THIAZOHALOSTATIN, A NEW CYTOPROTECTIVE SUBSTANCE PRODUCED BY *Actinomadura*

I. TAXONOMY, PRODUCTION, ISOLATION AND BIOLOGICAL ACTIVITIES

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Thiazohalostatin has been isolated from the culture broth of *Actinomadura* sp. by a screening program designed to find novel cytoprotective substances. It was purified by use of column chromatography on silica gel, reversed phase HPLC and then isolated as colorless powder. Thiazohalostatin prevented cell death caused by calcium overload and exhibited an inhibitory activity against lipid peroxidation.

Currently, therapy for ischemic heart disease is based on drugs such as β-adrenoreceptor antagonists, calcium antagonists and nitrates, which show pronounced hemodynamic effect. However, these drugs can have adverse reactions including systemic hemodynamic effects, leading to low blood pressure and peripheral edema in some patients. Recent observations that certain types of calcium antagonists such as flunarizine prevent the calcium overload in cardiac myocytes that occurs after ischemia have led to the design of a new type of antiischemic drugs that is cytoprotective, but has no (or little) hemodynamic effects[1].

During the course of a screening program for novel substances showing cytoprotective activity, we obtained a new metabolite of a fungus, rumbrin[2]. Further screening resulted in the isolation of a novel cytoprotective substance named thiazohalostatin (Fig. 1) from the culture broth of *Actinomadura* sp. HQ24. It prevented cell death caused by calcium overload in cells exposed to toxic concentrations of the calcium ionophore A23187. Additionally, thiazohalostatin exhibited an inhibitory activity against lipid peroxidation in rat brain homogenates.

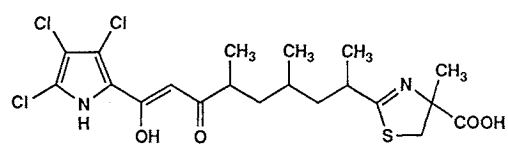
In this paper, we report the taxonomy of the producing strain, and production, isolation and biological activities of thiazohalostatin. Physico-chemical properties and structural studies of thiazohalostatin will be reported in the accompanying paper[3].

Materials and Methods

Chemicals

Chemicals employed were as follows: silica gel from Wako Pure Chemical Industries, Ltd., Tokyo, Japan; packed column of YMC D-ODS-7 from Yamamura Chemical Laboratories Co., Ltd., Kyoto, Japan; calcium ionophore A23187 and flunarizine from Sigma Chemical Co., St. Louis, U.S.A. All other chemicals were of analytical grade.

Fig. 1. Structure of thiazohalostatin.



Microorganism

Strain HQ24 was isolated from a soil sample

collected in the Kii peninsula, Japan, and has been deposited in the National Institute of Bioscience and Human Technology (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba-shi, Japan, under the accession No. FERM BP-4037.

Taxonomic Studies

Morphological and physiological properties of the strain were examined according to SHIRLING and GOTTLIEB[4]; several other tests were also used. The spore chain morphology and hyphae of the strain were determined using a light microscope and observed under a scanning electron microscope (S-800, Hitachi Co., Ltd., Tokyo, Japan). Whole-cell hydrolysates and menaquinones were analyzed by the method of LECHEVALIER and LECHEVALIER[5], and COLLINS *et al.*[6], respectively.

Fermentation

The seed medium and the production medium consisted of soluble starch 0.8%, glycerol 0.8%, soy bean meal 0.3%, fish meal 0.8% and $CaCO_3$ 0.2% (pH not adjusted).

Assays for Biological Activity

Biological activities were measured by the methods reported previously[2]. Cytoprotective activity against cell death caused by calcium overload was examined by a modified method of HIRAI *et al.*[7], as follows. 3T3-Swiss albino cells (mouse embryo cell line) were inoculated into each well of a 96-well collagen-coated plate containing 100 μl of DULBECCO's modified minimum essential medium supplemented with 10% fetal bovine serum (D-MEM). After overnight incubation at 37°C in an atmosphere of 5% CO_2 in air, the culture supernatant was discarded and the confluent cells were given 100 μl of D-MEM containing various concentrations of samples and 50 μl of D-MEM containing 5×10^{-5} M calcium ionophore A23187. After 90 minutes incubation at 37°C in an atmosphere of 5% CO_2 in air, cell viability was measured as the value of A_{570} by colorimetric MTT assay[8]. The percent of cell viability was calculated by the formula $[(C-B)/(A-B)] \times 100$, where A is the value of A_{570} in the system without A23187, B is that with A23187 and C is that with A23187 and a sample. The concentration range of a sample required for more than 50% of cell viability was determined.

Inhibitory activity against lipid peroxidation in rat brain homogenate was measured according to the method of KUBO *et al.*[9] in the presence of ascorbic acid (100 μM).

Results

Taxonomic Characterization of the Producing Strain

Strain HQ24 had branched substrate mycelia, and fragmentation of hyphae did not occur either on agar or under submerged growth conditions. The aerial mycelia were formed in abundance on inorganic salts - starch agar and oatmeal agar at 27°C for 21 days. Matured spore-chains contained 5 to 20 spores and were straight or looped. The spores were oval to ellipsoidal with a smooth surface, and were $0.4 \times 0.5 \sim 1.8$ μm in size as revealed by scanning electron microscopy (Fig. 2). Spores were not motile, and development of whirls, sporangia (including pseudosporangia), sclerotia or other special structures were not observed.

Fig. 2. Scanning electron micrograph of spore chains of strain HQ24.

Bar in the picture indicates 6 μm.

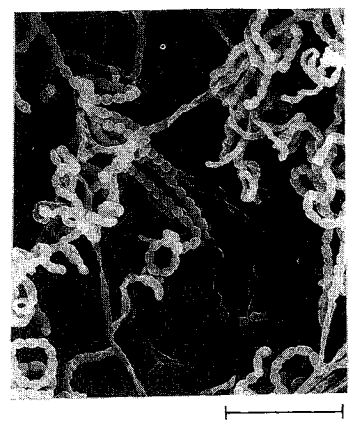


Table 1. Cultural characteristics of strain HQ24.

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Sucrose-nitrate agar	Abundant	Moderate, light olive gray	Yellowish brown	None
Glucose-asparagine agar	Abundant	Poor	Light olive gray	None
Glycerol-asparagine agar (ISP-medium 5)	Abundant	Poor, white	Yellowish brown	None
Inorganic salts-starch agar (ISP-medium 4)	Abundant	Abundant, white	Yellowish brown	None
Tyrosine agar (ISP-medium 7)	Abundant	Moderate, white	Yellowish brown	None
Nutrient agar	Abundant	None	Pale yellow	None
Yeast extract-malt extract agar (ISP-medium 2)	Abundant	Poor, white	Yellowish brown	None
Oatmeal agar (ISP-medium 3)	Abundant	Abundant, white	Yellowish brown	None

Table 2. Physiological characteristics of strain HQ24.

Temperature: Growth range	15~45°C
Melanin formation:	
Tyrosine agar (ISP-medium 7)	Negative
Peptone-yeast extract-iron agar (ISP-medium 6)	Negative
Tryptone-yeast extract broth (ISP-medium 1)	Negative
Hydrolysis of starch	Positive
Liquefaction of gelatine	Positive
Coagulation of skim milk	Negative
Peptonization of skim milk	Positive
Nitrate reduction	Positive

Table 3. Carbon utilization of strain HQ24.

Carbon source	Utilization	Carbon source	Utilization
L-Arabinose	+	D-Mannitol	−
D-Xylose	+	D-Mannose	+
D-Glucose	+	D-Galactose	+
D-Fructose	+	Maltose	+
Sucrose	+	D-Ribose	+
L-Rhamnose	+	Glycerol	+
Raffinose	±	D-Sorbitol	±
Inositol	±		

Symbols: +, good; ±, doubtful; −, poor or none.

Cultural characteristics of strain HQ24 grown at 27°C for 21 days are summarized in Table 1. Aerial mass color was white to light olive gray and the color of vegetative growth was pale yellow to yellowish brown. Distinctive soluble pigments were not formed on the media tested.

The observed physiological characteristics and carbon utilization of the strain are shown in Table 2 and Table 3, respectively. Whole-cell hydrolysates contained *meso*-diaminopimelic acid and a very small quantity of 3-hydroxy-diaminopimelic acid, but cell-wall hydrolysate contained no glycine. A small amount of madurose was found to be present in the whole-cell hydrolysates, but arabinose and xylose were not detected. An MK-9 (H_6) and MK-9 (H_8) were detected as the major and the minor components of menaquinones, respectively. From the above characteristics, we determined that the strain HQ24 belonged to the genus *Actinomadura*[10], and it was designated as *Actinomadura* sp. HQ24. Further studies for species identification will be reported elsewhere.

Production and Isolation of Thiazohalostatin

Strain HQ24 was inoculated into 100 ml of the seed medium in a 500-ml Erlenmeyer flask, and cultured at 27°C for 3 days on a rotary shaker (200 rpm) to give a seed culture. One hundred ml of this seed culture was inoculated into 25 liters of the production medium in a 50-liter jar fermentor and cultivated with agitation (150 rpm) at 27°C for 5 days under aeration (0.5 v/v/m). The cultured broth (50 liters) was filtered and the mycelial cake was extracted twice with acetone (15 liters × 2). After removal of acetone, this extract (5 liters) was adjusted to pH 4 with 3 N HCl and extracted twice with equal volumes of EtOAc. The organic

Table 4. Biological activities of thiazohalostatin.

	Thiazohalostatin	Flunarizine·2HCl
Cytoprotective activtiy[a] (μg/ml)	2.5~100	1.3~80
Inhibitory activity against lipid peroxidation IC_{50} (μg/ml)	6.6	55

[a] Concentration range required for more than 50% of cell viability.

layer was evaporated to dryness to give a residue (17.5 g). This residue was chromatographed on a column of silica gel (10 × 50 cm) packed with $CHCl_3$. After the column was washed with a mixture of $CHCl_3$ - MeOH (100 : 1), the active substance was eluted with a mixture of $CHCl_3$ - MeOH (15 : 1), and concentrated under reduced pressure to give crude thiazohalostatin (3.4 g). It was further purified by reversed phase HPLC using a packed column of YMC D-ODS-7 (2 × 25 cm) and MeOH as the developing solvent. After concentration of the active fraction, the pure colorless powder of thiazohalostatin was obtained (1.9 g). The purity of each preparation was confirmed by TLC and HPLC.

Biological Activities of Thiazohalostatin

As shown in Table 4, thiazohalostatin was as active as flunarizine in cytoprotective action. In addition, thiazohalostatin showed much higher inhibitory activity against lipid peroxidation than flunarizine.

Thiazohalostatin showed low toxicity; there was no death after ip injection in mice of 100 mg/kg.

Discussion

Flunarizine is one of the well-known brain protective agents, and it seems likely that a part of its anti-ischemic effect is the result of the prevention of membrane lipid peroxidation associated with abnormal calcium influxes into cells[9]. Our results suggest that thiazohalostatin has a quite similar mode of action to that of flunarizine. Therefore, thiazohalostatin is expected to be useful for the treatment of myocardial and cerebral ischemia[1,9] by the alleviation of tissue damage due to calcium overload and/or peroxidative disintegration of cell membranes. The *in vivo* activity of thiazohalostatin is now under study.

Acknowledgments

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