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Trypsin-specific inhibitors from the basidiomycete *Clitocybe nebularis* with regulatory and defensive functions

Petra Avanzo,[1] Jerica Sabotič,[1] Sabina Anžlovar,[1]† Tatjana Popovič,[1] Adrijana Leonardi,[2] Roger H. Pain,[1] Janko Kos[1,3] and Jože Brzin[1]

Correspondence
Jerica Sabotič
Jerica.Sabotic@ijs.si

[1]Department of Biotechnology, Jožef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia

[2]Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia

[3]Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, SI-1000 Ljubljana, Slovenia

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We have isolated serine protease inhibitors from the basidiomycete *Clitocybe nebularis*, CnSPIs, using trypsin affinity chromatography. Full-length gene and cDNA sequences were determined for one of them, named cnispin, and the recombinant protein was expressed in *Escherichia coli* at high yield. The primary structure and biochemical properties of cnispin are very similar to those of the *Lentinus edodes* serine protease inhibitor, until now the only member of the I66 family of protease inhibitors in the MEROPS classification. Cnispin is highly specific towards trypsin, with K_i in the nanomolar range. It also exhibited weaker inhibition of chymotrypsin and very weak inhibition of subtilisin and kallikrein; other proteases were not inhibited. Inhibitory activity against endogenous proteases from *C. nebularis* revealed a possible regulatory role for CnSPIs in the endogenous proteolytic system. Another possible biological function in defence against predatory insects was indicated by the deleterious effect of CnSPIs on the development of larvae of *Drosophila melanogaster*. These findings, together with the biochemical and genetic characterization of cnispin, suggest a dual physiological role for this serine protease inhibitor of the I66 MEROPS family.

INTRODUCTION

Serine proteases are found in all kingdoms of life, including viral genomes. They constitute more than one-third of all known proteolytic enzymes and, according to the MEROPS classification, are grouped into 13 clans, based on catalytic mechanism, and 40 families, based on common ancestry. Proteases of clan PA are widely represented in eukaryotes, with the exception of plants and fungi, in which other clans (mainly SB and SC) have more representatives (Page & Di Cera, 2008; Rawlings *et al.*, 2008). Serine proteases are also the predominant proteolytic enzymes in basidiomycete mushrooms. An even larger proportion of gelatinolytic activities could not be classified, thus representing potentially unique proteases with unknown types of catalytic mechanism, demonstrating mushrooms as an important source of different types of proteolytic enzymes (Sabotič *et al.*, 2007b).

Serine proteases are involved in various physiological and pathophysiological processes, including digestion, blood coagulation, fibrinolysis, development, fertilization, apoptosis, immunity and cancer progression, playing key roles in human health and disease (Nyberg *et al.*, 2006; Page & Di Cera, 2008). The serine proteases trypsin and chymotrypsin are responsible for the initial digestion of ingested proteins in humans (Whitcomb & Lowe, 2007) and in invertebrates (Muhlia-Almazan *et al.*, 2008). Similarly, serine proteases play very important nutritional roles in higher fungi. In addition to their obvious proteolytic role during growth in nitrogen-limiting conditions, serine proteases from *Pleurotus ostreatus* act as regulators of other extracellular proteases, and of laccases, which are involved in the degradation of lignin (Faraco *et al.*, 2005; Palmieri *et al.*, 2001). In *Agaricus bisporus*, serine proteases regulate activation of tyrosinases, enzymes that catalyse

†Present address: Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia.

Abbreviations: B, *N*-benzoyl; BAPNA, *N*-benzoyl-DL-arginine-*p*-nitroanilide; BOC, butoxycarbonyl; CD, circular dichroism; CnSPI, *Clitocybe nebularis* serine protease inhibitor; (r)Cnp, (recombinant) cnispin; LeSPI, *Lentinus edodes* serine protease inhibitor; MCA, 7-(4-methyl)coumarylamide; PAA, polyacrylamide; pNA, *p*-nitroanilide; UTR, untranslated region; Z, benzyloxycarbonyl.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are FJ478178 and GQ141891.

Supplementary data are available with the online version of this paper.

oxidation of phenols (Espin *et al.*, 1999). A serine protease from *A. bisporus* is involved in resource recycling during senescence in fruiting bodies, mobilizing nutrients from the stipe to reproductive spore-bearing tissues in the cap (Burton *et al.*, 1997). The nutritional role of this enzyme was confirmed by following the regulation of its expression in response to available nitrogen in *A. bisporus* and *Coprinopsis cinerea*. A role in fruiting body development was also suggested for serine proteases in both mushrooms (Heneghan *et al.*, 2009). Serine proteases are also important pathogenicity factors. For the mushroom pathogen *Verticillium fungicola*, subtilisin-type serine proteases are important for the attack of hyphal cell walls (St Leger *et al.*, 1997). Furthermore, dipteran larvae are major mushroom insect pests, and have serine proteases as the predominant digestive proteolytic enzymes (Coles *et al.*, 2002; Terra & Ferreira, 1994).

Specific protease inhibitors are essential in regulating endogenous proteolytic processes and protecting against exogenously introduced proteases of pathogens (Vandeputte-Rutten & Gros, 2002). Inhibitory activity against trypsin has been detected in fruiting bodies of basidiomycete fungi (Gzogian *et al.*, 2005; Vetter, 2000), yet only a few serine protease inhibitors from mushrooms have been isolated and characterized. These include two isomeric inhibitors of serine proteases IA-1 and IA-2 from *P. ostreatus* (Dohmae *et al.*, 1995) belonging to family I9 in the MEROPS classification, a serine proteinase inhibitor from *Lentinus edodes* (Odani *et al.*, 1999) that inhibits trypsin and chymotrypsin, belonging to family I66, and proteinase K inhibitor from *Trametes versicolor* (Zuchowski & Grzywnowicz, 2006), not assigned to a MEROPS inhibitor family.

We have previously isolated and characterized a novel family of inhibitors of cysteine proteases from the basidiomycetes *Clitocybe nebularis* (Brzin *et al.*, 2000; Sabotič *et al.*, 2006, 2007a) and *Macrolepiota procera* (Sabotič *et al.*, 2009). Here we describe biochemical properties and evidence for the biological function of new serine protease inhibitors, CnSPIs (*C*litocybe *n*ebularis *s*erine *p*rotease *i*nhibitors), isolated from *C. nebularis*, and genetic and biochemical characterization of one of these inhibitors, cnispin (Cnp), which has been heterologously expressed in *Escherichia coli*.

METHODS

Enzymes and substrates. Bovine trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), porcine kallikrein (EC 3.4.21.35) and porcine pepsin (EC 3.4.23.1) were from Sigma, bovine thrombin (EC 3.4.21.5) from Calbiochem, *Bacillus subtilis* subtilisin (EC 3.4.21.62) from Boehringer Mannheim and porcine elastase (EC 3.4.21.36) from Serva. 2× crystallized papain (EC 3.4.22.2) from Sigma was further purified by affinity chromatography (Blumberg *et al.*, 1970). Z-Phe-Arg-MCA [7-(4-methyl)-coumarylamide], Suc-Ala-Ala-Pro-Phe-MCA, Boc-Val-Pro-Arg-MCA, H-Pro-Phe-Arg-MCA, Suc-Ala-Ala-Ala-MCA and B-Arg-pNA (*N*-benzoyl-DL-arginine-*p*-nitroanilide, BAPNA) were from Bachem.

Fungal material. Fruiting bodies of *C. nebularis* were collected from their natural habitat in Kras forest (Slovenia) and frozen at −20 °C until use. Tissue from different parts of the basidiocarp and mycelium was prepared as described previously (Sabotič *et al.*, 2006). Mycelium was grown in 1.5 % (w/v) malt extract medium in the dark at 30 °C. The mycelium is kept in the collection of fungi, lichens and higher plants at the Slovenian Forestry Institute, Ljubljana, Slovenia.

Isolation of serine protease inhibitors. Frozen fruiting bodies or basidiocarps of *C. nebularis* were homogenized in buffer A (0.02 M Tris/HCl, pH 7.5, 0.3 M NaCl) containing 4 mM $Na_2S_2O_3$ (reducing agent). The homogenate was filtered and centrifuged for 15 min at 3400 ***g*** at 4 °C. The supernatant was applied to a trypsin affinity chromatography column (3.5 × 15 cm) prepared according to the manufacturer's instructions (Pharmacia) and equilibrated with buffer A. Bound inhibitory fractions were eluted with 0.01 M HCl, pooled, neutralized with 2 M Tris/HCl and concentrated by ultrafiltration (UM-10, Amicon). Serine protease inhibitors were additionally purified on a Sephacryl S-200 (Amersham Pharmacia Biotech) column (4 × 110 cm) in buffer A at a flow rate of 52.8 ml h^{-1}.

Analysis of endogenous serine proteases. Crude protein extract of *C. nebularis* basidiocarps was prepared by extraction with 0.5 M sodium acetate buffer, pH 4.5, containing 0.3 M NaCl, 50 mM EDTA and 0.05 % (w/w) $Na_2S_2O_3$. Insoluble material was removed by centrifugation at 9000 ***g*** for 15 min. The resulting supernatant was concentrated by ultrafiltration (UM-3, Amicon) and applied to a Sepharose S-200 size-exclusion chromatography column (1.5 × 130 cm) equilibrated with 0.1 M sodium acetate buffer, pH 5.5, containing 0.3 M NaCl and 10 mM EDTA, and eluted at a flow rate of 9.6 ml h^{-1}. Proteolytic activity in fractions was measured using Z-Phe-Arg-MCA as substrate in 0.1 M Tris/HCl, pH 9, and the influence of different inhibitors was tested. The following inhibitors were used at the indicated final concentration: 4 mM Pefabloc SC [AEBSF; 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, Roche], 100 μM chymostatin (Sigma), 30 μM E-64 ([(2*S*,3*S*)-3-carboxyoxirane-2-carbonyl]-L-leucine (4-guanidinobutyl)amide, Peptide Institute) and CnSPIs at 0.02 mg ml^{-1} and 0.2 mg ml^{-1}.

Insecticidal effect of CnSPIs on *Drosophila melanogaster*. Insecticidal effect was determined in feeding bioassays as relative mortality of larvae. Protease inhibitors were added to the rearing medium [3.2 % (w/v) sucrose, 8.5 % (w/v) yeast extract, 5 % (w/v) cornflour, 1.2 % (w/v) agar, 1.1 % (v/v) *p*-methylhydroxybenzoic acid, 0.5 % (v/v) propionic acid] before pouring into 1 × 5 cm tubes. Ten eggs of wild-type *D. melanogaster* strain Canton S (Indiana University, USA) were deposited on the medium and the tubes were maintained at 25 °C for 14 days to allow development of larvae into pupae, when their numbers were recorded. Each test was performed in five replicates. Rearing medium containing 0.5 mg BSA ml^{-1} was used as a reference assay. The following protease inhibitors were tested at a final concentration of 0.5 mg ml^{-1}: *C. nebularis* serine protease inhibitors (CnSPIs), serine protease inhibitor from pumpkin, *Cucurbita maxima* (CMTI) (Krishnamoorthi *et al.*, 1990), potato serine protease inhibitor (PSPI) (Brzin *et al.*, 1995), potato protease inhibitor 2 (PI2) (Pearce *et al.*, 1982), and *C. nebularis* cysteine protease inhibitor (clitocypin) (Brzin *et al.*, 2000). Relative mortality (RM) of larvae was determined as RM = 1 − [(test mortality)/(reference mortality)] and standard deviation was calculated.

SDS-PAGE and IEF analyses. Proteins were analysed on 12 % (w/v) polyacrylamide (PAA) gels in denaturing and non-reducing or reducing conditions and visualized using Coomassie brilliant blue staining. Low-molecular-mass markers 14.4–97 kDa (Amersham Pharmacia Biotech) were used for molecular mass estimation. Isoelectric focusing was carried out with a Pharmacia PhastSystem,

using commercial precast pH 3–9 gradient gels as described previously (Sabotič *et al.*, 2007a).

Protein sequencing. For the N-terminal sequence analysis, proteins separated by SDS-PAGE were electro-transferred to a PVDF membrane (Millipore), and visualized by Coomassie staining. Bands were excised and sequencing was performed on a Procise 492A Automated Sequencing System (Applied Biosystems).

For internal protein cleavage, the PAA gel was first negatively stained with Zn/imidazole (Ortiz *et al.*, 1992). The band of interest was excised, equilibrated in non-reducing sample buffer for 5 min and applied onto another PAA gel. SDS-PAGE was stopped when the protein entered the stacking gel. Immediately, 1 μg of *Staphylococcus aureus* V8 protease (EC 3.4.21.19, Miles) was applied and SDS-PAGE was run until proteins reached the boundary between stacking and resolving gel. The cleavage was left to proceed for 1 h at room temperature. Then SDS-PAGE was run to the end, after which the proteins were electro-blotted and the N-terminal sequences of the resulting fragments determined as described above.

HPLC analysis. The proteins were separated on an HPLC system using an Aquapore BU-300 column (PE Brownlee). The column was washed with 0.1 % (v/v) trifluoroacetic acid (TFA) in water and the proteins were eluted from the column with a linear gradient of acetonitrile in 0.1 % TFA (0–90 % in 30 min) at a flow rate of 1 ml min^{-1}.

Electrospray-ionization mass spectrometry. The molecular masses of the HPLC-purified proteins were determined by electrospray ionization (ESI) mass spectrometry as described previously (Pohleven *et al.*, 2009) on a Q-Tof Premier (Micromass MS Technologies/Waters) mass spectrometer.

Molecular cloning of the gene and cDNA encoding cnispin. Molecular cloning of the cnispin gene (*cnp*) and cDNA sequences followed the same steps as those for the *C. nebularis* lectin (Pohleven *et al.*, 2009). High-molecular-mass genomic DNA was isolated from frozen *C. nebularis* fruiting bodies as described by Moller *et al.* (1992), and cDNA synthesis was performed using total RNA, as described previously (Pohleven *et al.*, 2009). Partial *cnp* gene and cDNA sequences were amplified with forward (CnSPI-D-f) and reverse (CnSPI-D-r) degenerate primers that were constructed using the CADEPCO program (http://sabina.anzlovar.com/calc/cadepco.cgi) (Supplementary Table S1). On the basis of the resulting partial nucleic acid sequences, specific primers (Supplementary Table S1) were designed to amplify the complete gene and cDNA sequences.

To amplify the 3′ end of the cDNA sequence, 3′ rapid amplification of cDNA ends (3′ RACE) was carried out using the 3′ RACE adaptor primer (Supplementary Table S1) paired with a forward *cnp*-specific primer CnSPI-RACE-N1 (Supplementary Table S1) in the primary PCR. This was followed by secondary PCR using *cnp*-specific nested primer CnSPI-RACE-N2 (Supplementary Table S1). The complete *cnp* gene, with its promoter and terminator regions, was amplified using genome walking libraries as templates, constructed using the GenomeWalker Universal kit (BD Biosciences Clontech) as described previously (Pohleven *et al.*, 2009). In genome walking PCR amplifications, Advantage 2 Polymerase Mix (Clontech) was used with the PCR conditions suggested by the manufacturer. Adaptor primer AP1 and a nested primer AP2, provided by the manufacturer, were paired with nested forward gene-specific primers (CnSPI-term1 and CnSPI-term2; Supplementary Table S1) for downstream amplification, or nested reverse gene-specific primers (CnSPI-prom1 and CnSPI-prom2; Supplementary Table S1) for upstream amplification. Complete *cnp* gene and cDNA sequences were obtained using primers annealing to the 5′ untranslated region (5′ UTR) (CnSPI-f) and 3′ UTR (CnSPI-r).

All PCR products were cloned into pGEM-T Easy Vector System I (Promega) and sequenced using the Automated DNA Sequencing Service at Eurofins MWG Operon (Ebersberg, Germany).

Sequence analysis. Sequence analyses and multiple sequence alignments were performed in the BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Promoter analysis was performed using TESS (Transcription Element Search System) at http://www.cbil.upenn.edu/cgi-bin/tess/tess. The deduced amino acid sequence was analysed using online proteomics tools at the ExPASy server of the Swiss Institute of Bioinformatics (http://www.expasy.org/tools/). Similarity searches were performed using tBLASTn and BLASTP algorithms at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), and the FASTA3 and TFASTX algorithm at The European Bioinformatics Institute (EBI) server (http://www.ebi.ac.uk/Tools/similarity.html).

Expression and purification of recombinant cnispin (rCnp). *Nde*I and *Bam*HI restriction sites were introduced into the 5′ and 3′ ends of the cnispin cDNA clone (GenBank accession no. GQ141891) in PCR using *Pfu* DNA Polymerase (Promega). After *Nde*I/*Bam*HI (New England Biolabs) digestion of both insert and vectors the insert was subcloned into pET3a and pET11a vectors (Novagen) to generate recombinant proteins without tags. Both expression vectors were transformed into BL21(DE3) (Invitrogen) and BL21(DE3) pLysS (Novagen) strains of *E. coli*. Transformed *E. coli* strains were grown in Luria–Bertani (LB) medium supplemented with appropriate antibiotics at 37 °C. When OD_{550} reached 0.5–1, IPTG was added to a final concentration of 0.4 mM for strains transformed with the pET3a construct and 1 mM for strains transformed with the pET11a construct. Protein expression was monitored hourly to determine the time of maximum expression by analysing a sample of cells.

For expression of rCnp, the pET11a construct transformed into *E. coli* BL21(DE3) was used. Five hours after induction of expression, cells were harvested by centrifugation, resuspended in buffer C (50 mM Tris/HCl, 2 mM EDTA, 0.1 %, v/v, Triton X-100, pH 8), frozen and thawed three times, then sonicated at 4 °C. The insoluble fraction was separated by centrifugation (4000 ***g***, 15 min), resuspended in buffer C and subsequently in the same buffer containing first 2 M urea and then 8 M urea. The sample was applied to a Sepharose S-200 column (4 × 110 cm) equilibrated with buffer A and inhibitory active fractions were pooled.

Circular dichroism (CD). CD spectra of purified recombinant cnispin (0.45 mg ml^{-1} in 0.01 M phosphate buffer, pH 7) were acquired using an Aviv model 60 spectropolarimeter as described previously (Kidrič *et al.*, 2002). Equilibrium thermal unfolding transitions were obtained using the Aviv temperature scanning facility at 232 nm with 0.045 mg rCnp ml^{-1} in a 1 cm cell. Bandwidth was 2 nm, equilibration time 5 min, and data acquisition time 10 s, with measurements at 2 °C intervals.

Thermal and pH stability. To test thermal stability, cnispin (0.13 mg ml^{-1}) was incubated at 100 °C, 70 °C, 60 °C and 40 °C for 10 min and then at room temperature for 30 min. To test pH stability, cnispin (0.13 mg ml^{-1}) was incubated in 0.2 M Tris/HCl (pH 11), in 0.2 M Tris/HCl (pH 7) or in 0.2 M citric acid (pH 3) for 20 min and then neutralized. Residual inhibitory activity was measured against trypsin using BAPNA as substrate.

Inhibition assay. Inhibitory activities of samples during the isolation procedure were measured against trypsin (0.1 μM) in buffer B (0.05 M Tris/HCl, 0.02 M $CaCl_2$, pH 8). After 10 min of pre-incubation at room temperature, 10 μl of 0.1 M substrate BAPNA

was added and the mixture incubated for 20 min at 37 °C. The reaction was stopped with 0.2 M HCl and A_{405} measured.

Active-site titration. The molar concentration of active trypsin was determined by titration with *p*-nitrophenyl-*p'*-guanidinobenzoate (Chase & Shaw, 1969). Active concentration of rCnp was determined by titration of previously active-site-titrated trypsin, using BAPNA as substrate.

Determination of inhibition constants. Inhibition kinetics of trypsin were determined under pseudo-first-order conditions in continuous assays, as described for papain inhibition by clitocypin (Brzin *et al.*, 2000), using substrate Z-Phe-Arg-MCA and buffer B. Data were analysed by nonlinear regression analysis according to Morrison (1982), and k_d and k_a values were obtained using a K_m of 59 μM for trypsin.

The inhibition kinetics for the inhibition of chymotrypsin, subtilisin, thrombin, elastase and kallikrein were determined according to Henderson (1972), as described for cathepsin B inhibition by clitocypin (Brzin *et al.*, 2000), with the following modifications. Different amounts of the inhibitor (0.05–8 μM) were incubated with each of the enzymes for 10 min in buffer B in microtitre plates. Kallikrein was assayed in 0.05 M Tris/HCl, 0.05 M NaCl, 0.01 % (v/v) Tween buffer, pH 7.8, subtilisin in 0.1 M phosphate buffer, pH 8.8, and thrombin in 0.25 M phosphate buffer, pH 6.5. Reactions were initiated by adding substrate to a final concentration of 30 μM. Suc-Ala-Ala-Pro-Phe-MCA was used for chymotrypsin and subtilisin, H-Pro-Phe-Arg-MCA for kallikrein, Boc-Val-Pro-Arg-MCA for thrombin and Suc-Ala-Ala-Ala-MCA for elastase. After 10 min of incubation, the reaction was stopped with 0.2 M HCl. The released MCA was measured using a Safire microplate reader (Tecan).

Inhibitory activity against other classes of proteases. Inhibition of cysteine protease papain activity was assayed using substrate benzoyl-Arg-2-naphthylamide as described previously (Brzin *et al.*, 2000). Inhibition of porcine pepsin, an aspartic protease, was assayed in 0.1 M acetate buffer, pH 3.5, with the fluorogenic substrate FTC-haemoglobin, as described for FTC-casein (Twining, 1984).

Immunoblot analysis. The expression of cnispin in different parts of the basidiocarp and in cultured mycelium was analysed using aqueous extracts containing the whole soluble protein fraction (Sabotič *et al.*, 2006). Polyclonal antibodies produced against purified rCnp in rabbits (Biogenes) were used at 1 : 7000 dilution following the immunoblot procedure described previously (Sabotič *et al.*, 2006), modified by using nitrocellulose membrane (Porablot NCP, Macherey-Nagel) and chemiluminescence detection with Lumi-Light^{PLUS} (Roche).

RESULTS

Isolation and purification of *C. nebularis* serine protease inhibitors (CnSPIs)

CnSPIs were obtained from a crude protein extract of *C. nebularis* basidiocarps by trypsin-affinity chromatography. An additional purification step using size-exclusion chromatography yielded a single, symmetrical protein peak with trypsin-inhibitory activity. SDS-PAGE analysis of the inhibitory fraction revealed two protein bands with estimated molecular masses of 18 kDa and 16 kDa (see Fig. 4a, lane 1). Ion-exchange, hydrophobic interaction and HPLC chromatographies were not successful in separating them. N-terminal sequences were, however, determined from both bands. The upper band, with apparent molecular mass 18 kDa, yielded one main sequence, LPSDQYYIRNGRSFAGRALHEDHSL, and was named CnSPI1 or cnispin. In the lower band with apparent molecular mass 16 kDa, a 16 amino acid N-terminal sequence SRPGLYFIRNGESPAG was obtained (CnSPI2 in Fig. 2) that shows 50 % sequence identity to the corresponding 16 amino acid sequence of CnSPI1. In addition, a weaker sequence NLNNIVVIERTQAVH was detected in the lower band (CnSPI3 in Fig. 2). It shows no significant similarity to any of the CnSPI variants, but 33 % sequence identity to the sequence of the serine protease inhibitor from *Lentinus edodes* (Odani *et al.*, 1999). The internal sequence of CnSPI1 was determined by in-gel digestion with *S. aureus* V8 protease. Three peptides with apparent molecular masses of 4 kDa, 7 kDa and 10 kDa were obtained and their N-terminal sequences determined as LPSDQYY, DHSLNPKPVIXPTD and ALPNHGYILRA-RGAPTAE (underlined in Fig. 1). These sequences were used for degenerate primer design to obtain the nucleotide sequence coding for cnispin (Cnp).

Characterization of CnSPIs

ESI-mass spectrometry analysis of the HPLC-purified CnSPIs revealed two peaks with molecular masses of 16.380 kDa and 14.321 kDa, probably corresponding to the upper and lower bands resolved in SDS-PAGE analysis. The isoelectric points of the CnSPIs were 4.8 and 5.2. The native CnSPIs were stable across a wide pH range, retaining their inhibitory activity after incubation at pH 2 or pH 11. Their inhibitory activity was lost after heating at 100 °C for 10 min. CnSPIs proved to be very strong inhibitors of trypsin, while inhibition of chymotrypsin was about 40 times weaker. Elastase and thrombin were not inhibited by CnSPIs.

Inhibition of endogenous serine proteases

Size-exclusion chromatography of crude protein extract from basidiocarps of *C. nebularis* resolved the proteolytic activity measured against Z-Phe-Arg-MCA at pH 9 into four peaks (Supplementary Fig. S1) with estimated molecular masses ranging from approximately 50 kDa to 10 kDa. These proteases are of the serine catalytic type, proteolytic activity in all four peaks being completely inhibited by Pefabloc, while E-64 had no effect. Chymostatin completely inhibited activity in peaks 1 and 3 and only partially in peaks 2 and 4. CnSPIs inhibited activity of all four partially separated proteases, with 66 % inhibition in peak 1, 75 % in peak 2, 74 % in peak 3 and 55 % in peak 4.

Insecticidal activity against *D. melanogaster*

CnSPIs and other protease inhibitors were incorporated into rearing medium and their insecticidal activity against *D. melanogaster* was assessed. The relative mortality of larvae reared on medium containing CnSPIs was

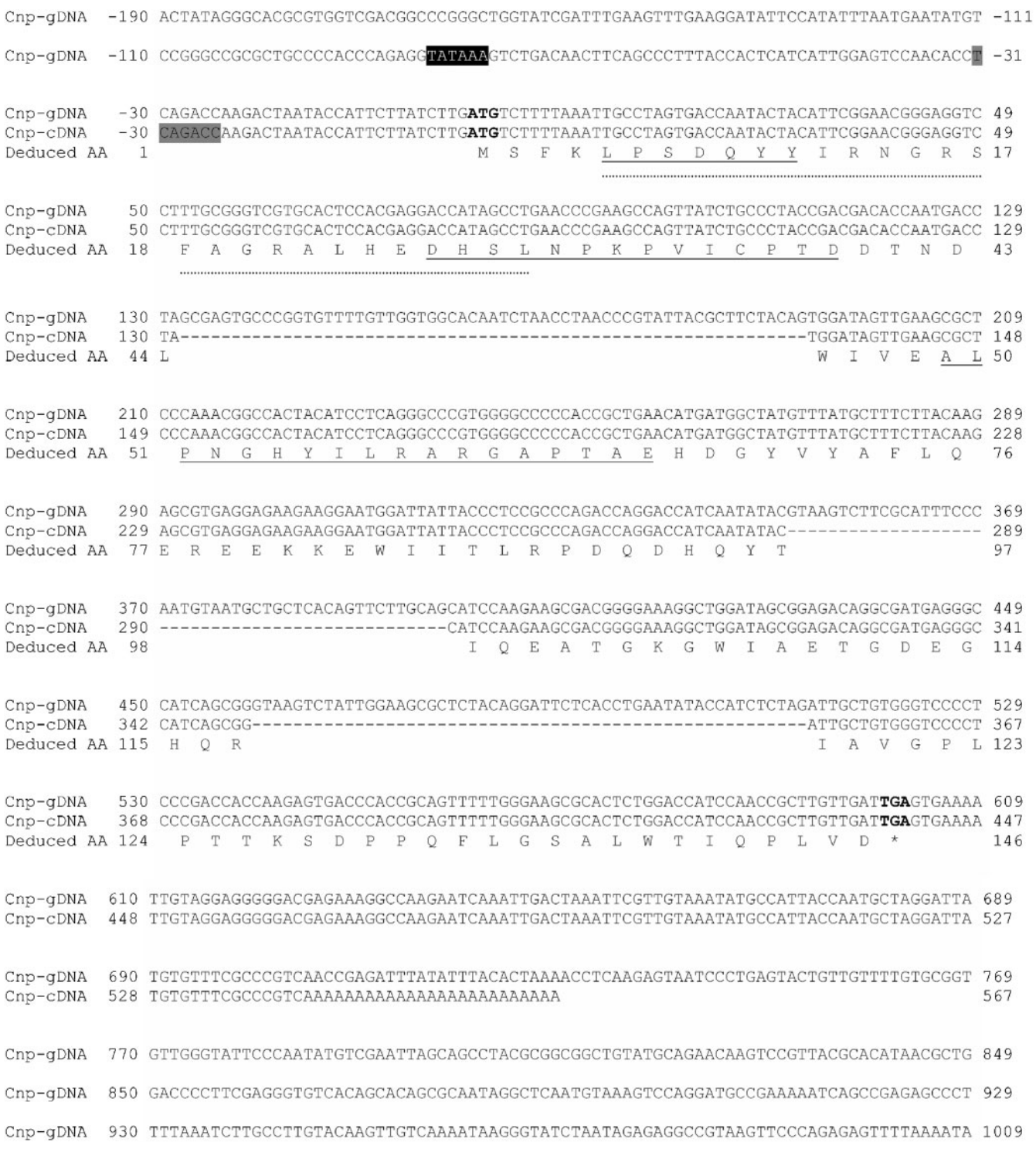

Fig. 1. Full-length cnispin (Cnp) gene and cDNA sequences. The genomic sequence is marked as gDNA and introns are indicated with hyphens in the cDNA sequence. The deduced amino acid sequence is shown below the nucleotide sequences. Nucleotides and amino acids are numbered starting with the start codon (ATG). The stop codon (TGA) is indicated by an asterisk. The predicted TATA box is shaded in black and the predicted transcription initiation site in grey. Underlined amino acid residues were determined by amino acid sequencing of peptides after digestion of CnSPI1 with V8 protease. The dotted line indicates the determined N-terminal amino acid sequence. GenBank accession numbers: Cnp-gDNA, FJ478178; Cnp-cDNA, GQ141891.

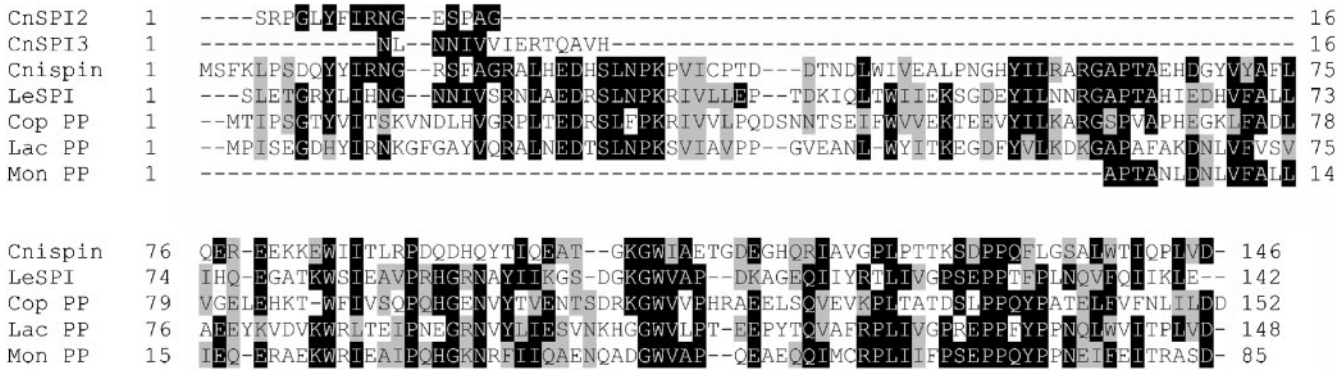

Fig. 2. Alignment of amino acid sequences of similar serine protease inhibitors from different basidiomycetes. Amino acid sequences of CnSPIs and the cnispin deduced amino acid sequence are aligned with the amino acid sequence of the *Lentinus edodes* serine protease inhibitor (Odani *et al.*, 1999) (LeSPI, Swiss-Prot P81639), and the predicted protein sequences annotated in the *Coprinopsis cinerea* genome (Cop PP, GenBank AACS01000321), *Laccaria bicolor* genome (Lac PP, GenBank DS547153) and *Moniliophthora perniciosa* genome (Mon PP, GenBank EEB87837). Identical amino acid residues are shaded in black and similar amino acid residues in grey.

0.59 ± 0.22. While potato protease inhibitor 2 (PI2) showed a very strong insecticidal activity, with no larvae surviving, the activity of CnSPIs was similar to that of another potato serine protease inhibitor, PSPI, that produced a relative mortality of 0.46 ± 0.08. In contrast, the serine protease inhibitor from pumpkin (CMTI) had almost no effect, with relative mortality 0.03 ± 0.00, which was similar to that of the cysteine protease inhibitor from *C. nebularis*, clitocypin, with relative mortality 0.07 ± 0.02.

Analysis of the cnispin gene and cDNA sequences

The complete sequence of the cnispin gene, with its promoter and terminator sequences, was obtained. Comparison of the full-length gene with the cDNA sequence revealed that the 603 bp coding region is composed of four exons (132, 158, 61, 90 bp) and three short introns (61, 47, 54 bp) (Fig. 1). The intron–exon boundaries in the second and third intron follow the GT-AG consensus splice sites, which are GURAGU for the 5′ splice site and WNYAG for the 3′ splice site in filamentous fungi (Kupfer *et al.*, 2004). The first intron, however, contains the variant GC-AG splice site pair, which is present in 1.89 % of introns in the basidiomycete *Cryptococcus neoformans* and in 0.86–1.19 % of introns in the ascomycetes *Neurospora crassa*, *Aspergillus nidulans* and *Saccharomyces cerevisiae* (Kupfer *et al.*, 2004). The cnispin promoter has a putative consensus TATA box sequence (TATAAA) at position −84 and a putative transcription initiation site (TCAGACC) at position −31 (Fig. 1), indicating transcriptional activity of the *cnp* gene. Similar positions were observed in the promoters of genes coding for a cysteine protease inhibitor, clitocypin (Sabotič *et al.*, 2006), and a ricin B-like lectin from *C. nebularis* (Pohleven *et al.*, 2009). As for these genes, no typical polyadenylation signal AAUAAA was found in the *cnp* 3′ UTR sequence.

Cnispin amino acid sequence analysis

The coding region of cnispin cDNA is 441 bp long (stop codon included) and translates into a 146 amino acid protein. The deduced amino acid sequence of cnispin matched exactly the corresponding parts of the amino acid sequence that was determined by protein sequencing (underlined in Fig. 1). Sequence similarity searches in databases were performed and a significant sequence similarity was found with a serine protease inhibitor from *Lentinus edodes* characterized at the protein level and a few hypothetical proteins annotated in genomes of basidiomycetes (Fig. 2). Cnispin exhibited the highest sequence identity (37 %) with *L. edodes* serine proteinase inhibitor (here termed LeSPI) (Odani *et al.*, 1999), the only member of the I66 family of protease inhibitors. Furthermore, it exhibited 34 % sequence identity with three predicted proteins in the *Coprinopsis cinerea* Okayama genome and 33 % sequence identity with three predicted proteins in the *Laccaria bicolor* S238N-H82 genome (Martin *et al.*, 2008). High sequence similarity was also found for limited regions of one other predicted protein in the *C. cinerea* genome and another four from the genome of *L. bicolor*. Similarly, sequence identity (35 %) was also found with the C-terminal half of a protein with a partial sequence annotated in the *Moniliophthora perniciosa* FA553 genome (Mondego *et al.*, 2008). In contrast to LeSPI and other putative serine protease inhibitors that do not have cysteines, cnispin contains a single cysteine in its amino acid sequence (C36).

Expression and purification of active recombinant cnispin

Purification of individual CnSPIs from basidiocarps could not be achieved due to their very similar biochemical properties; therefore, cnispin was heterologously expressed in *E. coli* for further characterization. The cDNA clone

(GenBank accession no. GQ141891; Fig. 1) was subcloned into expression vectors pET3a and pET11a and they were both transformed into *E. coli* BL21(DE3) and BL21(DE3) pLysS to determine the optimal expression system. Although cnispin was expressed in all the tested combinations, maximum expression of recombinant cnispin (rCnp) was achieved 5 h after induction using *E. coli* BL21(DE3) with expression vector pET11a::rCnp (Fig. 3). The inhibitor was expressed mainly as insoluble inclusion bodies (Fig. 3). Progressive solubilization of the pellet in 2 M and 8 M urea, followed by a one-step purification using size-exclusion chromatography, yielded a purified protein at a yield of 70 mg rCnp per litre of bacterial culture (Fig. 3). The purified rCnp exhibited a single 18 kDa band on SDS-PAGE under reducing conditions (Fig. 4a, lane 2). Under non-reducing conditions, however, it showed an additional band with an apparent molecular mass of 32 kDa, corresponding to a dimer, probably formed between the single cysteine residues.

Characterization of recombinant cnispin

Comparison of the molecular masses of rCnp and CnSPIs isolated from *C. nebularis* basidiocarps showed that rCnp indeed corresponds to the higher-molecular-mass protein CnSPI1 (Fig. 4a). Furthermore, polyclonal antibodies raised against rCnp recognized the upper CnSPI1 band specifically (Fig. 4b). The identity of the protein was confirmed by N-terminal sequencing (SFKLP). The calculated molecular mass of 16.407 kDa for rCnp lacking the initial methionine was confirmed exactly by ESI-mass spectrometry. IEF analysis confirmed the theoretical isoelectric point of 5.3.

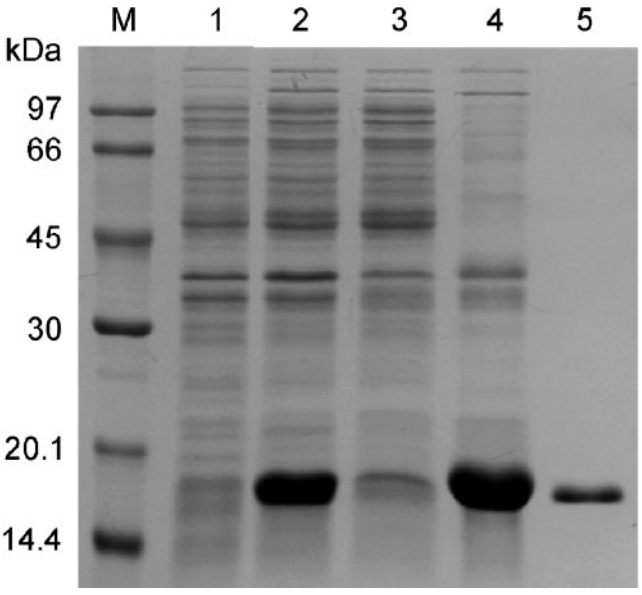

Fig. 3. Expression and purification of recombinant cnispin. Recombinant cnispin was analysed by SDS-PAGE on 12% (w/v) PAA gel under reducing conditions and stained with Coomassie blue. Lanes 1 and 2, crude cell extracts of *E. coli* BL21(DE3)/pET11a::rCnp before (lane 1) and 5 h after (lane 2) induction of expression. Lane 3, soluble fraction of cell lysate; lane 4, insoluble fraction of cell lysate dissolved in 8 M urea; lane 5, purified recombinant cnispin. M, protein molecular mass markers.

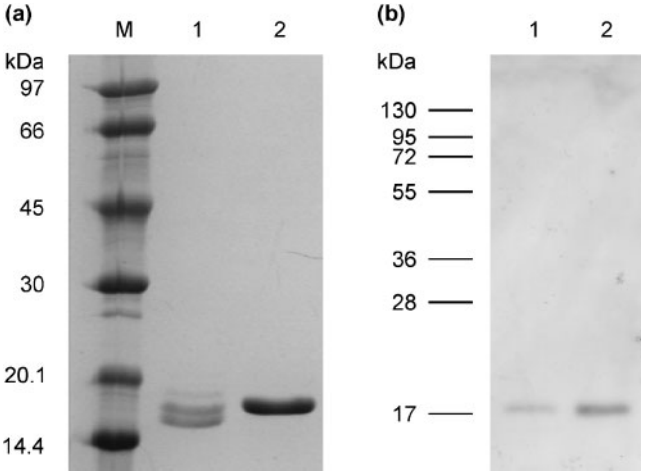

Fig. 4. Comparison of CnSPIs and cnispin by SDS-PAGE (a) and immunoblot (b) analyses. (a) PAA gel and Coomassie staining was used for SDS-PAGE analysis of CnSPIs and rCnp. (b) Proteins separated by SDS-PAGE under reducing conditions were transferred to nitrocellulose membrane and probed with polyclonal anti-rCnp antibodies. Immunoreactive bands were visualized by probing the blot with horseradish-peroxidase-conjugated goat anti-rabbit IgG secondary antibodies, followed by chemiluminescence detection. Lane 1, CnSPIs purified from basidiocarps by trypsin affinity and size-exclusion chromatographies; lane 2, purified recombinant cnispin. M, protein molecular mass markers.

The CD spectrum for rCnp in the far UV (Supplementary Fig. S2) showed marked similarity to that of clitocypin (Clt) (Kidrič *et al.*, 2002; Sabotič *et al.*, 2007a). The peak at 232 nm, which is a marker for tryptophan environment, is less marked than for Clt, but is still sensitive to unfolding (Supplementary Fig. S3). The only marked difference is that no peak is observed at 189 nm. While the contribution of tryptophan prevents an analysis of secondary structure in terms of β structure, there is clearly little or no α-helical structure.

The thermal stability of rCnp was examined by following its unfolding by CD. The temperature unfolding transition (Supplementary Fig. S3) shows a temperature midpoint of 56 °C, considerably less than that for the cysteine protease inhibitor clitocypin, at 67 °C (Kidrič *et al.*, 2002; Sabotič *et al.*, 2007a). The limited thermal stability of rCnp was also indicated by measurements of inhibitory activity. It exhibited full inhibitory activity after 10 min incubation at 40 °C, while at higher temperatures inhibition was reduced. No activity was retained after heating at 100 °C for 10 min. On the other hand, rCnp was stable across a

wide pH range, retaining its inhibitory activity after incubation at pH 3 and pH 11.

Kinetics of inhibition

Titration of trypsin with cnispin showed that complete inhibition was achieved at 1 : 1 molar stoichiometry. The pseudo-first-order rate constant, k, for binding of rCnp to trypsin increased linearly with inhibitor concentration. Kinetic constants of the inhibition of different proteases by rCnp are presented in Table 1. rCnp was most effective in inhibiting trypsin, with a K_i value of 3.1 nM, showing it to be a fast-acting (k_a 3.1×10^5 M^{-1} s^{-1}) and tight-binding (k_d 9.5×10^{-4} s^{-1}) inhibitor of this enzyme. The K_i value for the inhibition of chymotrypsin was in the micromolar range, while those for subtilisin and porcine kallikrein were significantly higher. rCnp showed no inhibitory activity against the other serine proteases porcine elastase and bovine thrombin, nor against the cysteine protease papain or the aspartic protease pepsin. It was also established that rCnp does not inhibit the human serine proteases β-tryptase, coagulation factors Xa and IXa, plasmin, urokinase, polymorphonuclear leukocyte elastase, thrombin or kallikrein 5 (not shown).

Expression of cnispin in the basidiocarp and in cultured mycelium

Detection of cnispin in different parts of the basidiocarp using polyclonal anti-rCnp antibodies revealed its presence in all parts of the basidiocarp. Analysis of the distribution showed less cnispin in the stipe and more in other parts of the basidiocarp, particularly in the cap and lamellae (Fig. 5). Cnispin was also expressed in cultured mycelium (Fig. 5), but was not secreted into the medium (not shown).

Table 1. Kinetic constants for the interaction of cnispin with different proteases

Kinetic and equilibrium constants for the inhibition of trypsin were determined under pseudo-first-order conditions in a continuous kinetic assay according to Morrison (1982). Equilibrium constants for the inhibition of chymotrypsin, subtilisin and kallikrein were determined according to Henderson (1972). Experiments were performed at 25 °C. Standard deviation is given where appropriate; ND, not determined; NI, no inhibition.

Enzyme	K_i (nM)	$10^{-5} \times k_a$ (M^{-1} s^{-1})	$10^4 \times k_d$ (s^{-1})
Trypsin	3.10 ± 0.66	3.10 ± 0.55	9.50 ± 0.47
Chymotrypsin	120 ± 20	ND	ND
Subtilisin	>1000	ND	ND
Kallikrein	>1000	ND	ND
Elastase	NI		
Thrombin	NI		
Papain	NI		
Pepsin	NI		

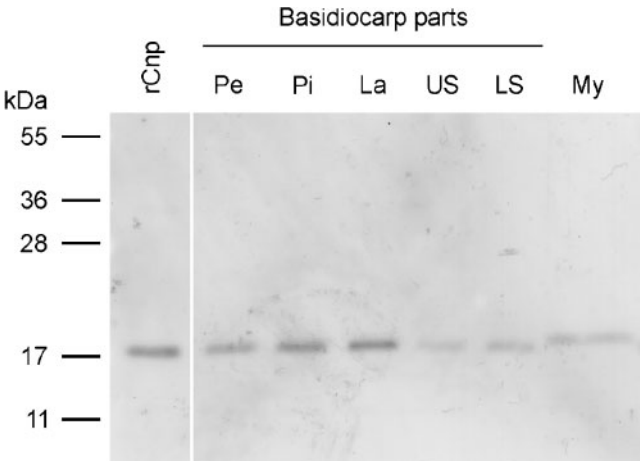

Fig. 5. Expression of cnispin in different parts of the basidiocarp and in cultured mycelium. Immunoblot analysis of cnispin expression in aqueous extracts was performed using polyclonal anti-rCnp antibodies. rCnp, recombinant cnispin; Pe, pellis; Pi, pileus; La, lamellae; US, upper stipe; LS, lower stipe; My, mycelium.

Similarity searches of the expressed sequence tags (EST) databases revealed a number of ESTs showing similarity to the cnispin amino acid sequence. They further support the expression pattern of cnispin-like serine protease inhibitors, as they were determined from both vegetative mycelia and fruiting bodies of the basidiomycete mushrooms *Laccaria bicolor*, *Coprinopsis cinerea* and *Lentinus edodes*.

DISCUSSION

Serine protease inhibitors, CnSPIs, were purified from basidiocarps of *C. nebularis* using trypsin affinity chromatography. On SDS-PAGE analysis (Fig. 4a) they were present as two bands which revealed three N-terminal sequences, all showing significant sequence similarity to the serine protease inhibitor from *Lentinus edodes* (LeSPI) (Odani *et al.*, 1999), until now the sole member of the I66 family of protease inhibitors in the MEROPS classification. The similar biochemical properties of the CnSPIs and the strong sequence similarity of their N-terminal sequences suggested that they all belong to the I66 protease inhibitor family. Since attempts to isolate the individual CnSPIs were not successful, CnSPI1, which showed the highest sequence similarity with LeSPI, was further characterized and named cnispin (Cnp). Based on the partial protein sequences obtained for cnispin, its coding gene and corresponding cDNA were amplified and sequenced, together with the promoter and 5′ UTR and 3′ UTR sequences (Fig. 1). Comparison of the full-length LeSPI protein sequence (142 amino acids) with the deduced amino acid sequence of cnispin (145 amino acids) revealed many conserved amino acid residues distributed throughout the sequence (Fig. 2), which are probably important for the inhibitory activity

and/or structure of the proteins. The single cysteine that is present in cnispin is probably responsible for the dimer formation observed for recombinant cnispin (rCnp) on SDS-PAGE under non-reducing conditions. A dimeric form of cnispin was also detected in basidiocarps by immunoblot analysis. Dimerization could represent a regulatory mechanism, since dimeric rCnp did not inhibit trypsin.

Biochemical characterization of CnSPIs and rCnp revealed them to have similar properties. On SDS-PAGE analysis under reducing conditions rCnp corresponded to the upper band of CnSPIs, which was confirmed by the similar molecular masses determined by ESI-mass spectrometry (16.407 kDa for rCnp, and 16.380 kDa and 14.321 kDa for CnSPIs). The apparent molecular masses observed on SDS-PAGE analysis are higher. CnSPIs and rCnp, like LeSPI (Odani *et al.*, 1999), showed similar isoelectric points, exhibited stability over a wide pH range and lost their inhibitory activity after incubation at high temperatures. However, the thermal stability of CnSPIs and rCnp is much lower than that of the thermally stable proteins clitocypin (Sabotič *et al.*, 2007a) and *Clitocybe nebularis* lectin (Pohleven *et al.*, 2009), also isolated from *C. nebularis*.

Cnispin inhibited trypsin with high specificity, with K_i in the nanomolar range. It inhibited chymotrypsin with K_i in the micromolar range, showed an even weaker inhibition of subtilisin and kallikrein, and no inhibition of the other serine proteases tested. The inhibitory profile of cnispin (Table 1) is very similar to that of LeSPI, which is also a strong inhibitor of trypsin and a weak inhibitor of chymotrypsin, while other serine proteases are not inhibited (Odani *et al.*, 1999). In addition, proteases of other catalytic classes are not inhibited by either cnispin or LeSPI. Both inhibitors inhibit trypsin at 1 : 1 molar ratio. Arginine is believed to be the primary reactive residue of LeSPI for trypsin (Odani *et al.*, 1999). Comparison of the amino acid sequences of cnispin, LeSPI and predicted serine protease inhibitors from other basidiomycetes (Fig. 2) revealed two conserved arginine residues, R21 and R60, either of which could be the inhibitory-active residue. An exception is the *Laccaria bicolor* predicted protein, which has a lysine at position 60 instead of an arginine, and shows a specificity for binding to the trypsin S_1 site similar to that for arginine (Bode & Huber, 1992).

Inhibitory activity against endogenous proteases and insecticidal activity against *D. melanogaster* larvae suggest regulatory and defensive biological functions for CnSPIs. A regulatory role in the endogenous proteolytic system of *C. nebularis* has been suggested for CnSPIs, based on specific inhibition of serine-type proteolytic activity from different basidiomycete mushrooms, including *C. nebularis* (Sabotič *et al.*, 2007b). The intracellular localization of CnSPIs supports an endogenous regulatory role. This was further confirmed by considerable inhibition of all partially purified serine proteases from *C. nebularis* by CnSPIs. Different levels of inhibition by Pefabloc, chymostatin and CnSPIs of the four separated peaks of proteolytic activity from the extract indicate a difference in specificity of these proteases. A role of CnSPIs in fruiting body development and/or resource recycling is thus probable.

The insecticidal effect against the model dipteran *D. melanogaster* suggests a defensive role of CnSPIs against predatory insects. Flies (Diptera) are major mushroom pests; most of them attack at early mushroom developmental stages while others, like flies of the genus *Drosophila*, feed on later developmental stages or mature mushrooms (Coles *et al.*, 2002; Krivosheina, 2008). Serine proteases constitute the predominant digestive proteolytic activity of dipterans (Terra & Ferreira, 1994). Analogously to plants, in which protease inhibitors play an important defensive role (Habib & Fazili, 2007), serine protease inhibitors probably play a defensive role against predation by insect pests and different pathogens also in mushrooms. The insecticidal effect of CnSPIs was compared with those of three plant serine protease inhibitors: PSPI and PI2, two serine protease inhibitors from potato (Brzin *et al.*, 1995; Pearce *et al.*, 1982), and CMTI, a trypsin inhibitor from pumpkin (Krishnamoorthi *et al.*, 1990). The CnSPIs showed an effect similar to that of PSPI, PI2 left no surviving larvae, whereas CMTI had no effect. A cysteine protease inhibitor, clitocypin, from *C. nebularis* was also used in the bioassay, since a putative digestive cysteine protease has been identified in *D. melanogaster* (Matsumoto *et al.*, 1995). Clitocypin had no effect on larval survival, confirming the predominant role of serine proteases in protein digestion of *Drosophila* larvae. Therefore, CnSPIs could offer an effective defence, with the possibility of adaptation and selection of the most appropriate isoforms.

In conclusion, a new serine protease inhibitor, cnispin, from the basidiomycete *C. nebularis*, has been characterized at the genetic and biochemical levels. Evidence is described that indicates its dual biological function. The similarity in primary sequence and biochemical properties to LeSPI indicates that cnispin belongs to the I66 family of protease inhibitors in the MEROPS classification. Analysis of the inhibition profile showed high specificity towards trypsin-like serine proteases. This strong and specific inhibition of trypsin, with many other serine proteases being unaffected, suggests that cnispin could prove an effective and useful tool in studying trypsin-dependent cellular processes in humans, such as cancer and inflammation of the cardiovascular, respiratory, musculoskeletal, gastrointestinal and nervous systems (Ramachandran & Hollenberg, 2008).

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