Trypsin-specific Inhibitors from the *Macrolepiota procera*, *Armillaria mellea* and *Amanita phalloides* wild mushrooms

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Wild growing mushrooms are a rich source of novel proteins with unique features. We have isolated and characterized trypsin inhibitors from two edible mushrooms, the honey fungus (*Armillaria mellea*) and the parasol mushroom (*Macrolepiota procera*), and from the poisonous death cap (*Amanita phalloides*). The trypsin inhibitors isolated: armespin, macrospin and amphaspin, have similar molecular masses, acidic isoelectric points and are not N-glycosylated. They are very strong trypsin inhibitors and weak chymotrypsin inhibitors. They are resistant to exposure to high temperatures and withstand extreme pH values. These exceptional characteristics are advantageous for their potential use in biotechnology, agriculture and medicine.

**Key words**: basidiomycete; inhibitor; trypsin inhibitor; mushroom; trypsin; thermostable

Received: 07 September, 2015; revised: 24 June, 2016; accepted: 26 June, 2016; available on-line: 03 March, 2017

**Abbreviations**: LeSPI, *Lentinula edodes* serine protease inhibitor; MCA, 7-(4-methyl)coumarylamide; PfTI, *Pleurotus floridanus* trypsin inhibitor

**INTRODUCTION**

Mushrooms, especially wild growing mushrooms, are consumed as a delicacy and are of high nutritional value in terms of high protein and fiber content and low energy content (Barros et al., 2008; Kalač, 2009). Mushrooms have also attracted interest as a rich source of proteins with unique features, displaying a great potential for use in biotechnology, medicine and agriculture (Erjavec et al., 2012). Protease inhibitors constitute a group of fungal proteins that possess exceptional characteristics (Sabotič & Kos, 2012; Dunaevsky et al., 2014). Trypsin inhibitory activity has been established in crude protein extracts of fruiting bodies of several species of mushroom (Vetter, 2000; Gzogyan et al., 2005), but only a few inhibitors have been isolated and characterized. These are the trypsin-specific inhibitors, such as cospin from *Coprinus cinereus* (Sabotič et al., 2012; Avanzo Caglič et al., 2014), crispin from *Clitocybe nebularis* (Avanzo et al., 2009; Avanzo Caglič et al., 2014), a serine protease inhibitor LeSPI from *Lentinula edodes* (Odani et al., 1999) and trypsin inhibitor PfTI from *Pleurotus floridanus* (Ali et al., 2014). Weak inhibition of trypsin has been also reported for macrocin 4, a cysteine protease inhibitor from *Macrolepiota procera* (Sabotič et al., 2009; Renko et al., 2010). While crispin and cospin display a trypsin-specific inhibitory profile, others show a broader inhibitory profile for serine proteases from different sources. Crispin, cospin and macrocin 4 possess a β-trefoil fold, but each utilizes a different loop for trypsin inhibition (Renko et al., 2010; Avanzo Caglič et al., 2014) demonstrating a remarkable diversity of fungal proteins.

Here, we report on the isolation and characterization of trypsin inhibitors from wild growing mushrooms, including the edible honey fungus *Armillaria mellea*, the parasol mushroom *M. procera*, and the poisonous death cap *Amanita phalloides*.

**MATERIALS AND METHODS**

**Enzymes and substrates.** Bovine trypsin, bovine chymotrypsin, porcine kallikrein, porcine pepsin and papain were obtained from Sigma, bovine thrombin from Calbiochem, subtilisin from Boehringer Manheim and porcine elastase from Serva. Substrates Z-Phe-Arg-MCA, Suc-Ala-Ala-Pro-Phe-MCA, H-Pro-Phe-Arg-MCA, Boc-Val-Pro-Arg-MCA and Suc-Ala-Ala-Ala-MCA were purchased from Bachem and MOCAc-APAKFFRLK(Dnp)-NH₂ was from Peptide Institute Inc.

**Isolation of trypsin inhibitors.** Fruiting bodies of *M. procera*, *A. mellea* and *A. phalloides* were collected in their natural habitat in Slovenia and frozen at –20°C. Thawed fruiting bodies were homogenized in 0.05 M Tris/HCl, pH 8, with 0.3 M NaCl (buffer A) containing reducing agent Na₂S₂O₃ (1 g/l), then centrifuged for 20 min at 8300 × g and 4°C. The supernatant was concentrated by ultrafiltration using a 3 kDa cut-off (UM-3, Amicon) and centrifuged for 10 min at 16 000 × g and 4°C. Proteins were separated on a preparative Sephacryl S-200 (GE Healthcare) column (4 × 110 cm, 63 ml/h flow rate with 21 ml fractions) equilibrated with buffer A. Fractions exhibiting trypsin inhibitory activity were pooled, concentrated by ultrafiltration and applied to a papain-affinity chromatography column containing papain coupled to CnBr-activated Sepharose prepared according to the manufacturer’s instructions (GE Healthcare). After washing with 50 mM Tris/HCl, 0.5 M NaCl, pH 7.5 (buffer B), the bound proteins were eluted with 10 mM NaOH in 4°C. The flow-through fraction was concentrated and applied to a trypsin-affinity chromatography column containing trypsin coupled to CnBr-activated Sepharose prepared according to the manufacturer’s instructions (GE Healthcare). The column was washed with buffer B and the proteins were eluted with 10 mM NaOH.

**SDS-PAGE and isoelectric focusing.** Proteins were analyzed on 12% polyacrylamide gels under denaturing and reducing conditions, and visualized using Coomassie brilliant blue staining. Low Molecular Weight markers of...
14.4–97 kDa (GE Healthcare) were used for molecular mass estimation.

Isoelectric focusing (IEF) was carried out with a Pharmacia PhastSystem using commercial precast pH 3–9 gradient gels following manufacturer’s instructions. Alternatively, precast Novex pH 3–7 IEF Protein Gels were used. Marker proteins with pI values ranging from 3.5–9.3 were used for calibration (GE Healthcare).

**Thermal and pH stability.** To test their thermal stability, trypsin inhibitors (0.2 mg/ml) were incubated at 25°C, 37°C, 50°C, 60°C, 70°C, 80°C or 100°C for 15 min and then for 40 min at room temperature. To test their pH stability, inhibitors (0.1 mg/ml) were incubated in 200 mM Tris/HCl (pH 11), in 50 mM Tris/HCl (pH 7.5) or in 30 mM citric acid (pH 2) for 15 min and then neutralized. Residual inhibitory activity was measured against trypsin using Z-Phe-Arg-MCA as a substrate.

**N-terminal sequence analysis.** Amino acid sequences were determined as described previously (Sabotič et al., 2007).

**Protein glycosylation analysis.** The macrospin, amphaspin and armespin proteins (2 µg) were denatured and analyzed for the presence of N-linked glycans using recombinant N-glycosidase F (Roche) and following the manufacturer’s instructions.

**Protease inhibition assays and determination of inhibition constants.** Inhibitory activities of samples were measured during the isolation procedure against trypsin, using N-benzoyl-DL-arginine-p-nitroanilide substrate as described in (Avanzo et al., 2009). Inhibitory activities against chymotrypsin, subtilisin, thrombin, elastase, kallikrein, papain and pepsin were assayed as described previously (Sabotič et al., 2012).

The kinetics of inhibition of trypsin and chymotrypsin were determined according to Henderson (Henderson, 1972) as described for cathepsin B inhibition by chlotoxyspin (Brzin et al., 2000). The Kᵢ values of 125 µM and 363 µM were determined and used for trypsin and chymotrypsin, respectively. Different amounts of the inhibitors (85 mM to 0.11 µM) were incubated with each of the enzymes for 10 min in 100 mM Tris/HCl (pH 8), 20 mM CaCl₂, prior to the addition of the Z-Phe-Arg-MCA substrate for trypsin or Suc-Ala-Ala-Pro-Phe-MCA for chymotrypsin. The released MCA was measured using an Infinite M1000 microplate reader (Tecan).

**RESULTS**

**Isolation and characterization of trypsin inhibitors**

A three step procedure was used to isolate trypsin inhibitors from crude protein extracts of *M. procera*, *A. mellea* and *A. phalloides*. Following size-exclusion chromatography, trypsin inhibitory fractions were subjected to papain-affinity chromatography to remove cysteine protease inhibitors of similar size having trypsin inhibitory activity. Finally, trypsin-affinity chromatography yielded the serine protease inhibitors named macrospin (Macrolepide pezona serine protease inhibitor) from *M. procera*, armespin (*Amullaria mellea* serine protease inhibitor) from *A. mellea* and amphaspin (*Ampullaria phalloides* serine protease inhibitor) from *A. phalloides*. SDS-PAGE analysis revealed two protein bands with estimated molecular masses of 17 kDa and 19 kDa in the macrospin sample (Fig. 1A). N-terminal sequences (16 amino acids) were the same for the proteins from these two macrospin bands, and were 25% identical and 50% similar to the

**Kinetics of inhibition**

Macropsin, amphaspin and armespin are strong inhibitors of trypsin, with *Kᵢ* values in the nanomolar range, and weaker inhibitors of chymotrypsin, with *Kᵢ* values in the micromolar range (Table 1). The most effective in inhibiting trypsin and chymotrypsin is macrospin, with *Kᵢ* values of 3.74 nM and 290 nM, respectively. The most specific for trypsin was amphaspin, which strongly inhibited trypsin and chymotrypsin with *Kᵢ* values of 21 nM and 190 nM, respectively. Macropsin was resolved into three bands on SDS-PAGE, with estimated molecular masses of 16 kDa, 18 kDa and 22 kDa (Fig. 1C), while amphaspin revealed only one band with an estimated molecular mass of 18 kDa (Fig. 1B). N-terminal sequences could not be determined for any of the protein bands of armespin and amphaspin, possibly because of the blocked N termini. Isoelectric focusing revealed similar isoelectric points of around pH 4.5 for each of the proteins. Amphaspin displayed a single band, macrospin had an additional band with pI 5.8, and armespin showed the greatest heterogeneity (Fig. 1D). To determine whether the observed heterogeneity is a result of the glycosylation variants, the proteins were deglycosylated with glycosidase F. For all three proteins, the SDS-PAGE patterns of treated and untreated proteins were similar (data not shown), indicating that they do not possess the N-linked glycans.

**Trypsin inhibitors are resistant to extreme conditions**

Thermal stability was examined by measuring residual inhibitory activity after heating for 15 min (Fig. 3A). Amphaspin retained its inhibitory activity after exposure to 100°C, while armespin and macrospin retained it after exposure to 80°C. 15 min exposure to 100°C resulted in 30% and 50% loss of inhibitory activity for armespin and macrospin, respectively.

The pH stability of trypsin inhibitors was measured by determining their inhibitory activities after incubation at extreme pH values (pH 2 and pH 11). All three inhibitors retained their full inhibitory activities upon return to neutral conditions (Fig. 3B).

**Figure 1. SDS-PAGE and isoelectric focusing analyses of purified macrospin, amphaspin and armespin.**

(A–C) SDS-PAGE analysis was performed in a 12% polyacrylamide gel, stained with Coomassie Blue. Lane M, protein molecular mass markers. D, precast pH 3–9 gradient gels were used for IEF analysis using the Pharmacia PhastSystem. S, standard protein IEF markers.

**Figure 2. Alignment of N-terminal amino acid sequences of macrospin and cnispin.**

Identical amino acid residues are shaded dark gray and similar ones in light gray.
inhibited trypsin, with a $K_i$ value of 8.8 nM, and only very weakly inhibited chymotrypsin, with a $K_i$ value of 606 μM.

Macrospin, amphaspin and armespin showed no inhibitory activity against other serine proteases – thrombin, kallikrein, elastase and subtilisin – nor against the cysteine protease papain or the aspartic protease pepsin.

**DISCUSSION**

Trypsin inhibitors with similar biochemical characteristics were isolated from fruiting bodies of *M. procera*, *A. mellea* and *A. phalloides*. After the first step of size-exclusion chromatography, fractions displaying trypsin inhibitory activity also exhibited inhibition of the cysteine protease papain, indicating the presence of mycocypin-like proteins. This was confirmed by an immunoblot analysis of the fractions of *M. procera* sample (data not shown). Papain-affinity chromatography was therefore employed as the second purification step, in which unbound fractions lacking papain inhibitory activity were collected for application to trypsin-affinity chromatography. The latter is the method of choice for isolating trypsin inhibitors from mushrooms and is complemented by a combination of other chromatography methods for trypsin inhibitor isolation. In addition to size-exclusion chromatography, used in the isolation procedures for cnispin (Avanzo et al., 2009) and PfTI, ammonium sulphate precipitation and ion-exchange chromatography were also included for the latter (Ali et al., 2014).

**Table 1. Kinetic constants for the interaction of macrospin, amphaspin and armespin with various proteases**

<table>
<thead>
<tr>
<th>Protease</th>
<th>Family</th>
<th>Macrospin $K_i$</th>
<th>Amphaspin $K_i$</th>
<th>Armespin $K_i$</th>
</tr>
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<tr>
<td>Trypsin</td>
<td>S1</td>
<td>3.74 ± 0.26 nM</td>
<td>8.81 ± 3.55 nM</td>
<td>50.8 ± 0.9 nM</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>S1</td>
<td>0.29 ± 0.01 μM</td>
<td>606 ± 132 μM</td>
<td>1.91 ± 0.36 μM</td>
</tr>
<tr>
<td>Thrombin</td>
<td>S1</td>
<td>Ni</td>
<td>Ni</td>
<td>Ni</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>S1</td>
<td>Ni</td>
<td>Ni</td>
<td>Ni</td>
</tr>
<tr>
<td>Elastase</td>
<td>S1</td>
<td>Ni</td>
<td>Ni</td>
<td>Ni</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>S8</td>
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<tr>
<td>Papain</td>
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<td>Ni</td>
<td>Ni</td>
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</tr>
<tr>
<td>Pepsin</td>
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<td>Ni</td>
<td>Ni</td>
<td>Ni</td>
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</tbody>
</table>

**Figure 3. Resistance of trypsin inhibitors to exposure to high temperature (A) and extreme pH (B) conditions.**

Residual trypsin inhibition relative to that at 25°C or at pH 7.5 is shown after the exposure to high temperature or extreme pH. Error bars represent standard deviation.

The trypsin inhibitory proteins isolated from mushroom fruiting bodies exhibit different degrees of heterogeneity. While amphaspin, LeSPI (Odani et al., 1999) and PfTI (Ali et al., 2014) display single bands in SDS-PAGE analysis, macrospin and cnispin (Avanzo et al., 2009) display double bands having similar N-terminal amino acid sequences. The greatest heterogeneity was observed for armespin with three bands and, at the genetic level, also for cospin, for which 4 isogenes have been identified (Sabotič et al., 2012). Nevertheless, they all have similar molecular masses of around 16 to 22 kDa, with the exception of PfTI with a molecular mass of 37 kDa. They all have isoelectric points between pH 4.4 and 5.2. The different degrees of heterogeneity are also reflected in their inhibitory profiles, where they display different levels of trypsin specificity. Cospin is the strongest trypsin inhibitor, but shows weak chymotrypsin inhibition (Sabotič et al., 2012). Macrospin, cnispin (Avanzo et al., 2009) and amphaspin inhibit trypsin with similar values of $K_i$ in the low nanomolar range, but cnispin and macrospin are also weak chymotrypsin inhibitors with $K_i$ values in the low micromolar range. Amphaspin inhibits chymotrypsin very weakly, with a $K_i$ value of $7 \times 10^4$ higher than that for trypsin. Armespin is the weakest of these trypsin inhibitors, albeit with a $K_i$ in the nanomolar range. It also weakly inhibits chymotrypsin. Strong trypsin inhibition and weak chymotrypsin inhibition has also been reported for LeSPI (Odani et al., 1999). Based on the similarity of biochemical properties and of the macrospin N-terminal sequence to that of cnispin, together with the very similar inhibitory profiles of LeSPI, cnispin, cospin and protease inhibitors as described in this study, these protease inhibitors may belong to the same I66 family in the MEROPS classification.

Trypsin inhibitors isolated from mushroom fruiting bodies are resistant to extreme temperatures and pH values. They all retained their inhibitory activity after exposure to 80°C and amphaspin even withstood 15 min exposure to 100°C. This type of exceptional thermal stability was observed for mycocypins, where it was shown that macrocypins and elitocypin do not resist thermal denaturation per se, but rather they reversibly unfold (Kidrič et al., 2002; Sabotič et al., 2009). This could be an intrinsic property of the symmetric globular proteins with the β-trefoil fold (Gosavi et al., 2008), including mycocypins, mycospins (Renko et al., 2010; Renko et al., 2012) and lectins with the β-trefoil fold (Zuľa et al., 2014).

Similarly, all three inhibitors described here are resistant to exposure to extreme pH, retaining inhibitory activity after exposure to acidic (pH 2) and alkaline (pH 11) pH. The same has been shown for cnispin (Avanzo et al., 2009) and cospin (Sabotič et al., 2012), while LeSPI withstood exposure to acidic...
pH only (Odani et al., 1999) and PrTI was stable between pH 4 and 10 (Ali et al., 2014).

Trypsin inhibitors from mushrooms show similar characteristics but display a considerable diversity. The trypsin inhibitors described from edible and poisonous mushrooms have similar biochemical properties, including low molecular mass, acidic isoelectric point, resistance to extreme pH and high temperatures, and specificity towards trypsin inhibition. On the other hand, they slightly differ in molecular mass and isoelectric point, and in resistance to extreme conditions. Furthermore, their specificity for trypsin inhibition differs in strictness, some weakly inhibiting other serine proteases. Since trypsins are mainly present in pathogenic fungi and are being secreted (St Leger et al., 1997; Dubovenko et al., 2010), the trypsin inhibitory activity is probably directed towards exogenous trypsins, indicating a defensive role against predators, parasites and pathogens. Cnispin and coxpin exert an entomotoxic activity against Drosophila melanogaster that is dependent on trypsin inhibitory activity, suggesting that digestive serine proteases are affected, leading to detrimental effects on growth and survival (Avanzo et al., 2009; Sabotič et al., 2012). This anti-nutritional effect of trypsin inhibitors that are resistant to thermal and pH denaturation could also affect mammals and possibly constitutes an important factor in the digestibility of wild mushrooms and, hence, their nutritional value for human consumption.

CONCLUSION

The exceptional characteristics of trypsin inhibitors from mushrooms are advantageous for their possible use in agriculture for crop protection and pest control, in biotechnology for purification processes and in medicine for drug development and studies on trypsin regulation in various diseases.

Acknowledgements

We are grateful to Dr Roger Pain for critical reading of the manuscript and language editing.

This work was supported by the Slovenian Research Agency under Grant P4-0127 (to J.K.).

REFERENCES


