Inhibitory Effects of Hot Water Extract of the Stevia Stem on the Contractile Response of the Smooth Muscle of the Guinea Pig Ileum

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The effects of a hot water extract of the stem of *Stevia rebaudiana* on the smooth muscle of isolated guinea pig ileum were investigated. The butyl alcohol layer of the extract antagonized the contractions of the isolated guinea pig ileum induced by histamine \(1 \times 10^{-5}\) M and acetylcholine \(1 \times 10^{-5}\) M in a concentration-dependent manner. The butyl alcohol layer of the extract also showed inhibition of \(\text{CaCl}_2 (1 \times 10^{-3}-3.8 \times 10^{-1}\) M)-induced contractions. The antagonism of the extract was considered to be non-specific, but this action might be related to an influx of extracellular \(\text{Ca}^{2+}\).

With column chromatography preparation, the active component was assumed to be stevioside. The antagonistic effects exerted by the stem extract of *Stevia rebaudiana* contributed to the gastroprotective activity of the extract in animals fed dietary histamine.

Key words: calcium channel blocker: spasmolytic; *Stevia rebaudiana*; stevioside

Stevia, *Stevia rebaudiana*, is a perennial plant that originated in Paraguay. It contains sweetening substances such as stevioside, rebaudioside A, B, D, E, and dulcoside A and B in the leaf. Stevioside is now used as a sweetening agent in Japan and Brazil. Recently it has become clear that stevioside has anti-tumor activity and antihypertensive activity.\(^2\,3\)

The hot water extract of stevia stem, which is used in agriculture, stockbreeding, and the fishery industry, has bactericidal activity, anti-rotavirus activity, antioxidant activity, and causes low oxygen tolerance in rainbow trout.\(^4\,8\) Stevia extract has no chronic toxicity in rats.\(^9\) The authors have found that stevia extract also has protective activity against gizzard erosion in broiler chicks and for gastric mucosal damage in rainbow trout against histamine.\(^10\,11\) The mechanisms of this protective activity and the active components are not yet clear. The antioxidant activity of stevia extract has been attributed to the attenuation of gastric damage,\(^11\) but other factors might strongly be involved in the protective activity. Administration of histamine and its agonists to animals is known to cause atrophy of gastric mucosa and hypoxia,\(^11\,13\) while stevia extract prevented the contraction of the smooth muscle of gastric mucosa.\(^11\) Hence, we assumed that there is a relaxant activity in stevia extract.

We investigated whether the spasmolytic substances were contained in the hot water extract of the stevia stem. The traditional medicine against the gastrointestinal disorders often contains spasmolytic substances.\(^14\,16\)

The aim of this study was to clarify the effect of a hot water extract of the stevia stem on the contraction of the isolated guinea pig ileum and to evaluate a possible contraction mechanism of the extract by comparing it with papaverine, which is known as a smooth muscle relaxant.

Materials and Methods

Chemicals. Acetylcholine chloride, histamine dihydrochloride, papaverine hydrochloride, and stevioside were purchased from Wako Pure Chemical Industries (Osaka, Japan), and all chemicals used were of the highest analytical grade available. All drugs except papaverine hydrochloride and stevioside were dissolved in distilled water. Papaverine dihydrochloride was dissolved in glacial acetic acid and stevioside in DMSO and they were diluted with water, because these substances are almost insoluble in water.

Stevia extract. The stevia extract was obtained from JBB Stevia Laboratory (Saitama, Japan). It was made mainly of stevia stems.\(^5\) The extract was dried on a rotary evaporator at room temperature and dissolved in water.

To remove the potassium ions in the stevia extract,\(^6\) the extract was fractionated with hexane, ethyl acetate, and butyl alcohol using a separator funnel. The extraction was repeated three times. Each water and solvent layer was concentrated and dried with a rotary evaporator at 25 °C. The fractions were dissolved in water.

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Abbreviations: DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography
Smooth muscle preparation. Male Hartley guinea pigs (body weight about 450 g) were purchased from Japan SLC (Hamamatsu, Japan). The animals were subjected to a 16 h fast and had free access to water ad libitum before the experiments. They were decapitated and the ileum was removed. A 2.0 cm length of the ileum was placed in 20 ml of organ bath containing Tyrode’s solution of the following composition (mM): NaCl 136.9, KCl 2.6, MgCl₂ 1.1, CaCl₂ 1.8, NaH₂PO₄ 0.4, NaHCO₃ 11.9, and glucose 5.6, bubbled with a mixture of 95% of oxygen and 5% of carbon dioxide at 32 °C under a resting tension of 1 g. The intestinal responses were recorded isotonically using an NEC Displacement Transducer and NEC DC Strain Amplifier (NEC, Tokyo, Japan). The animals were subjected to a 16 h fast and had free access to water ad libitum before the experiments. They were decapitated and the ileum was removed. A 2.0 cm length of the ileum was placed in 20 ml of organ bath containing Tyrode’s solution of the following composition (mM): NaCl 136.9, KCl 2.6, MgCl₂ 1.1, CaCl₂ 1.8, NaH₂PO₄ 0.4, NaHCO₃ 11.9, and glucose 5.6, bubbled with a mixture of 95% of oxygen and 5% of carbon dioxide at 32 °C under a resting tension of 1 g. The intestinal responses were recorded isotonically using an NEC Displacement Transducer and NEC DC Strain Amplifier (NEC, Tokyo, Japan).

Determination of calcium antagonist activity. To investigate the spasmyloytic activity of the extract of the stevia stems, histamine and acetylcholine were used as agonists to induce contractions against the isolated guinea pig ileum. Acetylcholine (from 1 × 10⁻⁹ to 1 × 10⁻⁴ M) and histamine (from 1 × 10⁻⁸ to 1 × 10⁻⁴ M) caused a concentration-dependent contraction of the guinea pig ileum; therefore the experiments were carried out with a maximal response dose of acetylcholine (1 × 10⁻⁵ M) and histamine (1 × 10⁻⁴ M). The water and organic solvent layer fractions of the stevia extract were added to the bath until the tissues equilibrated before the agonist was added to the bath.

To evaluate possible mechanisms of action of the extract, K⁺ was used as a depolarizing agent. The nutrient solution was replaced with a calcium-free hyperpotassiac Tyrode medium (K⁺ 80 mM). Cumulative dose-response curves induced by CaCl₂ (1 × 10⁻³ M to 3.8 × 10⁻² M) were obtained in the absence and presence of papaverine (2 × 10⁻⁵ M) and different doses of the fractions of the stevia stem extract.

Identification of the active component. The active fraction was taken for a fractionation step using a high performance liquid chromatography (HPLC) technique. The HPLC system consisted of two pumps (PU-987, JASCO, Tokyo), a solvent mixing module (HG-980-31, JASCO), a diode array detector (SPD-M10Avp, SHIMADZU, Kyoto, Japan), and a data analyzer (CLASS-vp, SHIMADZU, Kyoto, Japan). The mobile phases were 0.01 N HCl (A) and methanol (B). The mobile phase was delivered at a flow rate of 10.0 ml/min to a TSKgel ODS-80T S column (250 × 20.0 mm i.d., Tosoh, Tokyo). The linear gradient program for the separation procedure was from 100% to 70% of (A) in 10 min, 70% of (A) continued for the next 10 min, and then a decrease to 0% of (A) over the next 40 min. Peaks were detected at 216 nm. Six fractions were obtained by preparation with a HPLC (Fig. 5). The fractions were also applied to spasmyloytic assay with the isolated guinea pig ileum.

Analysis of data and statistics. Values of the results are given as means ± S.E.M. The significant differences between means were calculated using Student’s t-test.

Results

First we tried to get information about the active component of the stevia extract by solvent extraction, hexane, ethyl acetate, and butyl alcohol. It was found that the active component was recovered in organic phase only with butyl alcohol. The butyl alcohol fraction significantly exhibited an inhibitory effect on the contraction induced by histamine (1 × 10⁻⁴ M) and acetylcholine (1 × 10⁻⁵ M) on the isolated guinea pig ileum in a dose-dependent manner (Fig. 1, 2). The stevia extract showed relaxant activity not only against histamine but also against acetylcholine, which meant that the effects of the extract were non-specific for each

**Fig. 1.** Relaxant Effect of Different Doses of the BuOH-Fraction of the Stevia Extract on Histamine (1 × 10⁻⁵ M)-Induced Contractions in the Isolated Guinea Pig Ileum.

The guinea pig ileum was applied the magnus assay. The ileum contraction induced by histamine without stevia extract was used as a control (100), and the relaxant activity of the extract is represented with the percentage of the contraction against control. The experiments were done in triplicate. **P < 0.01 statistically different from control.
receptor of the contractile drugs. It was assumed that the stevia extract acted on the common signal in the contractions induced by histamine or acetylcholine. Hence, we tested whether the relaxant activity of the stevia extract was a calcium channel blocker.

The butyl alcohol fraction showed spasmolytic activities on the dose response curves induced by Ca\textsuperscript{2+} (1/C\textsubscript{20} to 3.8/C\textsubscript{10} M) on the smooth muscle with a calcium-free hyperpotassic medium (Fig. 3). Treatment of 1.0 mg/ml of the stevia extract attenuated the contraction more strongly than 2/C\textsubscript{10} M papaverine, which is a calcium channel blocker (Fig. 4).

These results show that the spasmyloytic effect of the extract of stevia stems inhibits calcium influx into the cells of the isolated guinea pig ileum.

Next we tried to identify the active component of the butyl alcohol fraction of the extract. Six fractions were obtained after preparative HPLC (Fig. 5A). These fractions were applied to spasmyloytic assay with the guinea pig ileum contracted by Ca\textsuperscript{2+}. Only fraction no. 6 showed spasmyloytic activity against the contract caused by Ca\textsuperscript{2+}.

Fraction no. 6 was purified with further column chromatography under the conditions described above. With repeated chromatography purification, fraction no. 6 showed a sharp single peak in the chromatogram (Fig. 5B), and the purity of the substance was about 99%. The purified fraction no. 6 was applied to FAB-MS analysis (Fig. 6). In FAB-MS spectrum (M + H\textsuperscript{+}) was observed at m/z 805, and (M + Na\textsuperscript{+}) was at m/z 827.
which coincided with the molecular weight of stevioside (Fig. 7). Stevioside is known for antispasmodic activity in the rat aorta. To confirm that fraction no. 6 was stevioside, authentic stevioside was applied for HPLC under the same conditions of isolation as fraction no. 6. The retention time and the absorption spectrum of stevioside coincided with fraction no. 6.

Fraction no. 6 was positive in orcinol–H$_2$SO$_4$. To compare the spasmolytic activity of fraction no. 6 and stevioside, purified fraction no. 6 and stevioside were applied for Magnus assay with contraction induced by $3 \times 10^{-2}$ M Ca$^{2+}$ under the depolarized condition. At the concentration of DMSO used in the present experiments, there was no effect of DMSO on the contraction of the guinea pig ileum. 0.75 mg/ml of purified fraction

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**Fig. 4.** Relaxant Effect of Different Doses of the BuOH-Fraction of the Stevia Extract on CaCl$_2$ ($1 \times 10^{-2}$ M)-Induced Contractions in Isolated Guinea Pig Ileum.

The contraction of the guinea pig ileum induced by $1 \times 10^{-2}$ M CaCl$_2$ without the stevia extract or papaverine was used as a control (100), and the effect of the extract or papaverine (final concentration $2 \times 10^{-3}$ M) on contraction was assessed. The experiments were done in triplicate. *P < 0.05 **P < 0.01 statistically different from control.

**Fig. 5.** HPLC Separation of the BuOH-Fraction of the Stevia Extract on the ODS Column Detected with UV at 214 nm.

A, The BuOH-fraction of the stevia extract are separated by preparative HPLC. The detail conditions for HPLC are described in “Materials and Methods.” B, The active fraction (no. 6) was further purified by preparative HPLC. Finally, purified fraction no. 6 was observed by chromatograph.

**Fig. 6.** FAB-MS Spectrum of Purified Fraction no. 6. (M + H$^+$) was observed at m/z 805 and (M + Na$^+$) at m/z 827.

**Fig. 7.** The Chemical Structure of Stevioside.
of the stem extract on the smooth muscle are not known, but this stevioside was purified from leaves. The effects of the stem extract was found to contain 28 mg of stevioside. Fraction no. 6 is probably stevioside. One gram of stevia extract was 150 times higher than that of crude stevia stem extract. From these results, the active component of fraction no. 6 is probably stevioside. One gram of stevia extract was found to contain 28 mg of stevioside.

Discussion

Dietary histamine and gizzerosine cause gastric mucosal damage in broiler chicks and fish with mucosal hypoxia, increases in gastric pepsin activity, and decreases in $\alpha$-tocopherol contents in the liver and other tissues.10–13,19,20 Our previous studies showed the gastroprotective activity of stevia extract in animals fed dietary histamine,10,11 but the mechanism of protection was not clear.

At the beginning of this study, we hypothesized that the stevia stem extract specifically blocked the histamine receptor, but the extract showed relaxant activity against contraction induced not only by histamine but also by acetylcholine. Histamine and acetylcholine bind to different receptors on the smooth muscle, but the signal pathway from each receptor is thought to be same: an activation of phospholipase C, increases of inositol triphosphate, and an influx of calcium.

In the present study, the extract inhibited contraction of the smooth muscle induced by CaCl$_2$ alone. 0.8 mg/ml of stevioside exhibited 59.8% of that contraction (Table 1). The relaxant activity of stevioside was as strong as that of fraction no. 6. The relative spasmylocytic activity of purified fraction no. 6 was 150 times higher than that of crude stevia stem extract.

From these results, the active component of fraction no. 6 is probably stevioside. One gram of stevia extract was found to contain 28 mg of stevioside.

Table 1. Comparison of Spasmylocytic Activity between Fraction no. 6 and Authentic Stevioside

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Relative contraction (%)</th>
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<tr>
<td>no. 6</td>
<td>0.75</td>
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<tr>
<td>Stevioside</td>
<td>0.80</td>
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Fraction no. 6 was purified with column chromatography from the BuOH-fracton of the stevia extract. Fraction no. 6 and stevioside were applied to magnus assay with contraction induced by $3 \times 10^{-4}$ M Ca$^{2+}$ under a depolarized condition. The contraction induced by $3 \times 10^{-4}$ M Ca$^{2+}$ was used as a control, and the percentage of contraction against control (100) was represented as relative contraction.

Table 1.

no. 6 exhibited 60.1% of the contraction induced by Ca$^{2+}$ alone. 0.8 mg/ml of stevioside exhibited 59.8% of that contraction (Table 1). The relaxant activity of stevioside was as strong as that of fraction no. 6. The relative spasmylocytic activity of purified fraction no. 6 was 150 times higher than that of crude stevia stem extract.

It is assumed that stevioside is an effective drug not only for hypertension but also for gastric disease based on our results. Moreover, calcium channel blocker is known to regulate the secretion of gastric acid and protect from gastric ulcer,22 which means stevioside can act on gastric secretions. Stevia extract inhibited the activation of pepsin activity in trout fed dietary histamine.13 Stevioside might inhibit the increase in acid secretion caused by histamine, and the pepsin activity might be decreased.

Takahashi et al. supposed that the gastric protective activity of stevia extract is due to (1) antioxidant activity, (2) inhibition of histamine incorporation from intestine, (3) activation of histamine catabolism, (4) blocking of H$_2$-histamine receptor, (5) inhibition of H$^+$/K$^+$ ATPase, and (6) inhibition of peptic activity.10 Though there is little information on the effects of exogenous histamine in animals,24 further research is needed to elucidate the gastroprotective activity of stevia extract. The spasmylocytic activity shown by stevia extract might be one of several gastroprotective activities.

In conclusion, stevia stem extract was found to be spasmylocytically active as a calcium antagonist. We also found that stevioside was the only spasmylocytic substance in the stevia stem extract that has gastroprotective activity in animals fed dietary histamine.

Acknowledgment

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References


