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Purification and characterization of a 43·5 kDa keratinolytic metalloprotease from *Microsporum canis*

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A keratinolytic protease secreted by a feline clinical isolate of *Microsporum canis* cultivated in a broth containing feline keratin as the sole nitrogen source was purified from the culture filtrate by affinity chromatography on bacitracin-agarose and by hydrophobic chromatography on octyl-agarose. The enzyme had an apparent molecular mass of 43·5 kDa and the pI was 7·7. It had a significant activity against keratin azure, elastin-Congo red and denatured type I collagen (azocoll). Using the latter substrate, the optimum pH was around 8 and the apparent optimum temperature around 50 °C. The protease was strongly inhibited by 1,10-phenanthroline, phosphoramidon and EDTA. The first 13 N-terminal amino acid sequence showed a 61% homology with that of the extracellular metalloprotease of *Aspergillus fumigatus* and with the neutral protease I of *A. oryzae*, confirming that this 43·5 kDa keratinase is a metalloprotease. This keratinolytic metalloprotease could be a virulence-related factor involved in pathophysiological mechanisms of *M. canis* dermatophytosis.

Keywords keratinase, metalloprotease, *Microsporum canis*

Introduction

The dermatophytes have the capacity to invade the keratinized skin layers, hair and nails, causing a cutaneous mycosis called dermatophytosis [1]. The main causative agent of dermatophytosis in cats and dogs is *Microsporum canis* [2]. This zoophilic dermatophyte causes a human infection that is on the increase in many European countries [3] and occurs mainly by direct contact with infected animals [4].

Pathophysiological mechanisms of dermatophytosis are poorly understood. It has been suggested that keratinases might be virulence-related factors, giving the ability to invade keratinized tissues and playing a role in the nutrition of the fungi [5,6] and in controlling host defense mechanisms [7,8]. Therefore, the characterization of keratinases would appear to be a major step towards a better understanding of the pathogenicity of the fungus and the host–fungus relationship. Some dermatophyte keratinases have been isolated from *Trichophyton rubrum* [6,9,10], *T. mentagrophytes* [11–13] and *M. canis* [14–17]. Mignon *et al.* [17] purified and characterized a *M. canis* 31·5 kDa keratinolytic subtilisin-like protease secreted *in vitro* in a minimal liquid medium containing cat keratin, and produced *in vivo* in naturally infected cats [18]. Keratinolytic activity was observed in the culture supernatant even in the presence of chymostatin, which completely inhibits the 31·5 kDa keratinolytic subtilisin-like protease [17], suggesting that there was at least one more keratinase belonging to another class of proteases [B. Mignon, unpublished results].

This paper describes the isolation, purification and characterization of a keratinolytic metalloprotease of 43·5 kDa produced by a feline clinical isolate of *M. canis* cultivated in a minimal liquid medium containing feline keratin as the sole nitrogen source.

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Material and methods

Strain and culture conditions

M. canis strain IHEM 15221 (Brussels, Belgium) was obtained from a hair-coat brushing of an infected cat. After propagation carried out at 27 °C on Sabouraud liquid medium containing chloramphenicol (0·05%) in 150 cm^2 culture flasks, the fungus was inoculated into 10 l of a minimal liquid medium containing feline keratin (5·2 g l^{-1}) as the sole nitrogen source, as described by Mignon [17]. The time-course production of the protease was monitored in duplicate cultures regularly sampled between days 4 and 11. Simultaneously, the fungus was grown in the same minimal liquid medium, except that keratin was replaced by peptone as nitrogen source.

Purification of the keratinolytic protease

The extracellular fluid was separated from hair and fungus by filtration through Whatman No. 1 filter paper and concentrated 357-fold by ultrafiltration using a Prepscale system (Millipore, Bedford, MA, USA), with a 10 kDa membrane cut-off. The filtrate was applied onto a bacitracin-agarose [19] column (16·5 × 1 cm) previously equilibrated in Tris buffer (50 mM Tris-HCl, 2 mM $CaCl_2$, pH 7·5). Elution was performed with a linear gradient of NaCl (65–65 ml, 1·5 M NaCl) at a flow rate of 18 ml h^{-1}. Fractions (2·7 ml) of the first peak with proteolytic activity were pooled and dialyzed against ammonium sulfate buffer (50 mM Tris-HCl, 2 mM $CaCl_2$, 0·5 M $(NH_4)_2SO_4$, pH 7·5). The solution was further loaded onto an octyl-agarose (Amersham Pharmacia Biotech, Uppsala, Sweden) column (10 × 1·5 cm) previously equilibrated with the same buffer. The bound protein was eluted with a linear decreasing gradient of ammonium sulfate (40–40 ml, 0 M $(NH_4)_2SO_4$) at a flow rate of 90 ml h^{-1} and fractions of 3·7 ml were collected. Purification was monitored by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [20] and azocoll degradation (see below). Protein concentrations were determined using the Pierce protein assay kit (Pierce, Rockford, IL, USA), based on the Bradford method [21]. All purification procedures were carried out at 4 °C.

Electrophoresis

SDS-PAGE was performed using the method of Laemmli [20] with a 12% resolving gel under reducing conditions. Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA). Molecular mass markers were the low range standards from Bio-Rad Laboratories.

Isoelectrofocusing was run in an immobilized 3–10 linear pH gradient (Immobiline DryStrip, Amersham Pharmacia), essentially as described by the supplier.

Protein N-terminal sequencing

The purified protease was subjected to SDS-PAGE and electroblotted to an Immobilon membrane (Millipore) according to standard protocols [22]. After staining, the band was cut and subjected to N-terminal sequencing on a Perkin Elmer sequencer type Procise (Applied Biosystems, Foster City, CA, USA). Sequence alignment search was performed using the BLASTP [23] and FASTA3 [24] methods.

Proteolytic assays

Standard assay of proteolytic activity was carried out using azocoll (Sigma, St Louis, MO, USA) as substrate in 50 mM Tris-HCl, 2 mM $CaCl_2$, pH 7·5 buffer. From a suitably diluted protease solution, 1 ml was incubated with 10 mg of substrate at 37 °C for 30 min under continuous shaking. The degradation of the substrate was estimated by measuring the absorbance at 520 nm after centrifugation. One unit of collagenolytic activity released 0·001 A_{520} units min^{-1} under the assay conditions.

Keratinolytic activity was measured using keratin azure (Sigma) as previously described [17]. Briefly, samples (1 ml) were incubated with keratin azure (5 mg) at 37 °C for 24 h in Tris buffer. One unit of keratinolytic activity was fixed as an increase of 0·001 A_{595} units h^{-1} under the assay conditions.

Elastin degradation was determined using elastin-Congo red (Sigma) as substrate. Samples (1 ml) were incubated with the substrate (10 mg) at 37 °C for 2 h in Tris buffer. The reaction was stopped by removing the substrate by centrifugation. A change of 0·001 A_{495} units min^{-1} corresponded to 1 unit of elastinolytic activity.

All proteolytic assays were run at least in triplicate, except for the standard assay on the fractions collected during the chromatographic steps.

pH and temperature optima and stability

The thermodependence of the activity and stability of the purified enzyme was determined using the standard assay as previously described. For determination of the apparent optimum temperature, the reaction was carried out at temperatures ranging 25–75 °C. The effect of temperature on the enzyme stability was investigated by measuring the residual activities after a 15-min to 2-h incubation of the protease solution at 25, 37 and 50 °C. The determination of the optimum pH was made using

the standard assay with the following buffers: 50 mM sodium acetate (NaAc), 2 mM $CaCl_2$, pH 3·8–7·2; 50 mM Tris-HCl, 2 mM $CaCl_2$, pH 7·4–9·1; and 100 mM glycine-NaOH, 2 mM $CaCl_2$, pH 9–12. The pH stability was evaluated using the standard assay and a pre-incubation of the enzyme in sodium acetate buffer at pH 4·6, in Tris buffer at pH 7·5 and in glycine-NaOH buffer at pH 11·2, for times ranging from 20 min to 6 h.

Inhibition assays

The inhibitors ethylenediamine-tetraacetic acid (EDTA), phosphoramidon, soybean trypsin inhibitor (SBTI), elastatinal and iodoacetamide were dissolved in water. 1,10-Phenanthroline, phenylmethylsulfonyl fluoride (PMSF) and pepstatin were dissolved in methanol. Chymostatin was dissolved in dimethyl sulfoxide (DMSO). All inhibitors were from Sigma except for SBTI from Fluka (Buchs, Switzerland). Each compound was pre-incubated with the purified enzyme for 20 min at 25 °C and tested for activity using the standard assay. An appropriate control, without inhibitor, was assayed simultaneously.

Results

Enzyme production

The enzyme production on minimal medium containing feline keratin was followed as a function of time (Fig. 1). Proteolytic activities using azocoll, keratin azure and elastin-Congo red were detected after several days. Keratinolytic and collagenolytic activities reached a maximum around day 8 and then decreased. Elastinolytic activity reached a maximum around day 11. In the medium with peptone instead of keratin, no proteolytic activity was detected (data not shown).

Purification of the protease

The results of the purification processes are summarized in Table 1 and Figure 2. Two major proteases, bound to bacitracin-agarose (Fig. 3a), were partially separated with the elution gradient (Fig. 2). Both peaks showed proteolytic activity. The second peak mainly contained the 31·5 kDa keratinolytic subtilisin-like protease previously purified by Mignon *et al.* [17] (Fig. 3a). Fractions of the first peak were pooled for the subsequent purification procedure. This first chromatographic step allowed a 54% recovery of the protease of interest with a 14-fold purification (Table 1). At this stage, SDS-PAGE revealed two contaminating bands of approximately 20 and 30 kDa (Fig. 2). After hydrophobic chromatography on octyl-agarose (Fig. 3b), SDS-PAGE revealed a single band with no significant contamination (Fig. 2). Contaminants were found in the flow-through (data not shown). The entire purification procedure resulted in a 75-fold purification of the protease from the concentrated supernatant, with a 42% yield (Table 1).

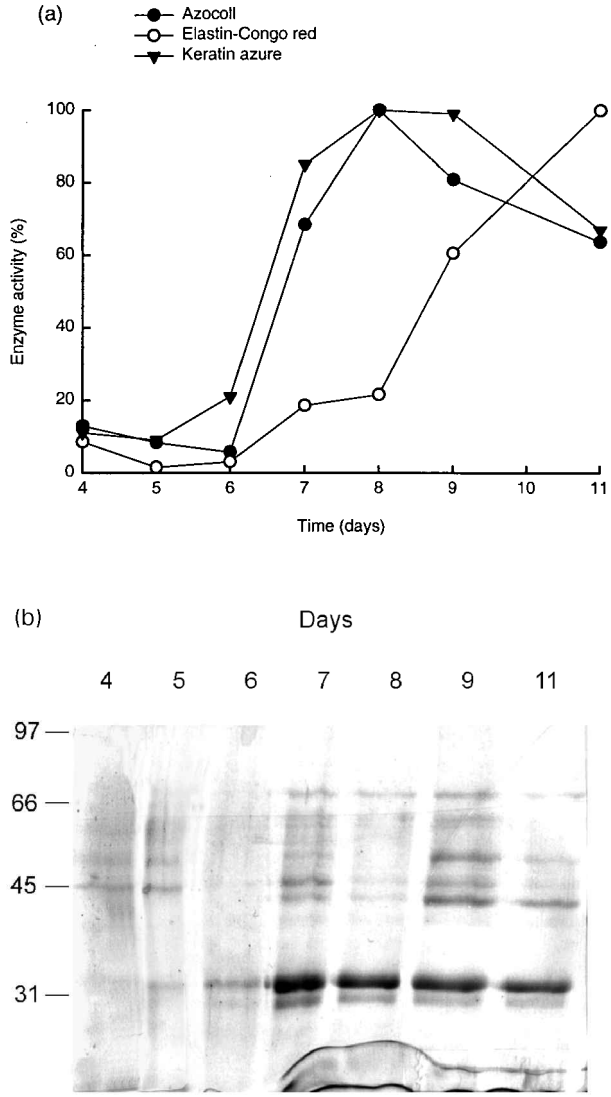

Fig. 1 Kinetics of extracellular enzyme production by *M. canis* IHEM 15221 grown in a minimal medium containing cat keratin. (a) Time course study of collagenolytic, elastinolytic and keratinolytic activities of *M. canis* culture filtrate. The maximum proteolytic activities of the culture supernatant were set at 100%. (b) Coomassie blue-stained SDS-PAGE (12% gel) temporal pattern of *M. canis* culture filtrate. Constant volumes (15 ml) of samples filtered and lyophilized were loaded. Molecular mass standards (in kDa) are shown on the left.

Molecular characterization

SDS-PAGE revealed a single band with an apparent molecular mass of 43·5 kDa (Fig. 2, lane 3). The

Table 1 Purification of the keratinolytic metalloprotease from *M. canis* IHEM 15221

Purification step	Volume (ml)	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Ultrafiltration	28	9·9	3308	332	100	1
Bacitracin-agarose	16	0·4	1800	4 500	54	14
Octyl-agarose	56	0·056	1400	25 000	42	75

Enzymatic activity was performed using the standard proteolytic assay on azocoll.

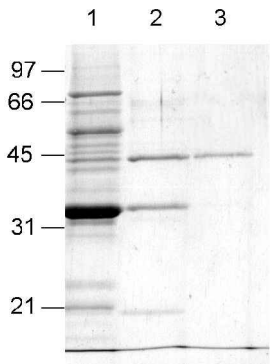

Fig. 2 Coomassie blue-stained SDS-PAGE (12% gel) pattern of the keratinolytic metalloprotease of *M. canis* at each step of purification. Lane 1, ultrafiltered culture supernatant; lane 2, fractions 23–28 from bacitracin-agarose; lane 3, fractions 38–52 from octyl-agarose. Molecular mass standards (in kDa) are shown on the left.

isoelectric point (pI) was 7·7. Partial N-terminal amino acid sequencing (13 residues) disclosed a 61% homology (Fig. 4) with a metalloprotease from *Aspergillus fumigatus* [25,26] and with the neutral protease I from *A. oryzae* [A. Doumas, unpublished results] which are both metalloproteases.

pH and temperature optima and stability

The protease was found to have an alkaline optimum pH around 8 (Fig. 5a). The residual activity remained above 80% between pH 6 and pH 9. Exposure of the protease to alkaline pH (>11) for less than 1 h resulted in a loss of more than 60% of its activity (Fig. 5b).

The apparent optimum temperature for enzyme activity was around 50 °C. The protease was stable at 25 and 37 °C for 2 h, but lost more than 80% of its activity after 1 h exposure at 50 °C (data not shown).

Inhibition studies

Results are summarized in Table 2. The enzyme was inhibited by metalloprotease inhibitors, such as 1,10-phenanthroline, phosphoramidon and EDTA. With EDTA, inhibition occurred in a dose-dependent manner. Specific cysteine-, aspartate- and serine- protease in-

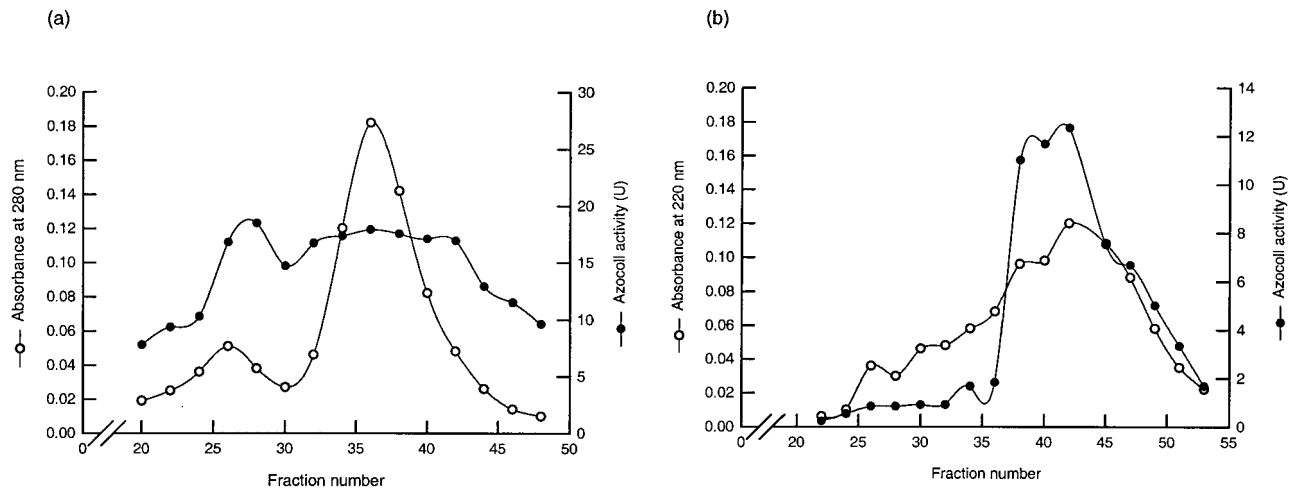

Fig. 3 (a) Chromatographic profiles on bacitracin-agarose. Fractions 23–28 with activity on azocoll were pooled, dialyzed against 50 mM Tris-HCl, 2 mM $CaCl_2$, 0·5 M $(NH_4)_2SO_4$, pH 7·5 and applied onto octyl-agarose. (b) Chromatographic profiles on octyl-agarose. Fractions 38–52 were pooled. Assays on azocoll were performed with an incubation time of 2 h.

1	A	E	F	K	V	F	A	W	G	L	N	D	P
2	A	D	Y	Q	V	Y	A	W	G	I	N	D	P
3	A	D	Y	Q	V	Y	A	W	G	I	N	D	P

Fig. 4 N-terminal amino acid sequence alignment of *M. canis* keratinolytic metalloprotease with other fungal metalloproteases. 1, *M. canis* 43·5 kDa metalloprotease; 2, *A. fumigatus* extracellular metalloprotease [25,26]; 3, *A. oryzae* neutral protease I [A. Doumas, unpublished results]. Boxes indicate sequence identity.

hibitors except PMSF had no or little effect on the enzyme activity. The activity was also strongly inhibited by 2-mercaptoethanol.

Enzymatic activity

The 43·5 kDa protease was active against azocoll, elastin-Congo red and keratin azure. Comparison between proteolytic activities of the 31·5 kDa subtilisin-like protease and the 43·5 kDa protease revealed that the latter was more collagenolytic than the 31·5 kDa subtilisin-like protease. Both proteases had quite similar activity on keratin azure. However, the 31·5 kDa subtilisin-like protease was not elastinolytic under the assay conditions (Table 3).

Discussion

Among the factors that could be implicated in the pathogenicity of *M. canis*, special attention has been paid to proteases [27,28]. In this study, the isolation, purification and characterization of a 43·5 kDa extracellular keratinolytic metalloprotease are reported. The protease was produced by a feline clinical isolate of *M. canis* grown in a minimal liquid medium containing keratin as the sole nitrogen source. Neither collagenolytic nor keratinolytic activities were detected in the medium containing peptone instead of keratin; moreover, SDS-PAGE analysis did not reveal any band corresponding to the 43·5 kDa metalloprotease (results not shown), indicating that the *M. canis* metalloprotease was probably induced by keratin. This result is in agreement with the observations of other authors suggesting that the *in vitro* production of keratinolytic proteases of *M. canis* [14,29,17], *T. rubrum* [5] and *A. fumigatus* [30] might be induced. It also strongly suggests that easily metabolized carbon and nitrogen sources could repress fungal keratinolytic protease secretion *in vitro* [5,10,29,31].

The protease was purified to a quite high, 42%, recovery by affinity chromatography on bacitracin-agarose followed by hydrophobic chromatography on octyl-agarose. The inhibition profile suggested that it was a metalloprotease. The high percentage of homology found at the level of the N-terminal amino acid sequence with two other metalloproteases of filamentous fungi confirmed that hypothesis. Therefore, this is, to our knowledge, the first dermatophyte keratinolytic metalloprotease to be purified and characterized. The enzyme, despite an apparent molecular mass of 43·5 kDa, appears

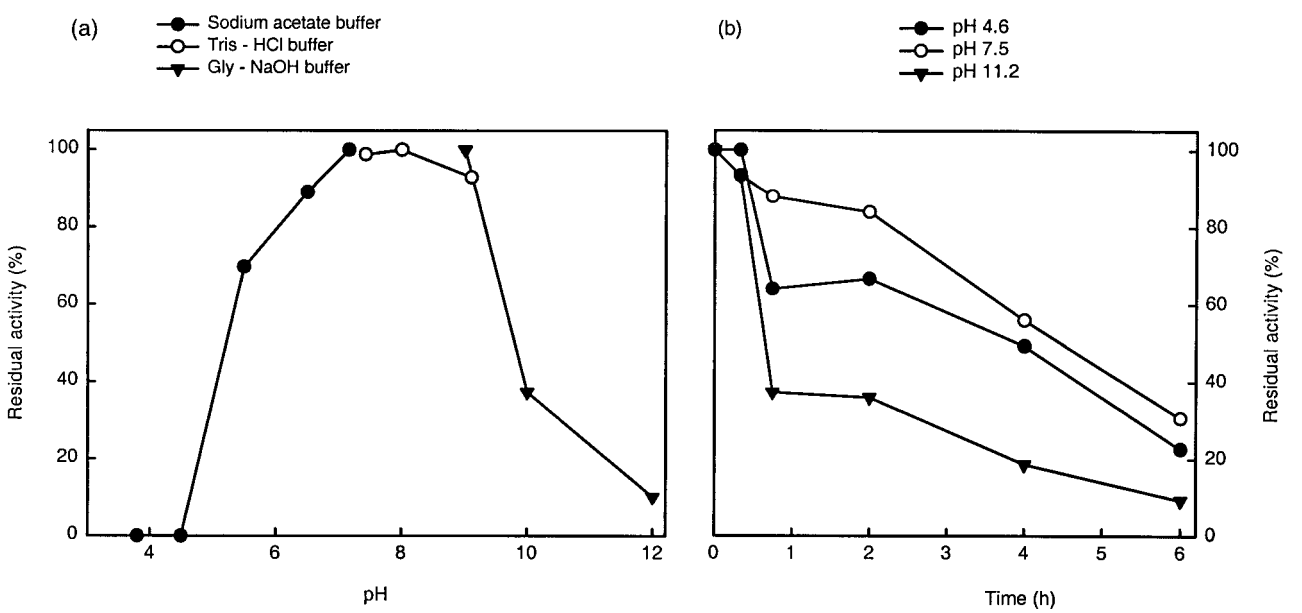

Fig. 5 pH dependence (a) and stability (b) of the activity of *M. canis* keratinolytic metalloprotease. The standard assay with azocoll was performed at 37 °C. Maximal activities were set as 100% relative enzyme activity.

Table 2 Effect of inhibitors on keratinolytic *M. canis* metalloprotease

Reagent	Final concentration	Residual activity (%) on azocoll
1-10 phenanthroline	1 mM	0
Phosphoramidon	10 μg ml^{-1}	0
EDTA	1–10 mM	81–53
PMSF	1–10 mM	92–43
SBTI	50 μM	95
Elastatinal	10 μM	100
Chymostatin	100 μM	100
Iodoacetamide	1 mM	88
Pepstatin	10 μM	100
2-mercaptoethanol	1%	14

to be different from the extracellular 45 kDa keratinase purified previously by Takiuchi *et al*. [14]. Indeed, these authors suggested that the enzyme they purified was a serine protease. Unfortunately, data are lacking about its biochemical characterization so further comparison with the keratinolytic metalloprotease reported here is impossible. Other keratinases have been purified from *M. canis* [16,17], *T. mentagrophytes* [11–13] and *T. rubrum* [9,10]. Most of them seem to belong to the serine protease family. The activity of the *M. canis* 43·5 kDa metalloprotease was optimal at a pH around 8, as is the case for most of the aforementioned keratinolytic proteases.

N-terminal amino acid sequencing of the 43·5 kDa keratinase revealed a 61% homology with metalloproteases from *A. fumigatus* [25,26] and *A. oryzae* [A. Doumas, unpublished results], whereas the 31·5 kDa keratinolytic subtilisin-like protease previously purified [17] disclosed marked homologies with other subtilases from *A. fumigatus* [32], *A. oryzae* [33], *A. nidulans* [34] and *A. flavus* [35]. These homologies between proteases from *M. canis* and other *Onygenales sensu lato* members, some of them opportunistic pathogens, would suggest that these fungi have fundamental similarities in their proteolytic systems even though the proteinases they produce may have adaptive specificity related to the substrates and tissues normally invaded by particular species.

It is interesting that the 43·5 kDa metalloprotease was both more collagenolytic than the 31·5 kDa subtilisin-like protease, and also showed elastinolytic activity while the latter protease did not. Further studies should investigate the possible specific roles of the metalloprotease in subcutaneous infections. Despite the fact that the 31·5 kDa subtilisin-like protease is produced *in vivo* in naturally infected cats [18], it seems that it is not a major antigen in *M. canis* infection in either cats [36] or guinea pigs [37]. However, an immune response to a crude *M. canis* exo-antigen containing the 31·5 kDa subtilisin-like protease has been demonstrated in both animals. Therefore, the 43·5 kDa metalloprotease could be a valuable candidate for additional immunological studies.

This is believed to be the first report which demonstrates that a metalloprotease is keratinolytic *in vitro*. Further investigations will be undertaken to determine if the 43·5 kDa metalloprotease is also secreted *in vivo*, especially in the cat, which is the natural host of *M. canis*. Furthermore, a molecular characterization of the genes encoding keratinolytic proteases of *M. canis* and the production of recombinant proteases could be of great interest for both fundamental studies on pathogenicity and for vaccine purposes.

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Table 3 Comparative collagenolytic and keratinolytic activities of the *M. canis* 43·5 kDa metalloprotease and the 31·5 kDa subtilisin [17]

	Specific collagenolytic activity (U min^{-1} mg^{-1})	Specific keratinolytic activity (U h^{-1} mg^{-1})	Specific elastinolytic activity (U min^{-1} mg^{-1})
Metalloprotease	61 500	27 000	906
Subtilisin	43 300	25 700	0

One specific collagenolytic activity unit = increase of 0·001 A_{520} units min^{-1} mg^{-1}; one specific keratinolytic activity unit = increase of 0·001 A_{595} units h^{-1} mg^{-1}; one specific elastinolytic activity unit = increase of 0·001 A_{495} units min^{-1} mg^{-1}.

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