Lack of Mutagenicity of Stevioside and Steviol in *Salmonella typhimurium* TA 98 and TA 100

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Abstract

Stevioside, a sweet-tasting diterpene glycoside derived from *Stevia rebaudiana*, and steviol, a product from enzymatic hydrolysis of stevioside, were tested for mutagenic activity by the *in vitro* Ames test, a preincubation method, using *Salmonella typhimurium* TA 98 and TA 100 as the tester strains, either in the presence or absence of metabolic activating system derived from the sodium phenobarbital and 5,6-benzoflavone pretreated liver S9 fractions from various animal species including rat, mouse, hamster and guinea pig. Stevioside and steviol at the concentrations up to 50 mg and 2 mg per plate, respectively showed no mutagenic effect on both tester strains either in the presence or absence of metabolic activating system. However, at the high concentration both stevioside and steviol showed some toxic effects on both tester strains. The toxic effect was decreased in the presence of the metabolic activating system.

Stevioside is a natural non-caloric sweetener from *Stevia rebaudiana* Bertoni which has been used for a long time by people of Paraguay and Brazil[1]. The purified extract is a white crystalline, odorless powder and approximately 300 times sweeter than sucrose. It is composed of steviol, a diterpenic carboxylic alcohol and three glucose molecules[2,3]. At present, stevioside has been widely used as a non-nutritive sweetening agent in various kinds of food and food products in many countries including Japan, Brazil and People's Republic of China[4-8]. More than 750 tons of stevia leaves per year are used as a source of crude extract for consumption[9]. In Thailand, the cultivation of the stevia plant is well established by farmers in Chiang Rai and Chiang Mai. Stevioside (95% purity) can be produced in both laboratory and industrial scales by Thai scientists. However, according to FDA (Thailand)'s regulation, it is not generally available for human use in Thailand.

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Stevioside has been subjected to various assessments for safety and no serious toxic effects have been reported[10-18]. Stevioside and crude extract of *S. rebaudiana* have been determined to be non-mutagenic in many bacterial test systems, such as some tester strains of *Salmonella typhimurium, Escherichia coli* and *Bacillus subtilis*, either in the presence or absence of metabolic activation which mostly derived from the rat liver S9 fraction[5,19,20]. However, it is known that stevioside is converted to its aglycone, steviol, by intestinal bacteria when it is orally administered to rats[21]. Pezzuto et al[22] demonstrated that steviol showed a dose-related positive response in the forward mutation assay using *S. typhimurium* TM677 in the presence of metabolic activation system (Aroclor induced rat liver S9 fraction). Later Temcharoen et al[23] had confirmed this positive result and also demonstrated that the liver S9 fractions from other laboratory animal species including hamsters, mice, guinea pigs and rabbits could activate steviol to be mutagenic against *S. typhimurium* TM677. Recently Suttajit et al[9] showed that stevioside at high dose (50 mg/plate) possesses a weak mutagenic activity to *S. typhimurium* TA 98 in the presence of Aroclor treated rat liver S9 fraction. This mutagenic effect may be caused by the impurities present in stevioside. So, in the present study the mutagenic activity of stevioside and steviol toward *S. typhimurium* TA 98 and TA 100 was reevaluated in the presence of metabolic activating system, liver S9 fractions derived from sodium phenobarbital and 5,6-benzoflavone pretreated rats and other laboratory animal species including hamsters, mice and guinea pigs using the Ames test, a preincubation method.

MATERIAL AND METHOD

Chemicals

Stevioside was obtained from the Thai Pharmacognocy Research Laboratory, Chiang Mai. Stevioside was extracted and purified from dried *Stevia rebaudiana* leaves as described by Adduci et al[24]. Steviol was obtained by oxidation of stevioside as described by Ogawa et al[25]. Both compounds were fine, white powder of 96 per cent purity. All chemicals and solvents used throughout the investigation were analytical grade. Sodium phenobarbital and 5,6-benzoflavone were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. 4-Nitroquinoline 1-oxide (4NQO) was purchased from Iwai Kagaru Co. Ltd., Japan. Sodium azide was purchased from J.T. Baker Chemical, New Jersey, U.S.A. Aflatoxin B_1(AFB_1) was purchased from Makor Chemicals Ltd., Jerusalem, Israel.

Animals

Adult male Swiss albino mice (30-35 g) and Wistar rats (200-250 g) were supplied by the National Animal Center at Salaya while Syrian golden hamsters (90-100 g) and albino guinea pigs (400-500 g) were from the Animal Center, Faculty of Science, Mahidol University, Bangkok, Thailand. All were used for the preparation of liver S9 fractions. They were housed individually in stainless steel cages in a room with a temperature at 25 ± 2°C with relative humidity at about 65 per cent. All animals were fed with rat or rabbit pellets (Gold Coins mills Co. Ltd., Singapore) and water *ad libitum*.

Treatment of animals and preparation of liver S9 fractions

Four to five animals of each species were treated with sodium phenobarbital and 5,6-benzoflavone following the induction procedure as described by Matsushima et al[26]. All animals were sacrificed by decapitation. The liver was removed quickly, weighed and washed several times with cold 0.15 M KCl. Details of liver S9 fraction preparation were given by Maron and Ames[27].

The Ames test

The Ames test was carried out with the use of a preincubation method as described by Maron and Ames[27]. *S. typhimurium* TA 98 and TA 100 were kindly supplied by Dr. Bruce N. Ames, University of California, Berkley, CA, U.S.A. Overnight cultures of bacteria in oxoid nutrient broth No. 2 were used in each test. Either stevioside or steviol was tested in two systems with or without metabolic activation (S9). In each experiment, 0.1 ml of bacterial culture, 0.1 ml of tested compound (stevioside was dissolved in distilled water and steviol was dissolved in DMSO in various concentrations), 0.5 ml of 0.2 M phosphate buffer, *p*H 7.4 (without metabolic activation) or 0.5 ml of S9 fraction (with metabolic activation) were mixed together and incubated at 37°C for 20 minutes in a shaking water bath. Afterwards 2 ml of soft agar containing 0.05 mM histidine and 0.05 mM biotin was added and mixed gently, then the mixture was

poured onto minimal top agar plates. The number of prototroph revertant colonies/plate was counted after incubation at 37°C for 48 hours. Each concentration was performed in triplicate and done repeatedly. The positive control consisted of sodium azide (0.5 μg/plate) and 4NQO (0.5 μg/plate) for *S. typhimurium* TA 100 and 98, respectively, in the absence of metabolic activation assay and AFB_1 (0.03 μg/plate) for both TA 100 and TA 98 in the presence of metabolic activation. Distilled water and DMSO (0.1 ml) were used as the negative control for stevioside and steviol, respectively. A number of revertant colonies in treated culture greater than twice the solvent control revertant colonies were considered as positive response.

RESULTS

Mutagenic activity of stevioside and steviol on *S. typhimurium* TA 98 and TA 100, in the presence or absence of sodium phenobarbital and 5,6-benzoflavone treated liver S9 fractions from rats, mice, hamsters and guinea pigs using the Ames test, a preincubation method, are shown in Tables 1 and 2 respectively. Stevioside at the concentrations of 12.5-50.0 mg/plate showed no mutagenic activity to *S. typhimurium* TA 98 and TA 100 either in the presence or absence of metabolic activating system (Table 1). The frequencies of spontaneous revertant of *S. typhimurium* TA 98 were 48 ± 11.4 his^+ revertant colonies/plate in the absence of liver S9 fractions and 54 ± 5.8, 57 ± 1.7, 56 ± 9.3 and 52 ± 6.2 his^+ revertant colonies/plate in the presence of liver S9 fractions from sodium phenobarbital and 5,6-benzoflavone treated rats, mice, hamsters and guinea pigs, respectively, whereas, 4 NQO, in the absence of liver S9 fraction and AFB1 in the presence of liver S9 fractions from various laboratory animal species showed positive results (Table 1). Similar negative results were also found with steviol at the concentrations of 0.25 to 2.0 mg/plate when assayed by the Ames test either in the presence or absence of liver S9 fraction from sodium phenobarbital and 5,6-benzoflavone treated animals (Table 1). However, steviol at the concentrations of 1 and 2 mg/plate in the absence of liver S9 fraction and at the concentration of 2 mg/plate

Table 1. Mutagenicity of stevioside and steviol to *S. typhimurium* TA 98 using Ames test (preincubation method).

Compound	Amount (mg/plate)	No. of his^+revertant colonies/plate (mean ± S.D., n=6)				
		-S9 mix	+S9 mix			
			Rat	Mouse	Hamster	Guinea pig
Stevioside	0	48 ± 11.4	54 ± 5.8	57 ± 1.7	56 ± 9.3	52 ± 6.2
	12.5	46 ± 10.5	38 ± 4.8	50 ± 5.0	52 ± 11.7	37 ± 67.5
	25.0	42 ± 8.0	38 ± 7.7	37 ± 6.0	45 ± 4.3	32 ± 2.2
	50.0	44 ± 14.6	32 ± 4.7	24 ± 2.0	36 ± 5.0	32 ± 4.6
Steviol	0	48 ± 11.4	54 ± 5.8	57 ± 1.7	56 ± 9.3	52 ± 6.2
	0.25	46 ± 15.6	54 ± 7.0	56 ± 4.3	51 ± 8.5	55 ± 3.6
	0.50	48 ± 11.3	46 ± 4.6	47 ± 4.5	53 ± 3.0	48 ± 6.2
	0.75	31 ± 6.8	47 ± 7.3	49 ± 3.0	55 ± 3.3	41 ± 4.5
	1.0	19 ± 5.9*	43 ± 5.3	40 ± 3.2	43 ± 6.4	36 ± 4.0
	1.5	17 ± 3.6*	41 ± 5.0	32 ± 1.1	42 ± 4.6	32 ± 2.6
	2.0	17 ± 2.1*	28 ± 1.4*	26 ± 3.6*	19 ± 3.8*	26 ± 2.0*
Positive control	μg/plate					
AFB_1	0.03	ND	1060 ± 25	605 ± 13	1146 ± 68	1936 ± 32
4-NQO	0.5	231 ± 20	ND	ND	ND	ND
B(a)P	5	ND	ND	32 ± 1.1	ND	ND

+S9 mix contained 100 μl/plate of liver S9 fractions from various animal species treated with sodium phenobarbital and 5,6-benzoflavone, and NADPH-generating system.
ND = not determine, * = killing effect

Table 2. Mutagenicity of stevioside and steviol to *S. typhimurium* TA 100 using Ames test (preincubation method).

Compound	Amount (mg/plate)	No. of his+revertant colonies/plate (mean ± S.D., n=6)				
		-S9 mix	+S9 mix			
			Rat	Mouse	Hamster	Guinea pig
Stevioside	0	163 ± 17	163 ± 18	156 ± 17	184 ± 10	154 ± 11
	12.5	142 ± 18	141 ± 6	147 ± 9	135 ± 4	137 ± 2
	25.0	134 ± 14	131 ± 4	138 ± 2	139 ± 9	129 ± 10
	50.0	119 ± 19	124 ± 2	117 ± 4	131 ± 5	102 ± 13
Steviol	0	163 ± 17	163 ± 18	156 ± 17	184 ± 10	154 ± 11
	0.25	162 ± 21	174 ± 14	193 ± 6	172 ± 7	156 ± 13
	0.50	138 ± 15	150 ± 18	188 ± 3	165 ± 18	141 ± 8
	0.75	124 ± 17	142 ± 13	131 ± 6	150 ± 16	114 ± 1
	1.0	120 ± 13	148 ± 6	126 ± 6	145 ± 14	114 ± 8
	1.5	101 ± 15*	125 ± 8	123 ± 8	127 ± 10	110 ± 7
	2.0	92 ± 10*	122 ± 9	120 ± 5*	108 ± 5*	100 ± 5
Positive control	µg/plate					
AFB_1	0.03	ND	1435 ± 65	724 ± 27	1883 ± 66	2002 ± 78
NaN_3	0.50	802 ± 20	ND	ND	ND	ND
B(a)P	5	ND	ND	859 ± 4	ND	ND

+S9 mix contained 100 µl/plate of liver S9 fractions from various animal species treated with sodium phenobarbital and 5,6-benzoflavone, and NADPH-generating system.
ND = not determine, * = killing effect

in the presence of liver S9 fractions from all laboratory animal species showed killing effect while stevioside at all concentrations showed no killing effect either in the presence or absence of liver S9 fractions obtained from all laboratory animal species (Table 1).

The results of the mutagenic activity of stevioside and steviol to *S. typhimurium* TA 100 either in the presence or absence of liver S9 fractions from sodium phenobarbital and 5,6-benzoflavone treated rats, mice, hamsters and guinea pigs are shown in Table 2. Both stevioside (at the concentrations of 12.5-50.0 mg/plate) and steviol (at the concentrations of 0.25-2.0 mg/plate) either in the presence or in the absence of the liver S9 fractions from all laboratory animal species showed no mutagenic activity to *S. typhimurium* TA 100. The frequencies of the spontaneous his^+ revertant were 163 ± 17 colonies/plate in the absence of liver S9 fraction and 163 ± 18, 156 ± 17, 184 ± 10 and 154 ± 11 colonies/plate in the presence of liver S9 fractions from sodium phenobarbital and 5,6-benzoflavone treated rats, mice, hamsters and guinea pigs, respectively (Table 2), while sodium azide (0.5 µg/plate) and AFB_1 (0.03 µg/plate) showed positive results absence and in the presence of liver S9 fractions from various laboratory animal species, respectively (Table 2). Stevioside at the concentrations of 1.5 mg and 2.0 mg/plate in the absence of liver S9 fraction and at the concentration of 2.0 mg/plate in the presence of liver S9 fraction from all laboratory animal species also showed killing effect, whereas, stevioside at all concentrations either presence or in the absence of liver S9 fractions from all laboratory animal species showed no killing effects (Table 2).

DISCUSSION

In the present study, our results showed that stevioside at the concentration as high as 50 mg/plate had no mutagenic activity towards *S. typhimurium* TA 98 and TA 100 by using the Ames test, a preincubation method, either in the presence or absence of liver S9 fractions from sodium phenobarbital and 5,6-benzoflavone treated rats, mice, hamsters and guinea pigs. These findings are in good agreement with the results from other investigators who reported that stevioside (up to 10 mg/plate) was not mutagenic to *S. typhimurium* TA 98, TA 100, TA 1535, TA 1537 and TA 1538 and

Escherichia coli either in the presence or absence of liver S9 fraction from rats by using the Ames test and the mutant frequency test, respectively (19,20). In addition, Pimbua et al(29) also demonstrated that stevioside at the concentration as high as 20 mg/plate had no mutagenic activity toward *S. typhimurium* TA 98 and TA 100 either in the presence or absence of metabolic activation systems derived from liver S9 fractions of various laboratory animal species including rats, mice, hamsters, guinea pigs and rabbits by using the Ames test, both standard plate incorporation method and a preincubation method. Recently Suttajit et al(9) reported that stevioside at the concentration of 50 mg/plate was mutagenic to *S. typhimurium* TA 98 but not in *S. typhimurium* TA 100 in the presence of liver S9 fraction from rats. They suggested that the mutagenic activity of stevioside might be due to the impurities present in the stevioside(9). Since this result was contradictory to our results, the mutagenic activity of stevioside at the dose of 50 mg/plate to *S. typhimurium* TA 98 was reevaluated in another laboratory. The tests were performed either in the presence or absence of liver S9 fractions from sodium phenobarbital and 5,6-benzoflavone treated rats and were done in duplicate and repeated independently. The results showed that stevioside at the concentration of 50 mg/plate was not mutagenic to *S. typhimurium* TA 98 either in the presence or absence of the liver S9 fraction from rats by using the Ames test, a preincubation method (Dr. Malyn Chulasiri, Dpt of Microbiology, Faculty of Pharmacy, Mahidol U., Personal communication). This result was consistent with previous findings from our laboratory. Therefore, the positive findings of Suttajit et al(9) which stevioside at the concentration 50 mg/plate was mutagenic to *S. typhimurium* TA 98 in the presence of the liver S9 fraction from rats reported by Suttajit et al(9) may be attributed to the unknown impurities in stevioside sample as they have suggested.

It has been demonstrated that stevioside was completely converted to steviol when incubated with rat colon microflora bacteria. When stevid was orally administered, it was nearly completely absorbed(21). Moreover, it was suggested that a similar degradation of stevioside to steviol and analogous absorption of steviol seemed to occur in human. Steviol has been demonstrated to be mutagenic toward *S. typhimurium* TM 677 in the presence of liver S9 fraction from Aroclor pretreated rats(22). Our previous report(23) showed that besides the liver S9 fraction from rats, the liver S9 fractions from other laboratory animal species including mice, hamsters, guinea pigs and rabbits could activate steviol to be mutagenic toward *S. typhimurium* TM 677 by using a bacterial forward mutation assay. In order to characterize the type of mutation induced by steviol, in this study, the Ames test, a preincubation method was used to detect the mutagenicity of steviol and the results showed that steviol at the concentration as high as 2.0 mg/plate had no mutagenic activity to *S. typhimurium* TA 98 and TA 100 either in the presence or absence of liver S9 fraction from sodium phenobarbital and 5,6-benzoflavone treated rats. This result was in agreement with that of Suttajit et al(9).

In the acute toxic study, the hamster has been shown to be the most susceptible animal species to the toxicity of steviol(30) and the liver S9 fraction from the hamsters also showed the highest efficiency in activating mutagenicity of steviol against *S. typhimurium* TM 677(23). Since there are species differences in the susceptibility to toxicity and mutagenicity of steviol, the liver S9 fractions from mice, hamsters and guinea pigs were tested in the present study. The results showed that steviol at the concentration as high as 2.0 mg/plate also had no mutagenic activity to *S. typhimurium* TA 98 and TA 100 in the presence of liver S9 fraction from mice, hamsters and guinea pigs. Although it has no mutagenic effect, steviol at a high concentration has a toxic effect as shown by reducing the number of surviving cells in the background lawn in both tester strains, particularly those incubated in the absence of metabolic activation. A toxic effect of steviol was observed in both *S. typhimurium* TA 98 and TA 100 at the concentrations higher than 0.75 and 1.0 mg/plate, respectively, when tested in the absence of metabolic activation (Tables 1 and 2). When the test was performed in the presence of liver S9 fractions from various animal species, it showed toxic effects to both tester strains at the concentrations higher than 1.5 mg/plate (Tables 1 and 2). The addition of liver S9 fractions from various animal species resulted in a reduction of the toxicity of steviol. It was possible that steviol was partially metabolized and its metabolite(s) seems to be less toxic than the parent compound.

Compadre et al(31) studied the metabolism of steviol by mammalian enzymes (rat liver S9

fraction) and identified the *in vitro* metabolites of steviol using gas chromatography/mass spectrometry (GC/MS). 15 α-Hydroxysteviol is a major metabolite of steviol and was not mutagenic and bactericidal when evaluated against *S. typhimurium* TM 677 either in the presence or in the absence of the metabolic activation (S9) by using forward bacterial mutation assay. So, it was possible that steviol itself was a direct-toxic substance and its structure for expression of its toxicity may be due to a hydroxyl group at position 13 and an unsaturated bond joining the carbon atom at position 16 and 17[22]. They proposed that 15 oxosteviol may play a role in the mutagenic activity of steviol and it was a direct acting mutagenicity. In contrast, the research of Procinska et al[32] could not confirm the direct acting mutagenicity of 15 oxosteviol and they suggested that the positive results of the previous studies[21] were due to misinterpretation of the data. From our study, the results of toxic effect of steviol to *S. typhimurium* TA 98 and TA 100 in the absence of liver S9 fraction was higher than the toxic effect of steviol in the presence of the liver S9 fractions from various animal species. Similarly, the results from Compadre et al[31] and Procinska et al[32] also showed the toxic effect of 15 oxosteviol to the tester bacteria. Therefore, we conclude that steviol might be a direct- toxic substance to bacteria.

In conclusion, stevioside and steviol were not mutagenic in the Ames test, a preincubation method, by using *S. typhimurium* TA 98 and TA 100 both in the presence and absence of metabolic activation. Steviol at the high concentrations also had a toxic effect to both strains of tester bacteria and its toxic effect was reduced in the presence of metabolic activating system. It has been known that the mechanisms of gene point mutation consisted mainly of base-pair substitution, frameshift mutation, insertion or deletion of the genes. In the Ames test, *S. typhimurium* TA 98 and TA 100 are used specifically for detecting frameshift and base-pair mutation, respectively[27]. It was possible that these two compounds, particularly steviol which was mutagenic to *S. typhimurium* TM 677 in the presence of the metabolic activation, by using bacterial forward mutation assay, may induce changes in the DNA and such alterations could not be detected by these two tester strains, *S. typhimurium* TA 98 and TA 100. Therefore, these two compounds should also be tested for their mutagenicity by using other strains of *S. typhimurium* such as TA 97, TA 102, TA 104 and TA 106, because each bacterial strain possesses different properties and is used to detect different kinds of mutations or mutagens.

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การศึกษาฤทธิ์ก่อกลายพันธุ์ของสารสตีวิโอไซด์ และ สตีวิออล โดยการทดสอบเอมส์

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สารสตีวิโอไซด์เป็นสารหวานที่สกัดได้จากใบของต้นหญ้าหวาน (*Stevia rebaudiana* Bertoni) สารสตีวิออลเป็นสารที่ได้จากการเปลี่ยนแปลงของสารสตีวิโอไซด์โดยเอ็นซัยม์ การศึกษาครั้งนี้เป็นการตรวจสอบฤทธิ์ก่อกลายพันธุ์ของสารทั้งสองชนิดโดยวิธีของเอมส์ (Ames test) โดยใช้แบคทีเรีย *Salmonella typhimurium* สายพันธุ์ TA 98 และ TA 100 เป็นตัวทดสอบ และทดสอบทั้งในระบบที่มี และไม่มีเอ็นซัยม์จากตับ (liver S9 fraction) ของหนูพุกขาว (rat) หนูถีบจักร (mouse) หนูแฮมสเตอร์ขนทอง (hamster) และหนูตะเภา (guinea pig) ที่ถูกกระตุ้นให้มีการเพิ่มการผลิต และการทำงานของเอ็นซัยม์ในส่วนของไมโครโซมภายในตับมาก่อนด้วยสาร sodium phenobarbital และ 5,6-benzoflavone ผลของการทดลองพบว่าสารสตีวิโอไซด์ และสารสตีวิออลในปริมาณความเข้มข้นสูงสุดที่ 50 และ 2 มิลลิกรัมต่อ plate ตามลำดับ ไม่มีฤทธิ์ทำให้แบคทีเรีย *S. typhimurium* ทั้งสองสายพันธุ์คือ TA 98 และ TA 100 เกิดการกลายพันธุ์เมื่อทดสอบโดยวิธีของเอมส์ ทั้งในระบบที่มี และไม่มีเอ็นซัยม์ แต่พบว่าทั้งสารสตีวิโอไซด์ และสตีวิออลในปริมาณสูงมีฤทธิ์ฆ่าแบคทีเรียที่ใช้ในการทดสอบทั้งสองสายพันธุ์ และฤทธิ์ฆ่าแบคทีเรียนี้ลดลงเมื่อมีเอ็นซัยม์จากตับของสัตว์ทดลองชนิดต่างๆอยู่ด้วย

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