HERBICIDINS A AND B, TWO NEW ANTIBIOTICS WITH HERBICIDAL ACTIVITY

II. FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL CHARACTERIZATION

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Herbicidins were produced in submerged fermentation by *Streptomyces saganonensis*. Isolation of the antibiotics from the culture broth was performed by adsorption on resinous adsorbent followed by elution with aqueous acetone. Herbicidins A and B were separated from each other by counter-current distribution on a Ronor column or by silica gel chromatography. Physico-chemical characterization revealed that herbicidins are new antibiotics having an adenine nucleoside moiety in their structures.

Taxonomic studies on the producing organism as well as biological activities, including a unique herbicidal effect of herbicidins, were described in the previous paper.[1] The herbicidin-producing organism was identified as a new species of *Streptomyces,* designated as *S. saganonensis*. It has Rectus-Flexibilis to Spira sporophores with smooth to warty spore surface and belongs to gray color series. Herbicidins A and B were distinguished from other known antibiotics in their characteristic biological activities, such as selective and contact herbicidal activity, protective activity against bacterial leaf blight and inhibitory activity of germination of plant seeds.

This report presents the fermentation, isolation and physico-chemical properties of these two new antibiotics. Structural elucidation of the antibiotics will be reported elsewhere.

Materials and Methods

Fermentation studies.

Production of herbicidins by *S. saganonensis* was primarily detected in a cultured broth fermented in 500-ml SAKAGUCHI flask as described in the previous paper.[1] Larger quantities of broth required for isolation and purification studies were produced in 600-liter fermentor containing 300 liters of the medium with the same composition as mentioned before.[1] Fermentation was performed by inoculation of 50 liters of seed culture fermented in 100-liter fermentor for 24 hours at 27°C, followed by agitation (190 rev/min) and aeration (300 liters/min) for 70 or 117 hours at 27°C.

The mycelial growth during fermentation was followed by the packed cell volume after centrifugation of 10 g of the broth at 3,000 rpm for 10 minutes.

Assay procedure.

Progress of the fermentation was monitored by extraction of the culture filtrate with *n*-BuOH and detection of herbicidins A and B on silica gel TLC (Silica Gel F_{254}, E. Merck, Darmstadt, Germany) using $CHCl_3$-MeOH (9: 1) as a solvent system for development. Quantitative determination of each of the components was performed by densitometry. The instrument used was Dual-Wavelength TLC Scanner (Model CS–900, Shimazu Co., Ltd., Japan) at 258 nm. Each authentic sample in *n*-BuOH

was applied on the same plate with samples to be tested. In addition to the TLC method, the antibiotic potency of broth or purified samples of herbicidins was also determined by their effect on germination of the seeds of Chinese cabbage as described in the previous paper.[1]

Isolation of herbicidins.

Fermentation broth (350 liters) containing herbicidins was adjusted to pH 5.0 with 6 N HCl and filtered with the aid of infusorial earth (Celite 545 from Johns-Manville Products Corp., Calif., U.S.A.). The filtrate (295 liters) containing about 85 and 30 μg/ml of herbicidins A and B, respectively, was adsorbed on 25 liters of Duolite S–30 (Adsorbent resin from Diamond Shamrock Chem. Co., U.S.A.) column. The spent broth and water washes (70 liters) contained very little activity and discarded. The adsorbate was eluted with 50% aqueous acetone, and 70-liter active fractions were collected and concentrated under reduced pressure to dryness. The remaining material was extracted with 5 liters of MeOH and the extract was concentrated to dryness under reduced pressure. The crude solid thus obtained was composed of approximately 25 and 8.5 g of A and B, respectively, and was further purified by two methods described below.

(1) The concentrate was applied to a counter-current distribution system for the separation of A and B from each other which consisted of Ronor columns (MVS RO–700, Ronor AG, Bern, Switzerland) equipped with 200 chambers. The solvent was $CHCl_3$ - MeOH - H_2O (5: 3: 5). The distribution coefficient for this solvent system for herbicidins A and B were 0.29 and 0.56, respectively. The rotating column (10 rev/min) was filled with the heavy phase using Beckman Solution Metering Pump Model 746 at 10 ml/min of flow rate and then the light phase was applied at the flow rate of 2.2 ml/min to equilibrate the heavy phase. After application of 50 g of the crude sample dissolved in 50 ml of the light phase at 2.2 ml/min flow rate, the light phase was continuously introduced into the column at the same flow rate. Each 15-ml fraction was collected automatically. Herbicidin A detected in cuts 180 to 400 was collected and concentrated to dryness. Fractions 20 through 130, containing herbicidin B, were combined and also concentrated to dryness. The crude powder of either herbicidin A or B thus separated was extracted again with MeOH to remove MeOH-insoluble impurities. Both A and B were further purified by chromatography on silica gel (Wakogel C–200, Wako Pure Chem. Ind. Ltd., Osaka) column. In the case of A, the column was developed with EtOAc and the effluent was assayed on silica gel TLC. The fractions containing A was combined and concentrated to dryness. The recovery of B from the column was performed by elution with EtOAc - acetone (9: 1). The yields of herbicidins A and B were 6.3 and 2.8 *g* (overall yield 20~30%), respectively.

(2) The alternate procedure for the isolation of herbicidins was as follows: The concentrate (3.7 liters) of Duolite S–30 eluate from 700-liter broth, containing both 60 g of A and B, was extracted 5 times with each 4 liters of *n*-BuOH. The antibiotic in the *n*-BuOH extract was transferred to 0.2 N HCl (2.5 liters × 4), adjusted to pH 5.5 with 6 N NaOH and concentrated under reduced pressure to dryness. The sample was then extracted with 2.5 liters of MeOH to remove MeOH-insoluble impurities and concentrated to yield 132 g of the crude powder. Further purification was carried out by chromatography on silica gel column. The adsorbate was eluted with solvent systems composed of different ratio of EtOAc and acetone and 550 ml fractions were collected. Fractions 38 through 65 of EtOAc - acetone (95: 5) eluant were combined, concentrated to dryness to yield 29 g of A, which was about 80% pure. Fractions 70 through 125 of EtOAc - acetone (80: 20) eluant were pooled to yield 30.5 g of B, 80% purity. In addition to these separated A and B samples, 10 g of a mixture of A and B (*ca* 1: 1 in ratio) was obtained from fractions 66 through 69 and 125 through 130 (acetone as an eluant).

Conversion of herbicidin A to B.

Biological conversion of herbicidin A to B was carried out by use of mycelia of herbicidin-producing organism harvested from four-day cultured broth in SAKAGUCHI-flask fermented as previously described.[1] After centrifugation at 3,000 rpm for 30 minutes at 5°C, the mycelial fraction from 500 ml of the culture broth was washed three times with each 30 ml of a cold 0.067 M, pH 7.0, phosphate buffer. The washed mycelia was resuspended into 50 ml of the same buffer containing 50 mg of herbicidin A and incubated at 27°C by continuous stirring. After 20 hours the rate of conversion reached about 60% as calculated from the amount of A and B on TLC plate.

Chemical conversion of A to B was performed by a mild alkaline hydrolysis. Hundred miligrams of herbicidin A was dissolved in 50 ml of 0.1 M, pH 8.5, phosphate buffer and incubated at room temperature by continuous stirring, while maintaining the pH of reaction mixture at 8.5 with 2 N NaOH by using a pH-titrator, Impulsomat E373 (Metrohm Herisau, Switzerland). After 48 hours the reaction mixture was diluted to 500 ml with distilled water and applied to Dowex 1×4 column ($HCOO^-$ form, 1.0×10 cm). After washing with distilled water the product which was adsorbed on the column was eluted with a linear gradient of HCOOH ($0.05\rightarrow0.2$ N). The eluates containing the reaction product were combined and concentrated to dryness under reduced pressure. White powder thus obtained was extracted with 30 ml of hot MeOH and treated with ethereal diazomethane to yield 40 mg of herbicidin B.

Results and Discussion

An example of a large-scale production of herbicidins in 600-liter fermentor is shown in Fig. 1. The time sequence of production of herbicidins followed by inhibitory activity of germination of Chinese cabbage seeds is almost parallel to the increase in mycelial growth and reaches a maximum of 110 μg/ml after 60 hours. The ratio of A to B at a maximum activity was about 3 to 1 when analyzed on TLC. The changes of pH of the broth during fermentation is gradual in the range between pH 7.25 and 7.45.

The isolation methods used for herbicidins are outlined in Figs. 2 and 3. For the first step purification the resinous adsorbent Duolite S–30 was selected because it removed considerable unrelated impurities, and gave a satisfactory recovery of the antibiotics in the eluate. Separation of herbicidins A and B was successfully performed by counter-current distribution, but *n*-BuOH extraction of the concentrate from Duolite S–30 eluate followed by separation of A and B on silica gel column was more efficient especially for the purification of a large quantity of the sample. Herbicidin A was obtained from the eluate of silica gel column as purified white powder indicating a single spot on TLC using several solvent systems described later. On the other hand, herbicidin B was recrystallized from MeOH-EtOAc to give colorless rod-shaped crystals.

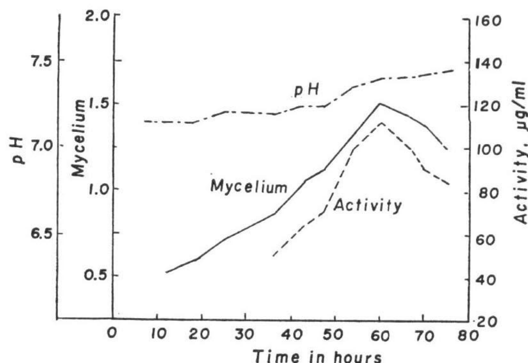
Herbicidin A is readily soluble in water, methanol and acetone, soluble in ethyl acetate, but insoluble in chloroform and benzene. The color reaction of herbicidin A revealed that it is positive for MOLISCH and anthrone, indicating the presence of poly-oxygenated moiety in its structure, but negative for EHRLICH, FEHLING, BIAL, carbazol-H_2SO_4, $FeCl_3$ and ELSON-MORGAN. Potassium permanganate was immediately decolorized by herbicidin A.

Herbicidin B is readily soluble in water and methanol, soluble in acetone, but insoluble in ethyl acetate, chloroform and benzene. Herbicidin B is also positive for the MOLISCH and anthrone reaction and negative for others tested for A. In contrast to A, potassium permanganate was slowly decolorized by B.

Herbicidins A and B behaved as basic substances with Rm value of 0.73 on high-voltage paper electrophoresis (60 volts/cm, 1.5 mA/cm) at pH 1.8 for 30 minutes according to the method by MAEDA *et al.*[2] Their molecular weights and molecular formulae were derived from the

Fig. 1. Time course of herbicidin production by *S. saganonensis*.

Mycelium: packed cell volume (ml/10 g culture broth)



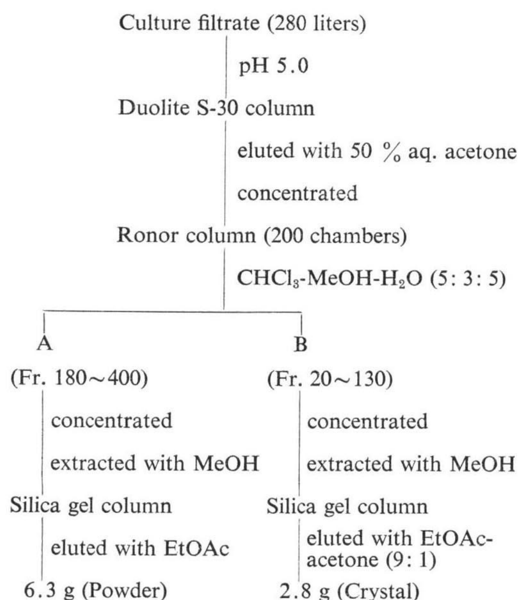


Fig. 2. Isolation and purification of herbicidins A and B (1).

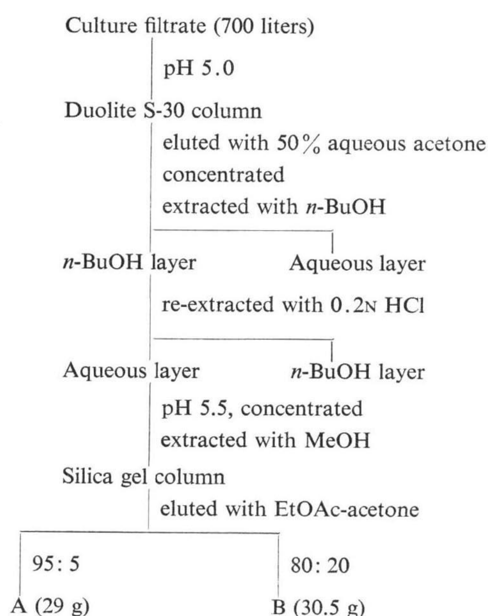


Fig. 3. Isolation and purification of herbicidins A and B (2).

analyses of mass spectra of each methanolysis product. These results as well as other physical properties are summarized in Table 1. The UV, IR and NMR spectra are shown in Figs. 4, 5 and 6, respectively.

Their physico-chemical properties, such as UV maxima at 258 nm, fragment ion peaks at 135 and 136 in mass spectra, two singlets due to heteroaromatic protons at δ 8.2~8.5 ppm and a doublet due to one proton at δ 6.2 ppm corresponding to the chemical shift of anomeric proton of usual nucleosides in NMR spectra, suggested adenine nucleoside moiety in the structures of both herbicidins A and B. The fact was further confirmed by isolation of adenine from their methanolysate with Amberlyst 15 (Rohm & Haas Co., U.S.A.).

Biological and chemical conversion of herbicidin A into B was performed with concomitant release of a quantitative amount of monocarboxylic acid with molecular formula of $C_5H_7O_3$, *m/e* 115, in proportion to the formation of B. In the conversion by alkaline treatment, another ester group in herbicidin A was also hydrolyzed. It was identified as methyl ester and remethylated to B by diazomethane treatment, which will

Table 1. Physico-chemical properties of herbicidins

	Herbicidin A	Herbicidin B
Nature	Basic, white powder.	Basic, white crystal.
Solubility	Sol. in H_2O, MeOH, EtOH, $(CH_3)_2CO$ & EtOAc.	Sol. in H_2O, MeOH, EtOH & $(CH_3)_2CO$.
M.P.	133°C (dec.)	155°C (dec.)
$[\alpha]_D^{20}$	+57.7° (*c* 1, MeOH)	+63° (*c* 1, MeOH)
Elementary analysis	Found C, 49.59; H, 5.32; N, 12.56% Calcd. for $C_{23}H_{29}O_{11}N_5 \cdot 1/2H_2O$ C, 49.28; H, 5.40; N, 12.49%	Found C, 46.68; H, 4.99; N, 14.80% Calcd. for $C_{18}H_{23}O_9N_5 \cdot 1/2H_2O$ C, 46.76; H, 5.23; N, 15.15%
M.W.	551 (calcd.)*	453 (calcd.)
UVmax. (ε)	258 nm (11,500) in MeOH	* 258 nm (9,200) in MeOH
IR (Nujol)	1750, 1725 cm^{-1}	1740~1750 cm^{-1}

* Calculated from the analyses of mass spectra of each methanolysis product.

Fig. 4. Ultraviolet spectra of herbicidins A and B.

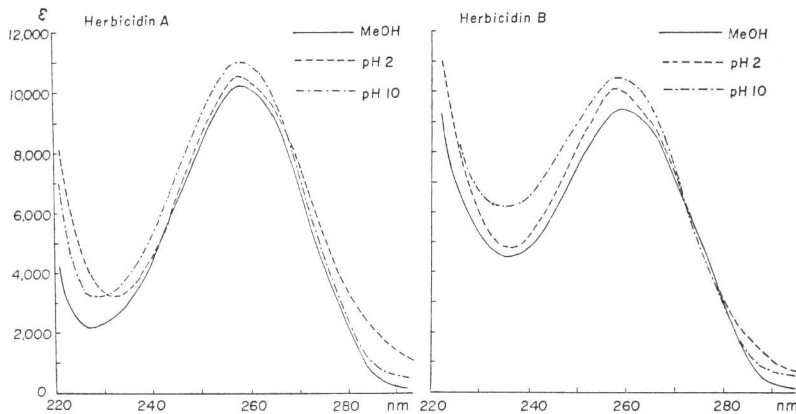
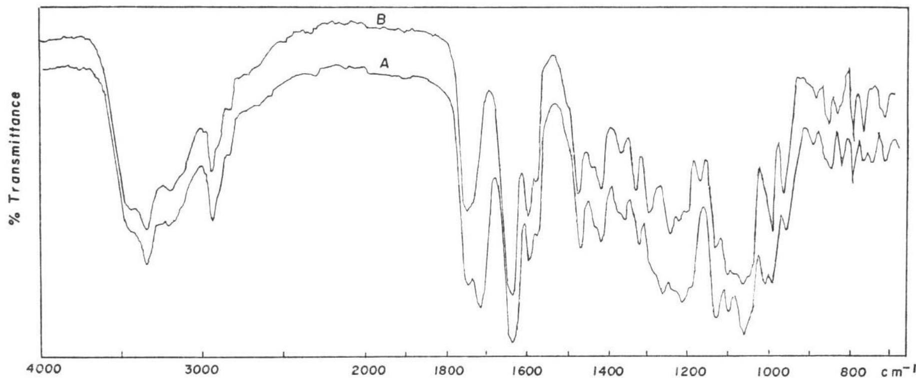
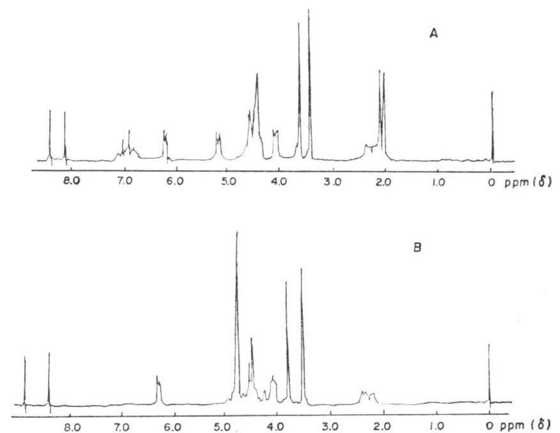


Fig. 5. Infrared spectra of herbicidins A and B (KBr).



be described in detail elsewhere. This monocarboxylic acid was identified as 2-hydroxymethyl-1,2-butenoic acid, an isomer of sarracinic, acid, synthesized by EDWARDS *et al.*[8] The NMR spectrum of herbicidin A exhibited some excessive signals than that of B, such as a doublet methyl group at δ 2.15 ppm coupled to a quartet due to one proton at δ 7.1 ppm with coupling constant 7.0 Hz. and broad singlet due to hydroxymethyl group at δ 4.4 ppm. These all signals indicated the presence of sarracinic acid isomer moiety in herbicidin A. Difference between A and B, therefore, reasonably ascribed only to the presence of ester linkage of sarracinic acid isomer in A and its absence in B.

Fig. 6. NMR spectra of herbicidins A and B in $CDCl_3$.



From the results described in this manuscript as well as from characteristic biological activities described in the previous report,[1] one can conclude that herbicidins A and B are two new antibiotics

with very closely related structures. Although details are not given in this paper, both herbicidins A and B are adenine nucleoside antibiotics, giving adenine moiety by acid hydrolysis. Among known antibiotics with herbicidal activity, toyocamycin, a pyrrolopyrimidine nucleoside antibiotic, is also an adenine nucleoside analog,[4] but is reported[5] to exhibit nonselective inhibitory activity against shoot and root growth of various plant seeds.

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