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Note

High-performance liquid chromatographic determination of macrosporin, altersolanol A, alterporriol A, B and C in fermentation of *Alternaria porri* (Ellis) Ciferri

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Macrosporin (Mac 1)[1], altersolanol A (As-A 2)[2], alterporriol A (Ap-A 3)[3], alterporriol B (Ap-B 3)[4] and alterporriol C (Ap-C 4)[5] have been found as metabolic pigments produced by *Alternaria porri* (Ellis) Ciferri, the causal fungus of black spot disease of stone-leek (Japanese name, negi). One of these pigments, As-A, inhibits elongation of the root in seeds of lettuce and stone-leek and shows antimicrobial activity against Gram-positive and -negative bacteria[6]. Also, Ap-A and Ap-B were found to be atropisomers of each other[3]. Cladofulvin[7] and asphodelin[8] have been reported as naturally occurring $\alpha\beta'$-bianthraquinone. The former is a metabolic product of *Cladosporium fulvum* and the latter is a component of *Aloe saponaria* HAW. Ap-C was found to be the second example of $\alpha\beta'$-bianthraquinone produced by fungi. Previously, we reported the high-performance liquid chromatographic (HPLC) determination of As-A, As-B and dactylariol, reduced anthraquinones produced during the fermentation period when *Alternaria porri* was cultured on Brian's medium T[9].

The structures of Ap-A, -B and -C show that they are modified bianthraquinones consisting of As-A and Mac (Fig. 1). So far as the biogenesis of Ap-A, -B and -C is concerned, two pathways can be considered, namely whether As-A and Mac are first metabolized and then bonded to alterporriols, or alterporriols are first metabolized and then their C–C linkages connecting the monomeric halves are cleaved to two halves of the molecules, As-A and Mac. This paper deals with the HPLC determina-

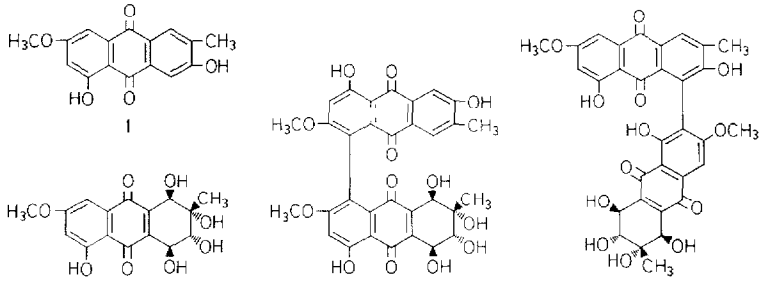

Fig. 1. Structures of pigments: 1 = macrosporin; 2 = altersolanol A; 3 = alterporriol A and B; 4 = alterporriol C.

tion of Mac, As-A and Ap-A, -B and -C during the period of fermentation in order to explore their metabolic pathways.

EXPERIMENTAL

Material

Mac, As-A and Ap-A, -B and -C were isolated as metabolic pigments of *Alternaria porri* (Ellis) Ciferri (IFO 9762), which was isolated and donated by the Institute for Fermentation, Osaka, Japan (IFO).

High-performance liquid chromatography

HPLC was performed on a Shimadzu LC-6A liquid chromatograph equipped with a UV detector, operating at 254 nm for all assays. Three solvent systems were used for the resolution of five metabolic pigments, because their polarities in HPLC differed. For Ap-A, -B and -C, 0.05 *M* ammonium dihydrogen phosphate (adjusted to pH 2.5 with phosphoric acid) acetonitrile (7:3) was used as the mobile phase. For As-A and Mac, the same components were used but in the ratios 4:1 and 1:1, respectively. The column used was a YMC A-312 (Yamamura Chemical Laboratories), commercially packed with reversed-phase octadecylsilica (5 μm) (150 mm $\times$ 6.0 mm I.D.), through which the above mobile phases were run at a flow-rate of 1.0 ml/min. Samples of 10 μl were injected on to the column.

Fermentation and extraction of pigments

A 2% sucrose solution of onion decoction was used as a culture medium. A number of 500-ml erlenmeyer flasks containing 200 ml of the medium were sterilized in an autoclave for 20 min at 2.3 bar and 120°C. The fungi, cultured on agar for 7–10 days, were inoculated into the flasks, which were then kept at 25°C. After fermentation for 1 day, 10 ml of the culture liquid were taken and extracted first with *n*-hexane to remove lipids and then with ethyl acetate (5 $\times$ 30 ml). After evaporation of the solvent, the coloured material obtained was denoted S-1 (1.1 mg). By a similar procedure, coloured material corresponding to fermentation periods of 2, 5, 7, 14, 21 and 28 days were obtained and denoted S-2 (2.1 mg), S-3 (4.6 mg), S-4 (5.8 mg), S-5 (7.2 mg), S-6 (7.8 mg) and S-7 (8.7 mg), respectively, plus S-8 for blank (0.7 mg).

RESULTS AND DISCUSSION

Quantitative analysis of Mac, As-A and Ap-A, -B and -C during the fermentation period

As shown in Fig. 2, the retention times (t_R) were 6.4 min (As-A, $k' = 1.29$), 14 min (Mac, $k' = 5.13$), 22 min (Ap-B, $k' = 7.93$), 23 min (Ap-A, $k' = 8.31$) and 46 min (Ap-C, $k' = 17.61$). We used the internal standard method for quantitation and α-naphthol (t_R 26 min, $k' = 9.89$) was used as the internal standard for Ap-A, -B and -C, and benzoic acid (t_R 14 min, $k' = 3.96$) and naphthalene (t_R 19 min, $k' = 7.53$) for As-A and Mac, respectively. For example, methanolic solutions of As-A (1 mg/ml) (0.4, 0.6, 0.8, 1.0 and 1.2 ml) were placed into five sample vials and 1-ml portions of methanolic solutions of benzoic acid (1 mg/ml) were added. After the volumes had been adjusted to 10 ml with methanol, 10-μl portions of each were subjected to HPLC under the condition mentioned above. By plotting the peak-area ratios against sample weight a calibration graph for As-A was obtained. The calibration graph for Mac was

TABLE I

ANALYTICAL RESULTS FOR PIGMENTS

(A) Peak-area ratio to I.S.; (B) weight ratio to I.S.; (C) concentration (mg/ml).

Sample	*Macrosporin*			*Altersolanol A*			*Alterporriol A*			*Alterporriol B*			*Alterporriol C*		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
S-1	$1.08 \cdot 10^{-3}$	–	–	0.132	0.16	0.016	–	–	–	–	–	–	–	–	–
S-2	$2.52 \cdot 10^{-3}$	–	–	0.420	0.51	0.051	–	–	–	–	–	–	–	–	–
S-3	0.566	0.31	0.031	1.192	1.46	0.146	0.677	0.50	0.0050	0.741	0.35	0.0035	0.660	0.26	0.0026
S-4	0.141	0.06	0.006	1.023	1.25	0.125	0.234	0.17	0.0017	0.438	0.16	0.0016	0.524	0.21	0.0021
S-5	1.056	0.59	0.059	0.896	1.10	0.110	0.486	0.36	0.0036	0.519	0.21	0.0021	1.261	0.49	0.0049
S-6	1.591	0.91	0.091	0.635	0.78	0.078	1.063	0.79	0.0079	0.924	0.47	0.0047	1.537	0.60	0.0060
S-7	2.083	1.20	0.120	0.337	0.41	0.041	2.126	1.59	0.0159	1.855	1.07	0.0107	1.264	0.49	0.0049
S-8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

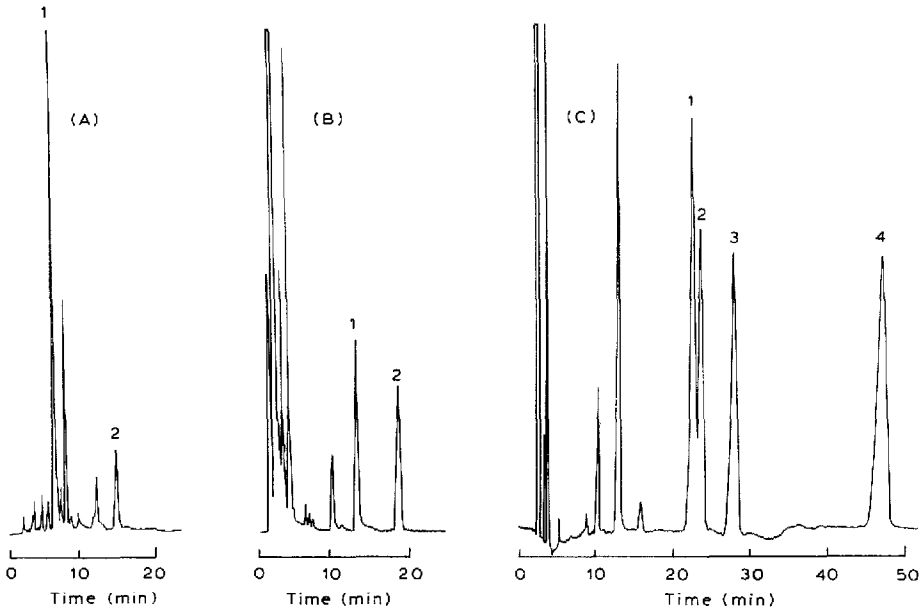

Fig. 2. Chromatograms of pigments and I.S. (A) 1 = Altersolanol A, 2 = benzoic acid; (B) 1 = macrosporin, 2 = naphthalene; (C) 1 = alterporriol B, 2 = alterporriol A, 3 = α-naphthol, 4 = alterporriol C.

obtained by using the same concentration as that of As-A, whereas for Ap-A, -B and -C the concentrations were reduced 10-fold. The limits of detection, based on a signal-to-noise ratio of 10, for Mac and As-A were 0.1 μg/ml and for Ap-A, -B and -C 1 μg/ml.

The concentrations of Mac, As-A and Ap-A, -B and -C were calculated from the detector responses (peak areas) by using the following linear equations for the calibration graphs obtained by using the method of least squares:

$$\text{Mac: } Y = (0.589X - 2.79 \cdot 10^{-2}) \cdot 0.1$$
$$\text{As-A: } Y = (1.232X - 6.70 \cdot 10^{-3}) \cdot 0.1$$
$$\text{Ap-A: } Y = (0.748X - 2.30 \cdot 10^{-3}) \cdot 0.01$$
$$\text{Ap-B: } Y = (0.647X - 1.26 \cdot 10^{-1}) \cdot 0.01$$
$$\text{Ap-C: } Y = (0.386X + 6.24 \cdot 10^{-3}) \cdot 0.01$$

where Y is the concentration of each pigment (mg/ml) and X is the ratio of peak area between each pigment and the internal standard (I.S.).

Extracts S-1 to S-8 were dissolved in methanol (10 ml) with the I.S. and then 10 μl of each were subjected to HPLC under the conditions given above. The results are shown in Table I and Fig. 3. Previously, we reported that As-A was not detected in the culture liquid of the onion decoction[2]. In this work, however, Aa-A was proved to be a metabolite by means of comparison of the retention time of the metabolite with that of an authentic sample.

As shown in Table I and Fig. 3, As-A and Mac were detected after fermentation for 1 day, whereas Ap-A, -B and -C were detected after 5 days. The content of As-A in

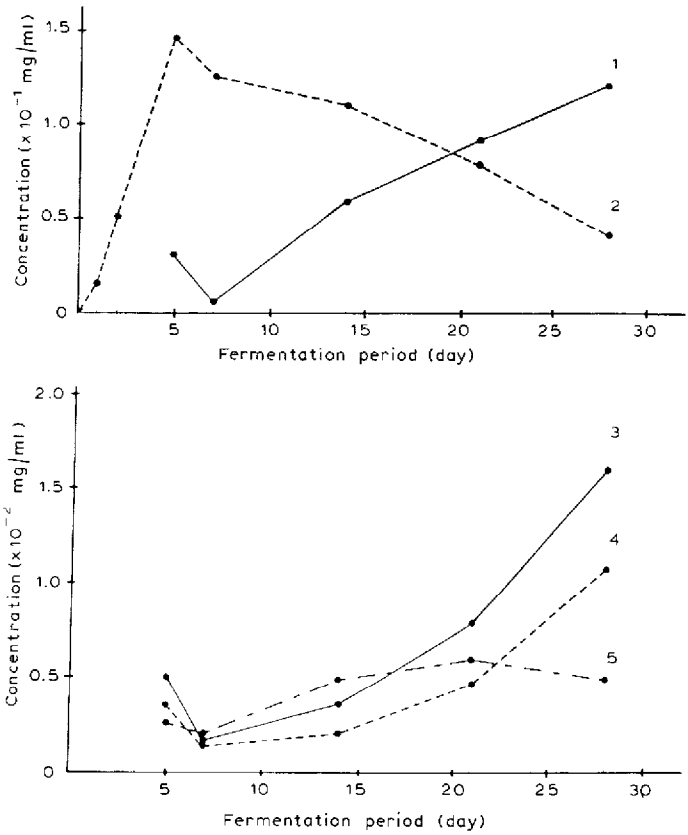

Fig. 3. Relationship between concentration of pigments and fermentation periods: 1 = macrosporin; 2 = altersolanol A; 3 = alterporriol A; 4 = alterporriol B; 5 = alterporriol C.

the culture liquid was found to increase continuously for 7 days and then gradually to decrease, whereas Mac and Ap-A, -B and -C were found to increase gradually for up to 28 days. Previously we reported[7] that in the early period of fermentation As-A is first formed, and then it is converted into As-B and dactylariol when *Alternaria porri* is cultured on Brian's medium T. The present results suggest that in the early period of fermentation As-A and Mac are first formed, and then these moieties are bonded to Ap-A, -B and -C when the fungus is cultured on onion decoction medium.

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