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DETERMINATION OF VALINE AND LEUCINE ISOMERS IN PEPTAIBOLS

Gábor Endre[1,2], Tamás Marik[1], László Kredics[1] Mónika Varga[1], Csaba Vágvölgyi[1], András Szekeres[a]

[1]*University of Szeged, Faculty of Science and Informatics, Departement of Microbiology, Közép fasor 52. Szeged H-6726*
[2]*Doctoral School in Biology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary*
e-mail: egabcy@gmail.com

Abstract
Peptaibols are oligomers, containing non-proteogenic amino acids. Some of them are considered as antifungal and antibiotic agents, therefore the elucidation of their structure is important. There have been a lot of efforts determining their amino acid sequences via mass spectrometry, but isobaric components cannot be determined using this technique.
In this work, a HPLC method development was carried out for the determination of leucine, isoleucine, valine and isovaline in one chromatographic run. Furthermore, a chiral separation of the optical isomers of these compounds has been also solved.

Introduction
Peptaibols are peptid-like oligomers produced as extracellular secondary metabolites by *Trichoderma* species. Some peptaibols function as antibiotic and antifungal agents [1]. They are amphiphilic, which allows them to form voltage-dependent ion channels in cell membranes, making them leak and leading to the cell death. These compounds contain non-coded, non-proteinogeic amino acids, like α-aminoisobutyric acid, ethylnorvaline, isovaline and hydroxyproline. Their N-terminus is acetylated, and the C-terminal amino acid is hydroxylated into an acid alcohol [2]. Due to their diverse nature, the structure elucidations of newly discovered peptaibols are important.
There have been a lot of efforts to determine their amino acid sequence by mass spectrometry [3-5], however, isobaric amino acids could be specified, and no configuration of the compounds could be observed. To apply liquid chromatography, problems could be solved-, e.g. isobaric amino acids could be separated and detected, as well as the D-, and L-isomers could be distinguished due to the proper chiral derivatisation.
For the chiral determination of the desired amino acids, Marfey's reagent can be used. The reaction was carried out by adding the chiral reagent into the amino acid sample forming two products, depending on the configuration of the connecting amino acid. When the reagent is reacting with an L-amino acid, the product could form an intramolecular H-bond that results in a ring-like system. In the case of D-amino acids reaction partners, another product occurs, that cannot form any H-bonds intramolecularly. This slight difference between these two products leaded to their baseline separation on the applied chromatographic column.

Experimental
Trichoderma pleuroti (SZMC 12454) was cultivated on solid medium. Peptaibols were extracted using $CHCl_3$/MeOH = 3/1 and the crude extract was separated on a normal phase column in a $CHCl_3$/MeOH gradient. One selected fraction was evaporated and hydrolysed in cc. HCl overnight, to release the amino acids. After setting the pH to 7, the achiral and chiral derivatisations were applied.
Derivatising agents, amino acid standards and other chemicals were purchased from Sigma Aldrich Inc. (Saint Louis, MO, USA). The achiral derivatisation of amino acids was done by

adding 125 µl of borate buffer (pH = 10.2) to 50 µl of amino acid solution. After mixing vigorously, 25 µl of OPA solution and 20 µl of FMOC solution were added. The solution was diluted with 780 µl HPLC water and mixed. The resulted solution was injected into a HPLC-UV system. Chiral derivatisation of amino acids was done by Marfey's reagent (Thermo Fischer Sicentific, Waltham, MA, USA). To 50 µl of amino acid solution, 100 µl of Marfey's reagent's solution (1 w% in acetone) and 20 µl solution of $NaHCO_3$ (1 M) were added. After mixing, the reaction mixture was thermostated at 35°C for 60 minutes, which was followed by neutralisation by 10 µl of HCl solution (2 M). The mixture was evaporated to dryness and was dissolved in 500 µl HPLC grade DMSO. The resulted solution was diluted ten times and injected into a HPLC-UV system.
HPLC method development and measurements were done using a Dionex Ultimate 3000 UHPLC instrument (Thermo Fischer Sicentific, Waltham, MA, USA). The instrument was equipped with a quaternary pump, a degasser, a well plate sampler, a column thermostat and a UV-VIS detector. Separation of achirally derivatised amino acids was performed using a Phenomenex Ez:faast AAA-MS 250 x 2 mm, 4 µm column (Phenomenex Inc., CA, USA). The mobile phase was the following: A: H_2O + 41 mM acetate buffer (pH = 4), B: MeOH + 41 mM acetate buffer (pH = 4). The elution was isocratic with the ratio of A/B = 45/55 for 33 minutes, while the flow rate was 0.35 ml/min at 45°C. The injection volume was 1 µl and peaks were detected at λ = 338 nm and λ = 262 nm. Separation of chirally derivatised amino acids was performed using same column as above and the mobile phase was the following: A: H_2O + 25 mM $HCOONH_4$, B: MeOH + 25 mM $HCOONH_4$. During the gradient elution the B % increased from 35% to 70 % for 30 minutes. The flow rate was 0.25 ml/min at 35°C during analyses and the injection volume was 5 µl and peaks were detected at λ = 340 nm and λ = 254 nm.

Results and discussion

In the first stage of our work, standard amino acid solutions were derivatised with o-phthaldialdehyde (OPA) and 9-fluorenylmethyloxycarbonyl chloride (Fmoc). A non-chiral HPLC-UV method was developed for the separation of leucin, isoleucin, valine and isovaline. The components were separated in an isocratic elution within 33 minutes. The chirally derivatised L-, and D-amino acids were also separated, but in this case, a gradient elution from 35 % B to 70% B was used. After the separation method optimization, the *Trichoderma pleuroti* (SZMC 12454) strain was cultivated. At the end of the fermentation the mycelia were extracted and the crude peptaibol mixture was purified by flash chromatography using $CHCl_3$/MeOH gradient. The peptaibol containing fractions eluted from 40% to 60% of MeOH and a fraction was selected from the collected ones contained the most amount of peptaibols. The oligomers were hydrolysed and the liberated amino acids derivatised achirally. The achiral amino acid separation resulted, that the peptaibol mixture contains valine and isovaline in 1 to 1 ratio and leucine and isoleucine in 1 to 1 ratio also as well. This peptaibol fraction was also chirally derivatised and analysed. Altogether L-isoleucine, D-isovaline and L-leucine were found, none of the two valine isomers could be detected.

Conclusion

Two successful HPLC-UV methods were developed to analyse isobaric amino acids and to define their configuration. Group of peptaibols were successfully purified, hydrolysed and analysed. After analysing the real samples, it could be concluded that further purification of the peptaibols is needed to have the pure compounds hydrolysed and analysed.

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