Environmental Health Perspectives Supplements
Vol. 101 (Suppl. 3): 53–56 (1993)

Mutagenicity and Human Chromosomal Effect of Stevioside, a Sweetener from *Stevia rebaudiana* Bertoni

by Maitree Suttajit,[1] Usanee Vinitketkaumnuen,[1] Umnat Meevatee,[2] and Duang Buddhasukh[3]

Leaves of *Stevia rebaudiana* Bertoni have been popularly used as a sweetener in foods and beverages for diabetics and obese people due to their potent sweetener stevioside. In this report, stevioside and steviol were tested for mutagenicity in *Salmonella typhimurium* strains TA98 and TA100 and for chromosomal effects on cultured human lymphocytes. Stevioside was not mutagenic at concentrations up to 25 mg/plate, but showed direct mutagenicity to only TA98 at 50 mg/plate. However, steviol did not exhibit mutagenicity in either TA98 or TA100, with or without metabolic activation. No significant chromosomal effect of stevioside and steviol was observed in cultured blood lymphocytes from healthy donors (n = 5). This study indicates that stevioside and steviol are neither mutagenic nor clastogenic *in vitro* at the limited doses; however, *in vivo* genotoxic tests and long-term effects of stevioside and steviol are yet to be investigated.

Introduction

Stevia rebaudiana Bertoni is a small herb (Compositae) (Fig. 1). The plant is native to South America and has been used for sweetening beverages and foods since 1600 (*1*). Stevia became popular and commercialized by Japanese. The plant has been distributed to southeast Asia including Thailand [as "Ya wan" (*2*)]. More than 750 tons of stevia leaves per year are used as crude extract for consumption. The sweetening compound was isolated from stevia leaves by Rebaudi and Resenac (*3*), and was named as "stevioside" (*4,5*). Stevioside has very high sweetening potency, 250–300 times that of sucrose, but little caloric value (*6*). Its sweetness is stable to heat and yeast fermentation. Stevia and stevioside have been applied as a sugar substitute and used by those with obesity, diabetes mellitus, heart disease, and dental caries (*7*). Stevioside can also inhibit the growth of certain bacteria (*8*).

Eight different sweetening ent-kaurene glycosides (from about 88 compounds in stevia leaves) were isolated (*9*). The common alycone of those glycosides is steviol, chemically ent-13-hydroxy kaur-16-en-19-oic acid. As shown in Figure 2, stevioside is the main sweet glycoside, 6–8%, in dry stevia leaves (*10*).

FIGURE 1. *Stevia rebaudiana* Bertoni.

[1]Department of Biochemistry, Chiang Mai University, Chiang Mai, Thailand.
[2]Department of Anatomy, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.
[3]Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.
Address reprint requests to M. Suttajit, Department of Biochemistry, Chiang Mai University, Chiang Mai, Thailand.

Toxicological Evaluation

The toxicological effects of stevia extract and stevioside have been pharmacologically and biochemically studied (*8,11–17*) and reviewed (*1,3,18*). In experimentally induced diabetic rabbits, stevioside can lower the level of blood glucose temporarily (*16*). It also lessened bradycardia (*16*).

It has been shown that neither stevia extract nor stevioside is toxic or teratogenic in mice, rats, hamsters, and guinea pigs by oral administration (*11–17*). However, acute toxicity can be seen in experimental animals when fatal doses of stevioside are administered IP or IV [LD_{50} values between 1 and >34 g/kg body weight (*1*)].

Mutagenicity

The mutagenicity of crude stevia extract, stevioside, and steviol have been reported (*18–23*). Stevioside showed no mutagenicity in bacterial systems (*18–20*); however, its aglycone, steviol, was mutagenic after metabolic activation in the forward mutation assay using only *Salmonella typhimurium* TA677 (TM677) (*21*) but not mutagenic in the reverse mutation test using *Salmonella typhimurium* TA100, TA98, TA102, or TA97. When steviol was metabolically activated with S-9 from Aroclor 1254-pretreated rats, 15-oxosteviol was found to be the mutagenic product in the forward mutation assay (*22*). It was suggested (*23*) that this major, oxidized steviol was responsible for the indirect mutagenicity of steviol in TM677, probably by selectively inducing a deletion or insertion of more than one base pair, which cannot be found in strains of TA98, TA100, and TA102, which are regularly used in the Ames test.

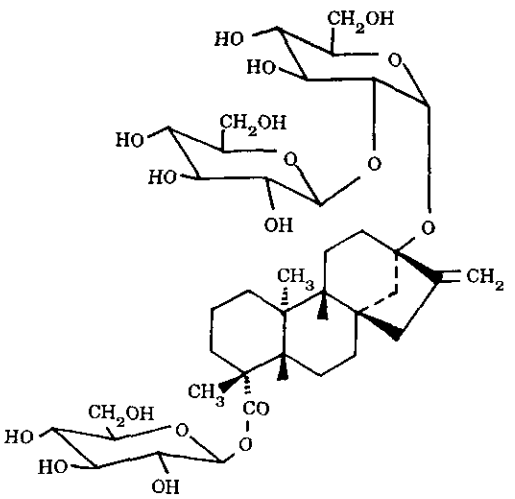

FIGURE 2. Chemical structure of stevioside.

Carcinogenicity

No evidence has been reported that stevioside and its metabolites are carcinogenic. No carcinogenicity was detected in hamsters given stevioside orally for 6 months and in long-term feeding for 2 years in rats (*1,17*).

Contraceptive Activity and Teratogenicity

It was shown that 5% water decoction of stevia leaves reduced the fertility of female rats by about 65% (*24*). Stevioside (95–98% pure) had no effects on the rate of pregnancy or the development of rat fetuses (*25*). Stevioside did not cause any abnormalities on mating, pregnancy, or the development of fetuses in the experimental animals (*15,26*).

Safety Assessment of Stevioside Consumption in Humans

Stevia has been used as a sweetening ingredient in foods and drinks by South American natives for many centuries, and there is no report of any plant toxicity to the consumers. The safety of stevia crude extract and stevioside have been well accepted and their various products commercialized in Japan as sweeteners for several foodstuffs (*7,8*). Stevia leaves have been used as herbal tea by mixing with other plant products for reducing sugar consumption in diabetic patients in Thailand (*27*). No side effects were observed in these patients after 5 years of continued consumption. Long-term genotoxicity and health risks in humans have not been completely assessed. In Thailand, stevia leaves or their crude extract have been legally permitted to be used commercially as a herbal tea by the Ministry of Public Health, but purified stevioside as an additive in foods and drinks has not yet been legalized. However, stevioside has been permitted to be exported. More evidence of the safety of products from stevia and stevioside and health risk assessment on their genotoxicity are needed. In this report, the mutagenicity and human-chromosomal effect of stevioside and its aglycone steviol were tested and considered for further health risk assessment.

Materials and Methods

Tested Compounds. Stevioside was isolated from stevia leaves by hot-water extraction, decolorized by electrolysis and ion-exchange chromatography, and crystallized by the method previously reported (*29*). The purity of the product was 99%. Steviol was obtained by periodate oxidation of stevioside, followed by acid hydrolysis and crystallization.

Mutagenicity Assay. The Salmonella mutation with preincubation was assayed using S-9 mix prepared from the livers of rats pretreated with sodium phenobarbital and 5,6-benzoflavone as previously described (*30*). The tester strains were *Salmonella typhimurium* TA98 and TA100. The bacteria were cultured in Oxoid nutrient broth no. 2–14 hr before each assay. The histidine$^+$ revertants were scored. All samples analyzed were in duplicate.

Chromosomal Aberration Test. Whole-blood samples from five healthy donors were used. Lymphocyte cultures were performed according to the standard method. After 24 hr of incubation, the cultures were added with different concentrations of stevioside (1, 5, and 10 mg/mL) or steviol (0.1 and 0.2 mg/mL). Mitomycin C at 1 μg/mL was used. Structural chromosomes aberrations were analyzed from 100 metaphases in each tested culture.

Results

Mutagenicity

As shown in Figure 3, at lower than 25 mg/plate either in the presence or absence of S-9 mix, stevioside was not mutagenic toward *Salmonella typhimurium* TA98. However, at the higher dose, 50 mg/mL, stevioside showed significant mutagenicity (four times the control) of TA98 without metabolic activation, while the same dose demonstrated a slight increase of bacterial mutation with the addition of S-9 mix. Under the same doses and conditions, stevioside did not exhibit any mutagenic activity toward TA100.

The spontaneous revertants were between 30 and 151 per plate. Two positive controls were done using 2-aminoanthracene (2-AA), 0.5 μg/plate, + S-9 mix and 2-(2-furyl)-3-nitro-2-furyl)acrylamide (AF-2), 0.1 μg/plate, S-9 mix. The former gave 789 revertants for TA98 and 997 for TA100 and the latter yielded 666 mutants for TA98 and 604 for TA100.

Steviol, 1–20 mg/plate, did not show mutagenic activity to either TA98 or TA100. Higher doses of steviol were not tested due to their strong cytotoxicity to both tester strains. The treatment with β-glucosidase on 50 mg stevioside did not significantly alter its mutagenic effect to TA98 either with or without metabolic activation (Fig. 3).

Chromosomal Aberrations

Stevioside at concentrations of 1, 5, and 10 mg/mL did not cause any significant aberrations of metaphasic chromosomes in all blood samples analyzed ($p > 0.05$). Similarly, steviol at 0.1 and 0.2 mg/mL did not show any significantly abnormal change of chromosomes in four blood samples, except in one case. Steviol (0.1 and 0.2 mg/mL) in the presence of S-9 mix in one tested blood sample did not alter the result. However, under the same conditions, mitomycin as the positive control caused remarkable genetic damage of chromosomes.

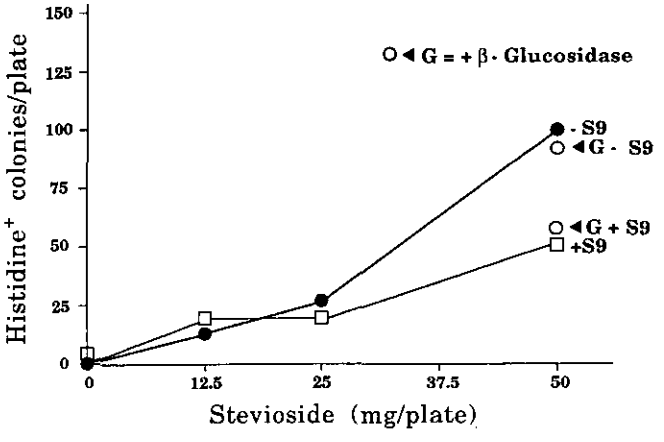

FIGURE 3. Reversion of *S. typhimurium* TA98 by stevioside.

Discussion

We have shown the lack of mutagenicity of stevioside and steviol at limited doses (up to 20 mg) toward *Salmonella typhimurium* strains TA98 and TA100 with or without metabolic activation. This confirmed the findings previously reported (*18,21*) that crude stevia extract and stevioside were negative toward TA98 and TA100 mutation. By using other various systems such as reversion mutation (*18*), bacterial recombination (*19*), host-mediated mutation (*20*), Salmonella mutation (*22*), forward mutation (*21*), chromosome aberration on human fetus fibroblasts (*8*), and dominant lethality (*8*), all the mentioned mutagenicity *in vitro* were negative both with and without S-9 mix. Pimbua et al. (*21*) also demonstrated that stevioside was not mutagenic, even after incubating with microsomal enzymatic fractions from livers of different animal species. Generally, stevioside per se is not a mutagen toward bacterial cells or a genotoxin to cultured mammalian cells, and it is not carcinogenic to experimental animals either.

Only at an unusually high dose, 50 mg/plate, was stevioside mutagenic to TA98 but not to TA100. The mutagenicity might be due to some impurities in the sample. Crude extract of stevia leaves was shown by other investigators to have some slight mutagenicity to TA100 (*8*) and induce weak chromosomal aberration in fibroblasts from Chinese hamsters (*20*). The mutagenic activity of stevioside at the high dose was more evident in the absence of its metabolic activation than with S-9 mix. The decrease of mutagenicity by the presence of S-9 mix might be due to some inactivation of such high amount of stevioside during the preincubation with rat-liver microsomal enzymes. If impurities were not responsible, stevioside seemed to be a very weak, direct-acting mutagen.

Steviol was shown to be nonmutagenic in TA98 and TA100 by the reverse mutation assay. In several mutagenic assays, only one forward mutation assay reported by Perzzuto et al. (*20*) and Pimbua et al. (*21*) with steviol at 5 mg/plate was shown to be mutagenic toward *Salmonella typhimurium* TA677 either with or without S-9 mix. The 15-oxo steviol was reported to be an active metabolite from steviol incubated with rat liver S-9 mix, and it was shown to be a direct-acting mutagen toward *Salmonella typhimurium* TM677 (*23*). No *in vitro* carcinogenicity of steviol has been studied. The health risk of long-term ingestion of stevioside has to be studied further. Experimentally, stevioside was converted into steviol by hydrolysis of endogenous or bacterial enzyme(s) in rats orally given with ^{3}H-stevioside (*31*). Steviol and dihydrosteviol could inhibit the mitochondrial translocation of adenine nucleotides and also inhibit energy metabolism, oxygen

consumption, and gluconeogenesis (*32*). It is still unknown whether steviol could be formed during ingestion and then absorbed in human gastrointestinal tract.

It was concluded that stevioside and steviol, at less than 20 mg/plate, are not mutagenic toward *Salmonella typhimurium* strains TA98 and TA100 with or without metabolic activation. At higher concentrations, stevioside showed weak mutagenicity to TA98, which might be due to contamination by impurities. An *in vitro* chromosomal effect on human lymphocytes of stevioside and steviol was not observed. However, other *in vitro* genotoxicity studies on stevioside and its metabolites and their long-term consumption in humans are yet to be investigated.

We sincerely appreciate generosity of Adul Sritep, Director of the Thai Pharmaconostic Research Institute, Chiang Mai, Thailand, who supplied stevia leaves for the preparation of stevioside and steviol, and of Veruree Sritep for financial support of this research project.

REFERENCES

1. Glinsukon, T., Pimbua, J., and Panichkul, T. Stevioside: a natural sweetener from *Stevia rebaudiana* Bertoni: toxicological evaluation. Thai J. Toxicol. 4: 1–22 (1988).
2. Sararom, P., Cheudmaungpuk, S., Hetamuruk, Y., Intarapanich, V., and Kijthum, V. Stevia rebaudiana. Kasetsart Extension J. 27: 38–52 (1982).
3. Crammer, B., and Ikan, R. Sweet glycosides from the stevia plant. Chem. Britain 22: 10915–10918 (1986).
4. Bell, F. Stevioside: a unique sweetening agent. Chem. Ind. 17: 897–898 (1954).
5. Bridel, M., and Lavielle, R. Le principe a saveur sucree du Kaa'-he'-e' (*Stevia rebaudiana* Bertoni). J. Pharm. Clin. 14: 99–154 (1931).
6. Kinghorn, A. D., and Sojarto, D. D. Current status of stevioside as a sweetening agent for human use. In: Economic and Medical Plant Research, Vol. 1 (H. Wagner, H. Hikiro, and H. R. Farnsworth, Eds.), Academic Press, New York, 1985, 1–52.
7. Fujita, H., and Edahiro, T. Safety and utilization of stevia sweetener. Shokukin Kogyo 82(22): 65–72 (1979).
8. Tama Biochemical Co. Ltd. Safety of Stevia. Tokyo, Japan, 1981, pp. 1–20.
9. Mosetting, E., Beglinger, U., Dolder, F., Lichti, H., Quilt, P., and Waters, J. A. Absolute configuration of steviol and isosteviol. J. Am. Chem. Soc. 85: 2305 (1963).
10. Vis, E., and Fletcher, H. G., Jr. Stevioside, IV. Evidence that stevioside is sophoroside. J. Am. Chem. Soc. 78: 4709 (1956).
11. Akashi, H., and Yokoyama, Y. Dried-leaf extracts of stevia, toxicological test. Shokuhin Kogyo 18: 34–43 (1975).
12. Lee, S. J., Lee, K. R., Park, I. R., Kim, K. S., and Tchai, B. S. A study of the safety of stevioside as a new sweetening source. Hanguk Sikpum Kwahakhoe Khi 11: 224–231 (1979).
13. Kurahashi, H., Yamaguchi, Y., Tsuzuki, S., and Maehashi, H. Pharmacological studies of stevioside. Matsumoto Shigaku 8: 56–62 (1982).
14. Glinsukon, T. Acute and subacute toxicity of stevioside in mice, rats, hamsters and guinea pigs. Report of the National Research Council of Thailand, Bangkok, 1987.
15. Chamnivikaipong, J., Samitasiri, Y., and Sukchotiratana, M. Effect of stevioside from stevia on the reproductive system of wistar female rat. In: Stevia Research, Proceedings from the First Stevia Research Symposium (M. Suttajit, A. Apisariyakul, D. Buddhasukh, M. Sukchotiratana, N. Chokethaworn, A. Monosroi, and Vinetketkumnuan, Eds.), May 9–10, 1990, Chiang Mai, Thailand, 1991, pp. 94–100.
16. Apisariyakul, A., Dhampipit, J., Sinchaisri, T., Rujanàvet, C., Chaipompatana, S., Kuntamuangli, N., and Punhom, A. A pharmacological study of stevioside from stevia. In: Stevia Research, Proceedings from the First Stevia Research Symposium (M. Suttajit, A. Apisariyakul, D. Buddhasukh, M. Sukchotiratana, N. Chokethaworn, A. Monosroi, and Vinetketkumnuan, Eds.), May 9–10, 1990, Chiang Mai, Thailand, 1991, pp. 72–86.
17. Panichkul, T., Glinsukon, T., Buddhasukh, D., Cheuychit, P., and Pimolsri, U. The plasma levels of urea nitrogen, creatinine and uric acid and urine volume in rats and hamsters treated with stevioside. Thai J. Toxicol. 4: 47–52 (1988).
18. Okumura, M., Fujita, Y., Imamura, M., and Aikawa, K. Safety of stevioside with rec-assay and reversion test. Shokuhin Eiseigaku Zasshi 19: 486–490 (1978).
19. Ishidate, M., Jr., Sofuni, T., Yoshikawa, Hayashi, M., and Nohmi, T. Primary mutagenicity screening of food additives currently used in Japan. Food Chem. Toxicol. 22: 633–636 (1984).
20. Pezzuto, J. M., Campadre, C. M., Swansan, S. M., Nanayakkara, N. P. D., and Kinghorn, A. D. Metabolically activated steviol, the aglycone of stevioside, is mutagenic. Proc. Natl. Acad. Sci. U.S.A. 82: 2478–2482 (1985).
21. Pimbua, J., Glinsukon, T., Rojanapo, W., Buddhasukh, D., and Cheuychit, P. Failure of stevioside to induce mutagenicity in *Salmonella typhimurium* TA98 and TA100 incubated with liver S9 fractions from various species. Thai J. Toxicol. 4: 31–38 (1988).
22. Compradre, C. M., Hussain, R. A., Nanayakkara, N. P., Pessuto, J. M., and Kinghorn, A. D. Mass spectral analysis of some derivatives and *in vitro* metabolism of steviol, the aglycone of the natural sweetners, stevioside, rebaudioside A, and rubusoside. Biomed. Environ. Mass Spectrom. 15: 211–222 (1988).