such as resistance to trypsin and temperature. This is the first report of the isolation of a hemolytic peptide from a mycophilic microfungus such as *S. chrysospermum*.

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