Agr. Biol. Chem., **40** (10), 2103～2104, 1976

Phytosterol from the Callus of *Stevia Rebaudiana* Bertoni

Kensuke NABETA, Teruyo KASAI and Hiroshi SUGISAWA

Department of Food Science, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa-ken, Japan

Received April 23, 1976

The intensely sweet constituent of *Stevia Rebaudiana* Bertoni[1] which is a wild shrub of *Compositae* family grown in Paraguay was designated as stevioside[2] and has attracted special attention in view of using for sweetening purpose. The cultivation of this plant has been attempted on a large scale in Japan[1] both as a source of natural sweetner and as an antidiabetic sweetner since 1971. The tissue culture of this plant must provide useful information on the formation of stevioside and other metabolites of this plant. We have thus induced the callus from the leaves of *Stevia* and proved the production of stigmasterol by this callus.

The plant of *Stevia* used for tissue culture was obtained from the Kagawa Agricultural Experiment Station (Takamatsu, Kagawa-Ken, Japan). The callus induction from the section (5 mm^2) of young leaves was carried out on the medium of Murashige and Skoog[3] in which 3.0% of sucrose, 0.9% of Difco Bacto-agar, 1.0 ppm of (2,4-dichlorophenoxy)acetic acid as auxin and 0.1 ppm of kinetin were provided. After four weeks the callus from the initial explant tissue was maintained in a similar medium. The growth was completed within 6 weeks. All cultures were grown at 25°C under continuous light.

Thirty three grams (dried weight) of callus (fourth culture) were extracted once with 200 ml of water, filtered and extracted three times with 200 ml of ethanol under reflux for 45 min. The ethanol solution was evaporated to dryness and then 50 ml of water was added to the residue. This water solution was then extracted three times with 20 ml of chloroform. The obtained chloroform extracts (83 mg) were chromatographed on silicic acid (5 g) column (eluent, ethyl acetate: chloroform 2: 8) and the fraction containing phytosterol was obtained. The existence of stigmasterol in this fraction was indicated by GLC on the column of 3 mm×2 m, 1% Silicon OV-17 on Chromosorb P AW 60/80 mesh (Fig.). The main peak having retention time 7.42 min was identical with that of an authentic stigmasterol. Moreover the crude powder of stigmasterol (0.3 mg) was obtained by crystallization from methanol. The MS spectrum of isolated stigmasterol indicated M^+ at *m/e* 412 and peaks at *m/e* 394 (M^+-H_2O), *m/e* 255 (M^+-H_2O-side chain) and *m/e* 213 (M^+-H_2O-side chain-42).

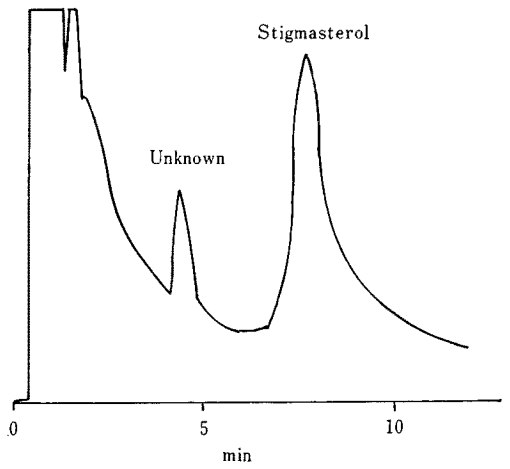

FIG. 1. Gas Chromatography of Phytosterol from the Callus of *Stevia*.

Column: 1% Silicon OV-17 on Chromosorb P AW 60/80 mesh
Column temp.: 250°C
Carrier gas: N_2 30 ml/min.

For the comparison of the phytosterol in *Stevia* callus with that in the original plant, the isolation of phytosterols from the leaves was carried out. Milled air-dried leaves (100 g) were extracted in 2 batches with a total 1 liter of methylene chloride for 24 hr at room temperature. The obtained methylene chloride extracts (5.92 g) was chromatographed on alumina (50 g) column (subsequent elution with hexane, hexane: benzene 4: 1, hexane: benzene 3: 2 hexane: benzene 2: 3, hexane: benzene 1: 4, benzene, chloroform and ethyl acetate). The phytosterols were eluted with ethyl acetate. Further purification of the phytosterols with chromatography on silicic acid column (5 g, ethyl acetate: benzene 2: 8) gave the phytosterol mixture (53 mg), mp 139°C～148°C. The MS ion peaks at *m/e* 412 and *m/e* 414 indicated coexistence of stigmasterol and sitosterol. The phytosterols were successively acetylated with Ac_2O/pyridine and the acetylated products were then separated by chromatography on silicic acid-$AgNO_3$ (10: 1) plate (solvent: chloroform). One of the products obtained, mp 141°C～142°C (mixed mp 141°C～142°C), MS ion peaks at *m/e* 394 (M^+-AcOH), *m/e* 255 (M^+-AcOH-side chain) and *m/e* 213 (M^+-AcOH-side chain-42), IR ν_{max}^{KBr} cm^-: 2900 and 1742 (ester C=O) was identified as stigmasterol acetate on the direct comparison of IR and MS of an authentic sample. The other product, mp 119°C～121°C, MS *m/e* 396 (M^+-AcOH), *m/e* 255 (M^+-AcOH-side chain) and *m/e* 213 (M^+-AcOH-side chain-42), IR ν_{max}^{KBr} cm^-: 2900 and 1742 (ester C=O)

was identified as sitosterol acetate on the comparison of IR and MS of an authentic sample of β-sitosterol acetate.

This result indicated that phytosterol was produced by the callus from *Stevia* as well as other plant.[4] Further investigation is in progress to ascertain the production of the secondary metabolites,[5] especially steviol and their derivatives including stevioside.

REFERENCES

1) T. Kawatani, R. Kanamoto and T. Tanabe, *Nettai Nogyo*, **17**, 125 (1973).
2) H. W. Wood, J. R. Allerton, H. W. Diehl and H. G. Fletcher, *J. Org. Chem.*, **20**, 875 (1955); E. Mosettig and W. R. Nes, *ibid.*, **20**, 884 (1955); E. Vis and H. G. Fletcher, *J. Amer. Chem. Soc.*, **78**, 4709 (1956); E. Mosettig, U. Beglinger, F. Dolder, H. Licht, P. Quitt and J. A. Walter, *ibid.*, **85**, 2305 (1963).
3) T. Murashige and F. Skoog, *Physiol. Plant*, **15**, 473 (1962).
4) "Plant Tissue Culture," ed. by M. Takeuchi, I. Ishihara and T. Furuya, Asakura Shoten, Tokyo, Japan, 1972, pp. 354~356.
5) H. Suzuki, T. Ikeda, T. Matsumoto and M. Noguchi, *Agr. Biol. Chem.*, **40**, 819 (1976).