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Short communication

High-performance liquid chromatography with photodiode array detection for analysis of the fungal metabolite sclerin

Alvin N. Starratt*, George Lazarovits

Agriculture and Agri-Food Canada, Pest Management Research Centre, 1391 Sandford St., London, Ont. N5V 4T3, Canada

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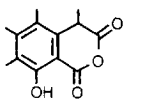
Abstract

A reversed-phase high-performance liquid chromatographic method has been developed for the analysis of sclerin produced by cultures of *Sclerotinia sclerotiorum*. Spectral data obtained with a photodiode array detector enables sclerin to be distinguished from its less hydrophobic hydrolysis and alcoholysis products.

Keywords: Sclerotinia sclerotiorum; Sclerin

1. Introduction

The pentaketide sclerin (structures of this and its derivatives are shown in Fig. 1), isolated from *Sclerotinia sclerotiorum* (*S. libertiana*) and *Aspergillus carneus* [1–3], has been shown to affect a wide spectrum of biological activities in diverse organisms such as fungi, plants and animals [1,4–6]. It stimulates the enzyme activity and growth of castor bean, mung bean and rice seedlings [1] and affects sclerotia formation and enzyme activities in the plant pathogenic fungus *S. sclerotiorum* [4]. Similar growth and enzyme stimulatory activities have been described in other microorganisms and animals [5,6]. However, despite the interest generated in its biological activity and its unusual chemical structure which has presented an intriguing biosynthetic problem [7], there is no chromatographic method to monitor stability, assess purity or to determine levels of sclerin in biological systems. Our recent results show that sclerin produced by fungal cultures can readily be analyzed by reversed-phase high-performance liquid chromatography (HPLC). The studies also show that, in aqueous or alcoholic environments, the anhydride ring of sclerin is rapidly



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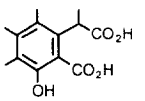

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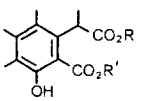

3 R = H or CH_3, R′ = CH_3 or H
4 R = H or C_2H_5, R′ = C_2H_5 or H
5 R = R′ = CH_3

Fig. 1. Structures of sclerin (**1**) and its derivatives, diacid (**2**), monomethyl ester (**3**), monoethyl ester (**4**) and dimethyl ester (**5**).

*Corresponding author.

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cleaved, yielding ring-opened products which recyclize upon removal of the hydroxylic solvent.

2. Experimental

2.1. Isolation of sclerin

Thirteen-day-old cultures of *S. sclerotiorum* on potato dextrose agar (Difco) were extracted by shaking for 24 h with ethyl acetate–methanol–acetic acid (50:50:1; 50 ml/Petri plate). The organic solvents were then removed by rotary evaporation and the aqueous solution remaining was extracted with ethyl acetate. Residue contained in this extract was chromatographed over silica gel (BDH, 60–120 mesh) using methylene chloride. Crystalline material eluted in early fractions was recrystallized from chloroform–diethyl ether to give sclerin: m.p. 122–123°C (Ref. [2], 123°C); ν_{max} (KBr) 3210, 1800, 1698, 1602, 1578 cm^{-1}; ^1H NMR (C^2HCl_3) δ 1.53 (3H, d, J=7 Hz), 2.15, 2.22, 2.27 (3H each, s), 4.13 (1H, q, J=7 Hz), 10.73 (1H, s); high-resolution electron-impact mass spectrometry m/z [M^+] 234.0896 (calcd. for $C_{13}H_{14}O_4$, 234.0892).

2.2. HPLC analysis

Reversed-phase HPLC analysis of sclerin was performed on Waters Nova-Pak C_{18} (300×3.9 mm I.D.) or Hamilton PRP-1 (150×4.1 mm I.D.) columns with a Waters system consisting of two M510 pumps controlled by a Millennium 2010 Chromatography Manager using acetonitrile–water linear gradients containing 10 m*M* acetic acid. The chromatography of sclerin in acetonitrile, acetonitrile–water mixtures, methanol and ethanol and related samples, introduced using a U6K injector, was monitored with a Shimadzu Model SPD-M6A photodiode array detector. The reversibility of the reactions with water and alcohol when samples were dried was studied by HPLC analysis of acetonitrile solutions of the residues. Analytical-scale reactions were conducted in polypropylene or acid-washed glass tubes to avoid possible effects of basic components associated with the glass. Sclerin in crude extracts of cultures of *S. sclerotiorum* (see above) was analyzed similarly after removal of the ethyl acetate and redissolving the residue in acetonitrile.

2.3. Dimethyl ester

Methylation of the product of sclerin in methanol with diazomethane in diethyl ether yielded the dimethyl ester: m.p. 112–113°C (Ref. [2], 113–116°C); ν_{max} ($CHCl_3$) 1726, 1663, 1592 cm^{-1}; ^1H NMR (C^2HCl_3) δ 1.49 (3H, d, J=7 Hz), 2.16, 2.20, 2.23 (3H each, s), 3.60, 3.81 (3H each, s), 4.20 (1H, q, J=7 Hz), 10.73 (1H, s).

3. Results and discussion

Early attempts to analyze sclerin on a Nova-Pak C_{18} column using linear gradients of 0 to 50% acetonitrile in water containing 10 m*M* acetic acid yielded two peaks, one eluting much later than the other. Varying the elution conditions affected the relative size of the peaks suggesting that the earlier eluting peak corresponded to the diacid resulting from hydrolysis of the anhydride ring and the later one was sclerin. Essentially a single peak corresponding to the retention time of sclerin could be obtained when the sample was rapidly injected onto the column and a linear gradient of 40 to 50% acetonitrile in water containing 10 m*M* acetic acid was used (Fig. 2A). Similar results were obtained with the Hamilton polymeric reversed-phase column. If the sample remained in the sample loop of the injector for even 2 min, a significant portion of the sample eluted at the earlier retention time indicating that rapid hydrolysis occurred. Apparently, sclerin is stable once it is in contact with the hydrophobic column packing despite the presence of water in the eluent. Addition of an equal volume of water to a stock solution of sclerin in acetonitrile also led to rapid formation of the earlier eluting substance (Fig. 2B; analysis performed 30 min after addition of water) which is consistent with hydrolysis. HPLC, after drying the diacid solution and redissolving in acetonitrile, indicated that the anhydride ring had reformed in the absence of water. Sclerin levels in cultures could easily be determined by HPLC of

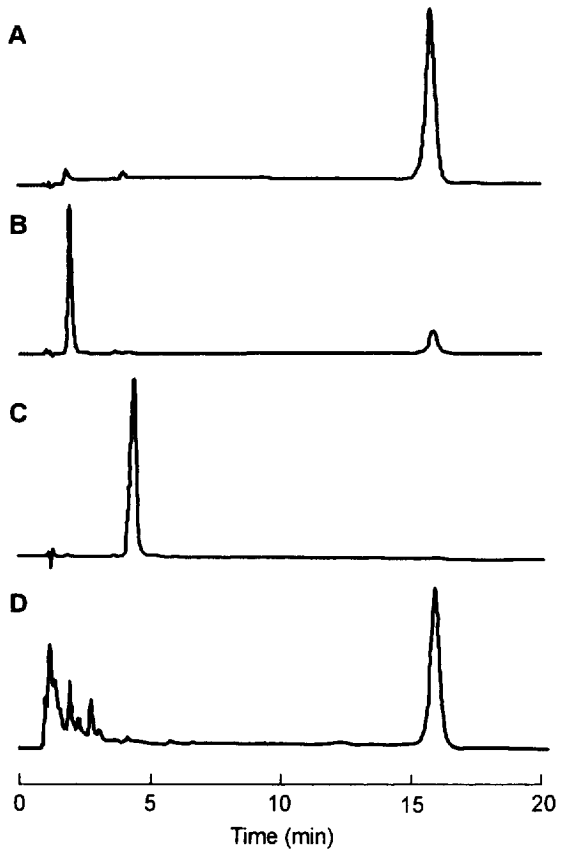

Fig. 2. Normalized HPLC plots (Nova-Pak C_{18} column) of sclerin (2 μg) dissolved in: (A) acetonitrile; (B) water–acetonitrile (1:1); (C) methanol; (D) analysis of sclerin in an extract of a culture of *S. sclerotiorum*. Chromatographic conditions: 30-min linear gradient from 40 to 50% acetonitrile in water containing 10 m*M* acetic acid at 1 ml/min. Detection: 215 nm.

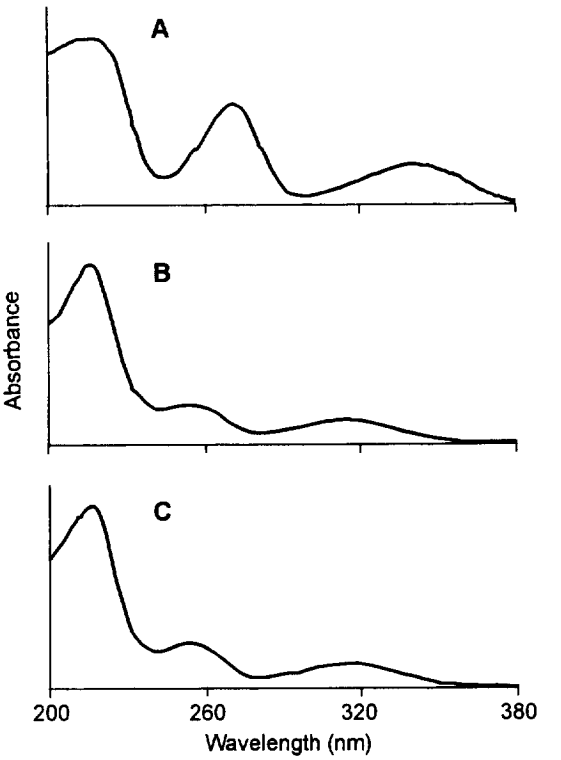

Fig. 3. Nomalized ultraviolet absorption spectra of sclerin and related compounds eluting from the Nova-Pak C_{18} column (photodiode array detection): (A) sclerin (major peak, Fig. 2A); (B) diacid (major peak, Fig. 2B); (C) monomethyl ester.

acetonitrile solutions of material contained in the ethyl acetate extracts (Fig. 2D).

Sclerin absorbed at higher wavelength than the diacid (Fig. 3A,B). Examination of spectral data acquired by the photodiode array detector indicated that the chromatographic peaks were homogeneous. The spectrum of the peak assigned to sclerin in the chromatograph of the culture extract (Fig. 2D) was identical to that of the peak for the standard prepared from crystalline sclerin. Using conditions which minimized hydrolysis (see Fig. 2A), results were very repeatable and a linear response was obtained for injections of sclerin in the range from 0.01 μg, near the practical limit of detection, to 8 μg, the upper level tested. Levels of sclerin produced by cultures of *S. sclerotiorum* could be determined by comparing peak areas of aliquots with responses given by external sclerin standards.

Evidence that alcoholysis of the anhydride ring occurs was obtained when sclerin was dissolved in methanol or ethanol. With conditions that eluted the hydrolysis product from the C_{18} column at 1.85 min (Fig. 2B) and sclerin at 15.80 min (Fig. 2A), the monomethyl and monoethyl esters, products of the reaction of sclerin with methanol and ethanol, eluted at 4.35 (Fig. 2C) and 6.60 min, respectively. Treatment of the methanolic solution with diazomethane yielded a dimethyl ester, eluting at 14.95 min under the same chromatographic conditions, with properties in good agreement with those reported [2].

The photodiode array spectra of the alcoholysis products were indistinguishable from that of the hydrolysis product and clearly different than that of

sclerin (see Fig. 3C for spectrum of the product formed when sclerin is dissolved in methanol). The occurrence of methanolysis provided an explanation for the variable results which we obtained when we measured the UV spectrum of sclerin in methanol with a Shimadzu UV-240 instrument. When scanned immediately after dissolving, the spectrum had peaks at wavelengths somewhat higher than reported [2,3] but repeated scanning showed that the wavelength of the peaks gradually shifted downward finally resulting in a spectrum similar to that shown in Fig. 3C. Removal of the alcohol and reanalysis by HPLC after dissolving in acetonitrile showed that a product with retention time and photodiode array spectrum identical to that of sclerin was again obtained.

Although it has been stated that the unusual stability of the anhydride ring appears to be without precedent [2], it has been reported that sclerin dissolves in sodium bicarbonate solution [1]. Therefore, it should not be unexpected that sclerin exists in aqueous or alcoholic solutions mainly in the ring-opened form, resulting from reaction of the anhydride with the solvent. The ease of hydrolysis of sclerin points to the diacid as being the substance exhibiting activity in the various reported biological tests.

In conclusion, our studies show that sclerin produced by fungal cultures can readily be analyzed by reversed-phase HPLC thereby enabling the quantitative determination of levels in extracts of cultures grown under various conditions. Spectra data generated by photodiode array detection provide a means to identify sclerin and distinguish it from its hydrolysis and alcoholysis products.

Acknowledgments

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