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Short Communication

High-performance liquid chromatographic determination of tentoxin in fermentation of *Alternaria porri* (Ellis) Ciferri

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ABSTRACT

Tentoxin is an interesting non-specific toxin produced by some *Alternaria* species, known as pathogenic organisms. The high-performance liquid chromatographic determination of tentoxin by using a YMCA-312 column, a reversed type of octadecylsilica, with 0.05 *M* ammonium dihydrogenphosphate–acetonitrile (7:3) as the mobile phase is described. The limit of detection is 0.1 μg/ml. One assay can be performed by using culture liquid without any other procedures within 30 min.

INTRODUCTION

In the course of investigations on the bioactive products of *Alternaria porri* (Ellis) Ciferri, the causal fungus of black spot disease in the stone-leek (Japanese name negi) and onion, we have previously reported three reduced anthraquinone derivatives, altersolanol A and B and dactylariol [1], which inhibit elongation of the root in the seeds of lettuce and stone-leek. Altersolanol A also shows antimicrobial activity against some Gram-positive and -negative bacteria [1] and altersolanol B shows high cytotoxicity towards HeLa [2] and Ehrlich ascites carcinoma [3]. Recently, we isolated another bioactive metabolite, tentoxin, a cyclic peptide phytotoxin, from the ethyl acetate-soluble part of the culture liquid of *Alternaria porri* cultured on onion decoction medium [4]. Tentoxin was first isolated by Meyer *et al.* [5] from *A. tenuis*, which is known as a pathogenic organism causing chlorosis in the cotyledons of many

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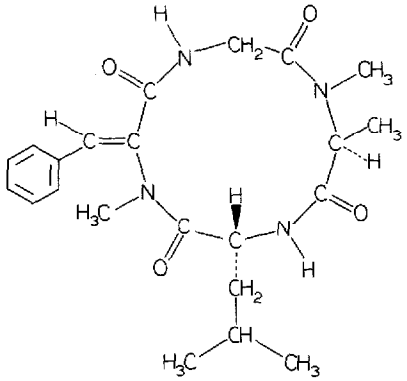

Fig. 1. Structure of tentoxin.

dicotyledonous plant species. The structure (Fig. 1), including the stereochemistry, has also been determined [5,6]. Tentoxin, other than as a metabolite of *A. tenuis*, is also known as a non-specific toxin produced by some *Alternaria* species known as pathogenic organisms, namely *A. mali* [7], *A. citri* [8] and *A. alternata* [9]. From the chemotaxonomic point of view, it is presumed that tentoxin might be found as a metabolite of some *Alternaria* species other than those mentioned above and any fungi related to *Alternaria*. Hence a convenient and accurate method for the determination of tentoxin was required.

Previously, we reported the high-performance liquid chromatographic (HPLC) determination of altersolanol A, macrosporin and alterporriol A, B, C [10] and D and E [11] in the fermentation of *Alternaria porri*. This paper reports the HPLC determination of tentoxin during the period of fermentation when *Alternaria porri* is cultured on onion decoction medium.

EXPERIMENTAL

Material

Tentoxin was isolated as a metabolite of *Alternaria porri* (IFO 9762), which was purchased from the Institute for Fermentation, Osaka, Japan (IFO).

High-performance liquid chromatography

HPLC was performed on a Shimadzu LC-6A liquid chromatograph with a UV detector (Shimadzu SPD-6AV) and integrator (Shimadzu C-R3A) operating at 254 nm in all assays. The solvent system used was 0.05 *M* ammonium dihydrogenphosphate (adjusted to pH 2.5 with phosphoric acid)–acetonitrile (7:3). The column used was a YMC A-312 (Yamamura Chemical Labs., Kyoto, Japan), commercially packed with reversed-phase octadecylsilica (5 μm) (150 mm × 6 mm I.D.). The mobile phase flow-rate was 1.0 ml/min and samples of 10 μl were injected onto the column.

Fermentation and extraction of tentoxin

A 2% (w/v) 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

sterillized in an autoclave for 20 min at 2.0 bar and 121°C. The fungi, subcultured on an agar medium for 7–10 days, were inoculated into the flasks, which were then kept at 25°C. After fermentation for 2 days, 10 ml of the culture filtrate were extracted with *n*-hexane (4 × 10 ml) to remove lipids. The aqueous layer obtained was called S-2. By a similar procedure, the aqueous layers corresponding to fermentation periods of 5, 7, 14, 21 and 28 days were designated S-3, S-4, S-5, S-6 and S-7, respectively; S-1 was the blank.

RESULTS AND DISCUSSION

Determination of tentoxin during the fermentation period

When the sample of S-6 was used, the chromatograms of tentoxin and the internal standard (I.S.) were as shown in Fig. 2, in which the retention time (t_R) was 17.4 min (tentoxin, capacity factor $k' = 4.3$). We used the internal standard method for quantitation and α-naphthol ($t_R = 21.7$ min, $k' = 9.8$) was used as the internal standard for tentoxin. Methanolic solutions of tentoxin (0.1 mg/ml) (0.4, 0.6, 0.8, 1.0

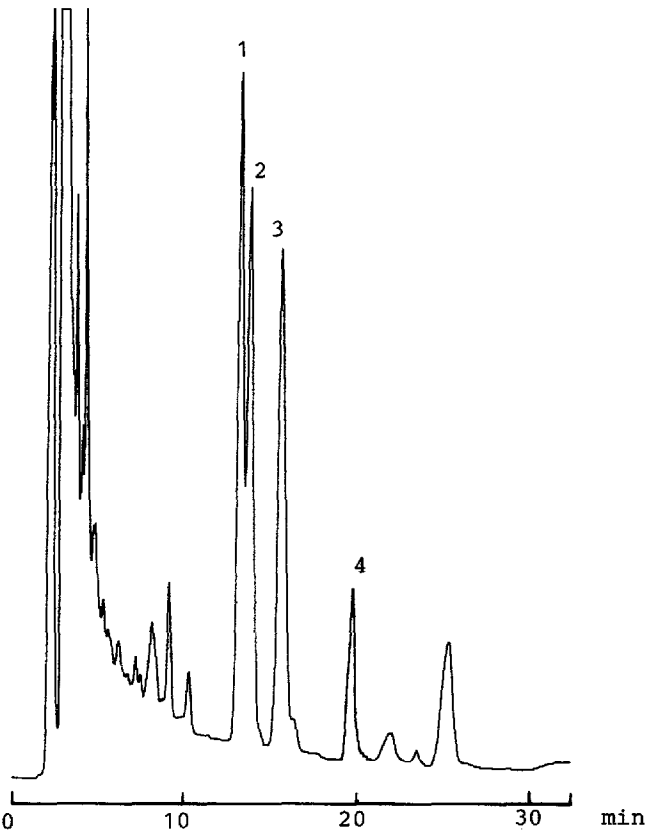

Fig. 2. Chromatograms of tentoxin and I.S. Sample: S-6. Conditions: flow-rate, 1.0 ml/min; detection, UV (254 nm). Peaks: 1 = alterporriol B; 2 = alterporriol A; 3 = tentoxin; 4 = α-naphthol.

and 1.2 ml) were placed in sample vials and 1-ml portions of methanolic solutions of α-naphthol (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 above conditions. By plotting the peak-area ratio against sample weight, a calibration graph for tentoxin was obtained. The limit of detection, based on a signal-to-noise ratio of 10 for tentoxin, was 0.1 μg/ml.

As a practical procedure, α-naphthol (1 mg) was dissolved in each of culture liquids S-1 to S-7 and then 10 μl of each were subjected to HPLC analysis under the above conditions.

The combined results obtained from eight fermentation experiments indicated that tentoxin was detected after fermentation for 5 days and increased continuously for up to 28 days, then levelled off at least for 56 days at 3.3 mg/l.

We conclude that the proposed HPLC method for the determination of tentoxin in fermentation of fungi may be potentially useful, facile and rapid, because it can be carried out by using culture liquid directly without any other procedures and an assay can be completed within 30 min.

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