Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytical Biochemistry 338 (2005) 326–331
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Proteolytic peptide patterns as indicators for fungal infections and nonfungal affections of human nails measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Klaus Hollemeyer[a,*], Sven Jager[b], Wolfgang Altmeyer[c], Elmar Heinzle[a]

[a] *Biochemical Engineering Institute, Im Stadtwald, Saarland University, D-66123 Saarbrücken, Germany*
[b] *D-66280 Sulzbach, Germany*
[c] *GENE-FACTS, Science Park Saar, Stuhlsatzenhausweg 69, D-66123 Saarbrücken, Germany*

Received 5 October 2004
Available online 13 January 2005

Abstract

The discrimination of onychomycoses from endogenous diseases showing macroscopically similar symptoms is difficult. Long-lasting but ineffective antifungal therapies using systemic medicaments with often severe adverse reactions may be the consequence. We introduce a novel mass spectrometric method for the discrimination of fungal infections and nonfungal affections. Horn samples from patients infected by *Trichophyton rubrum*, from patients with psoriasis affecting nails, and from healthy persons were investigated. Onychomycoses are basically associated with proteolytic attacks of the virulent fungi-secreting proteases partly hydrolyzing the horn material. Endogenous diseases lack these proteolytic activities, conserving intact structural proteins. Tryptical digestion of horn material produced cleavage peptides detectable by matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry. Mass spectra of horn material infected by *T. rubrum* were clearly different from those originating from healthy test persons and from patients with psoriasis. Two methods were successfully applied to quantify the differences between groups of samples. One is based on the Euclidean match factor, and the other is based on the identification of specific peptide peaks occurring exclusively within one group of persons. The Euclidean match factor distributions and the occurrence of specific peptide peaks allowed a clear differentiation of *T. rubrum* infections from psoriasis patients and healthy test persons. No differences were found between healthy test persons and psoriasis patients. The method is rapid and does not require any cultivation.

Keywords: Proteolytic peptides; *Trichophyton rubrum*; Psoriasis; MALDI–TOF MS; Euclidean match factor

Human fungal diseases can affect the whole surface of the body, including hair, fingernails, and toenails (*Tinea unguium*). The fungal infections of nails, called onychomycoses, account for approximately 30% of all superficial fungal infections and 50% of all nail disorders [1], with roughly 35 million people being affected in the United States. Several organisms are responsible for such onychomycoses. The dermatophytes *Trichophyton rubrum*, *Trichophyton interdigitale*, *Trichophyton mentagrophytes*, and *Microsporum gyoseum* are diagnosed most frequently [2,3]. The pathological yeasts *Candida albicans* and *Candida parapsiloris*, as well as the mold *Scopulariopsis brevicaulis*, are also diagnosed frequently [4]. Wearing nonsuitable shoes that produce physical nail deteriorations with subsequent fungal infections, as well as using public showers, saunas, and the like, creates the wide spread of the fungal diseases. Physical discomfort is experienced by 44% of these patients and leads to difficulties in doing manual work [5]. More than 50% of geriatric patients suffer from onychomycoses that may lead to secondary, and sometimes life-threat-

* Corresponding author. Fax: +49 681 302 4572.
E-mail address: k.hollemeyer@mx.uni-saarland.de (K. Hollemeyer).

0003-2697/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2004.12.019

ening, infections as erysipelas. These may be followed by sepsis, especially in patients who also suffer from diabetes [6]. Besides the fungal infections of nails, there are other diseases that are often mistaken for mycoses such as onychodystrophy, Reiter's syndrome, morbus darier, lichen planus, pityriasis rubra piloris, eczema nails, and psoriasis [7]. In addition, horn destruction may be caused by mite bites (i.e., scabies) and by other zoo parasites, aggressive chemicals such as acids and bases, and other proteolytic chemicals. Further reasons for horn anomalies include benign and malignant tumors such as melanomas affecting the nail-producing regions of fingers and toes. These sometimes appear very similar to fungal infections and may be fatal if they are not recognized in time [8]. Confusion often arises due to the similar macroscopic appearance of all these diseases with brittle and yellowish or brownish dyed fingernails and toenails. Endogenous diseases or environmentally caused anomalies being mistaken for mycoses may lead to long-lasting but ineffective antifungal therapies. Systemic antimycotica, such as the azole derivatives fluconazole and itraconazole as well as the naphthalene derivative terbinafine, are often used for mono systemic therapies. Severe adverse reactions, such as liver damage, allergies, and indispositions, are known to occur with these therapies; thus, such therapies are restricted to those patients suffering from certified mycoses. Routinely, suspicious samples from patients' horn are investigated microscopically to detect traces of fungi. Incubation of nail material for fungal growth lasts several weeks, usually with a success rate of less than 50%. Kanbe and coworkers [9] introduced a method for the identification of virulent fungi using polymerase chain reaction (PCR)[1] and PCR–restriction fragment length polymorphism (RFLP) techniques. This technique also involves cultivation.

We study the differentiation of fungal infection by *T. rubrum* from psoriasis affecting fingernails and toenails. We present a new application of an analytical method that was originally developed for the identification of animal species using matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry by measuring tryptic digests of feathers, down, or hair [10]. The novel application does not require any cultivation. The base of our method is the fact that keratinolytic proteases, secreted by the infecting fungi, partly hydrolyze and/or derivatize keratin and embedding proteins. Several species-specific proteases are known, including aspartic proteases of the pepsin family, serine proteases of the subtilisin family, and metalloproteases of two different families. Also, a non-pepsin-type aspartic protease and a chymotrypsin-like protease were found together with aminopeptidases, carboxypeptidases, and dipeptidyl-peptidases [11–19]. Tryptic digests of the horn originating from such infected samples should, therefore, contain cleavage peptides different from those from healthy persons and from patients with psoriasis. We studied cleavage peptide patterns originating from healthy test persons as well as from psoriasis-affected patients of different genders and ages. It was also of central interest whether peptide patterns varied with progressing damage by *T. rubrum* infections.

[1] *Abbreviations used:* PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; MALDI–TOF, matrix-assisted laser desorption/ionization time-of-flight; TFA, trifluoroacetic acid; CCA, α-cyano-4-hydroxycinnamic acid; MF, Euclidean match factor.

Materials and methods

Chemicals and analyzed samples

Trypsin from hog pancreas with an activity of 1645 U/mg was purchased from Fluka (Deisenhofen, Germany). Trifluoroacetic acid (TFA), α-cyano-4-hydroxycinnamic acid (CCA), 2-mercaptoethanol, and the calibration peptides human angiotensin II, substance P, human neurotensin, and the human adenocorticotropic hormone fragments ACTH(1–17) and ACTH(18–39) were obtained from Sigma (Deisenhofen, Germany). Ammonium bicarbonate and acetonitrile PA were obtained from Merck (Darmstadt, Germany). Acridine orange (10 mg/ml solution in water), Kimmig fungi agar base, and Kimmig selective supplement were purchased from Sigma [20,21].

Human horn samples from fingernails and toenails were obtained from the clinical practices of Sven Jager and Hans Peter Seidl of Technische Universität in Munich, Germany. Samples from healthy test persons ($n = 19$) of different genders and ages and from patients with *T. rubrum* ($n = 14$) or psoriasis ($n = 9$) were used.

Microscopic determination and culturing conditions

Native and cultured samples of suspicious horn material were examined by fluorescence microscopy using acridine orange as a fluorescent stain for microbial DNA ($\lambda_{exc} = 502$ nm/$\lambda_{emm.} = 526$ nm) [22]. Samples were cultured on Kimmig agar at room temperature and reinvestigated as soon as growth occurred, with a maximum culturing time of 4 weeks.

Sample preparation for MALDI–TOF mass spectrometry

Approximately 0.3–0.5 mg of horn was weighed, chopped to 0.2 mm, and transferred into eight-well PCR strips (Biozym Diagnostik, Hessisch Oldendorf, Germany) containing 50 μl of 25 mmol/L NH_4HCO_3 with 5% 2-mercaptoethanol (v/v) and carefully wetted.