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Use of α-aminoisobutyric acid and isovaline as marker amino acids for the detection of fungal polypeptide antibiotics. Screening of *Hypocrea*

H. Brückner, J. Maisch, C. Reinecke, and **A. Kimonyo**

Institute of Food Technology, University of Hohenheim, Stuttgart, Federal Republic of Germany

Summary. Filamentous fungi of the genus *Hypocrea* were grown on malt extract/peptone agar, the mycelia were extracted with dichloromethane/methanol, and the extracts were totally hydrolyzed with 6 N HCl (110°C, 24 h). The amino acids (AA) released from peptides were converted into their *N(O)*-pentafluoropropionyl 1-propyl esters and investigated by gas chromatography and gas chromatography/mass spectrometry for the presence of the nonprotein AA α-aminoisobutyric acid (Aib) and its homologue isovaline (Iva). In particular Aib served as specific marker compound for a particular group of fungal peptides named peptaibiotics, *i.e.* peptides containing Aib and having antibiotic activities. Screening of 24 species of *Hypocrea* revealed that the majority was capable of producing peptaibiotics. The reliability of the screening procedure was shown with the isolation of peptaibiotics from *Hypocrea muroiana* and *Hypocrea nigricans*. These findings extend the list of genera of fungi already known to produce Aib-containing peptides and also establish that Aib and Iva are fairly common in the biosphere.

Keywords: Amino acids – Nonprotein amino acids – α-Aminoisobutyric acid – Isovaline – Screening procedure – Gas chromatography – Polypeptide antibiotics (peptaibiotics) – Filamentous fungi

Introduction

It has been shown that various genera of moulds such as *Gliocladium*, *Trichoderma*, and *Stilbella* are capable of producing a group of polypeptide antibiotics which contain, together with protein amino acids (AA), relatively high amounts of the nonprotein α-aminoisobutyric acid, $H_2NC(CH_3)_2COOH$ (2-methylalanine, Aib) and, in many cases, isovaline, $H_2NC(CH_3)(C_2H_5)COOH$ (2-ethylalanine, Iva) (Dennis and Webster, 1971; Brückner and Przybylski,

1984; Przybylski et al., 1984; Brückner and Reinecke, 1988; Brückner et al., 1989; Brückner et al., 1989a).

It has been suggested, therefore, to name the peptides containing Aib and a C-terminal bonded phenylalaninol (Phol), or another amino alcohol, as peptaibophols or peptaibols, respectively (Pandey et al., 1977; Brückner and Reinecke, 1988). As very recently Aib-peptides have been characterized lacking the amino alcohol the comprehensive name peptaibiotics has been suggested (*i.e.* peptides containing Aib and having antibiotic activities) which include peptaibophols and peptaibols as special cases (Brückner et al., 1991). As some of the peptaibiotics cause lysis of mammalian cells (Jung et al., 1981) they might also be considered as polypeptide mycotoxins (Brückner et al., 1987). Typical sequences belonging to these groups of peptide antibiotics are exemplified with selected structures of peptides of the Trichobrachin (TB) and Trichovirin (TV) complex. **TB I B:** Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Iva-Gln-Gln-OH; **TB IIa B:** Ac-Aib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-OH, **TB IIb B:** Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Iva-Gln-Gln-Pheol; **TV I 4A:** Ac-Aib-Asn-Leu-Aib-Pro-Ala-Val-Aib-Pro-Aib-Leu-Aib-Pro-Leuol (Ac, acetyl; Leuol, leucinol; for other abbreviations see text) (Brückner et al., 1991). Application of the gas chromatographic screening procedure to *Hypocrea* revealed that most species and strains of this genus are able to produce Aib-containing peptides.

Materials and methods

Species and strains of *Hypocrea* were grown in petri dishes (9.5 cm diam.) for 7–14 d on malt extract/peptone agar [comprising 30 g malt extract (Serva, Heidelberg), 3 g soy peptone (Oxoid, Wesel), and 15 g agar (Merck, Darmstadt) in 1 l of water]. The mycelia were extracted with two 5 ml portions of 1:1 (v/v) dichloromethane/methanol, the extracts were evaporated to dryness, totally hydrolyzed by 6 N HCl (110°C, 24 h), and the hydrolysates investigated for the presence of the marker AA Aib and Iva (if present) by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). The configuration of Iva was determined by GC, Brückner and Langer (1991), and the use of synthetic D- and L-Iva as references.

For GC a Carlo Erba gas chromatograph Model HRGC 5160 was used equipped with a 25 m × 0.25 mm I.D. Chirasil-L-Val capillary column (Macherey-Nagel, Düren, Germany) and a flame ionization detector. GC/MS was carried out on a 25 m × 0.25 mm I.D. SE-30 capillary column (Macherey-Nagel) connected to a Varian MAT 44S mass spectrometer, electron impact mass spectra were recorded at an ionization energy of 70 eV. AA in hydrolysates of mycelial extracts were converted into pentafluoropropionyl 1-propyl esters by treatment with acetyl chloride in 1-propanol and, subsequently, pentafluoropropionic anhydride, Brückner and Reinecke (1988). *Hypocrea muroiana* IFO 31288 and *Hypocrea nigricans* IFO 31285 (cf. Table 1) were fermented in twentyfive 400 ml shake flasks each containing 200 ml of a sterilized medium [made up of 30 g malt extract, quality "light", and 3 g soy peptone (Serva) dissolved in 1 l of distilled water] with shaking on a rotary shaker (incubator shaker G 25, New Brunswick, Edison, USA) at 100 rpm and 25°C for 7 days. The microheterogeneous peptide mixtures were isolated from the filtered culture broths by XAD- and Sephadex LH-20 chromatography, purified by HPLC using standard procedures (Brückner and Przybylski, 1984), and, after total hydrolysis, investigated by GC.

Antibiotic activities of the peptide mixtures from *H. muroiana* and *H. nigricans* were tested against *Bacillus subtilis* ATCC 6633 (Difco Biotest Laboratories, Detroit, Mich., USA) grown on "standard 1—agar" (no 7881, Merck). Paper discs (6 mm diam.) were

soaked with 1% methanolic solutions of the respective peptide mixtures and agar plates with *B. subtilis* were incubated for 12 h at 4°C, followed by 24 h at 30°C, and then the inhibition zones were measured. The peptaibiotic Paracelsin (Brückner et al., 1984) was used as standard.

Results and discussion

The results of the screening of 24 species of *Hypocrea* for Aib and Iva are summarized in Table 1. As can be seen the majority of species of *Hypocrea* are capable of producing peptides containing Aib as well as, in most cases, Iva. Exceptions are *H. lutea*, *H. pulvinata*, and *H. splendens* of which single species have been examined. However, since in the case of *H. rufa* two strains were Aib-positive and one negative it is likely that Aib-positive strains of the above mentioned species will also be found. The gas chromatogram (GC) of *N*-pentafluoropropionyl (PFP) 1-propyl esters of amino acids from a total

Table 1. Screening of *Hypocrea* for the production of peptides containing Aib and Iva

Hypocrea species	Culture collection number		Marker amino acid Aib	Iva
H. albocornea Doi	IFO	30608	+	−
H. albofulva Berkeley & Broome	IFO	30609	+	−
H. ascoboloides Rehm	ICMP	1692	+	+
H. atrogelatinosa Dingley	ICMP	5429	++	−
H. cerebriformis Berkeley	IFO	30610	++	+
H. coprosma Dingley	ICMP	5543	++	n.d.
H. dichromospora Doi	IFO	8997	+	+
H. gelatinosa (Tode: Fries) Fries	ICMP	5417	+	+
H. lactea Fries	IFO	8434	+	+
H. lutea (Tode) Petch	IFO	9061	−	−
H. muroiana Hino & Katsumoto	IFO	31288	++	+
H. nigricans (Imai) Doi	IFO	31285	++	+
H. pachybasioides Doi	IFO	9464	+	+
H. pulvinata Fuckel	IFO	9385	−	−
H. rufa (Persoon ex Fries) Fries	IMI	90312	+	+
H. rufa (Persoon ex Fries) Fries	IMI	131883	−	−
H. rufa (Persoon ex Fries) Fries	ICMP	1697	+	n.d.
H. schweinitzii (Fries) Saccardo	IFO	9063	++	+
H. schweinitzii (Fries) Saccardo	ICMP	5421	+	n.d.
H. semiorbis Berkeley	ICMP	1693	+	n.d.
H. spinulosa Fuckel	IFO	9064	++	+
H. splendens Phillips & Plowright	IFO	7711	−	−
H. sublutea Doi	IFO	30156	+	+
H. vinosa Cooke	ICMP	5411	+	n.d.

Abbreviations: *IFO*, Institute for Fermentation, Osaka, Japan; *ICMP*, International Collection of Micro-Organisms for Plants, Auckland, New Zealand; *IMI*, International Mycological Institute, Kew, UK; abundance of Aib and Iva in mycelial extracts is designated by ++ (large amounts), + (significant amounts), − (not detected), n.d. (not detectable).

hydrolysate of a mycelial extract from *H. schweinitzii* IFO 9063 is shown in Fig. 1a and GC/MS of the PFP-Aib 1-propylester and PFP-Iva 1-propyl ester from the hydrolysate are shown in Fig. 1b and 1c, respectively. The MS and fragmentation scheme of the PFP-Aib 1-propyl ester shows the characteristic fragment at *m/z* 204 for $[CF_3CF_2CONHC(CH_3)_2]^+$ and the MS of the PFP-Iva 1-propyl ester with the typical fragment ion at *m/z* 218 for $[CF_3CF_2CONHC(CH_3)(C_2H_5)]^+$.

The proof of the widespread production of peptaibiotics by *Hypocrea* is of interest since up to now only one microheterogeneous peptaibiotic has been isolated and sequenced from *H. peltata* (Fujita et al., 1984). The reliability of the screening procedure was also confirmed by the isolation of 100 mg of a microheterogeneous peptide antibiotic (ca. 20 closely related compounds according to HPLC) from 5 l culture broth of *H. muroiana* IFO 31288, and of 40 mg of peptide mixture (composed of 8 major and 7 minor components according to HPLC) from 5 l culture broth of *H. nigricans* IFO 31285. Fig. 2 shows the GC of PFP AA 1-propyl esters and of (*N,O*)-PFP phenylalaninol of the total hydrolysate of the peptide mixture isolated (*cf.* Experimental) from *H. muroiana* IFO 31288. The GC of the peptide mixture from *H. muroina* shows the presence of Aib, Iva and Pheol, in addition to protein amino acids (*cf.* Fig. 2) while the peptide from *H. nigricans* contains Aib and Leuol, and Iva is a constitutent of only one peptide of the microheterogeneous peptide mixture (*cf.* Table 1, chromatogram not shown). The peptide mixture from *H. muroiana* showed in the filter disk test against *Bacillus subtilis* ATCC 6633 inhibitions zones of 15 (17) mm with 100 (200) μg peptide. The peptaibiotic from *H. nigricans* showed an inhibition zone of 10 mm under the same conditions when 200 μg were applied. The 20-mer peptaibiotic Paracelsin (Brückner et al., 1984), serving as standard, gave inhibition zones of 17–22 mm with 200 μg peptide. From the AA composition and antibiotic activity the isolated peptides can therefore be classified as belonging to the peptaibiotic family.

The results prove that the GC screening of *Hypocrea* for the nonprotein amino acids Aib (and Iva) is a highly suitable method for the detection of bioactive peptides of the peptaibiotic family and that these peptides are also common in this fungal genus. Together with results reported previously (Brückner and Reinecke, 1988; Brückner et al., 1989, 1989a), it has now been shown that the production of peptaibiotics takes place in most species of filamentous fungi of the genera *Emericellopsis*, *Gliocladium*, *Hypocrea*, *Stilbella*, and *Trichoderma* and in several or single species of *Byssochlamys*, *Ceratocystis*, *Dendrostilbella*, *Metarrhizium*, *Nectria*, *Paecilomyces*, *Penicillium*, *Samarospora*, *Sepedonium*, *Sphaerostilbella*, *Talaromyces*, and *Tolypocladium* (the latter genera have not yet been screened as extensively as the former). The production of peptaibiotics has now been proved in a total of 17 genera of filamentous fungi. These, in part very common, genera occur in habitats such as soil, decaying and growing plant materials, and dung. They are also found, in certain cases, as food and feed contaminants, and selected species are used in biotechnology (Brückner et al., 1987), or for food production (Brückner and Reinecke, 1988).

Screening for peptaibiotics should also attract great interest as a result of their broad range of bioactivities which include, in part, bactericidal, fungicidal,

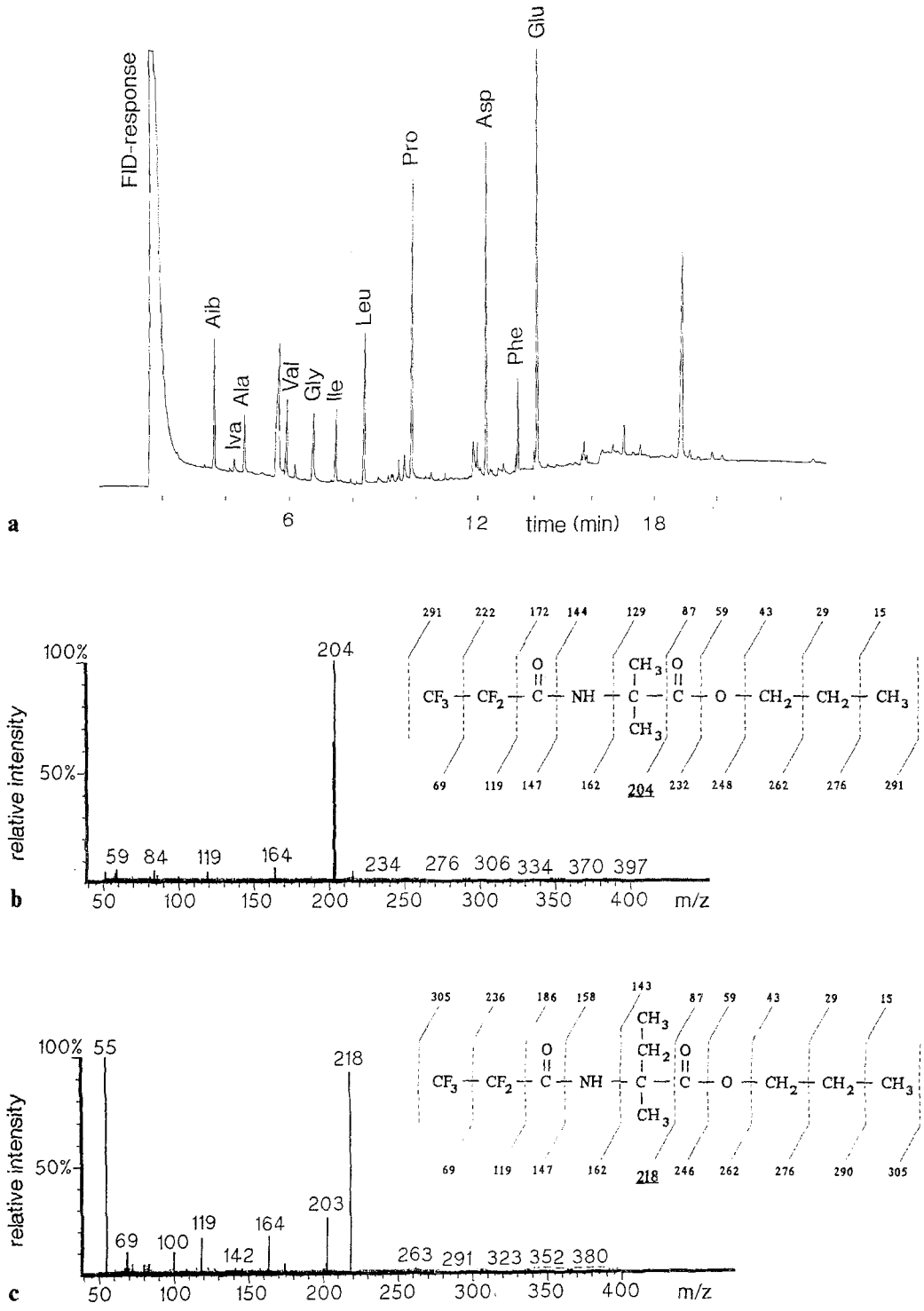

Fig. 1a–c. Gas chromatogram of (**a**) *N*-pentafluoropropionyl (PFP) 1-propyl esters of amino acids from a total hydrolysate of an organic mycelial extract from *Hypocrea schweinitzii* IFO 9063 and GC/MS of (**b**) the PFP-Aib 1-propyl ester and (**c**) PFP-Iva 1-propyl ester from the hydrolysate. Conditions: (**a**) Chirasil-L-Val column; temperature program, 5 min at 80°C, then at 6°C/min to 180°C; (**b**), (**c**), GC/MS SE-30 capillary column, temperature program, 5 min at 80°C, then at 10°C/min to 180°C; *FID* flame ionization detector

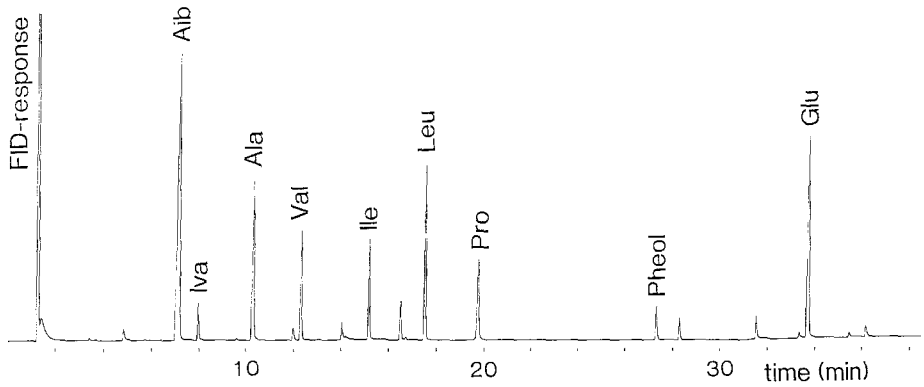

Fig. 2. GC of PFP-amino acid 1-propyl esters and (*N*,*O*)-phenylalaninol of a total hydrolysate of the peptide mixture isolated from *Hypocrea muroiana* IFO 31288. GC conditions: Chirasil-L-Val column; 5 min at 80°C, then at 3.5°C/min to 190°C; all components have the L-configuration with the exception of D-Iva

and amoebic activities, toxic properties such as hemolysis of erythrocytes, uncoupling of the oxidative phosphorylation of mitochondria, and the ability for the formation of voltage-depending ion conductances in lipid bilayer membranes (Boheim et al., 1983; Jung et al., 1981). Further, employing x-ray crystallography and nmr spectroscopy, unusual secondary structures of Aib-peptides have been reported (Hummel et al., 1987; Karle et al., 1988; Marshall et al., 1988; Rebuffat et al., 1989; Toniolo and Benedetti, 1988; Gessmann et al., 1991).

The findings that the production of peptides containing Aib and D- or L-Iva (Brückner et al., 1980; Bullough et al., 1982), is much more widespread in filamentous fungi than was assumed until recently contradicts also the generally held belief that these nonprotein AA are extremely rare in the biosphere. Taking geomicrobiological reports (Moore, 1969) into account, biotic origin would also provide a feasable explanation that Aib and Iva have been found in sediments below and above the cretaceous—tertiar (*K*/*T*) boundary, but not in the boundary itsself (Zhao and Bada, 1989).

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Authors' address: Prof. Dr. H. Brückner, Institute of Food Technology, University of Hohenheim, D-W-7000 Stuttgart 70, Federal Republic of Germany.