Veterinary Microbiology 159 (2012) 479–484

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Inhibition of the keratinolytic subtilisin protease Sub3 from *Microsporum canis* by its propeptide (proSub3) and evaluation of the capacity of proSub3 to inhibit fungal adherence to feline epidermis

A. Baldo [a], A. Chevigné [b], M.-E. Dumez [b], A. Mathy [a], P. Power [a,c], J. Tabart [a], L. Cambier [a], M. Galleni [b], B. Mignon [a,*]

[a] Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, B43 Sart Tilman, 4000 Liège, Belgium
[b] Macromolécules Biologiques, Center for Protein Engineering, University of Liège, 4000 Liège, Belgium
[c] Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junin 956 (1113), Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 12 February 2012
Received in revised form 29 April 2012
Accepted 30 April 2012

Keywords:
Dermatophyte
Feline epidermis
Adherence
Secreted subtilisin
Propeptide

ABSTRACT

Microsporum canis is a pathogenic fungus that causes a superficial cutaneous infection called dermatophytosis, mainly in cats, dogs and humans. Proteolytic enzymes have been postulated to be key factors involved in the invasion of the stratum corneum and keratinized epidermal structures. Among these proteases, the secreted subtilisin protease Sub3 was found to be required for adherence of *M. canis* arthroconidia to feline epidermis. This protease is synthetized as a preproenzyme consisting of a signal peptide followed by the propeptide and the protease domain. In order to assess whether the enzymatic activity of Sub3 could be responsible for the role of the protease in the adherence process, we expressed and characterized the propeptide of Sub3 and demonstrated that this propeptide is a strong inhibitor of its mature enzyme. This propeptide acts as a noncompetitive inhibitor with dissociation constants, K_I and K_I' of 170 and 130 nM respectively. When tested for its capacity to inhibit adherence of *M. canis* to feline epidermis using an *ex vivo* adherence model made of feline epidermis, the propeptide does not prevent adherence of *M. canis* arthroconidia because it loses its capacity to inhibit rSub3 following a direct contact with living arthroconidia, presumably through inactivation by fungal membrane-bound proteases.

1. Introduction

Pathogenic dermatophytes are ascomycete fungi able to invade keratinized structures, such as the superficial cornified skin layers, hairs and nails, causing superficial mycoses in humans and animals (Degreef, 2008; Vermout et al., 2008b; Chermette et al., 2008; Kraemer et al., 2012). *Microsporum canis* is the main agent of dermatophytosis in cats, its natural host (Scott et al., 1995), and is responsible for a frequent zoonosis (Ginter-Hanselmayer et al., 2007; Lunder and Lunder, 1992). Although, little is known about the physiopathology of the infection (Vermout et al., 2008b), proteolytic enzymes have been postulated as the key factors involved in the invasion of stratum corneum (Monod, 2008). Among these proteases, a family of five fungalysins (Brouta et al., 2002; Jousson et al., 2004), a family of three subtilisins (Sub) (Descamps et al., 2002), and two dipeptidyl proteases (Vermout et al., 2008a) have been characterized at the molecular level but their role in pathogenicity remains to be evaluated. However, we recently reported the involvement of *M. canis* Subs in adherence to feline corneocytes (Baldo et al., 2008) and

Abbreviations: DTT, dithiothreitol; AMC, 7-amino-4-methylcoumarin.

* Corresponding author. Tel.: +32 43664099; fax: +32 43664097.
E-mail address: bmignon@ulg.ac.be (B. Mignon).

0378-1135/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetmic.2012.04.041

demonstrated that Sub3 is required for adherence of arthroconidia to feline skin (Baldo et al., 2010).

To date, the mechanism by which Sub3 is involved in the adherence process remains unknown. This protease is synthetized as a preproprotein consisting of a signal peptide followed by the propeptide and the protease domain. In the last decade, the inhibition ability of several propeptides toward their corresponding enzymes has been demonstrated in different organisms (Billington et al., 2000; Chevigné et al., 2007; Maubach et al., 1997; Nakagawa et al., 2010). In fungi, propeptides of both a serine protease and a metalloprotease from *Aspergillus fumigatus* have shown to be highly selective inhibitors of their mature enzymes (Markaryan et al., 1996).

In the present study, we expressed and characterized the propeptide of Sub3 and demonstrated that it acts as a strong inhibitor of its mature enzyme. The propeptide was then tested for its capacity to inhibit adherence of *M. canis* arthroconidia to feline epidermis using an *ex vivo* adherence model made of feline epidermis, in order to assess whether the enzymatic activity of Sub3 could be responsible for the role of the protease in the adherence process.

2. Materials and methods

2.1. Arthroconidia production

The *M. canis* strain IHEM 21239 (Institute of Hygiene and Epidemiology-Mycology [IHEM], Brussels, Belgium) was used for arthroconidia production as previously described (Gupta et al., 2003). Briefly, *M. canis* was grown on 2% yeast extract/1% peptone agar (VWR) in an atmosphere containing 12% CO_2 at 30 °C for 15 days. Surface mycelium and conidia were scraped and filtered through Miracloth layers (22–25 μm pore size; Calbiochem) to obtain an arthroconidia suspension. Arthroconidia concentration was determined by serial dilutions on Sabouraud's agar medium. Arthroconidia were stored at 4 °C until use. In all cases, arthroconida were used within 1 month.

2.2. Construction, expression and purification of the propeptide of Sub3

cDNA corresponding to the propeptide of Sub3 (Descamps et al., 2002) was amplified by PCR, and the amplified product was cloned into the pCRII vector (Invitrogen). The following primers including sequences for endonuclease restriction sites (*Nde*I and *Xho*I) were used to amplify the propeptide sequence (5′–3′): CATATGCGCGCTTTCTTTCACAACCG (forward) and GTCCTCGAGCTAAGCCAGCTTGACAACACGGT (reverse). The DNA fragment encoding the propeptide was sequenced to verify the correct amplification. The pCRII vector was digested with *Nde*I and *Xho*I restriction enzymes and the insert was cloned into pET-22b(+) (Novagen) previously restricted with the same enzymes. Recombinant expression vector was transformed into *Escherichia coli* BL21 (DE3) pLys cells (Novagen). Transformants were selected on Luria-Bertani (LB) agar plates containing ampicillin (100 μg/ml). The expression was initiated by a (1/50) dilution of an overnight subculture in 2 L of Terrific broth medium containing ampicillin (100 μg/ml) until an A_{600} value between 0.5 and 1 was reached. The propeptide expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM; Immunosource). After 6 h incubation at 37 °C, cells were collected by centrifugation, resuspended in phosphate-buffered saline (PBS, pH 7.4) and lysed using a disintegrator (Constant Systems). The lysate was centrifuged for 20 min at 12,000 × *g*, and the pellet was discarded. The supernatant was loaded onto a Q-HP Sepharose column (60 ml; Amersham Biosciences, GE Healthcare) equilibrated with 20 mM Tris–HCl buffer (pH 8.5). The column was washed with 10 volumes of the same buffer, and the bound propeptide was eluted with a linear gradient of NaCl (0–1 M). The fractions containing the propeptide were pooled and concentrated by ultrafiltration on an Amicon YM 5 membrane (Amicon). Further purification was performed by gel filtration on a Sephacryl-100HR column (120 ml; Amersham Biosciences) equilibrated in 20 mM Tris–HCl buffer (pH 8; buffer A). Fractions containing the propeptide were pooled and purification was achieved by an anion-exchange chromatography on a MonoQ column (Amersham Biosciences) equilibrated with buffer A. The elution was performed with a linear NaCl (0–1 M) gradient. Fractions containing the pure propeptide were pooled and dialysed overnight against buffer A. The homogeneity of the preparation was assessed by 18% SDS-PAGE. The concentration of the purified propeptide was determined from the A_{280} value, using the calculated extinction coefficient value of 9970 M^{-1} cm^{-1}, and confirmed by a bicinchoninic acid assay (Pierce), using bovine serum albumin as standard.

2.3. Inhibition of M. canis Sub3 by its propeptide

The proteolytic activity of recombinant 31.5 kDa subtilisin 3 (Descamps et al., 2003) (rSub3, 11 nM) was measured at 25 °C in 50 mM Tris–HCl buffer (pH 7.5) containing 16 μM dithiothreitol (DTT) and 16 μM EDTA (buffer B) with AAPF–AMC (7-amino-4-methylcoumarin, Bachem) (120–600 μM), a specific substrate for subtilisin proteases including Sub3 (Mignon et al., 1998a), in the presence of increasing propeptide concentrations (100–400 nM), by following the increase of fluorescence emission resulting from the liberation of the AMC group upon hydrolysis of the substrate. The measured fluorescence intensity could be converted into product concentration, with the help of an AMC (0–0.6 μM) standard curve. Measurements were performed with an LS 50 B fluorimeter (Perkin Elmer) using excitation and emission wavelengths of 380 and 460 nm, respectively. The K_m value of rSub3 for AAPF–AMC was first determined separately using Hanes regression (0.1–1 mM). The model of inhibition and the inhibition constants K_I and K'_I by the propeptide were determined with the help of the Hanes plot using the equation:

$$V_i = \frac{V \times (S)}{\left(1 + \frac{(I)}{K'_I}\right) \times \left(K_m \times \left(\frac{1+(I/K_I)}{1+(I/K')}\right) + (S)\right)}$$

where V_i, V, (S), (I), K_m, K'_I and K_I represent the initial rate for product release, the maximal rate for product release, the concentration of substrate, the concentration of

inhibitor, the K_m of the enzyme (rSub3) for its substrate, the dissociation constant for enzyme–substrate–inhibitor (ESI) complex and the dissociation constant for the enzyme–inhibitor (EI) complex respectively.

2.4. Adherence assay

M. canis arthroconidia adherence assay was performed using an *ex vivo* model made of feline epidermis. Normal feline skin was obtained from an animal shelter immediately after euthanasia. Skin was harvested from the flanks and sheared. The pieces of skin, approximately 4 cm^2, with full epidermal thickness were placed on a gauze pad immersed in cold sterilized skin graft fluid (Duek et al., 2004). The role of Sub3 in adherence was assessed using the purified Sub3 propeptide. For each experiment, four pieces of skin were inoculated with arthroconidia exposed to Sub3 propeptide (13.4 μM) 1 h prior to the adherence assay and four pieces of skin were inoculated with PBS containing 16 μM DTT and 16 μM EDTA as control. The skin sections were inoculated with a final concentration of 5×10^4 arthroconidial suspension in a volume of 20 μl in PBS DTT–EDTA and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 4 h. After washing in PBS–Tween 0.1% (2 ml) for 10 min, each piece of skin with adherent arthroconidia was scraped with a sterile scalpel blade and the harvested material was spread onto Sabouraud's plates containing 0.05% chloramphenicol and 0.05% cycloheximide for 3 days. Colony forming units (cfu) were counted using a microscope. The experiment was performed in triplicate using skin samples from three different cats.

2.5. Stability of Sub3 propeptide

The stability of the propeptide was evaluated under several conditions mimicking those prevailing in the adherence assay. A temperature of 37 °C, the presence of Sub3, the contact with feline skin components and the contact with arthroconidia or arthroconidial secreted components were evaluated separately for their potential effect on Sub3 propeptide stability.

The stability of the Sub3 propeptide was tested by measuring the inhibition of the mature recombinant Sub3 activity using *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF-*p*Na, Sigma) as a substrate. An AAPF-*p*Na stock solution was prepared at 50 mM concentration in DMSO and stored at −20 °C. The reaction mixture contained a concentration of 5 mM substrate and the enzyme preparation (0.55 μM) in 190 μl of buffer B. After incubation at 37 °C for 20 min, the released *p*NA was measured by spectrophotometry at λ = 405 nm with a Multiscan RC spectrophotometer (ThermoLabsystems). A control with substrate but without enzyme was carried out in parallel.

To evaluate the stability of the propeptide at 37 °C and its putative inactivation by the protease Sub3, the propeptide (3.34 μM) was incubated alone or with rSub3 (0.55 μM) at 37 °C during 24 h in buffer B.

To evaluate the putative propeptide inactivation by *M. canis* arthroconidia themselves, the propeptide (3.34 μM) was incubated in the presence of 5×10^4 living or heat killed arthroconidia (5 min at 95 °C) at both 37 °C and 20 °C for 0–5 h.

To evaluate the putative inactivation of the propeptide by fungal components secreted by arthroconidia, the propeptide (3.34 μM) was incubated at 37 °C for 0–5 h in the presence of living arthroconidia (5×10^4) which were physically separated from the propeptide using a cell insert (Anopore 0.63 cm diameter, 0.2 μm diameter pore size; VWR). After incubation time, the propeptide was harvested and its inhibitory capacity was evaluated as described above.

2.6. Statistical evaluation

Inhibition of *M. canis* adherence was expressed as the residual percentage of adherence to feline epidermis in comparison with the controls. Significant differences between two data sets were assessed using a general linear model (GLM procedure of SAS), with significance defined as $P < 0.01$.

2.7. Ethics

Three cats were used for this study. They were euthanized in an animal shelter for reasons independent of our experiments. The skin was harvested on dead animals. As a consequence, no ethics protocol was needed.

3. Results and discussion

3.1. The propeptide of Sub3 made in E. coli is a strong inhibitor of its mature enzyme

In this study, the propeptide of the keratinolytic protease Sub3 was produced in *E. coli* as a cytoplasmic soluble protein. Nine milligrams of pure propeptide were obtained from 2 L of culture using three successive purification steps. The apparent molecular mass is 11 kDa (Fig. 1) which corresponds to the expected value (10.849 kDa) (Descamps et al., 2003). Initial rate measurements of the enzymatic activity of rSub3 in the presence of increasing concentrations of the propeptide indicated a strong inhibition of the enzyme. The Hanes plot suggested that the propeptide behaved as a noncompetitive inhibitor (Fig. 2), with dissociation constants K_I (for the rSub3–propeptide complex, EI) and K'_I (for the rSub3-AAPF–AMC–propeptide complex, ESI) of 170 ± 1 nM and 130 ± 1 nM respectively, while the K_m for this enzyme is 235 μM (data not shown) which was similar to the one obtained by Mignon et al. (1998a) for the natural Sub3 protease (370 μM). The affinity of the Sub3–propeptide complex is comparable to those reported for the interaction of subtilisin E of *Bacillus subtilis* (Ohta et al., 1991), *A. fumigatus* serine protease (Markaryan et al., 1996), and *Aspergillus niger* acid endopeptidase (Kubota et al., 2005) with their respective propeptide.

3.2. The use of Sub3 propeptide does not prevent adherence of M. canis arthroconidia to feline epidermis

The protease Sub3 is an important pathogenic factor as it was shown as highly keratinolytic and produced *in vivo*

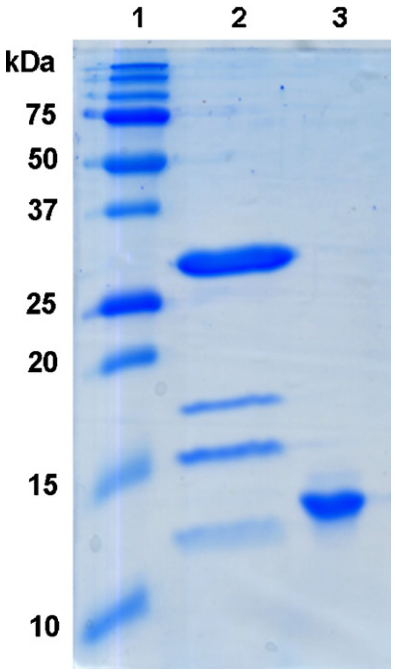

Fig. 1. SDS-PAGE (18%) showing rSub3 and the purified Sub3 propeptide. Lane 1, molecular weight marker (Precision Plus Protein™ Standards Dual Color, Biorad); Lane 2, rSub3 produced in *Pichia pastoris*; Lane 3, Sub3 propeptide produced in *E. coli* after 3 purification steps. Molecular weights (in kDa) are indicated on the left.

during infection (Mignon et al., 1998b). Moreover, the protease Sub3 is involved in adherence process to epithelial cells (Baldo et al., 2010) as it has been demonstrated for several microorganisms (Kumagai et al., 2005; Mann et al., 2006; Naglik et al., 2003). The propeptide of Sub3 produced in this study was thus tested for its capacity to inhibit *M. canis* adherence to feline epidermis. Despite the strong inhibition of Sub3 by its propeptide, the use of the latter at a high concentration (13.4 μM *i.e.* approximately $80 \times K_I$) still did not prevent adherence of *M. canis* arthroconidia to feline epidermis (Fig. 3). These results suggest that the propeptide did not interact with the protease under assay conditions.

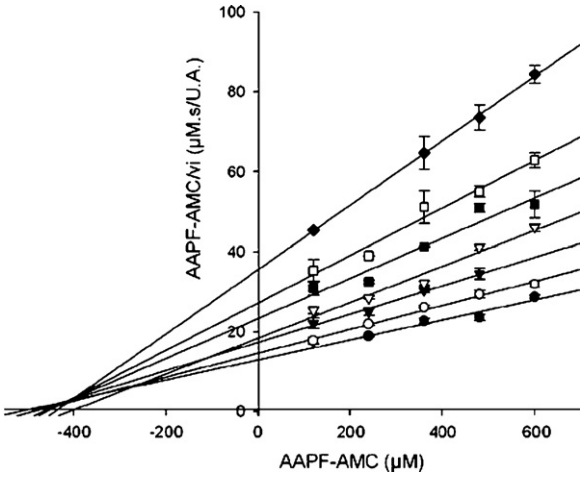

Fig. 2. Hanes plot of the inhibition of rSub3 (11 nM) by its propeptide showing that the propeptide strongly inhibits the mature enzyme activity as a noncompetitive inhibitor. Substrate concentration (AAPF – AMC)/initial hydrolysis rate *versus* substrate concentrations (120, 240, 360, 480, 600 μM) in the presence of increasing concentrations of propeptide are shown as follows: 0 nM (●), 100 nM (○), 200 nM (▼), 250 nM (▽), 300 nM (■), 350 nM (□), 400 nM (◆) in Tris buffer (50 mM, pH 7.5) containing 16 μM DTT and 16 μM EDTA, at 20 °C. Values are the means of three independent experiments ± SD.

To explain this discrepancy, the stability of Sub3 propeptide made in *E. coli* was tested as described in Section 2 under several conditions mimicking those prevailing in the adherence assay. Sub3 propeptide was found to be stable at 37 °C alone as well as in the presence of rSub3. Sub3 propeptide was also found to be stable after contact with feline skin at 37 °C for 4 h (data not shown). After contact with living arthroconidia, the propeptide of Sub3 lost 100% of its inhibitory capacity within 10 min at 37 °C while at 20 °C the inactivation occurred within 5 h (Fig. 4). In contrast, the propeptide was found to be stable after contact with heat killed arthroconidia both at 37 °C and at 20 °C (Fig. 4). The propeptide of Sub3 was also found to be stable after contact with fungal components secreted by arthroconidia (data not shown). These results suggest that the propeptide could be inactivated by a bound membrane protease present in arthroconidia. Our hypothesis is strengthened by the fact that the propeptide was found to be not degraded by a crude exoantigen which is a mixture of proteases secreted by *M. canis* in an inducing keratinolytic activity medium (Mignon et al., 1998a) (data not shown). The propeptide of various proteases is known to be digested by other concomitantly secreted proteases for which it represents a potential peptidic substrate. For example, the cathepsin B propeptide is degraded by papain (Fox et al., 1992) and the *Dermatophagoides pteronissynus* trypsin-like protease propeptide is hydrolyzed by a co-localized mite cysteine protease (Dumez et al., 2008).

In conclusion, Sub3 which is required for arthroconidia adherence to feline epidermis is strongly inhibited by its propeptide made by recombination in *E. coli*. However, the recombinant Sub3 propeptide appears to be inactivated after contact with living arthroconidia. This finding raises the question about the choice of peptidic inhibitors to

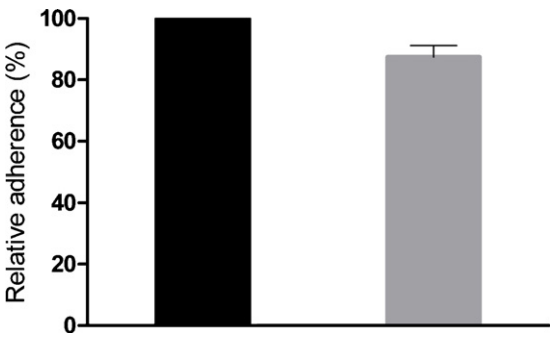

Fig. 3. The relative adherence of *M. canis* IHEM 21239 arthroconidia to feline epidermis is not decreased by the addition of the Sub3 propeptide. Adherence of arthroconidia in the presence of the propeptide (13.4 μM) (gray bar) is expressed as a percentage of the control without propeptide (black bar) over a 4 h incubation period under the same conditions. Values are the means of three independent experiments ± SEM.

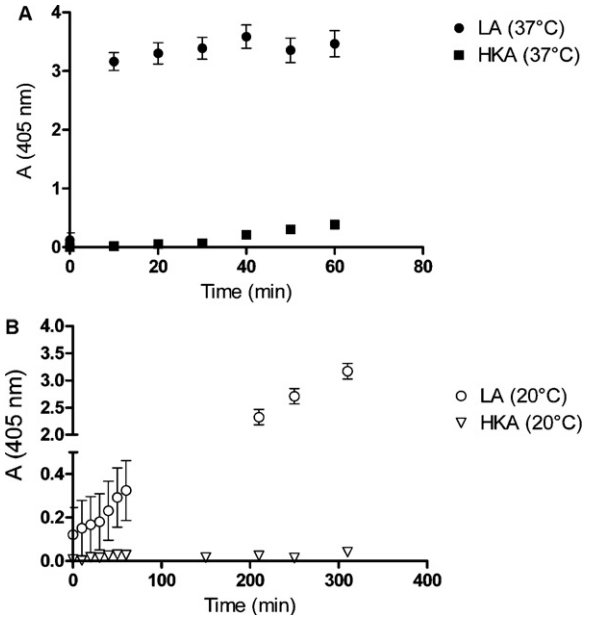

Fig. 4. The inhibitor activity of the Sub3 propeptide toward Sub3 is lost following direct contact with living *M. canis* arthroconidia. (A) Hydrolysis of AAPF-*p*Na (5 mM) by rSub3 (0.55 μM) in the presence of its propeptide (3.34 μM) preincubated with living arthroconidia (LA) (5×10^4) (●) and heat killed arthroconidia (HKA) (5×10^4, 5 min at 95 °C) (▼) at 37 °C. (B) Hydrolysis of AAPF-*p*Na (5 mM) by rSub3 (0.55 μM) in the presence of its propeptide (3.34 μM) preincubated with LA (5×10^4) (○) and HKA (5×10^4, 5 min at 95 °C) (▽) at 20 °C. Values are the means of three independent experiments ± SD.

target arthroconidia adherence since they could be digested by proteases on surface of the arthroconidia.

Conflict of interest statement

None.

Acknowledgements

This work was supported by grant 3.4595.04 from Fonds de la Recherche Scientifique Médicale (FRSM). A.B., A.M. and J.T. were and L.C. is the recipients of a studentship from FRIA (Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture, 1000 Bruxelles, Belgium). A.C. was a recipient of a PhD fellowship of the FRS-FNRS (Fonds de la Recherche Scientifique). P.P. was a recipient of a post-doctoral fellowship from FRSM-FNRS, and a current member of the Argentinean National Research Council (CONICET).

References

Baldo, A., Mathy, A., Tabart, J., Camponova, P., Vermout, S., Massart, L., Maréchal, F., Galleni, M., Mignon, B., 2010. Secreted subtilisin Sub3 from *Microsporum canis* is required for adherence to but not for invasion of the epidermis. Br. J. Dermatol. 162, 990–997.

Baldo, A., Tabart, J., Vermout, S., Mathy, A., Collard, A., Losson, B., Mignon, B., 2008. Secreted subtilisins of *Microsporum canis* are involved in adherence of arthroconidia to feline corneocytes. J. Med. Microbiol. 57, 1152–1156.

Billington, C.J., Mason, P., Magny, M.C., Mort, J.S., 2000. The slow-binding inhibition of cathepsin K by its propeptide. Biochem. Biophys. Res. Commun. 276, 924–929.

Brouta, F., Descamps, F., Monod, M., Vermout, S., Losson, B., Mignon, B., 2002. Secreted metalloprotease gene family of *Microsporum canis*. Infect. Immun. 70, 5676–5683.

Chermette, R., Ferreiro, L., Guillot, J., 2008. Dermatophytoses in animals. Mycopathologia 166, 385–405.

Chevigné, A., Barumandzadeh, R., Groslambert, S., Cloes, B., Dehareng, D., Filée, P., Marx, J.C., Frere, J.M., Matagne, A., Jacquet, A., Galleni, M., 2007. Relationship between propeptide pH unfolding and inhibitory ability during ProDer p 1 activation mechanism. J. Mol. Biol. 374, 170–185.

Degreef, H., 2008. Clinical forms of dermatophytosis (ringworm infection). Mycopathologia 166, 257–265.

Descamps, F., Brouta, F., Monod, M., Zaugg, C., Baar, D., Losson, B., Mignon, B., 2002. Isolation of a *Microsporum canis* gene family encoding three subtilisin-like proteases expressed *in vivo*. J. Invest. Dermatol. 119, 830–835.

Descamps, F., Brouta, F., Vermout, S., Monod, M., Losson, B., Mignon, B., 2003. Recombinant expression and antigenic properties of a 31.5-kDa keratinolytic subtilisin-like serine protease from *Microsporum canis*. FEMS Immunol. Med. Microbiol. 38, 29–34.

Duek, L., Kaufman, G., Ulman, Y., Berdicevsky, I., 2004. The pathogenesis of dermatophyte infections in human skin sections. J. Infect. 48, 175–180.

Dumez, M.-E., Teller, N., Mercier, F., Tanaka, T., Vandenbergue, I., Vandenbranden, M., Devreese, B., Luxen, A., Frere, J.-M., Matagne, A., Jacquet, A., Galleni, M., Chevigné, A., 2008. Activation mechanism of recombinant Der p 3 allergen zymogen: contribution of cysteine protease Der p 1 and effect of propeptide glycosylation. J. Biol. Chem. 283, 30606–30617.

Fox, T., De Miguel, E., Mort, J.S., Storer, A.C., 1992. Potent slow-binding inhibition of cathepsin B by its propeptide. Biochemistry 31, 12571–12576.

Ginter-Hanselmayer, G., Weger, W., Ilkit, M., Smolle, J., 2007. Epidemiology of tinea capitis in Europe: current state and changing patterns. Mycoses 50, 6–13.

Gupta, A., Ahmad, K., Porretta, I., Summerbell, M.R.C., 2003. Arthroconidial formation in *Trichophyton raubitschekii*. Mycoses 46, 322–328.

Jousson, O., Lechenne, B., Bontems, O., Capoccia, S., Mignon, B., Barblan, J., Quadroni, M., Monod, M., 2004. Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes *Trichophyton* and *Microsporum*. Microbiology 150, 301–310.

Kraemer, A., Mueller, R.S., Werckenthin, C., Straubinger, R.K., Hein, J., 2012. Dermatophytes in pet Guinea pigs and rabbits. Vet. Microbiol. 157 (1–2), 208–213.

Kubota, K., Nishii, W., Kojima, M., Takahashi, K., 2005. Specific inhibition and stabilization of aspergilloglutamic peptidase by the propeptide. J. Biol. Chem. 280, 999–1006.

Kumagai, Y., Yagishita, H., Yajima, A., Okamoto, T., Konishi, K., 2005. Molecular mechanism for connective tissue destruction by dipeptidyl aminopeptidase IV produced by the periodontal pathogen *Porphyromonas gingivalis*. Infect. Immun. 73, 2655–2664.

Lunder, M., Lunder, M., 1992. Is *Microsporum canis* infection about to become a serious dermatological problem? Dermatology 184, 87–89.

Mann, B., Orihuela, C., Antikainen, J., Gao, G., Sublett, J., Korhonen, T.K., Tuomanen, E., 2006. Multifunctional role of choline binding protein G in pneumococcal pathogenesis. Infect. Immun. 74, 821–829.

Markaryan, A., Lee, J.D., Sirakova, T.D., Kolattukudy, P.E., 1996. Specific inhibition of mature fungal serine proteinases and metalloproteinases by their propeptides. J. Bacteriol. 178, 2211–2215.

Maubach, G., Schilling, K., Rommerskirch, W., Wenz, I., Schultz, J.E., Weber, E., Wiederanders, B., 1997. The inhibition of cathepsin S by its propeptide-specificity and mechanism of action. Eur. J. Biochem. 250, 745–750.

Mignon, B., Swinnen, M., Bouchara, J.P., Hofinger, M., Nikkels, A., Pierard, G., Gerday, C., Losson, B., 1998a. Purification and characterization of a 31.5 kDa keratinolytic subtilisin-like serine protease from *Microsporum canis* and evidence of its secretion in naturally infected cats. Med. Mycol. 36, 395–404.

Mignon, B.R., Nikkels, A.F., Pierard, G.E., Losson, B.J., 1998b. The *in vitro* and *in vivo* production of a 31.5-kDa keratinolytic subtilase from *Microsporum canis* and the clinical status in naturally infected cats. Dermatology 196, 438–441.

Monod, M., 2008. Secreted proteases from dermatophytes. Mycopathologia 166, 285–294.

Naglik, J.R., Challacombe, S.J., Hube, B., 2003. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol. Mol. Biol. Rev. 67, 400–428.

Nakagawa, M., Ueyama, M., Tsuruta, H., Uno, T., Kanamaru, K., Mikami, B., Yamagata, H., 2010. Functional analysis of the cucumisin pro-peptide as a potent inhibitor of its mature enzyme. J. Biol. Chem. 285, 29797–29807.

Ohta, Y., Hojo, H., Aimoto, S., Kobayashi, T., Zhu, X., Jordan, F., Inouye, M., 1991. Pro-peptide as an intramolecular chaperone: renaturation of denatured subtilisin E with a synthetic pro-peptide. Mol. Microbiol. 5, 1507–1510.

Scott, D.W., Miller, W.H., Griffin, C.E., 1995. Fungal skin diseases. In: Muller, G.H. (Ed.), Small Animal Dermatology. 5th ed. W.B. Saunders, Philadelphia, pp. 329–391.

Vermout, S., Baldo, A., Tabart, J., Losson, B., Mignon, B., 2008a. Secreted dipeptidyl peptidases as potential virulence factors for *Microsporum canis*. FEMS Immunol. Med. Microbiol. 54, 299–308.

Vermout, S., Tabart, J., Baldo, A., Mathy, A., Losson, B., Mignon, B., 2008b. Pathogenesis of dermatophytosis. Mycopathologia 166, 267–275.