Scientific paper

Dermatophyte *Trichophyton mentagrophytes* Produces Cysteine Protease Inhibitor

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Abstract

The protein inhibitor of cysteine proteases was isolated from an important zoophilic dermatophyte species *Trichophyton mentagrophytes* (*T. mentagrophytes*) and partially characterized. The isolation process involved affinity chromatography, followed by ion-exchange chromatography and reverse phase high performance liquid chromatography. The fungal inhibitor appears to exist in a high (24 kDa) and low (12 kDa) molecular mass form. It inhibits proteolytic activity of papain, cathepsins B and L but not of cathepsin H or trypsin. Results of immunoblotting procedures indicate that sera of *T. mentagrophytes* infected rabbits contain antibodies against higher molecular mass forms of the inhibitor. Since no sequence homology has been found between partial protein sequences of *T. mentagrophytes* inhibitor and other known cysteine protease inhibitors so far, we can speculate that this inhibitor has some structurally unique characteristics. The *T. mentagrophytes* inhibitor shares some biochemical similarities (molecular mass, high and low molecular mass forms, inhibitory profiles) with clitocypin from *Clitocybe nebularis* and macrocypins from *Macrolepiota procera*.

Keywords: Dermatophyte, *Trichophyton mentagrophytes*, cisteine protease, inhibitor

1. Introduction

Dermatophytes are a group of closely related filamentous fungi that encompass anamorphic (asexual or imperfect) genera *Epidermophyton*, *Microsporum* and *Trichophyton,* of anamorphic class *Hyphomycetes* of the *Deuteromycota*.[1,2,3] They are the most common agents of fungal infections worldwide. They invade the *stratum corneum* of the skin, hair and nail and break down keratinized tissues *in vivo* and/or *in vitro*. Dermatophytosis is acquired from an exogenous source since dermatophyte fungi are not part of the normal skin flora.[4,5,6] Reactions to a dermatophyte infection in animals or humans may range from mild to severe as a consequence of the host's reactions to the metabolic products of the fungus, the virulence of the infecting strain or species, the anatomic location of the infection, and local environmental factors.[1] Although dermatophyte infections, including those with *Trichophyton mentagrophytes* (*T. mentagrophytes*), have a limited impact on the general health condition of animals, they are especially important from a zoonotic point of view.[7]

In vitro or during the infection process, hard keratin tissues have to be digested into short peptides and amino acids in order to be assimilated. It has been established that secreted proteolytically active enzymes are important for fungal virulence.[8,9,10,11] There are many reports on isolation and characterisation of secreted proteases from individual dermatophyte species which were often described simply as keratinases. It is now known that dermatophyte-secreted endoproteases are members of two large

protein families, the subtilisins (serine proteases) and the fungalysins (metalloproteases).[12] The genome of the dermatophyte group (including genus *Trichophyton*) encodes the same set of secreted proteases and the protein sequence of each orthologue is highly conserved. The difference between species is in their levels of secretion.[11] *Trichophyton rubrum* was shown to secret also leucine aminopeptidases and dipeptidyl-peptidases.[13] The proteolytic activity of these proteases is very likely to be endogenously regulated by specific inhibitors, produced by the fungi which might therefore be considered to have regulatory functions in the cells. That is why protease – protease inhibitor interaction is likely to be important in the process of invasion of the host cells.[10]

Cysteine proteases are one of seven classes of proteolytic enzymes and have a common catalytic mechanism – they all utilize a cysteine (Cys) residue as the nucleophile and a histidine (His) residue as the general base for proton shuttling. They are widely distributed and can be found in a variety of organisms, including viruses, eubacteria, fungi, animal and plant cells as well as humans.[14,15,16,17,18]

Protease inhibitors are in general important tools for regulating proteolytic activity of their target proteases, for blocking these in emergency cases, for signalling receptor interactions or clearance.[19] They can be separated into two general categories based upon their spectrum of activity: the non-specific and the class-specific inhibitors.[20] Inhibitors can act both intra- and extracellularly, forming complexes with their target cysteine proteases in order to maintain appropriate equilibrium of free enzymes and their complexes. When imbalances occur, cysteine proteases can be either under-activated or over-activated and the proper functioning of the organism is impaired. Since cysteine proteases are involved in so many pathological processes ranging from cardiovascular, inflammatory, neurological, respiratory, viral, musculoskeletal, immunological and CNS disorders or even cancer, the inhibitors are a prime target as a therapeutic tool.[17] Classification, nomenclature and other comprehensive information about all known proteases and their inhibitors can be found in the MEROPS database (http://merops.sanger.ac.uk/).[21]

To the best of our knowledge, no protein protease inhibitor of any class has so far been isolated from *Trichophyton mentagrophytes*. In the present paper the purification and properties of the first cisteine protease inhibitor isolated from this dermatophyte species, is reported.

2. Experimental

2. 1. Fungal Material

Isolates of a single strain of *T. mentagrophytes* were acquired and cultured at the Institute of Microbiology and Parasitology of the Veterinary Faculty, University of Ljubljana, Slovenia. The fungus was isolated during routine diagnostic procedures from hair of rabbits, infected with *T. mentagrophytes* after an outbreak on a farm. Primary isolation was performed on solid medium (Dermasel agar, Oxoid LTD) by incubating for two to three weeks at 26 °C. *T. mentagrophytes* colonies (1 cm^2) grown on solid medium were inoculated into a liquid medium (1 L) (Oxoid LTD) and cultivated for ten days at 26 °C. Mycelia were harvested, disintegrated and stored at –20 or –70 °C until use.

2. 2. Purification of Dermatophyte Cysteine Protease Inhibitor

A three-step procedure was developed for the purification of *T. mentagrophytes* inhibitor: papain-affinity chromatography, followed by ion-exchange and reverse phase high performance liquid chromatography.

Fungal mycelia were treated by several freezing and thawing cycles in liquid nitrogen and mechanically and ultrasonically disintegrated in 50 mM Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl (buffer 1). Fungal homogenate was centrifuged for 20 minutes at 10000 g and for 1 hour at 25000 g to remove cell debris (ultracentrifuge Beckman, Avanti J-301). pH of the supernatant was adjusted to 7.4 and applied on a column of carboxymethylpapain-Sepharose (Amersham Pharmacia Biotech) prepared according to the manufacturer's instructions. The column (2.5 × 9 cm) was equilibrated with buffer 1. Bound proteins were eluted with 10 mM NaOH, pooled and neutralized with dilute HCl. Fractions were tested for inhibitory activity against papain. Those with at least 75% inhibition were pooled and vacuum concentrated (Speed Vac, Savant).

The concentrate was then loaded onto CIM QA disk monolithic column (Bia Separations) equilibrated with 20 mM Tris/HCl buffer, pH 8.0. The absorbance was monitored at 280 nm and proteins were eluted using NaCl in Tris/HCl buffer (pH 8.0) in a linear gradient from 0 to 1 M, at a flow rate of 1.0 mL min^{-1}. Again, inhibitory fractions were vacuum concentrated (Speed Vac, Savant) and finally purified on an RP-HPLC.

The samples were applied onto Brownlee Aquapore Bu-300 column (30 × 4.6 mm) equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA). The column was eluted with a 30 minute linear gradient from 0–80% (v/v) of 0.1% (v/v) TFA in acetonitrile at a flow rate of 1.0 mL min^{-1}. Elution was monitored by absorbance at 215 nm.

2. 3. SDS-PAGE

SDS-PAGE with the method of Laemli was performed to monitor protein purity and determine approximate molecular mass of the isolated protein inhibitor using a 12% resolving gel on a mini-Protean II Slab cell apparatus (Bio-Rad). Prior to loading the samples onto the gel, concentrated inhibitory fractions were mixed (1:1) with SDS

Laemli buffer (100 mM Tris/HCl buffer, pH 6.8; 4% (w/v) SDS; 20% (v/v) glycerol; 20% (v/v) 2-mercaptoethanol; 0,025% (w/v) bromophenol blue) and boiled for 5 minutes. The samples were visualized with Coomassie brilliant blue R-350 or silver staining. Molecular masses were determined using molecular mass standards of 11–170 kDa (Fermentas).

2. 4. Western Blot Analysis

The separated protein samples were transferred from the SDS-PAGE gels to polyvinylidene difluoride membranes (Immobilon-P, Millipore) using the same apparatus as above. Blots were incubated overnight at 4 °C in a blocking solution containing 2% (w/v) Tween 20 (Sigma). After washing the membranes three times with 0.05% (w/v) Tween, they were incubated for 1.5 hours with serum of a commercial breed of rabbits, naturally infected with *T. mentagrophytes* (positive serum, diluted 1:200). Sera of 10 different rabbits were used. The clinical diagnosis of infected rabbits was confirmed by a microscopic examination of rabbit's hair and isolation of *T. mentagrophytes* on agar plates (Dermasel, Oxoid) after two weeks of incubation. Sera of conventional New Zeland white rabbits (Harlan) were used as a negative control (negative serum, diluted 1:200). After the first antibody was washed away, blots were incubated with secondary goat anti-rabbit IgG conjugated with horseradish peroxidase, diluted 1:750 (Jackson ImmunoResearch). Antigen – antibody complexes were made visible with 3-amino-9-ethylcarbazole (Sigma).

2. 5. Protein Sequence Analysis

The isolated protein inhibitor from *T. mentagrophytes* was enzymatically cleaved with β-trypsin, chymotrypsin, or *Staphylococcus aureus* protease V8. Hydrolysis of the inhibitor with β-trypsin was carried out at 37 °C in 0.1 M N-methylmorpholine, pH 8.2 for 2 hours and the enzyme was added every half hour. Proteolytic digestion with chymotrypsin was performed in 75 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1.5 M NaCl, pH 8.2 at 37 °C and the enzyme was added every half hour for 1.5 hours. The inhibitor was also cleaved with protease V8 in 0.1 M sodium phosphate buffer, 4 M urea, pH 7.8 for 24 hours at 37 °C. All three enzymatic cleavages were performed in final reaction volume of 100 μL and reactions were stopped by adding 500 μL of 0.1% (v/v) TFA. Deblocking of N-terminal residue in TFA/methanol was performed as described in Gheorghe.[22] Samples were incubated at room temperature for three days.

Hydrolisates were analysed with RP-HPLC on C(18) Chrompack column (100 × 3 mm) equilibrated with 0.1% (v/v) TFA in water. Peptides were eluted with a linear gradient of 0–60% (v/v) acetonitrile in water and 0.1% (v/v) TFA over 40 minutes at a flow rate of 1.0 mL min^{-1}.

The N-terminal sequences of the obtained peptides were determined using the Procise 492A Automated Sequencing System (Applied Biosystems).

2. 6. Assays of Inhibitory Activity

The inhibitory activities of samples and fractions during the isolation procedures were determined using a modified method of Barret, with papain as the enzyme in the assay.[23] Twenty μL of inhibitory fraction were preincubated with 60 μL of mixture of papain (0.2 μg in final solution, Sigma) and cysteine (final concentration 1.0 mM, Sigma) in 0.1 M sodium phosphate buffer with 1.5 mM EDTA, pH 6.0, in a 96-well microtitre plate (Costar) at room temperature for 10 minutes. The reaction was initiated by adding 20 μL of 6.0 mM BANA (N-α-benzoyl-D,L-arginine-β-naphthylamide, Sigma) in DMSO (Dimethyl sulfoxide, Merck) and the mixture was incubated at 37 °C for 10 minutes. Then 100 μL of 1:1 (v/v) mix of Fast Garnet GBC dissolved in 4% Brij 35 (Polyoxyethylene 23 Lauryl Ether) and 10 mM 4-chloromercuriobenzoic acid with 50 mM EDTA, pH 6.0 (all chemicals were from Sigma) were added. Absorbance was measured at 492 nm with a microwell reader (Rosys Anthos reader 2010, Anthos Labtec Instruments Ges. M. b. H.). All assays were made in duplicates and an established protease inhibitor, egg white cystatin was used as a control.

Inhibitory activities of purified inhibitors were also determined against human cathepsins B, L (Sigma) and H (Calbiochem). For cathepsin B (from bovine spleen) inhibition, 350 μL of purified inhibitor was preincubated for 5 minutes at 37 °C with the enzyme (concentration 100 μg/mL of 0.05 M phosphate buffer with 1.0 mM EDTA, pH 5.5) and 5 mM cysteine (Sigma). 100 μL of Z-Phe-Arg-PNA·HCl (Z-Phe-Arg p-nitroanilide, 2 mg/mL, Bachem) were added and the mix was incubated for 10 minutes at 37 °C. After the reaction was stopped with 2.0 mL of 1.2 mM iodoacetic acid (Sigma), the absorbance was read at 410 nm with a spectrophotometer (Lambda 12, Perkin Elmer).

For the determination of inhibition of cathepsin L (from human liver), 20 μL of purified inhibitor was incubated for 10 minutes at room temperature with 12 μL of enzyme (0.07 μg/μL) in 0.1 M acetate buffer with 1.0 mM EDTA, pH 5.5, and 8 μL of 17.5 mM cysteine. 10 μL of 217 μM Z-Phe-Arg-PNA·HCl (Sigma) were added. After 10 minutes of incubation at 37 °C, the reaction was stopped with 100 μL of 1.2 mM iodoacetic acid and the absorbance was measured with a microwell reader at 405 nm (Rosys Anthos reader 2010, Anthos Labtec Instruments Ges. M. b. H).

For determining the inhibitory activity against cathepsin H (from human liver), 20 μL of purified inhibitor was incubated for 10 minutes at 37 °C with 3 μL of enzyme (0.46 mg/mL) in 0.1 M phosphate buffer with 1.0 mM EDTA, pH 6.8 and 10 mM cysteine. As a substrate, 10 μL

of 27 mM H-Arg-PNA (H-Arg p-nitroanilide, Bachem) in methanol (Merck) was used. After 20 minutes of incubation at 37 °C the reaction was stopped with 1.2 mM iodoacetic acid and absorbance read at 405 nm with a microwell reader (Rosys Anthos reader 2010, Anthos Labtec Instruments Ges. M. b. H).

Isolated inhibitors were also tested against serine protease trypsin with a modified method of Erlanger [24]. Mixture of 200 μL of purified inhibitor and 50 μL of enzyme (143 μg/mL in 0.1 M acetic acid) was incubated for 10 minutes at room temperature. After the adition of 750 μL of 1.0 mM BAPNA (Nα-Benzoyl-DL-arginine p-nitroanilide; Sigma) in DMSO (Merck) and incubation at 37 °C for 10 minutes, the reaction was stopped using 0.2 M HCl. Absorbance was measured at 410 nm with a spectrophotometer (Lambda 12, Perkin Elmer).

The inhibitory activity was defined as follows. The activity of non-inhibited enzyme (incubated without any inhibitors) was taken as 100% activity. The activity of partially inhibited enzyme (incubated with the inhibitory fractions) was expressed in percent of residual activity compared to the uninhibited control.

3. Results and Discussion

3. 1. Isolation and Characterisation of the Fungal Inhibitor

Cysteine protease inhibitors have been found in various mammalian tissues and body fluids, as well as in other animals (insects, fishes), parasitic protozoa, plants, viruses and bacteria.[20,25,26,27,28,29] There are reports on mainly small molecular mass inhibitors of cysteine proteases from fungi.[8,30,31,32,33,34] Fungal protein inhibitors have so far been found in one of the most common pathogenic fungi *Candida albicans* and in the fruit bodies of edible mushrooms *Clitocybe nebularis* and recently in *Macrolepiota procera*.[10,35,36] In the present study, a protein inhibitor of cysteine proteases from a dermatophyte fungus *T. mentagrophytes* was purified and partially characterized. The inhibitor was found intracellularly but the inhibition was detected extracellularly in the growth medium as well (data not shown). Whether this inhibition was due to the same protein inhibitor is still to be determined.

After culturing the dermatophyte fungus *T. mentagrophytes* in Sabouraud dextrose liquid medium and mechanical disruption of the mycelial mass, the first step in the isolation of the cysteine protease inhibitor was affinity chromatography on immobilized papain. Bound inhibitory proteins were eluted and fractions showing at least 75% inhibition were pooled and concentrated. Since the eluate was not homogeneous (Figure 3, lane 2) it was subjected to anion-exchange chromatography which yielded a single major protein peak with inhibitory activity (PI, Figure 1).

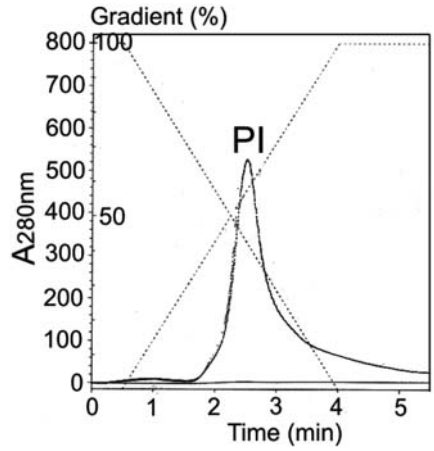

Figure 1: Anion-exchange chromatography of the *T. mentagrophytes* cysteine protease inhibitor (PI). Inhibitory fraction of *T. mentagrophytes* was applied on CIM QA disk monolithic column in Tris/HCl buffer, pH 8.0 and thoroughly washed. Retained proteins were eluted at a flow rate of 1.0 mL min^{-1} using a linear gradient of NaCl from 0 to 1.0 M (dashed line, 0–100%).

For further purification, the fraction was loaded onto an RP-HPLC column where two peaks with inhibitory activity were obtained (Figure 2).

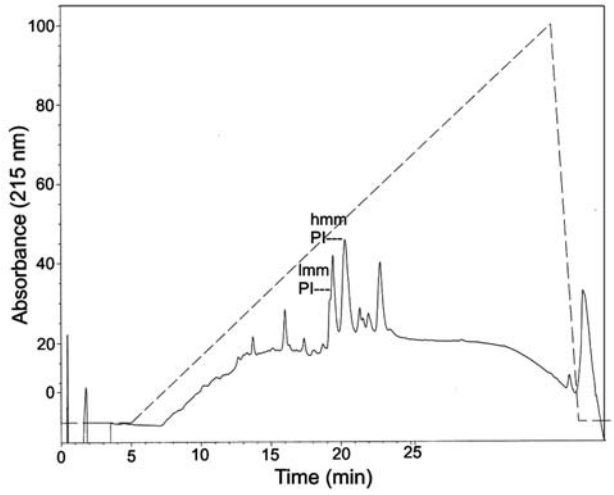

Figure 2: Reverse phase HPLC elution profile of the *T. mentagrophytes* cysteine protease inhibitor. The inhibitor of *T. mentagrophytes* was applied on the Brownlee Aquapore BU-300 column (30 × 4.6 mm) and eluted with a linear gradient of 0–80% (v/v) acetonitrile in water and 0.1% (v/v) TFA (dashed line) over 30 min at a flow rate of 1.0 mL min^{-1}. lmm PI and hmm PI designate low molecular mass and high molecular mass forms of the fungal inhibitor.

Again, peaks were collected and tested for inhibitory activity. After each chromatographic step, the fractions were also analysed by SDS-PAGE (Figure 3). The protein peak after ion-exchange chromatography showed two bands – lower at 12 and higher at 24 kDa (Figure 3,

lane 3). The two peaks with inhibitory activity against papain obtained after RP-HPLC separation again migrated in SDS-PAGE relative to apparent molecular mass of 12 (lmm PI) (Figure 3, lane 4) and 24 kDa (hmm PI) (Figure 3, lane 5).

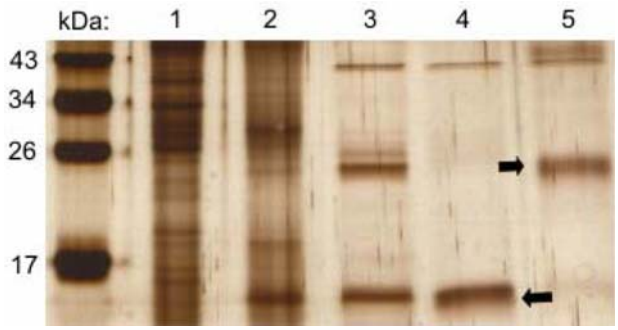

Figure 3: SDS-PAGE analysis of various *T. mentagrophytes* cysteine protease inhibitor purification steps. Whole *T. mentagrophytes* homogenate (lane 1); cysteine protease inhibitory fraction after affinity chromatography (lane 2); the inhibitor after ion-exchange chromatography (lane 3); low molecular mass form of inhibitor after RP-HPLC (lane 4) and high molecular mass form of inhibitor after RP-HPLC (lane 5). SDS-PAGE was performed on 12% polyacrylamide gel under denaturing conditions. The gel was silver stained.

The RP-HPLC and molecular mass analyses suggested that the *T. mentagrophytes* inhibitor can exist in a low molecular and a high molecular mass form, similar to what has been described for clitocypin and macrocypins.[35,36,37] The suggestion of a low and a high molecular mass form of the *T. mentagrophytes* cysteine protease inhibitor is supported by evidence of enzymatic digestions of a 12 and 24 kDa form of the inhibitor, prior to sequencing, which gave identical cleavage products after RP-HPLC (the data not shown). Whether the smaller and larger forms of the inhibitor are two differently folded proteins or in fact two or more monomeric units polimerising together, remains to be determined. Regarding the molecular mass of the studied protein, we can again draw some parallels with fungal clitocypin and macrocypins. They all have similar molecular masses of 16.8, 19 and 12 kDa respectively.[35,36,37]

3. 2. Western Blot Analysis

Dermatophyte colonization is characteristically limited to the dead keratinized tissue of the *stratum corneum* and results in either a mild or intense inflammatory reaction. Although the cornified layers of the skin lack a specific immune system, both humoral and cell-mediated reactions and specific and non-specific host defence mechanisms respond and eliminate the fungus.[2] The humoral immune response in rabbits infected with *T. mentagrophytes* has already been investigated using ELISA and its immunogenic proteins were identified by Western immunoblotting.[38,39,40,41] The same method was used to determine whether the sera of rabbits naturally infected with *T. mentagrophytes* contain antibodies against the isolated fungal inhibitor that was blotted onto a membrane (Figure 4). The results show that no reaction was observed around 12 kDa – that is in the region where low molecular mass form of the inhibitor is expected. At molecular mass of the expected high molecular mass form of *T. mentagrophytes* inhibitor (24 kDa), the overlapping cross-reactivity with *T. mentagrophytes* inhibitor antigen-like domains is possible, since there were bands detected with positive as well as negative rabbit's sera. Specific reactivity, on the other hand, can be contributed to even higher molecular mass forms of the inhibitor, since the positive reaction appears between 55 and 72 kDa only with the sera of *T. mentagrophytes* infected rabbits. It is possible that during the natural infection with dermatophyte *T. mentagrophytes* the high molecular mass forms of the fungal inhibitor are predominant. This would explain why we did not notice antibodies against the low molecular mass form of the inhibitor. To determine the immunogenic properties of the *T. mentagrophytes* inhibitor, the animals would have to be immunized with pure forms of inhibitors and the Western blot analyses repeated.

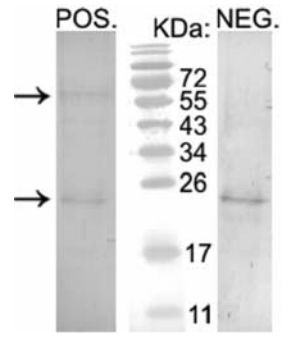

Figure 4: Western blot of isolated cysteine protease inhibitor of *T. mentagrophytes* with the serum of rabbits, infected with *T. mentagrophytes* (POS. – positive serum) and serum of non-infected rabbits (NEG. – negative serum). Representative results of Western blotting are given.

3. 3. Inhibition Assay

After each chromatographic step, the inhibitory activity of collected fractions was determined using papain as the standard enzyme in the assay. Inhibitory activities of purified proteins were determined against cysteine cathepsins B, L and H and also against serine protease trypsin. The *T. mentagrophytes* inhibitor was found to inhibit papain, cathepsins B and L but it had no effect on cathepsin H and serine protease trypsin (Table 1).

The profile of inhibition of the *T. mentagrophytes* inhibitor is similar to clitocypin – they both inhibit papain, cathepsin B and L, but not cathepsin H.[35] Macrocypins have similar but not identical inhibitory profiles. Recombi-

Table 1: Summary of the inhibition of a protein inhibitor from *T. mentagrophytes*.

ENZYME	SUBSTRATE	INHIBITION
papain	BANA	yes
cathepsin B	Z-Phe-Arg-PNA	yes
cathepsin L	Z-Phe-Arg-PNA	yes
cathepsin H	H-Arg-PNA	no
trypsin	BAPNA	no

nant macrocypins 1 and 3 were found to be effective inhibitors of papain and cathepsin L. Recombinant macrocypin 4 is also an effective inhibitor of papain, but on the other hand exhibited weaker inhibition of cathepsin L. Cathepsin B was inhibited in the micromolar range with all macrocypins, except recombinant macrocypin 3. Cathepsin H was inhibited by natural macrocypin and recombinant macrocypin 1 in the micromolar range, recombinant macrocypins 3 and 4 showed 10–fold lower values of inhibition.[36] The ability of the *T. mentagrophytes* inhibitor to inhibit proteases of other catalytic classes was also examined. The *T. mentagrophytes* inhibitor did not inhibit serine protease trypsin as was described for clitocypin and all but one of recombinant macrocypins. Interestingly, we found that *T. mentagrophytes* also produces very potent peptide inhibitors of cysteine proteases that can be found intracellularly and in the culturing medium. They appear to have identical inhibitory spectrum against selected cysteine proteases (unpublished data).

3. 4. Determination of Partial Amino Acid Sequences

Putative low and high molecular mass forms of the fungal inhibitor obtained after RP-HPLC were subjected to Edman degradation, but no signals were obtained, suggesting the N-terminal blockade. Both forms of the *T. mentagrophytes* inhibitor were then hydrolyzed enzymatically with β-trypsin, chymotrypsin and V8 and with mild acid hydrolysis in methanol/TFA. Obtained peptides were separated and N-terminally sequenced (Figure 5, Table 2).

The *T. mentagrophytes* inhibitor was found to be N-terminally blocked and very resistant to degradation with proteases. The cleavage with trypsin, chymotrypsin and V8 protease gave only a few peptides. The N-terminal blockade could not be removed with a high yield even after three-day incubation in methanol/TFA. Therefore, as a result of Edman degradation, only partial internal amino acid sequences were obtained.

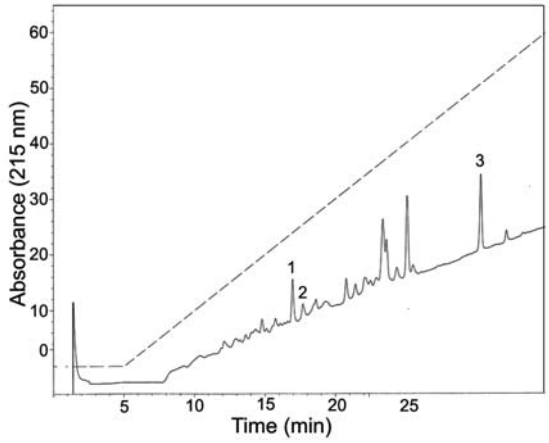

Figure 5: RP-HPLC of peptides obtained after digestion of the isolated *T. mentagrophytes* inhibitor with chymotrypsin. Analysis proceeded on a Chrompack C18 column (100 × 3 mm). The elution of peptides was achieved with a linear gradient of 0–80% (v/v) acetonitrile in water and 0.1% (v/v) TFA (dashed line) over 40 min at a flow rate of 1.0 mL min^{-1}. Peak 1 yielded a 13 amino acid sequence, peak 2 yielded an 8 amino acid sequence. Peak 3 corresponded to the N-terminally blocked inhibitor.

To investigate the possible homology of the isolated fungal inhibitor with other known cysteine protease inhibitors or proteins, the determined sequences were compared to non-redundant protein sequence databases using the blastp (protein – protein) algorithm at NCBI. Since no significant sequence homologies have so far been found between the obtained peptides and any known cysteine protease inhibitors, we can speculate that the *T. mentagrophytes* inhibitor has some structurally unique characteristics. This is not surprising since even the alignment of fungal inhibitor sequences of macrocypins with clitocypin showed only 17–21 % sequence identity.[36] For more detailed analyses the cDNA sequence of *T. mentagrophytes* inhibitor needs to be elucidated.

4. Conclusions

The *T. mentagrophytes* inhibitor exhibits some similarities in basic biochemical characteristics and inhibitory

Table 2: Peptide sequences obtained from *T. mentagrophytes* cysteine protease inhibitor after enzymatic hydrolysis with chymotrypsin and V8 or acid hydrolysis in methanol and TFA. X = unidentified amino acid residue.

Digestion	1	2	3	4	5	6	7	8	9	10	11	12	13
Chymotrypsin Peptide 1	V	N	E	N	A	P	T	V	Q	P	G	V	R
Chymotrypsin Peptide 2	V	V	T	H	F	Q	L	V					
V8	P	I	R	A	R	G	X	I	E	G	R		
MeOH/TFA	S	V	A	V	K	T	Y	L	V				

profiles with clitocypin from *Clitocybe nebularis* and macrocypins from *Macrolepiota procera*. However, for its classification into the same MEROPS family of fungal inhibitors (e.g. I48) additional structural analyses are needed. Also, a physiological target of the *T. mentagrophytes* inhibitor has not yet been found. Besides the regulatory role in the fungal endogenous proteolysis, its role may be expected in protection against host's immune cells that are responsible for elimination of the fungus from colonized skin.

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Povzetek

V prispevku opisujemo delno karakterizacijo proteinskega inhibitorja cisteinskih proteaz, izoliranega iz pomembne zoofilne vrste dermatofitnih gliv *Trichophyton mentagrophytes* (*T. mentagrophytes*). Pri izolaciji smo uporabili metodo afinitetne in ionsko – izmenjevalne kromatografije ter tekočinske kromatografije z visoko ločljivostjo na reverzni fazi. Izolirani glivni inhibitor se najverjetneje pojavlja v nizkomolekularni (12 kDa) in visokomolekularni obliki (24 kDa). Inhibira proteolitično aktivnost papaina, katepsina B in L, ne pa tudi katepsina H ali tripsina. Iz rezultatatov prenosa proteinov na membrano je razvidno, da serumi kuncev, naravno okuženih z glivo *T. mentagrophytes*, vsebujejo protitelesa le proti visokomolekularni obliki inhibitorja. Ker do sedaj nismo našli podobnosti v aminokislinskem zaporedju med pridobljenimi delnimi proteinskimi zaporedji inhibitorja in zaporedji drugih inhibitorjev cisteinskih proteaz, sklepamo, da ima inhibitor *T. mentagrophytes* določene strukturne posebnosti. Inhibitor iz *T. mentagrophytes* je v določenih biokemijskih značilnostih (molekulska masa, nizko- in visokomolekularna oblika, profil inhibicije) podoben clitocipinu iz glive *Clitocybe nebularis* in macrocipinom iz glive *Macrolepiota procera*.