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Note

Rapid identification and determination of herbicolin A and B by high-performance liquid chromatography

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Herbicolin A and B are antifungal antibiotics produced by a strain A 111 which was identified as *Erwinia herbicola* (Enterobacteriaceae)[1]. *Erwinia herbicola* is commonly found as a saprophyte on plants, in water and in soil. The structures of the antibiotics were determined as nonapeptides[2]. Herbicolin B is a lipodepsinonapeptide with the sequence DH–Abu–L–Thr–D–Thr–D–Leu–Gly–D–Gln–Gly–N–Me–L–aThr–L–Arg (DH-Abu = 2,3-dehydro-α-aminobutyric acid). The C-terminal Arg residue forms a lactone ring with the hydroxy group of L-Thr, while the N-terminus is acylated by an (*R*)-3-hydroxytetradecanoic acid residue. The main component, herbicolin A, differs from herbicolin B in having an additional D-glucose moiety linked as a 1-α-glycosidic bond to the 3-hydroxytetradecanoic acid residue. Thus herbicolin A constitutes a glycolipodepsinonapeptide antibiotic. Herbicolin A strongly inhibits yeasts, filamentous fungi[3] and sterol-requiring mycoplasmas[4,5], but not other bacteria[1].

For optimization of the fermentation of herbicolins, a high-performance liquid chromatographic (HPLC) method was developed which allows the concentration produced to be followed in a rapid and quantitative manner. Previous HPLC separations using LiChrosorb-NH_2 columns[2] were found to be inadequate for the present purpose and could be significantly improved using a C_8 column.

EXPERIMENTAL

Materials

Columns (125 × 4.6 mm I.D., metal) were obtained by Grom (Ammerbuch, F.R.G.) and octyl reversed-phase material (C_8 Nucleosil, 5 μm) was purchased from Macherey, Nagel & Co. (Düren, F.R.G.). All HPLC-grade organic solvents were purchased from Baker (Deventer, The Netherlands) and Merck (Darmstadt, F.R.G.). Sep-Pak C_{18} octadecyl cartridges for sample preparation were obtained from Waters Assoc. (Milford, MA, U.S.A.). Thin-layer chromatography (TLC) was performed in saturated glass chambers on precoated silica gel 60 (0.25 mm) F_{254}, 5 × 10 cm, purchased from Merck.

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Equipment

For all HPLC procedures the following apparatus were used: a Model 7125 valve for injection (Rheodyne, Cotati, CA, U.S.A.), a type 64 HPLC pump (Knauer, Bad Homburg, F.R.G.), a Model SP-2A UV detector (Shimadzu, Kyoto, Japan) and a Model CR-3A integrator (Shimadzu). Centrifugation was performed with a Minifuge GL centrifuge (Heraeus Christ, Osterode, F.R.G.).

Sample preparation

From the culture broth 10 ml were taken, the cells were removed by centrifugation for 15 min at 4000 *g* and 4–10 ml of the supernatant were passed through Sep-Pak cartridges. The Sep-Pak cartridges were conditioned by rinsing with 5 ml of methanol followed by 10 ml of water. After the supernatant had passed through, salts and hydrophilic components were removed with 10 ml of water. Elution was carried out with 2 ml of methanol. After appropriate dilution with methanol, this solution was used for HPLC.

HPLC procedure

The samples were injected onto the HPLC column, using a 20-μl sample loop. As the mobile phase for isocratic elution, was used acetonitrile–phosphoric acid (0.1%)–methanol (7:3:4, v/v/v) at a flow-rate of 1.5 ml/min. The herbicolins were detected by measuring the UV absorbance at 215 nm and 0.04 a.u.f.s. Integration of the peak area was performed with a CR-3A integrator.

Other chromatographic procedures

For TLC on precoated silica gel 60 F_{254} the solvent system was chloroform–methanol–water–acetic acid (65:25:4:3, v/v). The R_F values in this system are 0.12 for herbicolin A and 0.38 for herbicolin B. Other TLC systems are given in ref. 6. Herbicolins were detected using the tetramethyldiaminodiphenylmethane (TDM) method[7]. Spots of herbicolin A could also be detected with orcin reagent[8], owing the glucose moiety.

Enzymatic cleavage of herbicolin A

For enzymatic cleavage of the glucose residue of herbicolin A a solution of 0.5 mg/ml containing both herbicolin A and herbicolin B was used. Hydrolysis was performed in 0.1 *M* potassium phosphate buffer (pH 6.8) at room temperature. A 1-ml volume of buffered herbicolin solution was incubated for 1 h with 0.5 ml of buffer containing 6 units of α-glucosidase from yeast (Serva, Heidelberg, F.R.G.). The herbicolins were separated from the enzyme and desalted using the sample preparation procedure described above.

RESULTS

The chromatogram in Fig. 1 shows two main peaks designated 1 (herbicolin A) and 2 (herbicolin B) at 3.85 min, representing purified herbicolin A and B, separated by the method described under Experimental. Samples used for comparison purposes originated from an earlier batch, which represented a mixture of herbicolin A and B, purified according to the counter-current distribution method described previously[2].

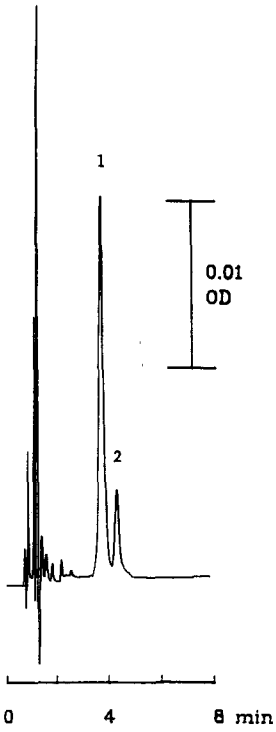

Fig. 1. High-performance liquid chromatogram of the purified forms of herbicolin A and B, detected at 215 nm using a C_8 reversed-phase column run with the isocratic solvent system acetonitrile–phosphoric acid (0.1%)–methanol (7:3:4, v/v/v). A 20-μl sample of concentration 10 μg/ml in methanol was injected. Peaks: 1 = herbicolin A; 2 = herbicolin B.

The ratio of herbicolin A to herbicolin B was approximately 4:1. This value varied depending on the fermentation conditions.

The proportions of herbicolin A and B could also be changed by using enzymatic splitting of the glucose residue of herbicolin A by commercial α-glucosidases (yeast). As shown in Fig. 2, the enzymatic treatment resulted in a reduction of the herbicolin A peak area whereas the herbicolin B peak area was increased. As herbicolin B has the same structure as the A form except for the 1-α linked glucose, splitting of this glucose should lead to herbicolin B. Further work is intended to optimize and analyse the enzymatic cleavage of the glucose moiety of herbicolin A.

In addition to identification with purified references (by HPLC and TLC), samples were scraped off from TLC and used as an additional proof that peak 1 corresponded to the glucose-containing herbicolin A whereas peak 2 corresponded to herbicolin B. Removal of the salts added to the enzyme assay was done using the Sep-Pak procedure described above. The main purpose of the present investigation, however, was to determine the content of herbicolin A in the fermentation broth. Fig. 3 shows a sample of culture broth measured according to the procedure described above. There are only a few other compounds remaining which do not interfere with quantification. Quantification was effected using pure herbicolin A as a reference.

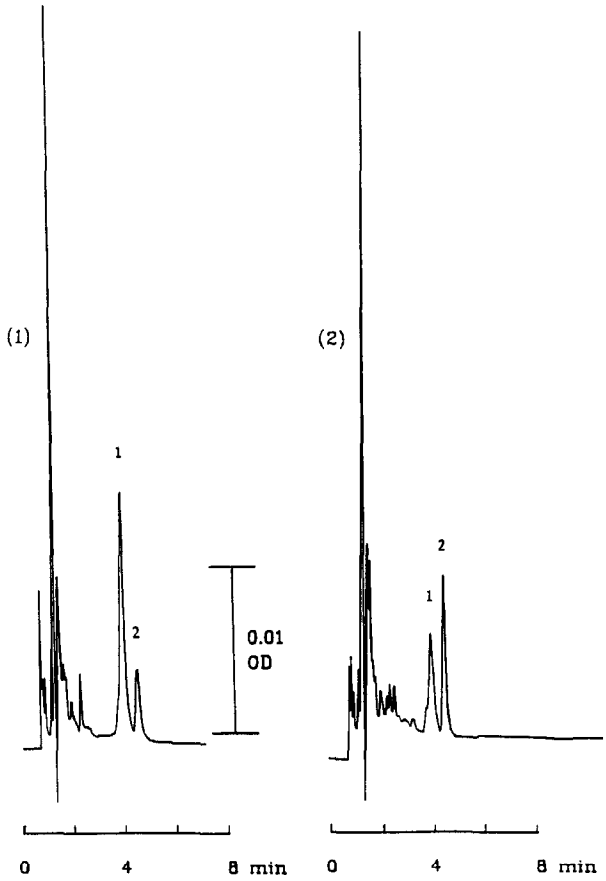

Fig. 2. HPLC of a sample of herbicolin A and B, (1) before and (2) after partial enzymatic hydrolysis of the glucose moiety from herbicolin A by α-glucosidase. Owing to the splitting off of the glucose residue from herbicolin A, the amount of herbicolin B is increased. Peaks: 1 = herbicolin A; 2 = herbicolin B.

Linearity was observed between 1 and 500 μg/ml with measurement of peak area. Fig. 4 shows the calibration graph obtained with herbicolin A, prepared as described previously[2]. The detection limit of herbicolin A was 0.1 μg/ml, at a signal-to-noise ratio of 3:1.

DISCUSSION

The HPLC method for the determination of herbicolin described here is a significant improvement over the TLC and other HPLC methods we have used previously in terms of sensitivity and time required. Attempts with C_{18} and C_4 columns were not as successful as with C_8 columns because of a higher retention time together with peak broadening or poorer resolution. Phosphoric acid (0.1%) and methanol were not suitable solvents to resolve all components. The described system of acetonitrile–phosphoric acid (0.1%)–methanol yielded the best separation properties.

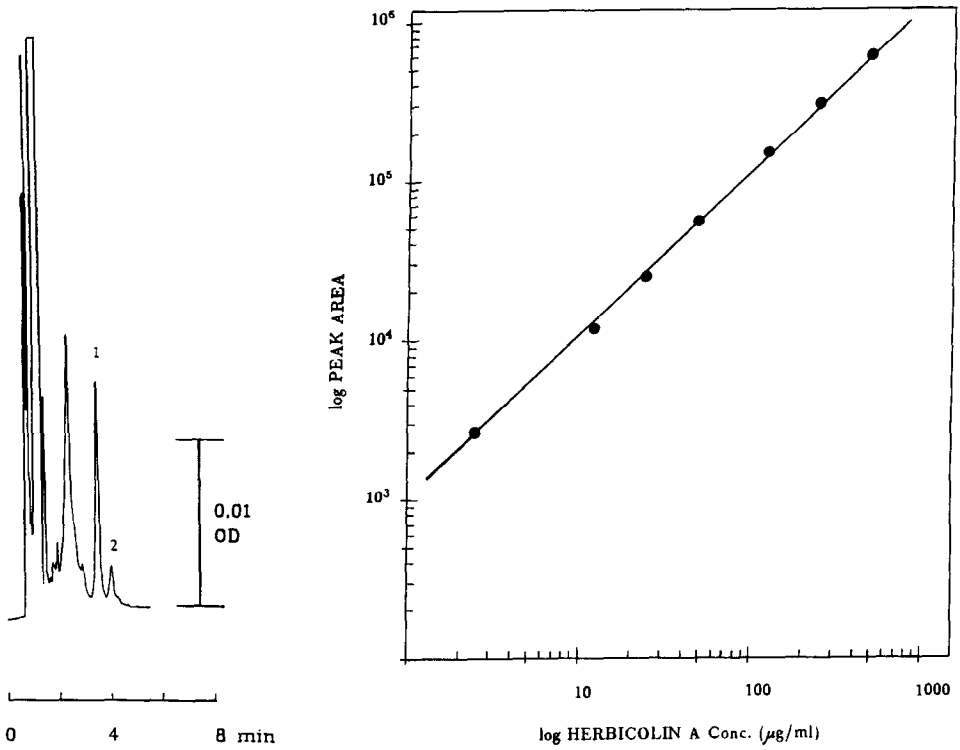

Fig. 3. HPLC of culture broth during the fermentation process after sample preparation with a Sep-Pak C_{18} cartridge. Detection at 215 nm; isocratic conditions as described. Peaks: 1 = herbicolin A; 2 = herbicolin B.

Fig. 4. Calibration graph for herbicolin A. Herbicolin A obtained from counter-current distribution according to ref. 2.

The isocratic system was found to be superior to the gradient system using phosphoric acid (0.1%)–methanol owing to easier handling, lower solvent consumption and shorter analysis time. Combined with sample preparation on Sep-Pak cartridges, the method is suitable for rapid control of fermentation and for monitoring the different fractions during the purification process.

Lipopeptides are widespread among microbial products. The diversity of molecular variations in bacterial surfactants has been described[9]. Most of these compounds, such as surfactin[10], bacillomycins[11], mycosubtilin[12], mycobacillin[13] and iturin A[14] possess antifungal activity. We have recently described the occurrence of long-chain iturin AL, which differs from iturin A by its predominant C_{16} β-amino acid[15]. Whereas the iturins have been shown to be separated by HPLC according to the chain length, the herbicolins represent compounds of constant chain length (C_{14}) but differ in the presence of a glucose residue. Hence the different polarities of the herbicolins allow their separation on reversed-phase materials. The biosynthetic origin of the herbicolins is still unresolved. They are obviously not secondary metabolic products of the stationary growth phase as is the case with peptide antibiotics of the bacillus group. As herbicolins are produced by strains of the non-spore-forming family

Enterobacteriaceae, a role in spore formation can be excluded. According to our observations, herbicolins are produced during the exponential growth phase, suggesting that herbicolins might be involved in membrane formation. The hydrophobic chain in the herbicolins represents β-hydroxymyristic acid, which is a common constituent of lipopolysaccharides of Enterobacteriaceae. Determination of herbicolins and its biosynthetic precursors by chromatographic analysis will possibly help in elucidating the biological role of herbicolins.

HPLC will be a very important tool for further investigation of lipopeptides and glycolipopeptides that are attaining not only scientific, but also therapeutic and biotechnological interest.

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