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Purification of antifungal lipopeptides by reversed-phase high-performance liquid chromatography

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ABSTRACT

A rapid procedure for the purification of antifungal lipopeptides from *Bacillus subtilis*, a potential agent for biocontrol of plant diseases, was tested. It consists of a solid-phase extraction on C_{18} gel followed by reversed-phase chromatography using a biocompatible PepRPC HR 5/5 column with a pharmacia fast protein liquid chromatographic system. This is a very effective method for isolating and fractionating iturin A and surfactin, two lipopeptides of different nature, co-produced by *Bacillus subtilis* strain S499. The presence of homologous lipopeptides was easily detected.

INTRODUCTION

Several strains of *Bacillus subtilis* produce various lipopeptides with antifungal activity. About ten lipopeptides have been isolated and their structures have been determined [1,2]. They are cyclic lipopeptides containing seven residues of D- and L-α-amino acids and one residue of a β-amino fatty acid or β-hydroxy fatty acid. Most of them were identified as mixtures of homologous series that differ in the length of the lipidic chain. This is the case with iturin A [3,4], bacyllomicin L, D and F [5–7] and mycosubtilin [8]. More recently, it was found that surfactin, a surfactant lipopeptide, was a mixture of two variants that differ in the terminal amino acid residue [9]. This kind of lipopeptide does not exhibit an antifungal activity but it enhances the action of iturin A if they are present in a mixture [10].

It is therefore of obvious interest to acquire an effective separation method in order to identify completely the composition of lipopeptides produced by a strain of *Bacillus subtilis*. Further, pure lipopeptides are necessary for the study of their structure–function relationship, which is still unclear.

Generally, the purification of these molecules requires three or more steps, including precipi-

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tation, extraction and chromatographic techniques. We describe in this paper a rapid and efficient fast protein liquid chromatographic (FPLC) technique for the purification of all lipopeptides produced by *Bacillus subtilis* strain S499.

EXPERIMENTAL

Strain and culture conditions

Lipopeptides were produced from a culture medium of *Bacillus subtilis* strain S499 isolated by Delcambe (1956). The cells were grown in a 20-l fermenter (Biolafitte, Poissy, France) at 30°C for 72 h with the medium described previously [11]. The stirrer speed was 300 rpm and the aeration rate was 0.3 vvm (*i.e.*, volumetric air flow per minute per working volume). The pH was maintained at 7 during the culture.

Isolation of lipopeptides

After 72 h of growth, the culture medium was centrifuged at 11 000 *g* for 25 min to remove the cells. The supernatant (500 mg of dry material) was applied to a Bond Elut C_{18} cartridge (5 g per 20 ml) from Analytichem International (Harbour City, CA, USA). The cartridge, which retained lipopeptides, was rinsed successively with 20 ml of water and 40 ml of 50% aqueous methanol and finally the lipopeptides were eluted from the cartridge with 20 ml of methanol. The eluate was evaporated and the residue was dissolved in 0.5 ml of methanol. The solution was analysed by FPLC as described below.

Antagonistic test

The antifungal activity of substances eluted from the Bond Elut C_{18} cartridge was evaluated by a classical antibiotic test using the agar diffusion method in Petri dishes. Each eluate was evaporated and the residue was lyophilized. The dry material was dissolved in 0.1 *M* $NaHCO_3$ to give a 5 mg/ml solution. Aliquots of 5 μl were placed in small open-ended cylinders placed on the surface of an agar plate pre-seeded with various phytophathogenic moulds: *Fusarium graminirum*, *Fusarium oxysporum*, *Rhizopus sp.*, grown on potato dextrose agar, and *Botrytis cinerea*, grown on Malt agar (both from Merck, Darmstadt, Germany). The plate was incubated at 37°C. Antifungal activity was detected by the presence of clear zones where fungi growth was inhibited. The minimum inhibitory concentration (MIC) was determined by decreasing the amount of substance tested.

FPLC purification

Instrumentation. A Pharmacia (Uppsala, Sweden) FPLC system, consisting of two Model P-500 high-precision pumps, a Model 3500 pump, a flow equalizer, an LCC-500 liquid controller, an MV-7A motor valve, a FRAC-100 fraction collector and a REC-482 recorder, was used throughout.

Chemicals. Acetonitrile was of HPLC grade from Alltech (Deerfield, IL, USA) and trifluoroacetic acid (TFA) was from Merck. Water was of Milli-Ro quality. All other reagents were of analytical-reagent grade.

Reversed-phase chromatography. The separation was performed on a PepRPC HR5/5 C_2/C_{18} column (Pharmacia). Lipopeptides were detected at 214 nm with a Waters Model 484 multiwavelength detector. The mobile phase components were (A) 0.1% TFA in Water and (B) 0.1% TFA in Water–Acetonitrile (30:70, v/v).

Thin-layer chromatography (TLC). TLC was performed on silica gel 60 plates (Merck), which were developed with chloroform–methanol–water (65:25:4, v/v/v). Lipopeptides were detected with a water spray, ninhydrin [12] and chlorination [13].

Amino acid analysis. The lipopeptide samples were hydrolysed with 6 *M* HCl at 110°C for 24 h. Water-soluble amino acids were reacted with phenyl isothiocyanate (PITC) to give phenylthiohydantoin (PTH) derivatives. The amino acid derivatives were analysed by HPLC (Pharmacia) using a PICO-TAG column (30 × 3.9 mm I.D.) (Waters–Millipore) and detected at 254 nm. The separation conditions used were those given in the Waters–Millipore manual.

RESULTS

The lipopeptides from the *Bacillus subtilis* strain S499 were isolated and purified according to the scheme shown in Fig. 1. Cells were

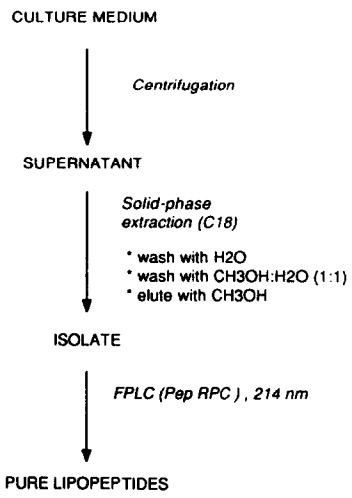

Fig. 1. Scheme of purification procedure.

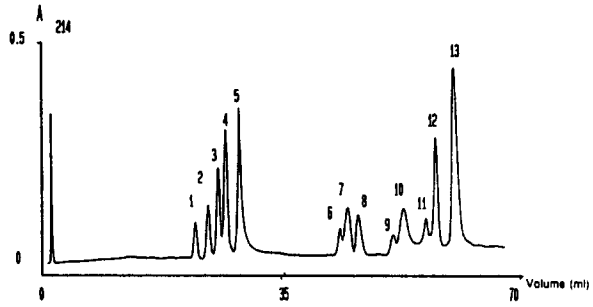

Fig. 2. FPLC separation of lipopeptides isolated from the Bond Elut C_{18} cartridge. Peaks: 1–5 = iturin A; 6–8 = unknown molecules; 9–13 = surfactin. Column, PepRPC HR 5/5 C_2/C_{18}; mobile phase, (A) 0.1% TFA in water and (B) 0.1% TFA in water–acetonitrile (30:70, v/v); gradient, 0% B in 5 ml, 0–45% B in 20 ml, 45–60% B in 11 ml, 60–80% B in 9 ml, 80–100% B in 15 ml, 100% B in 5 ml; flow-rate, 0.8 ml/min; detection, 214 nm.

removed by centrifugation and supernatant (500 mg of dry material) was passed through the C_{18} cartridge. The cartridge was then washed successively with 20 ml of water, 20 ml of 25–75% aqueous methanol and 20 ml of methanol. The elution of lipopeptides was monitored by TLC on silica gel as described above. They were detected as water spray-positive (white spot), ninhydrin-negative and chlorination-positive. The lipopeptides began to elute when the cartridge was washed with aqueous methanol of concentration >50% and were completely eluted with methanol. Antifungal activity against various phytopathogenic moulds was only detected in the fraction eluted with methanol. The MIC values for the supernatant and the isolate were determined and were of 500 and 50 μg/ml, respectively, on average.

A chromatogram of lipopeptides eluted from the Bond Elut C_{18} cartridge is presented in Fig. 2. The peaks were analysed by TLC on silica gel and their amino acid compositions were determined by HPLC after acid hydrolysis. Table I gives the results of these analyses. Each peak gave a single spot in TLC. According to the R_F values and α-amino acid composition, two groups of known lipopeptides, iturin A (peaks 1–5) and surfactin (peaks 9–13), were identified. A third group of unknown molecules (peaks 6–8) was also separated.

DISCUSSION

The present lipopeptide purification procedure includes an extraction step exploiting a solid-phase extraction technique and a chromatographic step involving reversed-phase chromatography with an FPLC system. The Bond Elut C_{18} phase is very efficient at adsorbing lipopeptides, owing to the important hydrophobic part of these molecules. Hence the majority of impurities such as inorganic materials and polar contaminants are easily removed by washing the Bond Elut C_{18} cartridge successively with 50% aqueous methanol. Lipopeptides are completely retained using less than 50% aqueous methanol as a washing agent. The TLC system used to monitor the elution of lipopeptides ensures a distinction between cyclic lipopeptides and other substances. Methanol is required to elute the lipopeptides completely. The antagonistic tests showed that the isolate is ten times more active than the crude supernatant by comparison of the MIC values. This illustrates the effectiveness of this clean-up step.

The solid-phase extraction on C_{18} shows some advantages over classical extraction by acid precipitation followed by extraction with methanol [4] or chloroform–methanol (2:1) [3]. It is rapid and no reactions such as hydrolysis or esterification of the lipopeptide functional groups were

TABLE I

AMINO ACID COMPOSITION AND R_F VALUES OF DIFFERENT FRACTIONS FROM FPLC SEPARATION

Peak	Amino acid composition										R_F [a]
	Ala	Val	Leu	Ileu	Ser	Tyr	Asx	Glx	Arg	Pro	
1	–	–	–	–	1	1	3	1	–	1	0.32
2	–	–	–	–	1	1	3	1	–	1	0.32
3	–	–	–	–	1	1	3	1	–	1	0.32
4	–	–	–	–	1	1	3	1	–	1	0.32
5	–	–	–	–	1	1	3	1	–	1	0.32
9	–	1	4	–	–	–	1	1	–	–	0.64
10	–	1	4	–	–	–	1	1	–	–	0.64
11	–	1	4	–	–	–	1	1	–	–	0.64
12	–	1	4	–	–	–	1	1	–	–	0.64
13	–	1	4	–	–	–	1	1	–	–	0.64

[a] TLC was carried out on silica gel G-60 with chloroform–methanol–water (65:25:4, v/v/v).

observed. Such reactions are possible in acidic conditions or during prolonged extraction with solvents [14].

The lipopeptides isolated from the Bond Elut C_{18} cartridge were well separated on the PepRPC C_2/C_{18} biocompatible reversed-phase column under the conditions given in Fig. 2. Two groups of lipopeptides were identified as iturin A and surfactin according to their R_F values on silica gel in chloroform–methanol–water (65:24:5, v/v/v), which were 0.32 and 0.65 respectively [14,15]. The α-amino acid composition of peaks 1–5 and 9–13 (Fig. 2) conform to the known heptapeptide moieties of iturin A and surfactin, as shown in Fig. 3, confirming the identification by the R_F values. Moreover, no minor components were detected with each peak, which indicates the high purity of the molecules.

In fact, *Bacillus subtilis* strain S499 has been shown to produce a high yield of iturin A and surfactin [14], but no details were given about

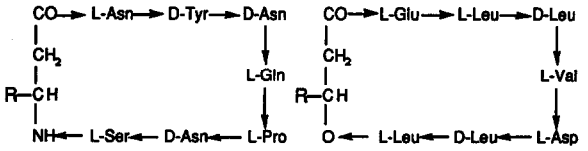

Fig. 3. Structures of iturin A and surfactin. R = Aliphatic chains.

their constituents. Although those lipopeptides are often produced together [16], they were purified by different chromatographic techniques. Chromatography on silicic acid has been used to purify iturin [3,4] but it does not allow the separation of homologous series. On the other hand, preparative HPLC using a C_{18} column enabled six homologues of iturin with different lengths of the side-chain to be separated [4]. Other chromatographic techniques such as ion-exchange [15] or gel permeation chromatography [17] have been used for surfactin purification. Our results show that iturin A and surfactin may be purified in one chromatographic step and both are constituted by a mixture of five homologous lipopeptides as the amino acid residues of the peptidic parts are identical. Confirmation should be obtained by lipidic chain analysis of each component. A third group of molecules also appeared in the chromatogram. They gave a white spot like iturin A and surfactin after a water spray on the TLC plate, with an R_F value of 0.11. A complete study of these molecules is in progress.

In conclusion, it is possible to purify simultaneously iturin A and surfactin including their homologues using a PepRPC column with an FPLC system after crude clean-up on a Bond Elut C_{18} cartridge. This purification procedure is rapid and efficient and provide molecules with a high degree of purity. The preparation of a large

amount of pure lipopeptides is easy by means of a direct scale-up with larger columns. In addition, the biocompatibility of this method allows the structure and function of lipopeptides to be maintained, which is essential for fundamental studies of these molecules. Such a method will be suitable for the identification and purification of other lipopeptides produced by different strains of *Bacillus subtilis*.

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