Journal of Chromatography B, 1009-1010 (2016) 25–33

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development and validation of LC–MS methods for peptaibol quantification in fungal extracts according to their lengths

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ARTICLE INFO

Article history:
Received 31 July 2015
Received in revised form 20 October 2015
Accepted 21 November 2015
Available online 4 December 2015

Keywords:
Trichoderma sp
Peptaibols
LC/ESI-IT-MS
Quantification
Validation
Biocontrol

ABSTRACT

Some terrestrial *Trichoderma* sp. strains are already used as biological control agents (BCAs). They all produce peptaibols, small antimicrobial peptides which are supposed to play a role in the anti-phytopathogenic activity of *Trichoderma* sp. *Trichoderma* strains producing high amounts of peptaibols could represent new potential BCAs. In this context, marine-derived *Trichoderma* strains from the marine fungal strain collection of the "Mer, Molécules, Santé" (MMS) laboratory were investigated for their peptaibol production. Previously, the quantification of peptaibols was performed using alamethicin, as standard (20-amino acid residues peptaibol). In this study, the development and validation of quantification LC/ESI-TI-MS methods using different standards of peptaibols (11-, 14- and 20-amino acid residues) was performed in order to quantify all of them, in a single analysis, in *Trichoderma* crude extracts according to their chain length. The developed and validated methods were used to study the peptaibol production kinetic of a marine-derived *Trichoderma* strain, *i.e., Trichoderma longibrachiatum* (MMS 151). The results showed the optimal culture time at the 9th day with concentrations reaching $1.4 \pm 0.2\%$ and $2.3 \pm 0.4\%$ of the fungal biomass respectively for 11- and 20-residue peptaibols. Then, the different peptaibol subgroups produced by 13 *Trichoderma* strains were quantified. According to their 18-, 19- and 20-residue peptaibol production, three strains referenced as MMS 1541, MMS 639 and MMS 151 seemed to be good candidates as potential new biological control agents with respective production of 0.4, 0.4 and 2.1%.

1. Introduction

During the last 20 years, the reduction of pesticide uses in crops has become a necessity for health protection and prevention. The search of alternative fungicides for the ornamental plants is becoming strategic. For example, phytosanitary products will be forbidden in the French public green spaces from 2020 (French program Plan Ecophyto 2018: (http://agriculture.gouv.fr/sections/magazine/focus/phyto-2018-plan-pour/#planECOPHYTO2018).

Some terrestrial *Trichoderma* sp. strains are already used as biological control agents (BCAs) [1]. An antagonistic action of these strains was highlighted on several phytopathogens like *Fusarium*, *Alternaria*, *Sclerotinia*, *Verticillium* or *Botrytis* [2]. *Trichoderma* strains are also used as plant growth-promoting agents. In fact, their use is recommended for cultivation of vegetables and ornamental plants, for plant and tree nursery and should lead to: (i) prophylactic protection of plants and scions against soil-borne plant pathogenic fungi; (ii) stimulation of root growth, including a higher percentage of root hairs; (iii) increased yield due to improved germination and rooting rates; (iv) increase of plant dry weight and number of flowers; (v) earlier onset of flowering and shortening of cultivation periods [2].

All *Trichoderma* strains produce peptaibols which are small linear antimicrobial peptides, characterized by molecular masses ranging from 500 to 2000 Da, thus containing 5–20 residues and a high amount of α-amino-isobutyric acid (Aib or U) [3]. They possess an acylated N-terminus and a C-terminus amino-acid residue reduced in amino-alcohol [4]. Peptaibols form a subgroup in the larger family called peptaibiotics [3,5,6]. Peptaibiotics have been studied for their potential biological activities particularly in the context of the alternative sources of antibiotics research [7] and as new therapeutic agents [8]. They had been shown to exhibit

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http://dx.doi.org/10.1016/j.jchromb.2015.11.039
1570-0232/

a wide range of biological activities including an antibacterial activity against *Gram-positive* bacteria [9–11], as well as dormant mycobacteria [12,13] and an antifungal activity [10]. They have also been widely studied for their potential use as biocontrol agents against plan fungal pathogens [14–16]. They have been reported to have an antiviral activity, particularly against infections caused by the virus of tobacco mosaic [17–19], and an antiparasitic activity against amoebae (*Dictyostelium* sp.) [20] and protozoa (*Plasmodium falciparum*) [21]. Peptaibols are supposed to play a role in the anti-phytopathogenic activity of *Trichoderma* sp. [15,22]. If they are long enough, they are able to form voltage-dependent ion channels in the lipid membranes [23,24]. Consequently, *Trichoderma* strains producing high amounts of long-chain peptaibols (18–20-residues) could represent potential new BCAs. Moreover, peptaibols could have not the same efficiency according to the targeted phytopathogen. It thus necessary to quantified separately each produced peptaibol subgroups in order to select the most efficient *Trichoderma* strain to inhibit the targeted pathogen.

Peptaibols could be classified according to their amino acids chain length and are produced in microheterogenous mixtures. The main subgroups are 11-, 14-, 18-, 19- and 20 amino acid residue peptaibols [25]. Until now, the LC–MS quantifications of peptaibols have been performed using alamethicin, as standard [26,27]. Currently, all peptaibol subgroups (11-res–20-res peptaibols) were quantified using only one standard: the commercial alamethicin mixture. Commercial alamethicin is a mixture of four 20-residue peptaibols, being the only current peptaibol commercialized mixture. Moreover, according to the study from Degenkolb, this commercial mixture contain a mycotoxin: the trichothecene [28] In the present study a LC/ESI-IT-MS methods was developed using different standards of peptaibols (11-, 14- and 20-residues) to quantify each peptaibol subgroup separately. It seems necessary to use standards with different chain lengths since they probably lead to different ionizations in the mass spectrometer (ESI-IT) and consequently to different sensitivities.

In the present study, after development and validation of the analytical methods of the quantification of the different peptaibol subgroups, 12 marine-derived *Trichoderma* strains of the marine fungal strain collection of the laboratory were screened in order to select the best potential BCAs.

2. Experimental

2.1. Chemical

Dichloromethane (CH_2Cl_2) and methanol (MeOH) used for peptaibol extractions and purifications were purchased from Carlo Erba (Val de Reuil, France) and distilled prior to use. The HPLC quality grade methanol for MS analyses was obtained from Biosolve (Valkenswaard, Netherlands).

2.2. Peptaibol standards

Alamethicin F50/5, hypomurocin A-III and a bergofungin D analogue were synthesized using microwave-assisted one shot synthesis according to the protocol of Ben Haj Salah and Inguimbert [29]. They are respectively named PEP-20 (20-residue peptaibols), PEP-11 (11-residue peptaibols) and PEP-14 (14-residue peptaibols) in this paper. Hypomurocin A-III (PEP-11) was observed in *Hyprocrea muroina* [30] and the similar sequence of bergofungin D (PEP-14) isolated from *Emericellopsis donezki* [31] contain proline residues in positions 9 and 12 instead of hydroxyproline. Their sequences are:

PEP-11: AcUQVLUPLLUPLol

PEP-14: AcVUUVGLUUPQUPUFol

PEP-20: AcUPUAUAQUVUGLUPVUUQQFol

Q = Glutamine V = Valine U = Aib P = Proline L = Leucine A = Alanine Fol = Phenylalaninol Lol = Leucinol G = Glycine.

2.3. Fungal strains

Twelve marine-derived and one commercial strain of *Trichoderma* sp. were selected for this study. Their specificities are given in Table 1. Their qualitative peptaibol productions have been determined using mass spectrometry analyses in infusion mode as described by Carroux et al. [32].

2.4. Culture

Strains were inoculated onto *Petri* dishes (10-cm diameter) containing 20 mL of DCA medium (Dextrose 40 g/l, enzymatic digest of Casein 10 g/l, Agar 15 g/l, DIFCO, France) prepared with reconstituted seawater. Cultures were incubated in natural light at 27 °C.

2.5. Extraction and purification

After incubation, mycelia and conidia were scraped from the agar surface. The harvested biomass was steeped twice in CH2Cl2/MeOH 1:2 then 2:1 (v/v, 30 mL total volume) for 30 min at room temperature. The combined extracts were filtered, washed with distilled H2O (20 mL) and evaporated to dryness to provide crude extracts.

2.6. LC–MS analysis

The samples were analyzed using a high performance liquid chromatography (HPLC) system consisting on a spectraSYSTEM P1000XR pump, a spectraSYSTEM AS3000 as autosampler (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Kromasil-C18 5 μm 250 mm × 2 mm column (Interchim, France) heated at 40 °C. The autosampler was maintained at room temperature and was programmed to inject 5 μL of sample in the chromatographic column. The mobile phase consisted on a MeOH/H_2O mixture (85:15, v/v) in isocratic mode at a constant flow rate of 0.2 mL/min. Mass analyses were performed using a Finnigan Matt LCQ^{TM} ESI-IT mass spectrometer (Thermo Fisher Scientific) in positive ionization mode with a capillary temperature of 160 °C, a capillary voltage and a spray voltage of 3 V and 4.50 kV, respectively. Nitrogen flow rates were 60 and 0 (arbitrary units) respectively for sheath gas and auxiliary gas. The parameters of ion optic transmission were 50 V, −3.25 V, −5.5 V and 400 V for tube lens offset, multipole 1 offset, multipole 2 offset and multipole RF amplifier respectively.

Analyzed under neutral conditions and in positive mode by electrospray ionization-ion trap-mass spectrometry (ESI-IT-MS), long chain peptaibols appear principally as doubly charged sodium adduct ions $[M + 2Na]^{2+}$ and short chain peptaibols appear as singly charged sodium adduct ions $[M + Na]^{+}$. All data acquisitions and reprocessings were performed using LCQ Xcallibur 1.3 software (Thermo Fisher Scientific). For each standard, the analyzed ions were:

- PEP-11: $[M + Na]^{+} = 1197$
- PEP-14: $[M + Na]^{+} = 1417$ and $[M + 2Na]^{2+} = 720$
- PEP-20: $[M + Na]^{+} = 1985$ and $[M + 2Na]^{2+} = 1004$.

Concerning the peptaibols produced by *Trichoderma* sp., the expected diagnostic ions of each subgroup are presented in Table 2.

The short chain peptaibols were quantified using the standard calibration curve performed with PEP-11 and PEP-14 for 11- and 14-residue peptaibols respectively. The long-chain peptaibols