

Peptaibiotics: an advanced, rapid and selective analysis of peptaibiotics/peptaibols by SPE/LC-ES-MS

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Received August 5, 2005

Accepted October 1, 2005

Published online April 20, 2006; © Springer-Verlag 2006

Summary. “Proteomics” and “peptidomics” are used as technical terms to define the analysis and study of all proteins and peptides expressed in an organism or tissue. In analogy we propose the name *peptaibiotics* for the analysis of a group of fungal peptide antibiotics (peptaibiotics) containing the characteristic amino acid Aib (α -aminoisobutyric acid). In analogy to the peptidome the complete expression of peptaibiotics by fungal multienzyme complexes should be named the *peptaibome*.

Peptaibiotics are defined as peptides containing Aib and exerting a variety of bioactivities. They comprise the sub-groups of *N*-acetylated peptaibols, characterized also by a *C*-terminal amide-linked 2-amino alcohol, and lipopeptaibols having in place of an acetyl group a lipophilic fatty acid acyl group. Furthermore, lipoaminopeptides are also known with long-chain fatty acid on the *N*-termini, a lipoamino acid in position three and a strongly basic secondary or tertiary amine form a subgroup of mixed forms which could not be integrated in one of these three previously mentioned groups.

Here we present a specific and rapid screening method on the peptaibome applicable directly onto filamentous fungi cultured in a single Petri dish. The method comprises solid-phase extraction (SPE) of peptaibiotics followed by on-line reversed-phase HPLC coupled to an ion trap electrospray tandem mass spectrometer (ES-MS). The presence of these peptides is indicated by characteristic mass differences of $\Delta m = 85.1$ Da representing Aib-residues which can be observed in the *b*-series of acylium fragment ions resulting from ES-MS. Partial sequences can be deduced from the data and compared with structures compiled in electronic peptaibol data bases. The judgement is possible whether or not structures are novel, already known or related to known structures. Suitability of the method is demonstrated with the analysis of strains of *Trichoderma* and its teleomorph *Hypocrea*. New sequences of peptaibiotics are presented and those being related to established 10- to 18-residue peptaibols trichovirin, trichogin and trichotoxin, which have been described in the literature.

Keywords: α -Aminoisobutyric acid – Electrospray mass spectrometry – Fungal secondary metabolites – Peptide libraries – Solid-phase extraction

Introduction

The *proteome* is defined as the entirety of proteins expressed in an organism, tissue or a body fluid. The term “*proteomics*” is defined as the analytical methodology used to study these proteins (Baggermann et al., 2004;

Soloviev and Finch, 2005). In analogy to the *proteome* and the *peptidome* we define the *peptaibome* as the entire expression of fungal *peptides* containing the characteristic non-protein amino acid Aib. Accordingly, *peptaibiotics* is the analytical methodology for structural characterization of all *peptaibiotics* expressed in fungal cells.

A *peptaibiotic* is defined as fungal *peptide* containing Aib and exerting *antibiotic* or other bioactivities. The subgroup of *peptaibols* are *peptides* containing Aib and a 2-amino alcohol.

Peptide antibiotics of the group of peptaibiotics/peptaibols are fungal secondary metabolites characterized by the occurrence of the non-protein Aib and in many cases the additional presence of other α -alkylated amino acids like α -ethylalanine (isovaline, Iva) or in a single case of α -ethylnorvaline (Etnor).

These unique peptides are produced by a widespread group of filamentous fungi comprising soil and aquatic species, wood decaying, plant pathogenic, fungicolous and coprophilous species. Known members of this group consist of 5 up to 20 residues (Degenkolb et al., 2003).

The structural diversity of the peptaibiotics is caused by the varying amounts of protein and non-protein amino acids and varying substitution on *N*- and *C*-termini. The *N*-termini might be acetylated or acylated by a fatty acid of medium chain length; then the resulting peptaibols are terminated lipopeptaibols (Toniolo et al., 2001; Auvin-Guette et al., 1992). The *C*-termini of most peptaibiotics consists of an amide bound 2-amino alcohol like Pheol, Valol or Leuol or, less frequently, complex heterocyclic residues, an amino acid with free or methylated carboxy group or a sugar alcohol, amine, amide or diketopiperazine (Brückner et al., 1991a, b; Degenkolb et al., 2003).

Peptaibiotics exert a broad range of biological activities depending on their chain length and particular structural features. They have been described to exhibit antibacterial activity primarily against gram-positive bacteria (Leclerc et al., 2001; Dornberger et al., 1995; Gräfe et al., 1995), and display antifungal activity (Berg et al., 1996; Dornberger et al., 1995), antiviral activity (Kim et al., 2000; Yun et al., 2000), antimycoplasmic activities (Leclerc et al., 2001; Beven et al., 1998) and pigment induction in *Phoma destructiva* (Ritzau et al., 1997; Berg et al., 2003). Bio-activity includes also haemolysis of erythrocytes and leucocytes (Irmscher and Jung, 1977), insecticidal action on larvae (Matha et al., 1992; Bandani et al., 2001; Landreau et al., 2002), inhibition of the mitochondrial ATPase and uncoupling of oxidative phosphorylation in mitochondria (Krishna et al., 1990; Okuda et al., 1994; Gupta et al., 1991; Bullough et al., 1982). Furthermore, neuroleptic activities including induction of hypothermia in mice have been reported for ampullosporins and trichofumins (Ritzau et al., 1997; Kronen et al., 2001; Berg et al., 2003).

Amphiphilic peptaibols of sufficient chain-length are membrane-active polypeptides. They form voltage-dependent or independent ion channels or pores in natural and artificial bilayer membranes, and show, owing to the presence of Aib, high preference for α -helical conformations including 3_{10} -helical motifs (Boheim, 1974; Chugh and Wallace, 2001; Chugh et al., 2002; Whitmore and Wallace, 2004; Gessmann et al., 2003; Grigoriev et al., 2002, 2003; Duclohier et al., 2004; Jung et al., 2003).

Presently, the family of peptaibiotics comprises more than 300 sequences many of which are structurally closely related. Informations and overviews on structures and taxonomy of fungal producers of peptaibiotics are available from the Peptaibol Database (Whitmore et al., 2003) or databases such as Antibase 2.0 (Laatsch, 1997). Occurrence, structures and sequencing methods of peptaibiotics and peptaibols have been compiled in a review by Degenkolb et al. (2003).

The development of a method is required which (i) provides sufficient and reliable diagnostic information on peptaibiotic production and (ii) enables a rapid analysis and differentiation among new and already known peptaibiotics. This is of major interest because a still growing number of peptaibiotics is discovered.

In continuation of our work on screening fungi for the production of Aib-peptides (Brückner et al., 1991b; Jaworski and Brückner, 2001; Kirschbaum et al., 2004; Krause et al., 2005; Psurek et al., 2005), here we present a chromatographic screening method which enables the

rapid and sensitive detection and sequential characterization of this particular group of fungal peptides. The method is applicable directly onto filamentous fungi grown on single agar plates. The method comprises solid-phase extraction (SPE) followed by on-line reversed-phase high performance liquid chromatography (HPLC) coupled to electrospray ionization tandem mass spectrometry (ES-MS). Mass spectral data are acquired with an ion trap mass spectrometer operating in the positive ion mode. The presence of the marker amino acid Aib, detected by characteristic mass differences of fragment ions of $\Delta m = 85.1$ Da (Aib-H₂O), together with diagnostic ions from other constituents, confirm the biosynthesis of peptaibiotics. By comparing partial sequences deduced from the data with those compiled in databases the judgment is possible whether or not structures are novel, identical or related to structures already known. The method has been applied on species and strains of *Trichoderma* and its teleomorph *Hypocrea*. The peptaibiome, i.e. the entire expression of peptaibiotics might be also considered a natural peptaibiotic library (Jung, 1996).

Peptaibiomics will allow the determination of peptaibiotics biosynthesized by the multienzyme complexes (Raap et al., 2005) of a multitude of filamentous fungi grown on a single agar plate without time-consuming isolation and purification procedures. This method can be extended to analyze fungi growing in (sub)liquid media, or in natural habitats such as: soil, water, decaying organic material, dung, faeces, or as parasites of plants, mushrooms, toadstools and insects.

Peptaibiomics comprises growth of fungi on suitable media, solid-phase extraction of the entirety of peptaibiotics, HPLC profiling and complete or partial ES-MS/MS sequencing of peptaibiotics, and finally sequence matching using data bases.

Materials and methods

Chemicals

Acetonitrile (MeCN) and methanol (MeOH) (all of gradient grade quality) were purchased from Merck (Darmstadt, Germany); dichloromethane (DCM) was from Carl Roth (Karlsruhe, Germany); trifluoroacetic acid (TFA) was obtained from Fluka (Deisenhofen, Germany).

Culture of fungi

Fungi were supplied as growing cultures or lyophilized spores from the culture collections CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) and BCCM/MUCL (The Belgian Co-ordinated Collections of Micro-organisms/(Agro) Industrial Fungi & Yeasts Collection, Louvain-la-Neuve, Belgium). The filamentous fungi investigated are listed in Table 1.

Table 1. Filamentous fungi screened for peptaibiotic production

Filamentous fungi	Culture collection number
<i>Trichoderma asperellum</i> Samuels, Lieckfeldt & Nirenberg	CBS 433.97
<i>Trichoderma inhamatum</i> Veerkamp & W. Gams	CBS 345.96
<i>Trichoderma aggressivum</i> f. <i>europaeum</i> Samuels & W. Gams	CBS 100526
<i>Trichoderma stromaticum</i> Samuels & Pardo-Schultheiss	CBS 101875
<i>Hypocrea semiorbis</i> Berkeley	CBS 244.63
<i>Hypocrea vinosa</i> Cooke	CBS 247.63
<i>Hypocrea dichromospora</i> Yoshim. Doi	CBS 337.69
<i>Hypocrea gelatinosa</i> (Tode: Fries) Fries	CBS 724.87
<i>Hypocrea nigricans</i> (Imai) Doi	MUCL 28439
<i>Hypocrea muroiana</i> Hino & Katsumoto	MUCL 28442
<i>Hypocrea lactea</i> (Fries: Fries) Fries	CBS 853.70

Malt-extract agar was prepared by dissolving 30.0 g malt extract (Oxoid, Wesel, Germany), 3.0 g soy peptone (Oxoid) and 15.0 g agar (Fluka) in 1 l demineralized water (final pH 6–6.5), followed by sterilization for 20 min at 121 °C.

Freeze dried cultures were suspended in sterile water (1.5 ml) and, after soaking for 15 min, transferred to Petri dishes (9.5 cm diameter) with malt extract agar. This medium was also used for storage of growing moulds at 4 °C in the refrigerator. For analyses the moulds were transferred under sterile conditions to three Petri dishes with malt-extract agar and were grown for 7 days under daylight at ambient temperature.

Solid phase extraction of peptaibiotics

Fungal cultures were grown for seven days on malt-extract agar in Petri-dishes of 95 mm diameter. Extraction was performed with three 5 ml portions of a mixture of MeOH and DCM (1/1, *v/v*). Extracts were combined, centrifuged at 3500 rpm for 15 min and the supernatant was evaporated to dryness using a rotary evaporator. Amounts of 10 ml MeOH/H₂O (1/2, *v/v*) were added and the resulting solution was passed with the aid of a syringe through a conditioned Sep-Pak C-18 cartridge, (1.5 cm × 1.0 cm I.D.; particle size 80 µm; Waters, Milford, MA). Conditioning was performed by washing successively with 10 ml portions of MeOH, H₂O and MeOH/H₂O (1/2, *v/v*). Then the cartridge was washed with 10 ml water and 10 ml MeOH/H₂O (1/2, *v/v*).

Finally, peptides adsorbed were eluted with 10 ml MeOH (100%). The effluent was collected in a tarred round-bottomed flask and evaporated to dryness using a rotary evaporator. The weight of the re-

maining residue was determined gravimetrically and MeOH was added in order to yield an about 1% solution (*w/v*). Aliquots of 10 µl were analyzed by HPLC-ESI-MS.

Testing of the efficiency of SPE

The efficiency and completeness of the SPE procedure described above was proved with test solutions of the 11- to 20-residue peptaibols anti-amoebin (Jaworski and Brückner, 2000), paracelsin (Brückner et al., 1984), alamethicin F50 and alamethicin F30 (Kirschbaum et al., 2003), trichobrachin (Brückner et al., 1993) and acretocin (Kirschbaum et al., 2004). Amounts of 1.0 mg of the peptaibols were dissolved in 1 ml MeOH, then 2 ml H₂O were added and the resulting solutions were applied to the Sep-Pak cartridges. Then the cartridges were washed with each 10 ml of H₂O and MeOH/H₂O (1/2, *v/v*), then the peptaibols eluted with 10 ml MeOH. Each step was analyzed by HPLC. The sum of the peak areas of the respective peptaibol before and after treatment with Sep-Pak cartridges were determined and compared. Under the conditions of the controlled washing procedure of the cartridge with water and the MeOH/H₂O mixture, no peptaibiotic eluted. Complete elution was accomplished with 100% MeOH (10 ml).

Chromatography

For analytical HPLC a HP 1100 series instrument comprising a Model G1322A degasser, G1312A binary pump, G1313A autosampler, G1316A column thermostat, G1314A UV/VIS detector, and software HP Chem Station for LC (Rev. A.04.02) were used (all from Agilent, Waldbronn, Germany or Palo Alto, CA, USA). Analysis of the extracts by HPLC was performed on a Kromasil KR100 column, 150 mm × 4.6 mm i.d., 3.5 µm particle size (EKA Chemicals, Bohus, Sweden) using a binary gradient (see Table 2) at a detector wavelength of 205 nm. Eluent A consisted of MeCN/MeOH/water (32/32/36, *v/v/v*), and eluent B consisted of MeCN/MeOH (1/1, *v/v*); to both eluents 0.1% TFA (*v/v*) was added. The column temperature was set on 35 °C. For gradient program and flow rate see Table 2.

Mass spectrometry

For ES-MS a LCQTM MS (Thermo Finnigan MAT, San Jose, CA, USA) was used. Extracts were analyzed by online HPLC-MS. Nitrogen was used as sheath and auxiliary gas and helium (purity >99.9990%, Messer-Griesheim, Krefeld, Germany) as collision gas. Sequence analysis was carried out in the positive ionization mode. The *m/z* values were recorded in centroid mode and have an accuracy of ±0.5 Da. Conditions for positive ionization mode were: spray voltage 4.00 kV, heated capillary temperature 230 °C, capillary voltage +3.0 V, tube lens offset +30.0 V, sheath gas 50 units, auxiliary gas 5 units, maximum ion time 1000 ms. For online HPLC-MS the temperature of the heated capillary was set to 250 °C, sheath gas to 65 units, auxiliary gas to 20 units. For automatic mass calibration a mixture was used comprising caffeine (*m/z* 195.1), Met-Arg-Phe-Ala (*m/z* 524.3) and the

Table 2. Gradient program for analytical HPLC separation

Time (min)	Eluent A (%)	Eluent B (%)	Flow rate (ml/min)	Time (min)	Eluent A (%)	Eluent B (%)	Flow rate (ml/min)
0	100	0	0.8	75	0	100	1.0
5	100	0	0.8	76	100	0	1.0
45	50	50	0.8	85	100	0	1.0
65	0	100	0.8				

perfluorinated Ultramark 1621 (m/z 1022.0, 1122.0, 1222.0, 1322.0, 1422.0, 1522.0, 1622.0, 1722.0, 1822.0, 1921.9).

A collision induced dissociation (CID) energy of 0% was used for scanning of molecular masses and fragments resulting from cleavage of the extremely labile Aib-Pro bond. A CID energy of 45% was used for generating series of characteristic fragment ions.

Results and discussion

Use of the Kromasil KR100 column together with the gradient system consisting of MeOH, ACN and water provided best separations for short-chain (11–16 residues) and long-chain (17–20 residues) peptaibiotics of varying lipophilicity.

The purified peptides resulting from SPE were analyzed using HPLC-ES-MS. Molecular ions and more or less complete series of characteristic fragment ions were generated. From the mass differences (Δm) of fragment ions the presence of the marker amino acid Aib, characterized

by $\Delta m = 85.1$ Da, as well as other peptide constituents could be deduced. If mass fragments could not be assigned unambiguously only the mass differences were registered.

The isomeric amino acids in extracts cannot be distinguished by HPLC-ESI-MS. Therefore Val and Iva are denoted Vxx, and Leu and Ile designated as Lxx in partial sequences of peptides shown in Figs. 1 and 3.

The mass differences of $\Delta m = 213$ Da in many partial sequences presented in Figs. 1 and 3 result from the Gln-Aib bonds which are particularly stable under the fragmentation conditions of ES-MS (Jaworski et al., 1999; Kirschbaum et al., 2003).

The observed fragment ions of $\Delta m = 197$ Da of the protonated dipeptide Pro-Vxx and of $\Delta m = 211$ Da of Pro-Lxx result from the cleavage of the C-terminal prolyl peptide (Figs. 1 and 3). The particular lability of the Aib(Iva)-Pro bond has been recognized previously (Jaworski et al., 1999; Jaworski and Brückner 2000, 2001) and is of diagnostic value.

The peptaibome of *Trichoderma* species

In all extracts of the *Trichoderma* strains analyzed characteristic mass differences of $\Delta m = 85.1$ Da could be detected by ES-MS and partial sequences of peptaibiotics could be assigned.

Sequences resulting from analyses of *Trichoderma asperellum*, *Tr. inhamatum*, *Tr. aggressivum* f. *europaeum* and *Tr. stromaticum* are compiled in Fig. 1. Comparison of these sequences with those of peptaibiotics compiled in the Peptaibol Database (Whitmore et al., 2003) provided novel and already known structures.

The HPLC elution profile of the purified extract of *Trichoderma asperellum* (CBS 433.97) and the MS (CID 45%) of the peptide eluting at 54.5 min is shown as an example in Fig. 2.

Sequence no. 7 from *Tr. asperellum* might be identical with trichotoxin A50I from *Trichoderma viride*, strain NRRL 5242 (Przybylski et al., 1984). For sequences see Fig. 5. Sequences no. 3 to 6 are most likely new analogues of the trichotoxin A50 group.

The partial sequence no. 4 of *Tr. aggressivum* f. *europaeum* is similar to hypomurocin BI (Fig. 5) isolated from *Hypocrea muroiana* Hino et Katsumoto (Becker et al., 1997). The fragment ion of $\Delta m = 142$ Da indicates the presence of Gly-Aib. Sequences no. 5 and 6 are considered to be new analogues of hypomurocin B.

Peptides nos. 3 to 7 of *Tr. inhamatum* correspond to partial sequences of trichovirins (Jaworski et al., 1999).

<i>Trichoderma asperellum</i> (CBS 433.97)		MW
1	[227]-Aib-Val-Aib-Ala-Ser-[70]	809
2	[227]-Aib-Lxx-Aib-Ala-Aib-[86]	837
3	[270]-Lxx-Aib-213-Aib-Ala-Aib-Aib-210-Aib-Aib-Gln-Valol	1689
4	[270]-Lxx-Aib-213-Aib-Ala-Aib-Ala-Aib-210-Aib-Aib-Gln-Valol	1689
5	[270]-Lxx-Aib-213-Aib-Aib-Ser-Aib-Aib-210-Aib-Vxx-Gln-Valol	1733
6	[270]-Lxx-Aib-213-Aib-Aib-Ala-Aib-Aib-210-Aib-Aib-Gln-Valol	1703
7	[270]-Lxx-Aib-213-Aib-Aib-Ala-Aib-Aib-210-Aib-Vxx-Gln-Valol	1717
<i>Trichoderma aggressivum</i> f. <i>europaeum</i> (CBS 100526)		MW
1	[184]-Gln-Lxx-Aib-Aib-Leuol	796
2	[184]-Gln-Lxx-Aib-Ser-Lxx-Leuol	826
3	[184]-Gln-Lxx-Aib-Ala-Lxx-Leuol	810
4	[286]-Lxx-Aib-213-Vxx-Aib-142-Aib-210-Aib-Aib-Gln-Valol	1719
5	[286]-Lxx-Aib-213-Gly-Aib-Lxx-Aib-Aib-210-Aib-Aib-Gln-Valol	1733
6	[286]-Lxx-Vxx-Gln-Aib-170-Ala-Vxx-Aib-210-Aib-Aib-Gln-Valol	1747
<i>Trichoderma inhamatum</i> (CBS 345.96)		MW
1	[242]-Lxx-Aib-Lxx-Aib-Aib-242-Aib-210-Aib-Aib-Gln-Lxxol	1675
2	[256]-Lxx-Aib-Lxx-[579]	1146
3	[185]-Ala-Aib-Aib-Gln-Vxx-Vxx-Aib-Ala-Aib-Aib-210-Aib-Aib-Gln-Lxxol	1703
4	[185]-Ala-Vxx-Aib-Gln-Vxx-Vxx-Aib-Ala-Aib-Aib-210-Aib-Aib-Gln-Lxxol	1717
5	[185]-Ala-Aib-Aib-Gln-Vxx-Vxx-Aib-Ala-Vxx-Aib-210-Aib-Aib-Gln-Lxxol	1717
6	[256]-Vxx-Aib-Gln-Vxx-Vxx-Aib-Ala-Vxx-Aib-210-Aib-Aib-Gln-Lxxol	1731
7	[185]-Ala-Vxx-Aib-Gln-Vxx-Vxx-Aib-Ala-Vxx-Aib-210-Aib-Aib-Gln-Lxxol	1731
<i>Trichoderma stromaticum</i> (CBS 101875)		MW
1	[184]-Ala-Lxx-Aib-Ser-Lxx-Vxx-Ala-Vxx-[616]	1538
2	[368]-Vxx-Ala-213-Vxx-299-210-Aib-Aib-Gln-Lxxol	1774
3	[284]-Ala-Vxx-Vxx-213-Vxx-Aib-213-210-Aib-Aib-Gln-Lxxol	1788
4	[284]-Ala-Vxx-Vxx-213-Vxx-Aib-213-210-Aib-Vxx-Gln-Lxxol	1802

Fig. 1. Examples of partial sequences from *Trichoderma* strains screened for peptaibiotics; abbreviations according to the standard three-letter code; Aib α -aminoisobutyric acid; Lxx Leu or Ile; Vxx Val or Iva (isovaline); Leuol leucinol; Valol valinol; Lxxol leucinol or isoleucinol; MW molecular weight. Fragment ions, the structure of which could not be assigned, are put in squared brackets. Chirality of constituents (exception Iva) was determined by enantioselective GC-MS according to procedures described (Pätzold and Brückner, 2005)

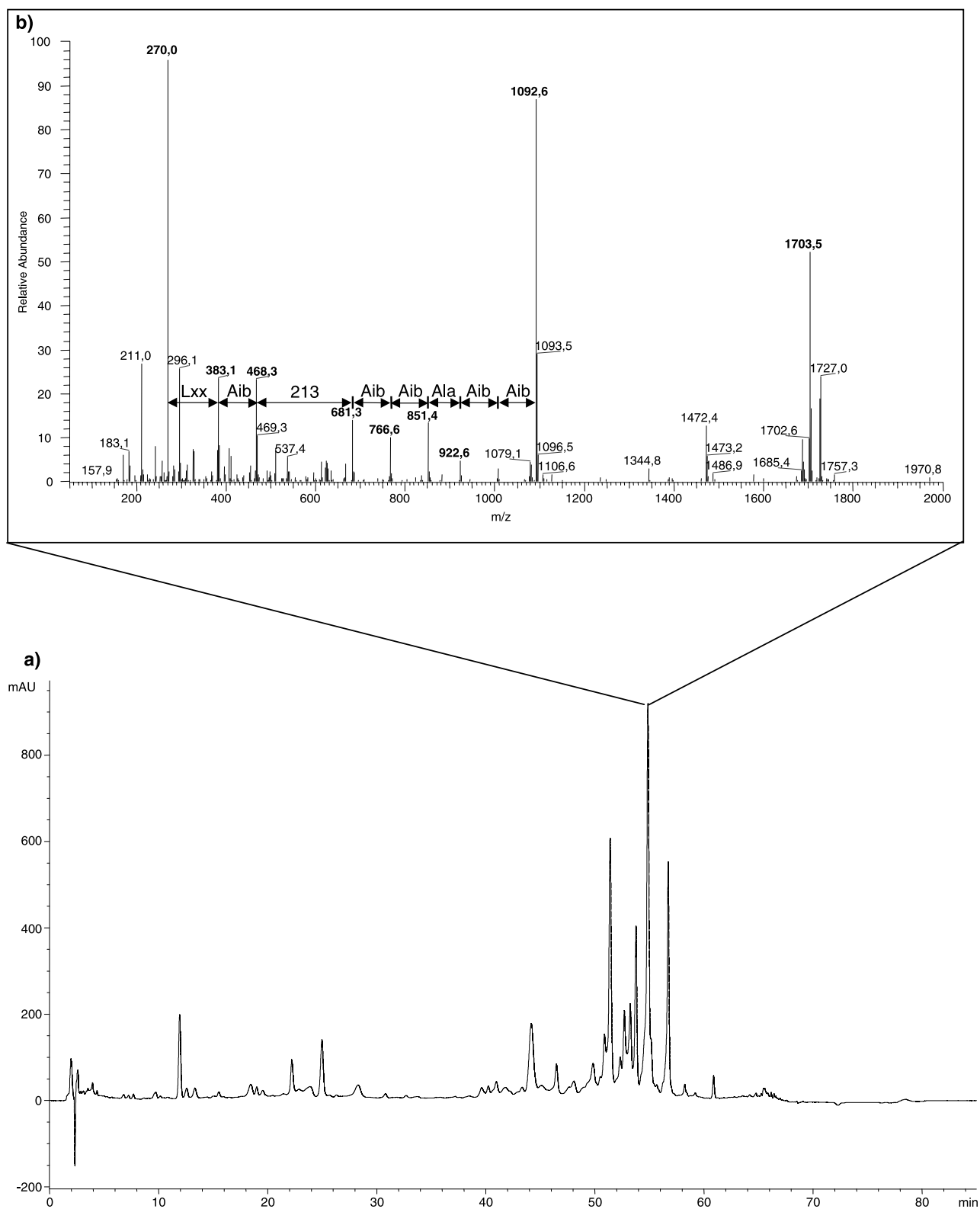


Fig. 2. **a** Analytical HPLC of the purified mycelial extract of *Trichoderma asperellum* after clean-up with Sep-Pak cartridge, **b** fragment ions resulting from ES-MS of the peptide eluting at 54.5 min. Characteristic masses are in bold characters. The partial sequence deduced therefrom is shown in Fig. 1 (sequence no. 6). For chromatographic conditions see Materials and methods

<i>Hypocrea muroiana</i> MUCL 28442	
1	[291]-Phe-Aib-Lxx-Aib-Lxx-[186]
2	[199]-Ala-Aib-Aib-213-Aib-Aib-Ser-Aib-Lxx-[842]
3	[199]-Ala-Aib-Aib-213-Aib-Aib-Ser-Vxx-Vxx-[816]
4	[199]-Ala-Aib-Aib-213-Aib-Aib-Ser-Aib-Lxx-196-Aib-Lxx-Gln-Gln-Pheol
<i>Hypocrea nigricans</i> MUCL 28439	
1	[157]-Vxx-Vxx-Vxx-Aib-[623]
2	[270]-Vxx-Aib-Lxx-[623]
<i>Hypocrea gelatinosa</i> CBS 724.87	
1	[157]-Vxx-Lxx-Lxx-Aib-Vxx-[510]
2	[142]-Gln-Lxx-Lxx-Aib-[623]
<i>Hypocrea dichromospora</i> CBS 337.69	
1	[184]-Ala-Vxx-Aib-Aib-Aib-Leuol
2	[184]-Lxx-Aib-Gly-Lxx-Leuol
3	[184]-Ala-Lxx-Aib-Ala-Lxx-Leuol
4	[255]-Lxx-Aib-Gly-Lxx-Vxx-[247]
<i>Hypocrea vinosa</i> CBS 247.63	
1	[212]-Gly-Vxx-Aib-Gly-Gly-Vxx-Aib-Gly-Lxx-Leuol
2	[212]-Gly-Lxx-Aib-Gly-Gly-Vxx-Aib-Gly-Lxx-Leuol
3	[212]-Gly-Vxx-Aib-Gly-Gly-Lxx-Aib-Gly-Lxx-Leuol
4	[212]-Gly-Lxx-Aib-Gly-Gly-Lxx-Aib-Gly-Lxx-Leuol
<i>Hypocrea semiorbis</i> CBS 244.63	
1	[284]-Ala-Aib-Ala-213-Aib-Aib-Leu-Gly-Aib-[788]
2	[284]-Ala-Aib-Ala-213-Vxx-Aib-Aib-Gly-Leu-[774]
3	[284]-Ala-Aib-Aib-213-Vxx-Aib-Aib-Aib-[774]
4	[284]-Ala-Aib-Aib-213-Ala-Leu-Aib-Gly-Leu-[788]
<i>Hypocrea lactea</i> CBS 853.70	
1	[212]-Gly-Lxx-Aib-Gly-Gly-Vxx-Aib-Gly-Vxx-Leuol
2	[212]-Gly-Vxx-Aib-Gly-Gly-Lxx-Aib-Gly-Vxx-Leuol
3	[212]-Gly-Vxx-Aib-Gly-Gly-Lxx-Aib-Gly-Lxx-Leuol
4	[212]-Gly-Lxx-Aib-Gly-Gly-Lxx-Aib-Gly-Lxx-Leuol

Fig. 3. Examples of partial sequences from *Hypocrea* strains screened for peptaibiotics; abbreviations according to the standard three-letter code; *Aib* α -aminoisobutyric acid; *Lxx* Leu or Ile; *Vxx* Val or Iva (isovaline); *Leuol* leucinol; *Pheol* phenylalaninol; *MW* molecular weight. Fragment ions, the structure of which could not be assigned, are put in squared brackets. Chirality of constituents (exception Iva) was determined by enantioselective GC-MS according to procedures described (Pätzold and Brückner, 2005)

Amino acid exchanges could be located in position nos. 4 (Leu) and 10 (Gly).

Other partial sequences resulting from analyses of *Tr. aggressivum* f. *europaeum*, *Tr. inhamatum*, *Tr. asperellum* and all sequences identified from *Tr. stromaticum* did not correspond with structures compiled in databases. Thus, the fungi are producers of novel microheterogeneous peptaibiotics.

The peptaibiome of Hypocrea species

In extracts of the filamentous fungi *Hypocrea semiorbis*, *Hyp. vinosa*, *Hyp. dichromospora*, *Hyp. gelatinosa*, *Hyp.*

nigricans, *Hyp. muroiana* and *Hyp. lactea* peptides containing Aib could be detected. Partial sequences of these peptides could be deduced from series of fragment ions. Examples of partial sequences of peptaibiotics are shown in Fig. 3.

The HPLC elution profile of the purified extract of *Hypocrea muroiana* and the MS (CID 45%) of the peptide eluting at 51.7 min is presented in Fig. 4.

Hypocrea vinosa and *Hyp. lactea* produce peptaibols, which are considered to be identical with trichogin GA IV and trikoningin KB I or to be novel analogues of these peptaibols. Sequences no. 4 from *Hyp. vinosa* and no. 4 from *Hyp. lactea* are identical with trichogin GA IV isolated from *Tr. longibrachiatum* (Auvin-Guette et al., 1992) and the partial sequence no. 1 from *Hyp. vinosa* might be trikoningin KBI from *Tr. koningii* (Auvin-Guette et al., 1993). For comparison of sequences see Fig. 5.

For the other fragments and partial sequences shown in Fig. 3 no similarity could be validated by comparison with sequences stored in databases. Consequently, the sequences determined are assumed to be represent new structures of the peptaibol family.

Conclusions

A method named peptaibiomics is presented aimed on the complete analysis of all peptides containing Aib (i.e. peptaibiotics) from growing fungal cultures on agar plates. The method might be extended on solid-phase substrates or material from the natural habitats.

Factors such as composition of growth medium (chemically defined or complex medium), physico-chemical parameters such as temperature, light or radiation, aeration, stress factors etc. governing the peptaibiome can be investigated and correlated with sequences of peptaibiotics.

Extracts from a single Petri dish applied to SPE and LC-ES-MS provide sufficient diagnostic information on the entire of peptaibiotics production. Using characteristic *N*- and *C*-terminal mass fragments, among peptaibiotics and subgroups such as peptaibols, lipopeptaibols or lipopeptides can be distinguished. Moreover, as structures of almost 300 peptaibiotics are known, the use of peptaibiomics will enable a decision whether or not structures are novel or related or identical to already known structures.

The peptaibiome might also be an important tool for establishing or supporting taxonomic species concepts in fungi (Druzhinina and Kubicek, 2005).

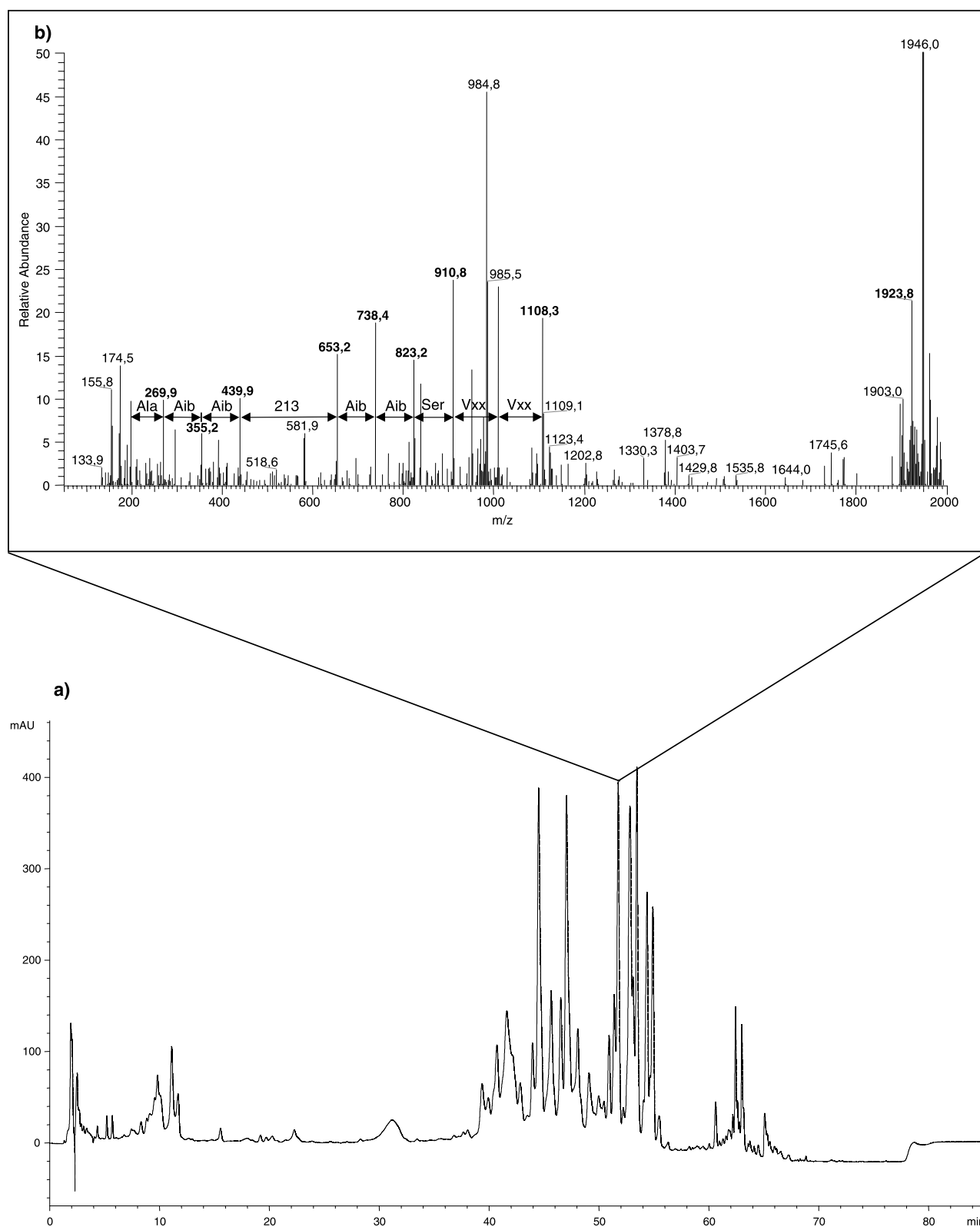


Fig. 4. **a** Analytical HPLC of the purified mycelial extract of *Hypocrea muroiana* after clean-up with Sep-Pak cartridge, **b** fragment ions resulting from ES-MS of the peptide eluting at 51.7 min. Characteristic masses are in bold characters. The partial sequence deduced therefrom is shown in Fig. 3 (sequence no. 3). For chromatographic conditions see Materials and methods

Trichotoxin A50I	Ac Aib Gly Aib Leu Aib Gln Aib Aib Aib Ala Aib Aib Pro Leu Aib Iva Gln Valol
<i>Tr. asperellum</i> sequence 7	[270] Lxx Aib [213] Aib Aib Ala Aib Aib [210] Aib Vxx Gln Valol
Hypomurocin BI	Ac Aib Ser Ala Leu Aib Gln Aib Val Aib Gly Aib Aib Pro Leu Aib Aib Gln Valol
<i>Tr. aggressivum</i> f. <i>europaeum</i> sequence 4	[286] Lxx Aib [213] Vxx Aib [142] Aib [210] Aib Aib Gln Valol
Trichogin GA IV	Oc Aib Gly Leu Aib Gly Gly Leu Aib Gly Ile Leuol
<i>Hyp. vinosa</i> sequence 4	[212] Gly Lxx Aib Gly Gly Lxx Aib Gly Lxx Leuol
<i>Hyp. lactea</i> sequence 4	[212] Gly Lxx Aib Gly Gly Lxx Aib Gly Lxx Leuol
Trichokoningin KBI	Oc Aib Gly Val Aib Gly Gly Val Aib Gly Ile Leuol
<i>Hyp. vinosa</i> sequence 1	[212] Gly Vxx Aib Gly Gly Vxx Aib Gly Lxx Leuol

Fig. 5. Comparison of sequences of previously described peptaibiotics and sequence data resulting from SPE/HPLC-ES-MS. For abbreviations see Figs. 1 and 3; Ac acetyl; Oc *n*-octanoyl

Acknowledgements

We thank our students Susanne Skrbek and Sabine Kempff, who contributed to parts of the experimental work. We also thank Dr. Thomas Degenkolb for valuable comments.

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