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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*

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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).

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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.

Caps were sealed, and after transferring the samples into a boiling water bath for 20 min, the samples were cooled down on ice and 50 µl of 25 mmol/L NH_4HCO_3 containing 5 mg trypsin/ml was added to each tube. Tubes were incubated for 2 h in a water bath at 37 °C. Then 5 µl of each sample was transferred into a fresh tube, previously filled with 45 µl saturated CCA solution in 50% acetonitrile and 1% TFA, and was mixed by pipetting. Then 1 µl of each sample was manually pipetted on a polished MALDI–TOF steel target plate and allowed to dry.

Mass spectrometry

Analysis was performed on a Reflex III TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with the SCOUT 384 probe ion source. The system uses a pulsed nitrogen laser (337 nm, model VSL-337ND, Laser Science, Boston, MA, USA) with 400 µJ/pulse energy. The ions were accelerated under delayed extraction conditions in the positive mode with an acceleration voltage of 20 kV and a reflector voltage of 22.5 kV. A 6.9-kV potential difference between the target and the extraction lens was applied with a time delay of 1 µs. A Lecroy 9384C 1-GHz digital storage oscilloscope was used for data acquisition (Chestnut Ridge, NY, USA). The detector signals were amplified in two stages, digitized, and transferred to the XACQ program on an NT 4 workstation (Microsoft, Redmond, WA, USA). Autoexecute mode was chosen for automated measurement with 26 to 32% laser power, a resolution higher than 1400, a signal-to-noise (S/N) ratio of more than 4, and a noise range of 100, leading to the maximum number of detectable peaks. Spectra with saturated peaks were not used. For each spectrum, 6 subspectra from more than three spots consisting of 25 successful laser shots were added. The data were further processed with the program XMASS 5.15 (Bruker Daltonics) using the SNAP algorithm for exclusive detection of monoisotopic masses. From the identified monoisotopic peaks, the 99 most intensive ones were selected between 1 and 3 kDa with a threshold of 250 a.i.

Peak selection

First, monoisotopic peak masses were rounded to integer values. The frequency of occurrence of peaks was determined by the inspection of 24 single spectra of one sample, and only those peaks were accepted for further calculations occurring in at least 10 of these single spectra. These accepted peaks resulted in an average spectrum per sample containing relative intensity ($u_{i,\mathrm{rel}} = u_i/\Sigma(u_j)$) and absolute intensity (u_i, s_i). Specific peptide masses that occurred in the spectra of samples originating from *T. rubrum*-infected horn, but not in the spectra of other samples ($m/z_{\mathrm{T\text{-}HP}}$, where T = *T. rubrum*-infected patients, H = healthy test persons, and P = patients with psoriasis), were determined. Similarly, peptides detected only in the groups of healthy test persons and patients suffering from psoriasis, $m/z_{\mathrm{HP\text{-}T}}$, were identified.

Calculation procedure

Euclidean match factors (MF) of two average mass spectra with absolute intensities were calculated according to Alfassi [23]:

$$\mathrm{MF} = \left[1 + \sum \left(\frac{u_i}{\sqrt{\sum u_i^2}} - \frac{s_i}{\sqrt{\sum s_i^2}}\right)^2\right]^{-1},$$

where u_i and s_i are the absolute intensities of the i^{th} components of the two compared samples u and s, respectively. Each individual vector represents a single point on a sphere with unit radius in a hyperspace of n dimensions, where n is the number of components of the vector. The matching factor ranges from 0.333 to 1, with the latter indicating identity of two spectra compared. Each average spectrum was compared with all of the other average spectra within its own group and with all members of the two other groups, leading to a matching factor table. Matching factors of members of one group are called homologous ($\mathrm{MF_{X–X}}$ and $\mathrm{MF_{Y–Y}}$). They were classified, and the frequency of occurrence resulted in Euclidean match factor distributions $\mathrm{MFD_{X–X}}$ and $\mathrm{MFD_{Y–Y}}$, respectively. These distributions were measures for the particular homogeneity of the two groups, where $\mathrm{MF_{X–X}}$ and $\mathrm{MF_{Y–Y}}$ mean values more than 0.8 with standard deviation (SD) values less than 0.08 were accepted as highly homogeneous groups. Similarity considerations were performed between the homologous distribution $\mathrm{MFD_{X–X}}$ or $\mathrm{MFD_{Y–Y}}$ and the heterologous distribution $\mathrm{MFD_{X–Y}}$.

Results

A total of 984 single raw spectra were measured from healthy persons, from patients with successfully cultured and undoubtedly identified *T. rubrum* infections, and from patients with psoriasis affecting nails. Average spectra of these three groups are depicted in Fig. 1. Spectra of healthy test persons were very similar, irrespective of gender or age.

Comparison of samples by Euclidean match factor distributions

The average MF of the group of healthy test persons was 0.87 with an SD of 0.04, indicating a high similarity. The average of $\mathrm{MF_{P–P}}$ was 0.88 with an SD of 0.08, and the average of $\mathrm{MF_{T–T}}$ was 0.77 with an SD of 0.08,

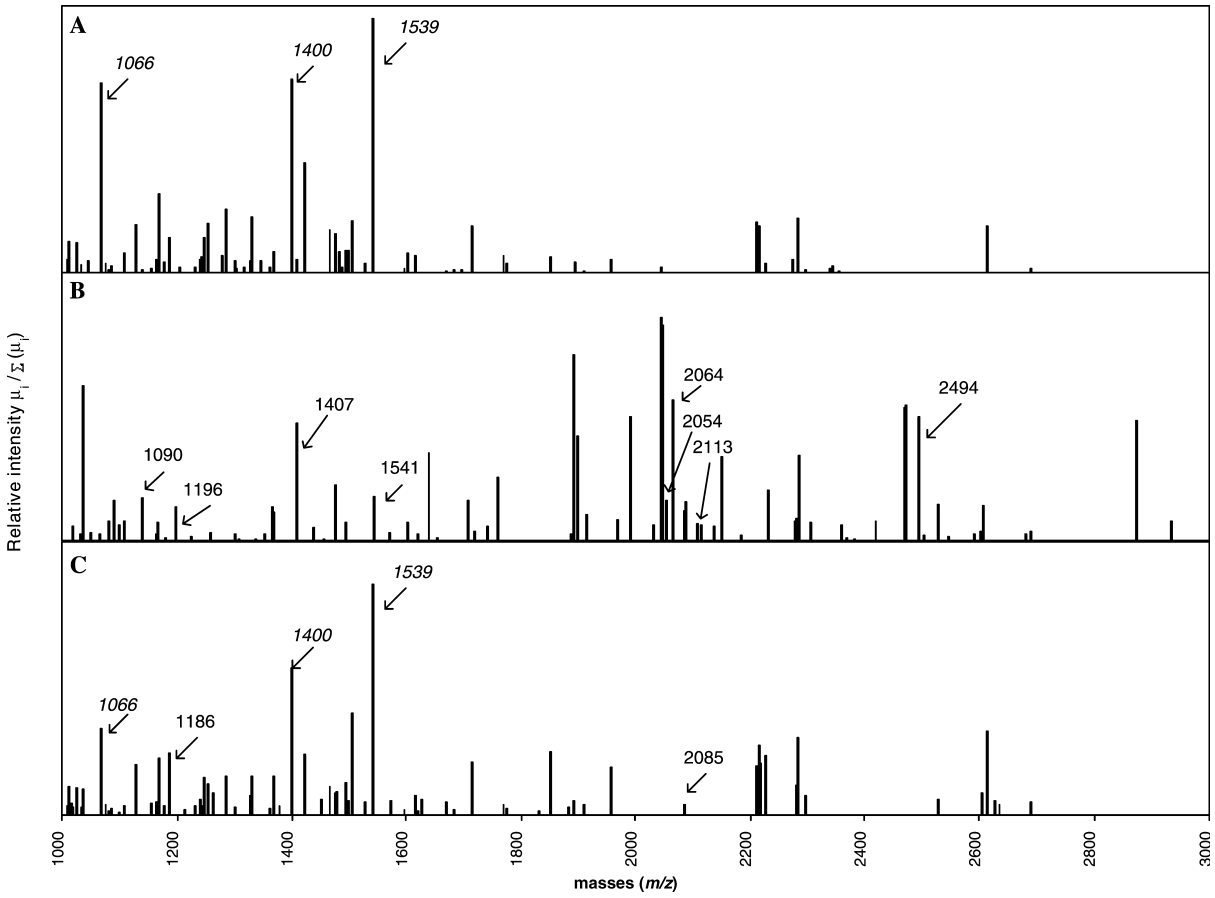

Fig. 1. Average mass spectra of tryptical digest peptides of healthy horn material (A), horn material infected by *T. rubrum* (B), and horn material affected by psoriasis (C) from one typical test person each. Peptides specific for *T. rubrum* infection, m/z_{T-HP}, and having a frequency of observation of 100% are marked in (B). Three peaks dominant in healthy and psoriasis-affected persons are marked in (A and C) (shown in italic).

showing high similarity within these groups as well. Contrary to this, the mass spectra of healthy persons differed significantly from patients suffering from the fungal disease, as can be seen in Table 1, where match factors are classified into groups. In addition, the average MF_{H-T} was only 0.41 with an SD of 0.04, indicating a poor relationship between the two groups. No overlapping regions were identified between the two distributions, showing the usefulness of the method for distinguishing between healthy samples and samples originating from *T. rubrum* infections. The comparison of psoriasis patients with *T. rubrum* patients resulted in an average value of MF_{P-T} of 0.44 and an SD of 0.05. The corresponding distribution MFD_{P-T} did not overlap with either MFD_{P-P} or MFD_{T-T}, indicating complete dissimilarities (Table 1). Therefore, psoriasis-affected horn is clearly distinguished from *T. rubrum* infections. The homologous distribution MFD_{T-T} had the smallest mean value as well as the largest SD of all homologous distributions. This indicates variations of the mass spectra due to the stage of infection. MFD_{P-P}, MFD_{H-H}, and MFD_{H-P}, as given in Table 1, indicate a high similarity within and between groups. The average MF_{P-H} value was 0.83 with an SD of 0.05. The distributions of these groups completely overlap. This is also an indication that the endogenous disease psoriasis does not cause alterations in the primary structure of horn proteins affected. Obviously, there were not any proteolytic or other chemical activities in these cases. The visible anomalies of horn growth of patients suffering from psoriasis are caused by other effects.

Identification of T. rubrum infections by specific masses

Fungal infections cause alterations of horn that produce tryptic peptides detectable by mass spectrometry that could not be detected in the spectra of healthy test persons or patients suffering from psoriasis (m/z_{T-HP}). On the other hand there were also peptides detected exclusively in the spectra of healthy persons and psoriasis patients but not in *T. rubrum* -infected samples (m/z_{HP-T}). These peaks, however, were usually rather small compared with those found after infections with *T. rubrum.* The major reason for this observation is that, in most cases, nondegraded horn material was also present in the samples. The specific peptides were used for a sec-

Table 1
Classification of homologous and heterologous Euclidean match factors: frequencies of occurrence (percentages)

Class of MF	MF_{H-H}	MF_{H-T}	MF_{T-T}	MF_{P-T}	MF_{P-P}	MF_{P-H}
0.95–1.00	13.8	0	0	0	24.4	0
0.90–0.95	28.7	0	15.2	0	53.7	19.4
0.85–0.90	34.7	0	27.2	0	0	45.6
0.80–0.85	17.4	0	21.7	0	0	25.0
0.75–0.80	5.4	0	0	0	19.5	4.4
0.70–0.75	0	0	18.5	0	2.4	5.0
0.65–0.60	0	0	10.9	0	0	0.6
0.60–0.65	0	0	6.5	0	0	0
0.55–0.60	0	0	0	2.5	0	0
0.50–0.55	0	9.7	0	27.6	0	0
0.45–0.50	0	47.6	0	32.5	0	0
0.40–0.45	0	8.7	0	26.0	0	0
0.35–0.40	0	34.0	0	11.4	0	0
0.30–0.35	0	0	0	0	0	0
N	167	206	92	123	41	180
Average mean	0.88	0.41	0.77	0.44	0.88	0.83
SD	0.04	0.04	0.08	0.05	0.08	0.05

Note. MF_{X-X}, comparison within own group; MF_{X-Y}, comparison between two different groups of persons. Samples originated from healthy test persons (H), from patients with *T. rubrum* infections (T), or from patients with psoriasis (P).

ondary differentiation (Table 2). From all m/z_{T-HP} found, 25 different peptides were observed with a frequency larger than 50% in *T. rubrum* samples. Using these exclusive peptide peaks, it was possible to completely differentiate between *T. rubrum*-derived spectra and spectra derived from healthy persons or patients with psoriasis. The selected specific masses m/z_{T-HP} were found in the mass spectra of *T. rubrum*-infected individuals, with an average frequency of 62% and variations between 29 and 79%.

Some peptide peaks occurred in all three groups but had significantly different relative intensities. Whereas spectra $m/z = 1539$, $m/z = 1400$, and $m/z = 1066$ were the major peaks in healthy persons and patients with psoriasis, these were very small or even not observable in spectra derived from *T. rubrum*-infected individuals. The occasional existence of these peaks in *T. rubrum*-infected samples may be attributed to the presence of residual nondegraded material. These findings also support the hypothesis of there being proteolytic cleavage of the structural proteins during fungal infection but absence of such activities during psoriasis affection.

Table 2
Specific peptide mass spectral peaks (m/z_{T-HP}) observed exclusively in *T. rubrum*-infected samples but not in the groups of healthy test persons and those suffering from psoriasis

Frequency of observation (%)	m/z_{T-HP}
100	1090, 1196, 1541, 2054, 2064, 2113, 2494
80–99.9	1167, 1223, 2359,
70–79.9	1029, 1049, 1456
60–69.9	1043, 1338, 1364, 1443, 1068
50–59.9	1352, 1886, 1993, 2151, 2418, 2571, 2872

Note. Group sizes: *T. rubrum*-infected persons (T), 14 test persons; healthy and psoriasis-affected persons (HP), 28 test persons.

Discussion

The investigation of human horn material after enzymatic digest with trypsin and subsequent determination of the arising cleavage peptides using MALDI–TOF mass spectrometry led to distinct results. First, the digests of human horn material originating from healthy test persons of different genders and ages showed a high homogeneity of the cleavage peptide patterns despite the large variation of sources. Second, the mass spectra of tryptic digests of samples infected by *T. rubrum* were clearly different from spectra of healthy test persons and of patients suffering from the endogenous disease psoriasis. The homogeneity of the spectra within the group of the spectra originating from *T. rubrum* infections was not as high as it was within the other two groups. This indicates a progressive degradation of structural proteins during the progression of the fungal infection. Spectra originating from psoriasis-affected patients could not be differentiated from those originating from healthy persons by the geometric Euclidean match factor distributions MFD_{P-P} or MFD_{H-H} combined with MFD_{P-H}. Moreover, it was also not possible to identify any peptide peak occurring exclusively in either of the two groups. The conformity of both groups of spectra indicates intact structural proteins of nail-affecting psoriasis despite visual macroscopic appearance. A proper identification was also possible using the seven specific peptide masses, being present in all of the

investigated spectra originating fungal infections. This second way of analyzing the data even further improved the reliability of the method.

The integrity of the horn material is the measure for discrimination between psoriasis and the proteolytic fungus *T. rubrum.* This is a fundamentally new concept for measuring the damage of proteolytic activities by fungal infections. Contaminations of the samples with components originating from fungal mycelium or from secreted proteins are unlikely due to the high dilution that is applied during preparation of the hydrolyzed cleavage peptides solution. Possible contaminations originating from trypsin are known and were excluded from calculations. Incubation of suspicious material takes up to 4 (or more) weeks, with a high probability of false results. The novel method properly identifies fungal infections, detecting their biochemical traces in their protein substrates within hours.

It seems that the method is not restricted to *T. rubrum* infections and could also potentially differentiate among infecting organisms differing in their individual sets of proteases attacking keratin and embedding proteins. This would lead to different types of damage for the structural proteins. Endogenous diseases without proteolytic activities, such as psoriasis, could be distinguished from those diseases showing proteolytic activities. Endogenous diseases damaging formerly healthy horn material, or diseases disturbing the proper syntheses of nail material, may also be detected and differentiated from each other. Creating libraries containing specific peptide patterns of causative organisms and of diseases may lead to clear and quick identifications of the origin of the affection.

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