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ARTICLE

Mass spectrometric investigation of alamethicin

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ABSTRACT The peptaibols, such as alamethicin are secondary metabolites belonging to the family of fungal peptide antibiotics. These compounds are linear, amphipathic oligopeptides classified into 4 subfamilies and composed of 5-20 amino acids. Their backbones usually contain several nonproteinogenic amino acid residues representing characteristic building blocks of the structure. In our present work, the mass spectrometric analysis of alamethicin has been investigated by on-line reversed-phase high performance liquid chromatography (HPLC) coupled to electrospray ionization tandem mass spectrometry (ES-MS). Initially, the parameters of the MS were optimized by the continuous infusion of an alamethicin standard solution directly into the ESI source. Then, the proper HPLC method was developed for the analysis of the alamethicin components, which was capable to separate the peaks of F50-5, F50-6a, F50-7 and F50-8b from each other, which could be identified based on their mass spectra.
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KEY WORDS

alamethicin
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A number of economically important antibiotics, secondary metabolites and extracellular enzymes are produced by species of the genus *Trichoderma*, including a unique peptide group of antibiotics (Sivasithamparam and Ghisalberti 1998). Although these peptide-type molecules are produced by other organisms as well, most of them are produced by this genus or by species from closely related genera (Degenkolb et al. 2003). They have characteristic molecular weights of 500-2200 Da and contain numerous non-proteinogenic, α,α-dialkylated amino acids like isovaline (Iva) and α-aminoisobutyric acid (Aib), an acetylated N-terminus, and an amino alcohol, mostly phenylalaninol at the C-terminal end (Szekeres et al. 2005). The name peptaibol comes from the words peptide, Aib, and amino alcohol. The peptaibols produced by *Trichoderma* species regularly contain 18-20 amino acid residues, and their unusal amino acid content is synthesized through non-ribosomal biosynthesis (Marahiel et al. 1997). The bioactivities of peptaibols are mainly related to formation of channels in lipid membranes, which are containing several hydrophobic transmembrane helices surrounding a central pore (Duclohier 2004; Whitmore et al. 2004).

The first described peptaibol was reported in 1967, which was isolated from the ferment broth of *Trichoderma viride* (later reidentified as *T. arundinaecum*) and named alamethicin (ALM) (Meyer and Reusser 1967; Leitgeb et al. 2007; Kredics et al. 2013). Based on their retention factor values in thin layer chromatography (TLC), ALM is composed of two major classes, an acidic and a neutral group distinguished by Glu/Gln18 exchange and named ALM F30 (85%) and ALM F50 (12%), respectively, and an additional minor component (ALM F20) (Kirschbaum et al. 2003; Degenkolb et al. 2007). Moreover, some further groups of ALM (ALM-F40, ALM-F60 and ALM-F70) were also recognised by TLC in low concentrations (Melling and McMullen1975).

In our earlier work we described a sensitive and low-cost biological pre-screening for the detection of peptaibol compounds (Marik et al. 2013). The present study is dealing with the establishment of a proper analytical method enabling the sensitive detection and identification of these fungal peptides.

Materials and Methods

Materials

Methanol, acetonitrile for sample preparation and eluents were purchased from VWR (Hungary). The trifluoroacetic acid (TFA) and ALM standards were obtained from Sigma-Aldrich (Hungary). Membrane-filtered, deionized water for HPLC runs was produced by Millipore water purification equipment (Merck, Hungary) with a resistivity of 18MΩ. Reversed phase (RP) – high performance liquid chromatography (HPLC)-UV/electrospray ionization (ESI) – mass spectrometric (MS) investigations were performed on an Agilent 1100 (Palo Alto, USA) modular HPLC system coupled to a Varian 500 (Agilent, USA) ion trap (IT)-MS. The HPLC system was equipped with a degasser (G1379A), a binary pump (G1376A), a micro-well plate autosampler (G1229A) and a diode array detector (G1315B). The HPLC and the MS were controlled by the Chemstation B.02.01 and MS Workstation 6.6 softwares, respectively.

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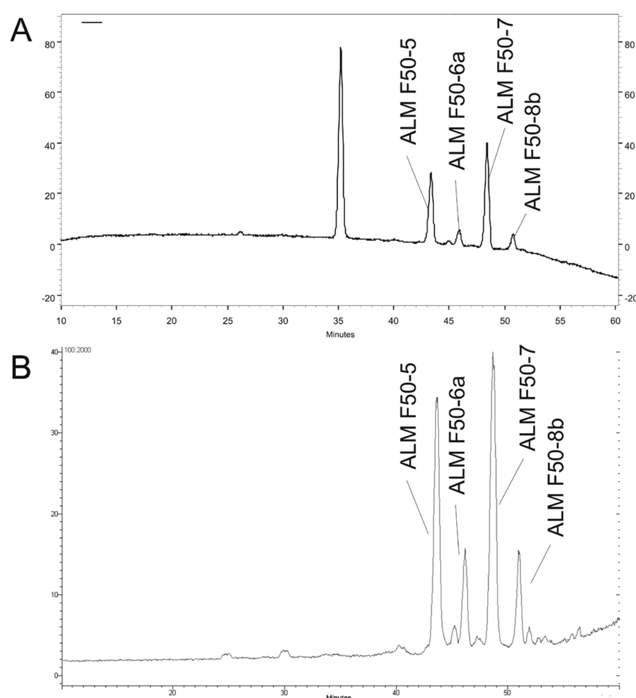

Figure 1. HPLC-UV (A) and HPLC-ESI-MS chromatogram of ALM components.

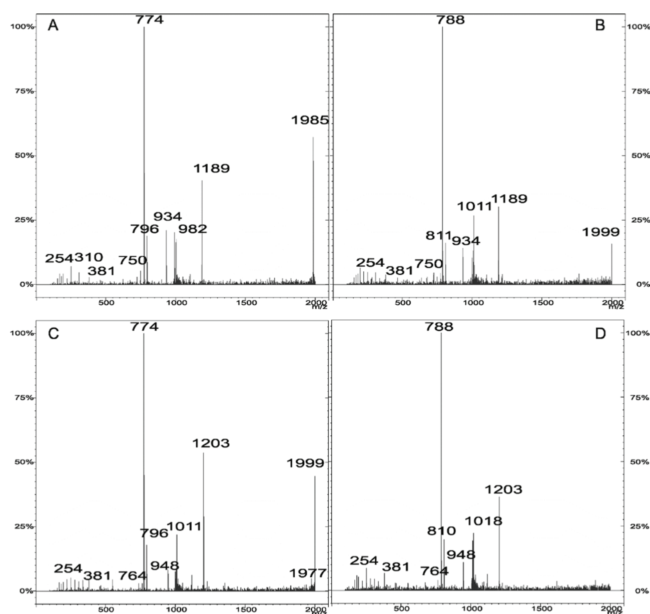

Figure 2. HPLC-ESI-MS spectra of ALM-F50-5 (A), ALM-F50-6a, ALM-F50-7 and ALM-F50-8b.

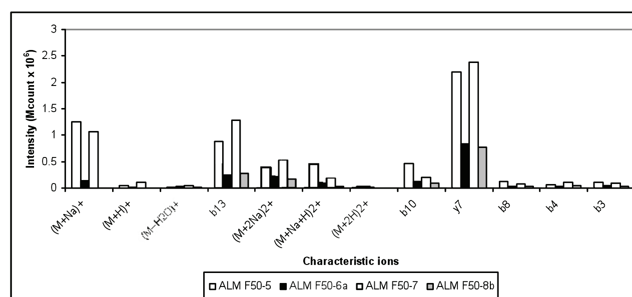

Figure 3. Abundances of the characteristic fragments of ALM components appearing on the HPLC-ESI-MS spectra.

HPLC parameters

The HPLC system solvent A was water with 0.05% (v/v) TFA, while solvent B was MeCN/MeOH 1/1 (v/v) with 0.05% (v/v) TFA. Separations were performed using a Phenomenex Gemini NX-C18 (Gen-Lab Kft., Hungary) (150 mm x 2.0 mm, 3 µm) HPLC column with mobile phase flow rate of 0.2 mL/min. The column temperature was maintained at 40°C using a Jones Model 7990 Space column heater (Jones Chromatography, UK). The length of the connecting capillaries (127 µm ID) between injector and column and column to MS nebulizer were mineralized by placing the column heater in optimal position. The gradient elution started with 65% B held for 5 min and increased linearly to 80% B at 45 min, and then to 100% B at 70 min, which was decreased to the initial percentage until the pressure stabilized. The injection volume was 5 µl.

Ion trap mass spectrometric conditions

The MS examinations were made with a Varian 500MS Ion Trap mass spectrometer equipped with an atmospheric-pressure ESI source in scan mode at normal scan speed. Positive and negative ESI Parameters were: spray chamber temperature, 50°C; drying gas (N_2) pressure and temperature, 30 psi and 350°C, respectively; nebulizer gas (N_2) pressure, 50 psi; needle voltage, 5704 V/-5000 V; spray shield voltage, 600 V/-600 V. General Parameters were: maximum scan times, 2.78; µScans averaged, 2 µScans; data rate, 0.36 Hz; multipier offset, 0. Ionization Control Parameters were: target TIC, 100%; max ion time, 250000 µsec. The full scan parameters were: capillary voltage, 66 V; RF loading, 147%; Low mass, 100 *m/z*; High mass, 2000 *m/z* high.

Result and Discussion

Initially, in order to optimize the ESI-IT-MS, the mass spectrometric parameters including RF loading, capillary- and needle voltages were tuned in both positive and negative modes by continuous infusion (5 µl/min) of an ALM standard solution (100 ng/µl) with the built-in syringe pump of the instrument. Based on the reported data the purchased standard mainly represents definitely the neutral ALM F50 peptides (Kirschbaum et al. 2003), thus during the optimization, the

Table 1. Identifications of characteristic ions (*m/z*) of ALM components acquired in positive mode.

	ALM components			
	5	6a	7	8b
R_t (min)	43.7	46.2	48.5	51.1
$(M+H)^+$	1963	1977	1977	1991
$(M+Na)^+$	1985	1999	1999	Out of range
$(M+2Na)^{2+}$	1004	1011	1011	1018
$(M+Na+H)^{2+}$	993	1000	1000	1007
$(M+2H)^{2+}$	982	989	989	996
$(M-H_2O)^+$	1945	1959	1959	1973
b_{13}	1189	1189	1203	1203
b_{10}	934	934	948	948
b_8	750	750	764	764
b_4	381	381	381	381
b_3	310	310	310	310
y_7	774	788	774	788

following protonated/deprotonated molecular ions ($(M+H)^+$/$(M-H)^-$) of microheterogenity mixture of ALM components were monitored in positive/negative mode: *m/z* 1963/1961 for ALM F50-5, *m/z* 1977/1975 for ALM F50-6a/6b/7 and *m/z* 1991/1989 for ALM F50-8b/8c. After the setup of ion optic parameters, the optimal ion source conditions such as nebulizer gas- and drying gas pressures and source temperatures were estimated via flow injection analysis in the mobile phase constitution 1/1 (v/v) of eluent A/B.

For further investigations, ALM was separated by HPLC on an analytical ODS column equivalent to the stationary phase used by Krause et al. (2006) as the general method for the peptaibol analysis, but other than applied by Kirschbaum et al. (2003) specifically for the ALM separation (Fig. 1).

Mass spectrometric measurements of peptaibols usually generate molecular ions and more or less complete series of characteristic fragment ions (Kirschbaum et al. 2003; Krause et al. 2006). However, in our case, the molecular ions showed much lower abundance due to their easily disintegration than the sodium adduct form in positive mode with exception of ALM-F50-8b falling its correspondent *m/z* value out of the examined mass range (Fig. 2, Fig. 3), which could make the identification of the unknown peptaibols difficult in our further screening studies. With the ESI negative mode ionization the $(M-H)^-$ masses were visible only with moderate intensity.

The most abundant ions were the y_7 and b_{13} fragment ions resulting from cleavage of the extremely labile Aib-Pro bond of the 20 residue peptides at position 6-7 from the carboxyl terminus in positive mode. Thus, the summation of the proper y and b ions could serve the mass of hydrogen adduct of the molecular ion. It is interesting that the double charged adduct ions showed high intensity in the used MS instrument such as $(M+2Na)^{2+}$ and $(M+Na+H)^{2+}$ (Fig. 2, Fig. 3), which was not observed earlier. Relatively high were the signals of the b_{10} fragments due to the break of the Aib-Gly bond, although their y_{10} counter-fragments were not detected. At the lower *m/z* values with lower intensity, but detectable level, the series of shorter b fragments were recorded including b_{10}, b_8, b_4 and b_3 ions (Fig. 2, Fig. 3). From the mass differences (Δm) of fragment ions, the presence of the marker amino acid Aib, characterized by *m/z* 85.1 Da (Krause et al. 2006) could not be deduced. The fragment ions and the acquired corresponding masses are shown in Table 1.

The resulting peaks were in accordance with the ALM components reported previously (Kirschbaum et al. 2003), eluted with proper resolutions (Fig. 1). Furthermore, based on the mass spectra acquired from certain peaks, the different components could be identified, some of which were denoted earlier only as mixture of isomers including ALM-F50-4b/5, ALM-F50-6a/6b and ALM-F50-8b/8c (Kirschbaum et al. 2003). In the case of ALM-F50-4b/5 eluted at 43.7 min, the characteristic ions of the 4b component were not presented regarding to the single charged and double charged sodium adducts at the *m/z* 1971 and 998, respectively (Fig. 2A). The peak of ALM-F50-6a/6b at 46.2 min contains also only the 6a component, because of the absence of *m/z* 930 (b_{10} fragment) regarding the ALM-F50-6b (Fig. 2B). Similarly, in the case of ALM-F50-8b/8c at 51.1 min, only the ALM-F50-8b was involved due to the lack of y_7 (*m/z* 744) and b_{10} (*m/z* 962) fragments originated from the fragmentation of ALM-F50-8c (Fig. 2D) while the ALM-F50-8c eluted at 48.5 min showing the highest peak area (Fig. 2C). There was a peak with a remarkable area on the UV chromatogram at approx. 35 min, but it was not active mass spectrometrically.

The results achieved in this study could contribute to the identification of other unknown peptaibol molecules isolated from ferment broth of different *Trichoderma* species based on the gathered data about the MS fragmentation properties, which are in the scope of our further research activities.

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