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Sweet and Sweetness-inducing Activities of New Triterpene Glycosides, Strogins

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Abstract

In a previous study we isolated homologues of new oleanane-type triterpene glycosides from leaves of *Staurogyne merguensis* Kuntze and named them strogins. Strogins themselves have a sweet taste (sweet activity), which diminishes in a few minutes. Subsequent application of cold water to the mouth then elicits a sweet taste (sweetness-inducing activity). In the present study we systematically examined the properties of the sweet and sweetness-inducing activities of strogins. Strogins **1**, **2** and **4** had both the sweet and sweetness-inducing activities, while strogins **3** and **5** had no activities. The sweetness-inducing activity in response to cold water lasted for 1 h for strogin **2** and 2 h for strogins **1** and **4**. The sweetness-inducing activity was immediately diminished by application of γ-cyclodextrin to the mouth after strogins were held in the mouth. It seems that the strogins were adsorbed on the gustatory receptor membranes and eliminated by inclusion activity of γ-cyclodextrin. The structure of strogin resembles that of gymnemic acid, which has antisweet activity. There was competition between strogin **1** and gymnemic acid; treatment of the tongue with strogin 1 before application of *Gymnema* extract to the mouth reduced the antisweet activity. While the sweetness-inducing activity of curculin in response to water was suppressed by the presence of divalent cations such as Ca^{2+} or Mg^{2+}, that of strogin was not suppressed by the divalent cations. The changes in the inactive complex between strogin and the sweet receptor site in the adaptation state into the active complex induced by cold stimulation were discussed.

Introduction

Staurogyne merguensis grows wild in Malaysia. The leaves have a sweet taste. After chewing the leaves, application of cold water to the mouth elicits a sweet taste. In a previous study (Hiura *et al.*, 1996) we purified five new oleanane-type triterpene glycosides from the leaves and named them strogins **1–5**. Their structures were determined on the basis of chemical and spectral evidence. Strogins **1–5** have an oleanene skeleton and glucuronic acid at C-3 of the aglycone. Thus, structural studies on strogin have greatly progressed, but the sweet activity and the sweetness-inducing activity have been examined only preliminarily in the previous paper (Hiura *et al.*, 1996) and no systematic study has been carried out.

In the present study the sweet activity and the sweetness-inducing activity of strogin analogues were evaluated systematically. Strogins **1**, **2** and **4** had both the sweet and the sweetness-inducing activities, while strogins **3** and **5** had no activities. We examined the temperature dependence of the sweet and sweetness-inducing activities, and showed that the sweetness-inducing activity was maximal at 4–10°C and decreases sharply with an increase of temperature. The structure of the strogins resembles that of gymnemic acid, which has antisweet activity. Hence we examined whether or not strogin **1** and gymnemic acid are adsorbed on the gustatory receptor membranes in a competitive manner. Based on the results obtained in the present study, a possible mechanism of the sweetness-inducing action of strogins was discussed.

Materials and methods

Materials

Strogins **1–5** were isolated from leaves of *S. merguensis* Kuntze as described previously (Hiura *et al.*, 1996). Air-dried leaves of *Gymnema sylvestre* was extracted with 50% aqueous ethanol at 60°C (Yoshikawa *et al.*, 1989). The extract was chromatographed on a column of Diaion HP-20 (H_2O, 30% aqueous methanol, 100% methanol). We used the fraction eluted by methanol as the *Gymnema* extract.

Procedure of the psychophysical experiments

The sweet taste of strogin was evaluated using four subjects

by a method reported previously (Ugawa *et al.*, 1992). The subjects were paid volunteers drawn from the staff and students of the Faculty of Education, Yokohama National University, and the research members of Lotte Central Laboratory Co. Ltd. The experiments were carried out in a quiet and clean room regulated at 22°C. A series of standard sucrose solutions (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 and 0.40 M) were prepared. Five millilitres of each solution was added to paper cups coated with polyethylene. For the training, subjects were required several times to compare the sweetness of a fixed concentration (1 mM) of strogin **1** with that of the standard sucrose solutions and to select a standard solution with an intensity of sweetness equivalent to that of the given test solution. After tasting each solution, the mouth was rinsed repeatedly with deionized water. There was an interval of at least 3 h between each test.

Evaluation of the sweet activity of strogin

The trained subjects held 2 ml of 1 mM strogin solution in the mouth and compared its sweetness with that of standard sucrose solutions. Water elicited a sweet taste after the subject had held the strogin solution in the mouth for 3 min. To eliminate this effect, the subjects were requested to taste the strogin solution for no longer than 30 s.

Evaluation of the sweetness induced by water after strogin

Two millilitres of 1 mM strogin solution were held in the mouth for 3 min and spat out. During the 3 min holding of the strogin solution the sweetness of strogin diminished. Then subjects tasted 5 ml of deionized water and compared the sweetness induced by water with that of standard sucrose solution.

The effects of cyclodextrin on the sweetness induced by the cold water were examined. Two millilitres of solutions containing 5% α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD) or soluble starch was held in the mouth for 2 min after 1 mM strogin **1** was held in the mouth for 3 min and then subjects evaluated the sweetness induced by cold water (4°C).

To examine the effects of divalent cations on the sweetness induced by cold water, solutions at 4°C containing various concentrations of $CaCl_2$ or $MgCl_2$ were tasted instead of deionized water after 1 mM strogin had been held in the mouth for 3 min.

Competition between strogin 1 and gymnemic acid

Sucrose solutions of different concentrations were tasted and its threshold concentration was determined. To examine the effects of *Gymnema* extract on the sucrose threshold, 2 ml of *Gymnema* extract solution (10 mg/ml) was held in the mouth for 3 min and then the sucrose threshold was determined. The effect of strogin **1** on the antisweet activity of *Gymnema* extract was examined as follows. Two millilitres of strogin **1** solution (1 mM) was held in the mouth for 3 min and then 2 ml of *Gymnema* extract solution (10 mg/ml) was held in the mouth for 3 min, after which the threshold of sucrose was determined. To eliminate the contribution of the sweet taste of strogin itself, all the solutions used in the above experiments were adjusted to 50°C because the sweet taste of strogin **1** disappears at this temperature.

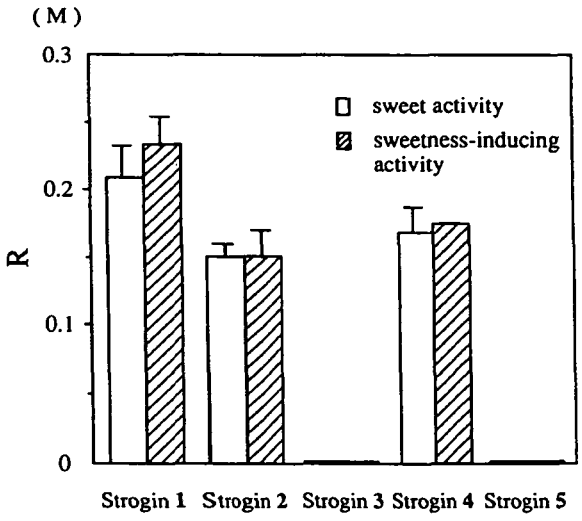

Figure 1 The sweet and sweetness-inducing activities of strogin analogues. The strogin analogues **1–5** were dissolved in deionized water. The sweetness of 1 mM strogin solution at 4°C was evaluated by comparing its sweetness with a standard sucrose solution. *R* on the ordinate represents the sucrose concentration whose sweetness is equivalent to that of the test solution. The sweetness-inducing activity of each strogin analogue was evaluated by tasting deionized water at 4°C after a strogin solution was held in the mouth for 3 min. Each value in the figure is the mean ± SE of data obtained from four subjects.

Results and discussion

Stability of strogin 1

The sweet and the sweetness-inducing activities of strogin **1** were unchanged by boiling for 3 h. The activities of strogin 1 were also unchanged between pH 2 and 11 at room temperature. Strogin 1 was precipitated at pH 2, but the activities recovered by neutralization. Thus, strogin **1** is rather stable against changes in temperature and pH.

Sweet and sweetness-inducing activities of strogin analogues

Figure 1 shows the sweet and sweetness-inducing activities of strogins **1–5** at 1 mM. The sweetness of 1 mM strogin **1** is equivalent to that of 0.20 M sucrose. The molecular weight of strogin **1** is 1070, hence strogin **1** is 200 times sweeter than sucrose on a molar basis and 64 times sweeter than sucrose on a weight basis. After the sweetness of strogins diminished due to adaptation, application of cold water induced a sweet taste. The sweetness induced by cold water (4°C) was

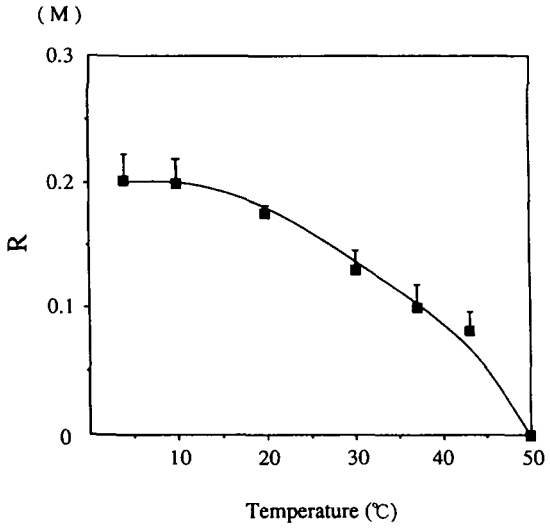

Figure 2 Temperature dependence of the sweet activity of strogin **1**. Strogin **1** solution (1 mM) at different temperatures was tasted and its sweetness compared with the sweetness of standard sucrose solution. *R* on the ordinate represents the sucrose concentration whose sweetness is equivalent to that of the test solution. Each value in the figure is the mean ± SE of data obtained from four subjects.

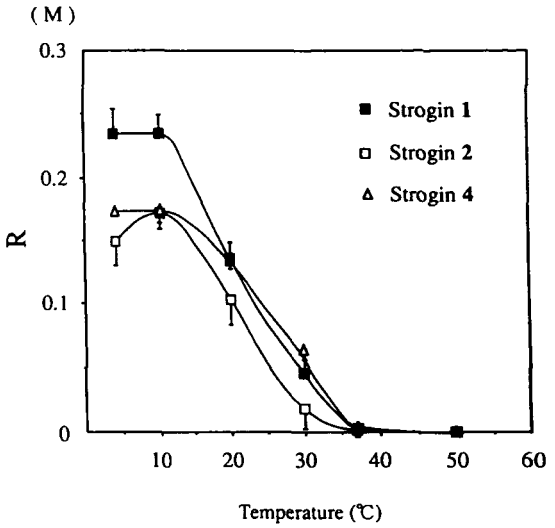

Figure 3 Temperature dependence of the sweetness-inducing activity of strogins **1**, **2** and **4**. The strogin analogues were dissolved in deionized water. Each solution at 4°C containing 1 mM strogin analogue was held in the mouth for 3 min and then the sweetness induced by water at different temperatures was evaluated. *R* on the ordinate represents the sucrose concentration whose sweetness is equivalent to that of the test solution. Each value in the figure is the mean ± SE of data obtained from four subjects.

equivalent to that of 0.23 M sucrose after 1 mM strogin **1** was held in the mouth for 3 min. Strogins **2** and **4** also had the sweet and sweetness-inducing activities, but they were weaker than those of strogin **1**. Strogins **3** and **5** had no sweet and sweetness-inducing activities.

Temperature dependence of sweet and sweetness-inducing activity

Figure 2 shows the sweetness of strogin **1** itself as a function of temperature. The maximum sweetness was elicited at 4–10°C, and the sweetness decreased with an increase in temperature. The sweetness vanished above 50°C. Figure 3 shows that the temperature dependence of the sweetness induced by water after strogins **1**, **2** and **4** were held in the mouth. The maximum sweetness was induced by cold water at 4–10°C. The sweetness-inducing activities of these strogins decreased sharply with an increase of temperature, disappearing completely above 37°C. The sweetness-inducing activity disappeared at much lower temperature than the sweet activity (see Figure 2).

Persistence of sweetness-inducing activities

Figure 4 shows the time course of the sweetness-inducing activity after strogins **1**, **2** and **4**. The sweetness-inducing activity persists for 1 h for strogin **2** and 2 h for strogins **1** and **4**. This persistence suggests that strogin is strongly bound to gustatory receptor membranes.

Gymnemic acid, which has antisweet activity, is known to be bound strongly to the gustatory receptor membranes and its activity persists for a long time. Imoto (1990) showed that

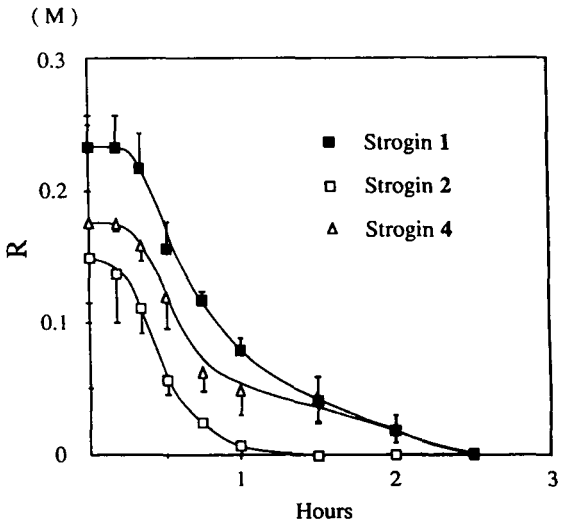

Figure 4 Persistence of the sweetness-inducing activity of strogins **1**, **2** and **4**. Strogin analogues (1 mM) were dissolved in deionized water. The sweetness induced by water (4°C) was assayed after each strogin solution was held in the mouth for 3 min. The abscissa shows the time after the strogin was spat out from the mouth. *R* on the ordinate represents the sucrose concentration whose sweetness is equivalent to that of the test solution. Each value in the figure is the mean ± SE of data obtained from four subjects.

the antisweet activity of gymnemic acid is suppressed by γ-cyclodextrin, which has the ability to include many compounds in its cavity. Hence we examined the effects of

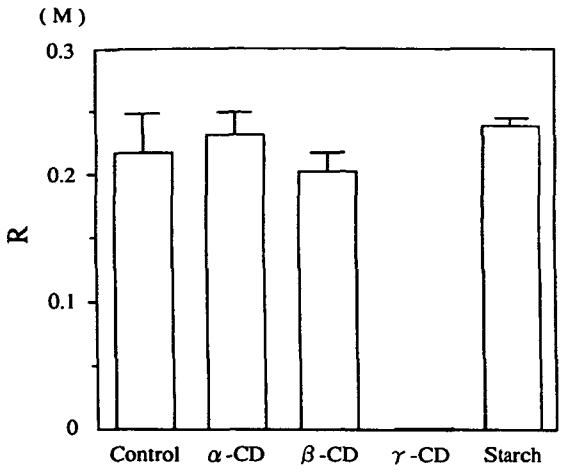

Figure 5 Effects of various types of cyclodextrin on the sweetness-inducing activity of strogin **1**. Strogin **1** solution (1 mM) was held in the mouth for 3 min. After the mouth was rinsed with deionized water, solutions containing 5% cyclodextrin analogues and soluble starch were held in the mouth for 2 min. Then the sweetness induced by cold water (4°C) was evaluated. *R* on the ordinate represents the sucrose concentration whose sweetness is equivalent to that of the test solution. Each value in the figure is the mean ± SE of data obtained from four subjects. Columns indicate control (no cyclodextrin), α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD) and soluble starch.

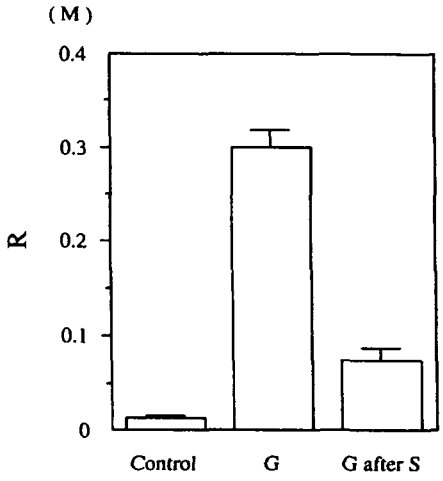

Figure 6 Competition between strogin **1** and gymnemic acid. *R* on the ordinate shows the threshold concentration of sucrose. G, sucrose threshold after *Gymnema* extract; G after S, sucrose threshold after strogin **1** and *Gymnema* extract were held in the mouth. The detail of the experimental procedure is described in Materials and methods. Each value in the figure is the mean ± SE of data obtained from four subjects.

cyclodextrin analogues on the persistence of the sweetness-inducing activity of strogin **1**. Figure 5 shows the effects of α-, β- and γ-cyclodextrins and soluble starch. Only γ-cyclodextrin suppresses the sweetness-inducing activity of strogin **1**. It seems that strogin **1** fits the cavity size of γ-cyclodextrin and that strogin **1** bound on the gustatory receptor membranes is eliminated by the inclusive action of γ-cyclodextrin. These results gave evidence that strogin is adsorbed on gustatory receptor membranes.

Competition between strogin and gymnemic acid

The structure of strogin **1** resembles that of gymnemic acid (Yoshikawa *et al.*, 1989). Both compounds have an oleanene skeleton and glucuronic acid at C-3 of the aglycone. There is a possibility that both gymnemic acid and strogin bind to the same sweet receptor site. We applied *Gymnema* extract containing gymnemic acid to the mouth and examined its effect on the antisweet activity of gymnemic acid. The threshold of sucrose was ~0.01 M and became ~0.3 M after *Gymnema* extract (Figure 6). Treatment of the tongue with strogin **1** before *Gymnema* extract led the sucrose threshold to ~0.08 M, suggesting that pretreatment with strogin **1** prevents binding of gymnemic acid to the sweet receptor site. It therefore seems that strogin **1** and gymnemic acid bind to the same site on the gustatory receptor membranes.

Effects of divalent cations on the sweetness-inducing activity

The taste-modifying protein curculin itself has a sweet taste.

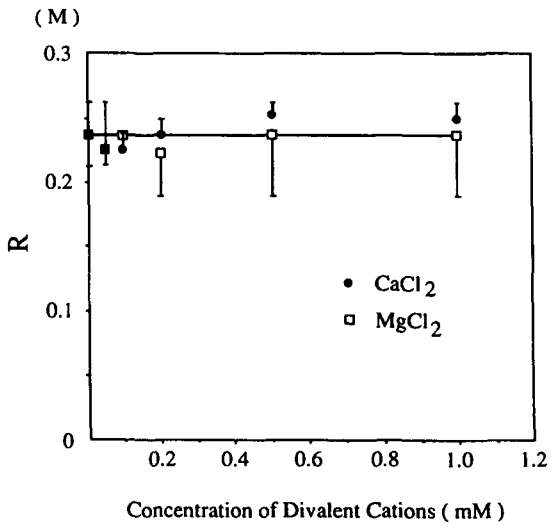

Figure 7 Effects of divalent cations on the sweetness-inducing activity of strogin **1**. Strogin **1** solution (1 mM) was held in the mouth for 3 min. After the mouth was rinsed with deionized water, solutions at 4°C containing different concentrations of $CaCl_2$ or $MgCl_2$ were tasted. *R* on the ordinate represents the sucrose concentration whose sweetness is equivalent to that of the test solution. Each value in the figure is the mean ± SE of data obtained from four subjects.

After the sweetness of curculin diminished, application of water to the tongue induced a sweet taste. The sweetness induced by water was completely suppressed by the presence of 1 mM $CaCl_2$ or $MgCl_2$ (Yamashita *et al.*, 1995). We examined the effects of divalent cations on the sweetness-inducing activity of strogin **1**. As shown in Figure 7, both $CaCl_2$ and $MgCl_2$ had no effect on the sweetness-inducing

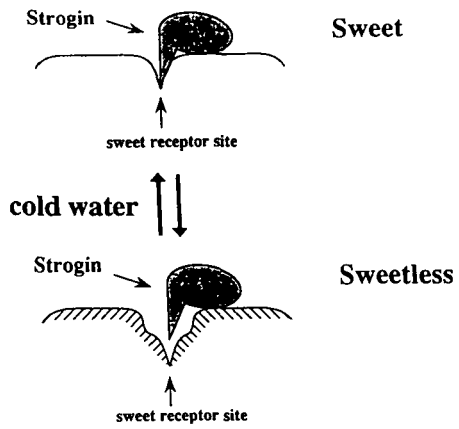

Figure 8 Schematic diagram illustrating the sweetness-inducing action of strogin.

activity. This result suggests that the mechanism of the sweetness-inducing activity of strogin is different from that of curculin.

Mechanism of the sweetness-inducing activity

The sweetness-inducing activity of curculin was explained as follows (Yamashita *et al.*, 1995). The sweetness of curculin was suppressed by divalent cations in saliva. Application of deionized water to the tongue washes out saliva and eliminates divalent cations from the tongue surface. Then the sweetness recovers. The sweetness-inducing activity of strogin is different from that of curculin on two points. First, in the case of strogin, only cold water induced a sweet taste, while the sweetness-inducing activity of curculin had no temperature dependence. Second, the sweetness-inducing activity of curculin was suppressed by divalent cations, while that of strogin was not.

Figure 8 shows a schematic diagram of the sweetness-inducing activity of strogin. Strogin is assumed to have two binding sites: one site binds to an active site of sweet receptor protein and another binds to a different site. The latter binding is rather strong, and hence once strogin is applied to the tongue it is not easily detached from the receptor membranes. Binding of strogin to the active site of sweet receptor protein induces a sweet taste, which is easily diminished due to adaptation. The mechanism of the taste adaptation is unknown in general. One possibility is that an active complex between the active site of the receptor protein and strogin is changed into an inactive complex due to a conformational change of the receptor protein or the receptor membranes. Application of cold water to the inactive complex leads to the active complex. It is unknown whether cold stimulation itself induces the sweet taste or the application of water is necessary for induction of a sweet taste. However, a touch of the tongue with a cold glass induced a sweet taste, and so cold stimulation itself may be essential for the induction of the sweet taste. It seems that a rapid decrease in temperature of the receptor membranes effectively recovers the conformation of the receptor sites from the inactive form in the adaptation state into the active form, which induces the sweet taste.

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