Structural Basis of Trypsin Inhibition and Entomotoxicity of Cospin, Serine Protease Inhibitor Involved in Defense of Coprinopsis cinerea Fruiting Bodies*§

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Background: Mushrooms are a rich source of novel proteins with unique features. Results: Cospin, a trypsin-specific protease inhibitor, has a β-trefoil fold and is toxic against the fruit fly. Conclusion: Cospin represents one type of fungal protein-mediated defense against fungivorous insects. Significance: Cospin, the first fungal trypsin inhibitor with determined three-dimensional structure, utilizes a different loop for trypsin inhibition compared with other β-trefoil inhibitors.

Cospin (PIC1) from Coprinopsis cinerea is a serine protease inhibitor with biochemical properties similar to those of the previously characterized fungal serine protease inhibitors, cnispin from Clitocybe nebularis and LeSPI from Lentinus edodes, classified in the family I66 of the MEROPS protease inhibitor classification. In particular, it exhibits a highly specific inhibitory profile as a very strong inhibitor of trypsin with $K_i$ in the picomolar range. Determination of the crystal structure revealed that the protein has a β-trefoil fold. Site-directed mutagenesis and mass spectrometry results have confirmed Arg-27 as the reactive binding site for trypsin inhibition. The loop containing Arg-27 is positioned between the β2 and β3 strands, distinguishing cospin from other β-trefoil-fold serine protease inhibitors in which β4-β5 or β5-β6 loops are involved in protease inhibition. Biototoxicity assays of cospin on various model organisms revealed a strong and specific entomotoxic activity against Drosophila melanogaster. The inhibitory inactive R27N mutant was not entomotoxic, associating toxicity with inhibitory activity. Along with the abundance of cospin in fruiting bodies of C. cinerea and the lack of trypsin-like proteases in the C. cinerea genome, these results suggest that cospin and its homologs are effectors of a fungal defense mechanism against fungivorous insects that function by specific inhibition of serine proteases in the insect gut.

Higher fungi belong to phyla Basidiomycota and Ascomycota and form sexual reproductive structures termed fruiting bodies or mushrooms. Fruiting bodies of Basidiomycetes are a rich source of proteases with unique characteristics (1–3). Interestingly, they also contain a great number of protease inhibitors with unique features. It is still unclear whether they are directed against endogenous or exogenous proteases (1, 4–6).

Only a few serine protease inhibitors from basidiomycete fruiting bodies have been isolated and characterized, although serine proteases constitute the predominant proteolytic activity in these structures (1). These inhibitors include two isomeric inhibitors of serine proteases, IA-1 and IA-2, from Pleurotus ostreatus, POIA1 and POIA2 (7), belonging to family I9 in the MEROPS classification, a serine protease inhibitor from Lentinus edodes (8), and cnispin, a trypsin-specific inhibitor from Clitocybe nebularis (4), the latter two belonging to family I66 (9). In addition, proteinase K and trypsin inhibitors have been isolated from mycelia of white rot basidiomycetes Trametes versicolor (10) and Abortiporus biennis (11), respectively, but have not yet been assigned to a MEROPS inhibitor family. The P. ostreatus proteinase A inhibitor 1, a homolog of subtilisin propeptide, is the only fungal serine protease inhibitor for which the three-dimensional structure has been reported (12). Although it has been extensively studied not only as a subtilisin inhibitor but also as an intramolecular chaperone (12, 13), little is known about its biological function. Based on the inhibition of proteases IA-1 and IA-2 from the same organism, a role in controlling misplaced endogenous proteases has been proposed (7).

The two well characterized serine protease inhibitors of family I66 in the MEROPS classification, LeSPI from L. edodes (8) and cnispin from C. nebularis (4), are small proteins (16 and 16.4 kDa) with similar acidic isoelectric points and are stable over a wide pH range. They inhibit trypsin with high specificity ($K_i$ in the low nanomolar range) but chymotrypsin less strongly ($K_i$ in the micromolar range for cnispin and nanomolar range for LeSPI).
for LeSPI), whereas other proteases are not inhibited (4, 8). A dual biological role has been proposed for cospin in regulating unidentified endogenous proteases and in defense against fungivorous insects (4).

Protease inhibitors and lectins constitute one of the natural defensive strategies of plants against herbivorous insects as well as other parasitic organisms and pathogens (14–17). Several serine protease inhibitors from plants have been shown to possess entomotoxic activity, which is mediated by inhibition of digestive proteolytic enzymes, resulting in reduced availability of the amino acids necessary for growth and development (16, 18, 19). Several lectins (20) and cysteine (5, 6) and serine (4, 8) protease inhibitors of higher fungi have been suggested to play a role in the defense of fruiting bodies against predatory, parasitic, and/or pathogenic organisms.

Serine proteases, and specifically trypsin-like enzymes, play very important nutritional roles and are involved in various physiological and pathophysiological processes. Novel inhibitors of serine proteases can thus find various applications ranging from pest management and crop protection to drug development and design for therapeutics as well as in basic medical research.

A hypothetical protein (CC1G_09480.3) with high homology to the previously described serine protease inhibitors from the mushrooms L. edodes (8) and C. nebularis (4) was identified in the genome of Coprinopsis cinerea (21) and designated PIC1. Here we describe the genetic background and biochemical properties of this serine protease inhibitor from the inky cap mushroom C. cinerea named cospin (PIC1) and report its three-dimensional structure and mechanism of inhibition. In addition, we present evidence for its biological role in defense against fungivorous insects in C. cinerea fruiting bodies.

**EXPERIMENTAL PROCEDURES**

**Enzymes, Substrates, and Inhibitors**—Bovine trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), porcine kallikrein (EC 3.4.21.35), porcine pepsin (EC 3.4.23.1), and soybean trypsin inhibitor (EC 3.4.21.35), porcine pepsin (EC 3.4.23.1), and soybean trypsin inhibitor were from Sigma, bovine thrombin (EC 3.4.21.5) was from Roche Applied Science, porcine elastase (EC 3.4.21.36) and stricter benzoyl-DL-arginine-7-(4-methyl)coumarylamide), Suc-Ala-Ala-Pro-Phe-MCA, were from Bachem.

**Cloning, Heterologous Expression, and Purification of Recombinant Cospin**—RNA was isolated from lyophilized fruiting bodies of C. cinerea using the RNaseasy Lipid Tissue Mini kit (Qiagen) according to the manufacturer’s protocol. cDNA encoding cospin (pic1) was synthesized and amplified using the OneStep RT-PCR kit (Qiagen) with total RNA and oligonucleotide primers pic1-seq-fwd and pic1-seq-rev (supplemental Table S1). The PCR product was sequenced after cloning into the pGEM-T Easy vector (Promega). Plasmids for heterologous expression of untagged and C-terminal His-tagged cospin in E. coli were constructed by PCR amplification of the coding region of the cDNA clone using primers carrying NdeI and BamHI restriction sites (pic1-N-fwd, pic1-C-rev, pic1-CHis-rev). The PCR products were subcloned into pGEM-T easy, and the inserts were released with NdeI and BamHI and ligated into appropriately linearized pET24b (Novagen), resulting in the expression plasmids pET24-pic1 and pET24-pic1-CHis. For heterologous expression, E. coli BL21(DE3) transformed with these plasmids was cultivated at 37 °C in LB medium supplemented with 50 mg/liter kanamycin, induced with 1 mM isopropyl-β-D-thiogalactoside at an A600nm between 0.5 and 1, and incubated further at either 37 °C for 4 h or at 23 °C for 16 h. Solubility was checked as described previously (24).

Untagged recombinant cospin was purified as described below. His-tagged cospin was purified by metal affinity chromatography. E. coli cells expressing pET24-pic1-CHis were harvested and resuspended in cold PBS (10 mM Na2HPO4, 17.5 mM KH2PO4, 135 mM NaCl, 2.5 mM KCl) containing 1 mM PMSF. The cells were lysed using a French press (SLM Aminco; SLM Instruments, Inc.), and the lysate was cleared by centrifugation at 4 °C, first for 15 min at 7,000 × g and then for 30 min at 15,000 × g. The supernatant was applied to a cobalt affinity resin (TALON, Clontech) following the manufacturer’s instructions, except that the resin was equilibrated with PBS, washed after binding with PBS containing 5 mM imidazole, and eluted with PBS containing 200 mM imidazole. After elution, imidazole was removed using a PD-10 Desalting column (Amersham Biosciences).

**Construction, Expression, and Purification of Mutant Forms of Cospin**—The cospin (pic1) cDNA sequence (GenBank™ accession number Q903329) was the basis for the design of mutagenic oligonucleotides (supplemental Table S1) that were used in PCR site-directed mutagenesis using KOD Hot Start DNA Polymerase (Novagen) and expression plasmid pET24-pic1 as template. The DpnI endonuclease (Fermentas) was used for digestion and recovery of the vectors containing mutated inserts (25). Based on the sequence alignment of cospin to the other fungal protease inhibitors with experimentally established trypsin inhibitory activity LeSPI (8) and csnip (4), putative reactive site residues on cospin were selected, and mutants R21A and K61A prepared. Furthermore, the zdock server was used to predict a model of a cospin–trypsin complex, and a few additional reactive site residues of cospin were suggested as binding into the active site of trypsin. Mutants R27N, E106W, E106Q, and Y132W were, therefore, prepared.
Expression vectors pET24 with subcloned mutated cospin (pic1) inserts were transformed into the BL21(DE3) (Invitrogen) strain of E. coli. The transformed strain was grown in LB medium supplemented with appropriate antibiotics at 37 °C. When optical density at 600 nm reached 0.5–1, isopropyl-β-d-thiogalactoside was added to a final concentration of 0.5 mM. Four hours after induction of expression, cells were harvested by centrifugation, resuspended in buffer A (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). The supernatant was fractionated on a Sepharose S-200 column (4 × 110 cm) equilibrated with buffer B (0.02 M Tris-HCl, 0.3 M NaCl (pH 7.5)), and inhibitory active fractions were pooled.

**SDS-PAGE**—Proteins were analyzed by 12% (w/v) polyacrylamide gels under denaturing reducing and non-reducing conditions and visualized using Coomassie Brilliant Blue staining. Low molecular mass markers 14.4–97 kDa (GE Healthcare) were used for estimating molecular mass.

**Inhibition Assay**—Inhibitory activities of samples during the isolation procedure were measured against trypsin (0.1 μM) in buffer C (0.05 M Tris-HCl, 0.02 M CaCl₂ (pH 8)) in microtiter plates. After 10 min of preincubation at room temperature, 2 μl of 0.1 M substrate BAPNA was added, and the mixture was incubated for 20 min at 37 °C. The reaction was stopped with 0.2 M HCl, and absorbance was measured at 405 nm (A₄₀₅).

**Active Site Titration**—The molar concentration of active trypsin was determined by titration with p-nitrophenyl-p′-guanidinobenzoate (26). Active concentrations of cospin were 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 clitorcycin (27) using substrate benzoylcarbonyl-Phe-Arg-MCA and buffer C. Data were analyzed by nonlinear regression analysis according to Morrison (28), and kᵣ and kₐ values were obtained using kₐ of 59 μM for trypsin.

The kinetics of inhibition of chymotrypsin, subtilisin, kallicrein, elastase, and thrombin were determined according to Henderson (29), as described for cathepsin B inhibition by clitorcycin (27), with the following modifications. Various amounts of the inhibitor (0.01–40 μM) were incubated with each of the enzymes for 15 min in microtiter plates. Chymotrypsin and elastase were assayed in buffer C, kallicrein was assayed in 0.05 M Tris-HCl, 0.05 M NaCl, 0.01% (v/v) Tween buffer, (pH 7.8), subtilisin was assayed in 0.1 M phosphate buffer (pH 8.8), and thrombin was assayed 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 was used for kallicrein, and t-butoxycarbonyl-Val-Pro-Arg-MCA was used for thrombin and Suc-Ala-Ala-Ala-Arg-MCA for elastase. The released MCA was measured using a microplate reader (Tecan Infinite M1000).

**Inhibitory Activity against Other Classes of Proteases**—Inhibition of cysteine protease papain activity was assayed using substrate benzyoyl-Arg-2-naphthylamide as described (27).

Inhibition of porcine pepsin, an aspartic protease, was assayed using the fluorogenic substrate fluorescein isothiocyanate-he-moglobin as described (6).

**Mass Spectrometry and Blue Native PAGE**—Trypsin and cospin were mixed in molar ratio 2:1 (final concentrations 0.034 and 0.068 mM, respectively) and incubated at 37 °C. Samples were taken at times ranging from 5 min to 30 days and frozen until analysis. They were analyzed using the Novex NativePAGE™ Bis-Tris Gel System (Invitrogen) and mass spectrometry (MALDI-TOF spectrometer; Bruker Daltonics) following the manufacturer’s recommendations.

**pH Stability**—To test pH stability, recombinant cospin (0.035 mg/ml) was incubated for 30 min 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) and then adjusted to pH 7. Residual inhibitory activity was determined against trypsin using BAPNA as substrate.

**Crystallization, Structure Solution, and Refinement**—Cospin was concentrated to 30 mg/ml in 10 mM Tris-HCl buffer, and crystals were grown in 0.1 M Mes (pH 6.0), 20% (v/v) 2-methyl-2,4-pentanediol. Before flash-freezing in liquid nitrogen they were soaked briefly in mother liquor containing 10% (v/v) glycerol. All data sets were collected on the in-house Rigaku rotating anode (RU 200) using Xenon mirrors and processed using the HKL2000 package (30). The structure was partially solved using the data set from the crystal, iodinated by the hyper-VIL method (31). The resolution of that set was 2.2 Å, and positions of four iodine atoms were obtained with automated SOLVE/RESOLVE scripts incorporated in the AutoSol module of the PHENIX suite (32), which builds approximately half of the structure. The native data set, collected to 1.80 Å, was phased by molecular replacement with AMoRe (33) with the partial structure of the iodinated protein as a search model, and an almost complete structure was built by ARP/warp (34). The structure was refined with Refmac (35) and MAIN (36). Soybean trypsin inhibitor (STI) in complex with trypsin (37) was used for modeling the cospin inhibitory reactive site in MAIN (36).

**Quantitative Real-time PCR**—Gene expression levels of cospin in fruiting bodies relative to those in vegetative mycelium were evaluated by quantitative real-time PCR. RNA was extracted from lyophilized fruiting bodies and mycelium of C. cinerea using the RNeasy® Lipid Tissue Mini kit (Qiagen). Vegetative mycelium was collected after growing for 3 days in the dark at 37 °C on solid YMG overlaid with sterile cellophane discs. For collection of fruiting bodies, mycelium was pregrown as above and, after 3–4 days, plates were transferred to 28 °C, 12 h light/dark cycles and 90% humidity. Primordia were collected after 5–8 days. RNA extraction, cDNA synthesis, and quantitative real-time PCR were carried out as described (20) using amplification primers Pic1-RTpcr-fwd and Pic1-RTpcr-rev. S.E. of the mean are based on four technical replicates of each cDNA template and gene.

**Sequence Analysis**—Sequence analysis and multiple sequence alignments were performed in the BioEdit Sequence Alignment Editor. Similarity searches were performed using blastp and tblastn algorithms at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) at the Joint Genome Institute Fungi Portal and at the Broad Institute of Harvard and MIT servers.
Biotoxicity Assays—Assays for biotoxicity against the insect Aedes aegypti, the nematode Caenorhabditis elegans, and the amoeba Acanthamoeba castellanii were performed as described (24). For biotoxicity assays against D. melanogaster, purified proteins were incorporated into the rearing medium. Transparent cylindrical tubes (1 cm diameter × 11 cm height) were filled with 500 ml of solid rearing medium (0.8% agar, 10% fresh yeast, 7.5% glucose, 5.5% cornmeal, 1% flour, 0.05% methylparaben, 0.1% propylparaben) containing a final concentration of 100 g/ml of either purified protein (cospin wild-type or mutant R27N) or bovine serum albumin (BSA; as control). Twenty eggs of D. melanogaster strain Canton S were added per tube (5 replicates per treatment) and incubated at 23 °C under 12 h light/day cycles. Development was monitored, and the total numbers of pupae and flies were determined after 9 and 13 days, respectively. Significant differences were evaluated by one-way analysis of variance and Dunnett's post hoc multiple comparisons versus the control group.

RESULTS

Identification and Characterization of Cospin—We cloned and sequenced the gene and the cDNA coding for the PIC1 hypothetical protein from fruiting bodies of the C. cinerea strain AmutBmut and named it cospin, Coprinopsis cinerea serine protease inhibitor. The deduced amino acid sequence of cospin (pic1) from the AmutBmut strain (GenBank™ accession number ACX48485) differs by 8 amino acids from that of the sequenced monokaryotic strain, Okayama 7 (Fig. 1). Interestingly, 3 more isogenes are found in the genome of C. cinerea, exhibiting 38–95% sequence identity to cospin. The ectomyccorrhizal fungus Laccaria bicolor also contains 4 isogenes for cospin-like proteins with 17–30% sequence identity, and the plant pathogen Moniliophthora perniciosa contains one iso-
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Differential Expression of Cospin in C. cinerea—To determine the differential expression of cospin (pic1) in C. cinerea, we quantified by quantitative real-time PCR the ratio of expression of the gene in fruiting bodies to that in vegetative mycelium of the homokaryotic strain AmuBmmt. The expression of cospin (pic1) was found to be 696 ± 75-fold higher in fruiting bodies than in vegetative mycelium, a ratio in the range of that observed for fruiting body lectins from the same organism (38).

Expression and Purification of Active Recombinant Cospin and Cospin Mutants—For a detailed biochemical analysis of its biochemical properties, cospin was produced recombinantly in E. coli BL21(DE3) and purified. It was expressed as a soluble protein. Size exclusion chromatography yielded a purified protein at a yield of 180 mg of cospin/liter of bacterial culture. The recombinant cospin exhibited a single 18-kDa band on SDS-PAGE under reducing conditions (supplemental Fig. S1). Under non-reducing conditions it showed an additional band with an apparent molecular mass of 36 kDa, corresponding to a dimer (supplemental Fig. S2). Expression of cospin mutants yielded soluble proteins of the same molecular mass.

pH Stability—The stability of recombinant cospin to pH was measured by determining its inhibitory activity after incubation at extremes of pH (supplemental Fig. S3). Cospin retained its inhibitory activity after incubation in acidic (pH 3) and alkaline (pH 11) conditions.

Kinetics of Inhibition—Titration of trypsin with cospin showed that complete inhibition is achieved at a 1:1 molar stoichiometry. The pseudo-first order rate constant, $k$, for binding of cospin to trypsin increased linearly with inhibitor concentration. Kinetic constants of the inhibition of different proteases by cospin are presented in Table 1. Cospin was most effective in inhibiting trypsin, with a $K_i$ value of 22 ps, showing it to be a fast-acting ($k_d$ $5.28 \times 10^6$ M$^{-1}$s$^{-1}$) and tight binding ($k_d$ $1.26 \times 10^{-4}$ s$^{-1}$) inhibitor of this enzyme. The $K_i$ value for the inhibition of chymotrypsin was 116 nm. Inhibition of subtilisin, porcine kallikreins, and elastase was very weak. Furthermore, cospin showed no inhibitory activity against the serine protease bovine thrombin, the cysteine protease papain, or the aspartic protease pepsin.

Inhibitor Binding Site and Mechanism of Inhibition—To determine the trypsin binding site, several single amino acid mutants were prepared based on sequence similarity to LeSPI and cospin and on models of the cospin-trypsin complex. Of all the cospin mutants prepared, only the mutant R27N lacked the inhibitory activity toward trypsin, exhibiting an $\sim 2 \times 10^4$ higher $K_i$ value (Table 1). The inhibition constant for cospin R27N mutant against chymotrypsin was in the micromolar range, whereas subtilisin, kallikreins, and elastase were not inhibited (Table 1). All the other mutants (R21A, K61A, E106W, E106Q, Y132W) showed the same inhibitory profile as cospin.

The binding of Arg-27 into the S1 pocket of trypsin was additionally confirmed by the mass spectrometry analysis of cospin preincubated with trypsin (2:1 ratio). Small amount of cospin was cleaved after Arg-27 (Fig. 2A). The peak corresponding to a molecular mass of 13,782 Da correlates well with the theoretical molecular mass (13,775 Da) and was absent in all control samples (including cospin alone and the substantially autodegraded trypsin sample) (Fig. 2B). Furthermore, native-PAGE analysis revealed that cospin forms a very stable complex with trypsin that remained stable at 37 °C for over 14 days (Fig. 2D).

Crystal Structure—Data collection and refinement statistics are summarized in Table 2. Cospin crystallized in the C2 space group with one molecule per asymmetric unit. The complete sequence was visible in the crystals, from Met-1 to Asp-150. Positioning of nearly all the residues was clearly revealed by the electron density maps. The exceptions are Met-1 and the C terminus (Glu-148—Asp-150), where no interpretable electron density was observed, and the side chains of Arg-27 and Glu-95.

Cospin is based on a β-trefoil fold (Fig. 3, A and B), also present in proteins such as Kunitz-type STI (r.m.s.d. 2.2 Å for 110 aligned residues) (37), interleukins-1α and 1β (r.m.s.d. 1.9 Å for 109 aligned residues) (39), fibroblast growth factors (r.m.s.d. 1.8 Å for 109 aligned residues) (40), myocynins, the cysteine protease inhibitors also isolated from basidiomycete fruiting bodies (r.m.s.d. 1.8 Å for 123 aligned residues) (41) and lectins (42, 43). The cospin fold resembles a tree-like structure with 2 loops in the root region, a stem comprising a six-stranded β-barrel, and two layers of loops in the crown region. The stem is an up- and-down β-barrel composed of six antiparallel β-strands that are laid at an angle of less than 45 degrees to the axis of the barrel. The N and C termini are in the root region. There are two very long loop regions in the tree crown comprising 35 (β1-β2) and 18 (β11-β12) residues.

Modeling of Cospin-Trypsin Complex—After aligning the inhibitory loop Glu-25—Leu-29 in cospin with the inhibitory loop Ser-60—Arg-65 of STI in complex with trypsin (37), cospin fitted well in the active site cleft (Fig. 3, C and D).

The model of the cospin-trypsin complex (Fig. 3, C and D) reveals that although there are no critical clashes between cospin and trypsin, the positions of side chains of cospin are not optimized for binding to trypsin. Arg-27 fits well to the S1 pocket, but Asp-26 does not fit to the S2 binding site and thus most probably undergoes a conformational change on binding.
to trypsin (Fig. 3D). If the side chain of Asp-26 were to assume the conformation of side chain of Tyr-62 in STI, a hydrogen bond between its side chain carboxyl group and the backbone carbonyl of Ser-124 could be formed. The side chain of Ser-28 in cospin, which corresponds to Ile-64 of STI in the S1′ binding site, is most probably rotated in the complex so that the hydroxyl group points toward the trypsin core. In this orientation a hydrogen bond could be formed between the hydroxyl group in Ser-28 and the carbonyl of Phe-41. We cannot rule out a rotation of the side chain of the active Ser-195 in trypsin and formation of a hydrogen bond between the two serine hydroxyl groups. In contrast to the other residues, Leu-29 in cospin exhibits better binding to the S2 pocket of trypsin than Arg-65 in STI.

Cospin Biotoxicity—Cospin was tested for toxicity against the amoeba A. castellanii, the nematode C. elegans, and the two dipteran insects A. aegypti and D. melanogaster. No toxicity was observed against A. castellanii, C. elegans, and A. aegypti (supplemental Fig. S4). However, it showed significant (p < 0.05 with respect to the control) toxicity against D. melanogaster by causing developmental delay in both pupae and flies (Fig. 4). The cospin mutant R27N was not toxic to D. melanogaster (p > 0.05 with respect to the control), strongly suggesting that entomotoxicity is mediated by specific inhibition of serine proteases in the fly.

TABLE 2
Data collection and refinement statistics

| Data collection | 3N0K |
|-----------------|------|
| PDB code        |      |
| Crystallization conditions | 0.1 M MES, pH 6.0, 20% MPD |
| Space group     | C2   |
| Cell dimensions | 78.63, 38.19, 55.089 |
| α, β, γ (°)     | 90, 96.87, 90 |
| Resolution (Å)  | 17.0-1.80 |
| Rmerge (%)      | 3.5 (11.2) |
| I/σI            | 76.5 (21.4) |
| Completeness (%)| 95.0 (83.4) |
| Redundancy      | 7.1 (3.8) |

Refinement

| Resolution | 17.0-1.80 |
| No. of reflections (work/free) | 13,046/727 |
| Rwork/Rfree | 16.7/19.4 |
| B factors | |
| Protein | 20.6 |
| Water | 35.8 |
| No. of atoms | |
| Protein | 1180 |
| Water | 251 |
| r.m.s.d. | |
| Bond length (Å) | 0.021 |
| Bond angle (°) | 1.761 |

FIGURE 2. Mechanism of trypsin inhibition by cospin. A, mass spectrometry analysis of the cospin-trypsin complex were incubated at 37 °C for 30 days. AU, arbitrary units. B, a zoom on peak 5 from panel A, corresponds to cospin lacking the first 27 residues (solid line). Dotted and dashed lines represent controls, cospin and substantially autodegraded trypsin, respectively. The units are the same as in panel A. C, shown is a list of peaks from panel A, with their corresponding determined and theoretical masses. D, shown is a blue native PAGE analysis of formation of the complex between cospin and trypsin, incubated at 37 °C for the indicated periods of time. M, molecular mass marker.
DISCUSSION

A serine protease inhibitor, cospin (PIC1), highly expressed in fruiting bodies of the inky cap mushroom (C. cinerea), has been cloned and characterized at the molecular and functional levels. Based on the high sequence similarity of cospin to the previously characterized serine protease inhibitors LeSPI from L. edodes (8) and cnispin from C. nebularis (4), it was assigned to family I66 of the MEROPS protease inhibitor classification. In addition to sequence similarity, cospin also exhibits biochemical properties similar to those of cnispin (4) and LeSPI (8). They are all small proteins with acidic isoelectric points and exhibit very similar inhibitory profiles. Cospin is a highly specific trypsin inhibitor, with a $K_i$ in the picomolar range. It also inhibits chymotrypsin (family S1), albeit with a $K_i$ in the micromolar range, and shows even weaker inhibition of kallikrein, elastase (family S1), and subtilisin (family S8). Other serine proteases and proteases of other catalytic classes are not inhibited. Furthermore, cospin, similarly to cnispin (4), retains inhibitory activity after exposure to extremes of pH.

The three-dimensional structure of cospin shows it to be a $\beta$-trefoil-fold protein that supports the assignment of family I66 of the MEROPS classification to the clan IC together with the Kunitz-type serine protease inhibitors (family I3) and mycocypins (families I48 and I85) (9, 41). To determine the cospin binding site for trypsin, several cospin mutants were prepared based on sequence and literature data and finally based on the crystal structure. Inhibitors of trypsin and trypsin-like proteases have arginine or lysine at the P1 position (44), and by chemical modification, arginine was shown to be the key residue for binding of the homologous inhibitor LeSPI to trypsin (8). Site-directed mutagenesis of cospin revealed that Arg-27 is the corresponding primary reactive residue of cospin. Cospin mutant R27N exhibited weaker inhibition of trypsin, with a 200 higher $K_i$ value, whereas inhibition of chymotrypsin, whose active site accommodates large residues at the P1 position, including Asn (44), was almost unchanged.

The mutagenesis, native-PAGE, and mass spectrometry analyses along with modeling of the complex show clearly that cospin is a classic canonical inhibitor that binds to the active site in a substrate-like manner and forms a tight and stable complex with trypsin. The cleaved cospin was observed with MALDI-TOF after only 5 min of incubation and to a similar degree also after 30 days (not shown), strongly suggesting that the peptide is not a consequence of nonspecific degradation but, rather, of the well defined dynamic equilibrium.

Protease inhibitors with the $\beta$-trefoil fold can utilize their loops in various ways for the inhibition of serine proteases (Fig. 5). The trypsin-specific inhibitor cospin from C. cinerea utilizes

![FIGURE 3. Three-dimensional structure of cospin (A and B) and a model of cospin binding to trypsin (C and D). A and B, shown is a crystal structure of cospin. The $\beta$-barrel forming the trunk is shown in red, and Arg-27 is in blue. C, a model of cospin binding to trypsin is shown. D, a detailed comparison of cospin (orange) and STI (red) binding to trypsin is shown. S2, S1, S1', and S2' binding sites are shown in green and cyan, and the active site Ser-195 is in yellow. The cospin residues involved in binding are labeled.](image)

![FIGURE 4. Toxicity of cospin wild-type and trypsin binding mutant (R27N) against D. melanogaster. BSA was used as control. Error bars represent the S.E.](image)
D. melanogaster pin toward saprophytes like genes (family S1) in fungal genomes are associated with patho-
tical in the inhibition of chymotrypsin by the winged bean chymotrypsin inhibitor (45), trypsin by STI (37), and porcine pancreatic elastase and human neutrophil elastase by BbCI (2), and asparag-
inyl endopeptidase or trypsin by mycocypins (3).

The strong, highly specific inhibition of trypsin, viewed alongside the absence of trypsin-like protease genes in the C. cinerea genome (9), suggests that cospin is directed in vivo mainly against exogenous proteases and thus plays a defensive role against predators and parasites. Trypsin-like protease genes (family S1) in fungal genomes are associated with patho-
genic and symbiotic fungi, whereas they are mostly absent from saprophytes like C. cinerea (47, 48). The distinct toxicity of cospin toward D. melanogaster is thus in agreement with such a defensive function and suggests that the main targets of cospin are dipteran insects as serine proteases constitute the predom-
antly digestive proteolytic activity of dipterans (49). The reported pH values of midgut contents in different dipteran larvae range from very acid (pH 3) to very alkaline (pH 9–12), and the pH varies along the digestive tract (50); therefore, the broad pH stability displayed by cospin would be advantageous for a defensive protein directed to digestive proteases.

Of the two dipteran insects used in the biotoxicity assays, cospin showed toxicity only against D. melanogaster. Although the β2–β3 loop of the crown region, which is a different loop from those involved in other β-trefoil-fold protease inhibitors. Macrolecypin 4 from Macrolepiota procera utilizes the crown region loop β5–β6 for inhibition of trypsin and asparaginyl endopeptidase (41), whereas the root region loop β4–β5 is func-
tional in the inhibition of chymotrypsin by the winged bean chymotrypsin inhibitor (45), of trypsin by Kunitz-type STI (37), and of porcine pancreatic elastase and human neutrophil elastase by Bauhinia bauhinioides cruzipain inhibitor (46). This indicates that the β-trefoil fold can serve as a fundamental scaffold to which different inhibitory loops can be attached at differ-
et positions.

The β-trefoil fold loops involved in inhibition of serine proteases. Involvement is shown of different loops of β-trefoil proteins in inhibition of trypsin by cospin (1), chymotrypsin by the winged bean chymotrypsin inhibitor (45), trypsin by STI (37), and porcine pancreatic elastase and human neutrophil elastase by BbCI (2), and asparag-
inyl endopeptidase or trypsin by mycocypins (3).

FIGURE 5. Schematic representation of the β-trefoil fold loops involved in inhibition of serine proteases. Involvement is shown of different loops of β-trefoil proteins in inhibition of trypsin by cospin (1), chymotrypsin by the winged bean chymotrypsin inhibitor (45), trypsin by STI (37), and porcine pancreatic elastase and human neutrophil elastase by BbCI (2), and asparag-
inyl endopeptidase or trypsin by mycocypins (3).

β-Trefoil Trypsin Inhibitor Involved in Fungal Defense

development of several families of flies has been associated with fruiting bodies of higher fungi, including the Drosophilidae (51), that of the mosquito has not. Furthermore, a lack of toxi-

Many dipteran insects utilize fruiting bodies of Basidiomycetes for breeding (51). The high toxicity of cospin toward D. melanogaster, manifested as delayed egg development, can be attributed to inhibition of digestive trypsin-like proteases as the poorly inhibitory cospin R27N mutant was not toxic. Delayed development of eggs laid in the fresh fruiting bodies that hatch and feed on the fruiting body tissue only when the latter starts to decay has been observed for Drosophilidae flies in their natural habitat (51). These observations are in accord with the fruiting body-specific expression of cospin. Further-
more, the ubiquitous occurrence of inhibitors for trypsin-like proteases in homobasidiomycete fruiting bodies (56, 57) sug-

In addition to a defensive function against predatory insects, an endogenous role has been proposed for cnispin (4). We have shown that endogenous proteases may also be targeted by cospin (supplemental Fig. S5). However, these proteases are not trypsin-like enzymes, as no such proteases are encoded in the genome and because a similar inhibitory pattern was observed with the cospin R27N mutant. We suggest that, like in mycocypins (41), other loops of the β-trefoil fold may be responsible for the inhibitory activity against these endogenous proteases. Because most of the proteolytically active bands were not inhib-

Because most of the proteolytically active bands were not inhib-

Based on the absence of a signal sequence for the classical secretion of cospin, these proteases would have to be cytoplasmic to be inhibited by cospin.
β-Trefoil Trypsin Inhibitor Involved in Fungal Defense

It is interesting that the β-trefoil fold has been observed in several proteins proposed to be involved in fruiting body defense in higher fungi, including several lectins (20), mycocytales (41) (a family of fungal cysteine protease inhibitors) and defense in higher fungi, including several lectins (20), mycocyteal reading of the manuscript.

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