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Induction of Salivary Gurmarin-binding Proteins in Rats fed *Gymnema*-containing Diets

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Abstract

Gymnema sylvestre, a tropical plant, contains gurmarin that selectively suppresses sucrose responses of the chorda tympani nerve in rats and mice. We investigated preference for taste solutions and saliva composition in rats fed a diet containing this plant (gymnema diet). Preference for 0.01 M sucrose and a mixture of 0.03 M sucrose and 0.03 mM quinine-HCl significantly decreased at 1–2 days after the start of the gymnema diet and subsequently returned closely to the control levels within about a week. There was no significant change in preference for NaCl, monosodium glutamate and quinine-HCl during feeding trials. Submandibular saliva of rats fed the gymnema diet for 4 and 14 days showed an inhibitory effect on immunoreaction between gurmarin and antigurmarin serum. Analyses using electrophoresis and affinity chromatography indicated that the saliva contains gurmarin binding proteins with molecular weights of 15, 16, 45, 60 and 66 kDa. These results suggest that reduction of preference for sucrose was probably caused by gurmarin contained in the gymnema diet and subsequent restoration of the preference may be due to suppression of the effect of gurmarin by salivary gurmarin-binding proteins induced by the gymnema diet.

Introduction

Leaves of a tropical plant, *Gymnema sylvestre*, contain two types of specific inhibitors of sweet taste, gymnemic acid and gurmarin. Gymnemic acid, a mixture of triterpene glycosides, is a most potent inhibitor of sweet taste in humans and responses of the chorda tympani nerve to sucrose in chimpanzees (Hellekant *et al.*, 1985; Kurihara, 1992). Gurmarin, a peptide of mol. wt 4209, is reported to suppress selectively chorda tympani responses to sweeteners in rats (Imoto *et al.*, 1991; Miyasaka and Imoto, 1995) and certain strains of mice (Ninomiya and Imoto, 1995; Ninomiya *et al.*, 1997, 1998). This inhibitory effect of sweetener responses occurs at a low concentration ($>1 \times 10^{-6}$ M) and lasts for >3 h in the rat (Imoto *et al.*, 1991). These sweet-taste inhibitors may function as a defense against vermin damage by making the plant unpalatable to many species of animals.

Our previous study (Ninomiya *et al.*, 1994) demonstrated that when fed diets containing papain (cysteine protease), rats did not soon consume the required amounts of diet to maintain or increase body wt. However, some animals started to ingest sufficient amounts of such diets at a few days after the start of feeding. At this time, cystatin S (a cysteine protease inhibitor) was concomitantly induced in the submandibular saliva of animals. It has been shown that cystatin S binds to papain and reduces its activity (Bobek and Levine, 1992). We therefore proposed that cystatin S may participate in the reduction of nociceptive stimulation of the oral mucosa by papain and improve food intake.

In this report, we investigate changes in taste preference and salivary composition in rats fed diets containing powder of leaves of *G. sylvestre* (gymnema diet). The results demonstrate that preference for sucrose transiently decreased and subsequently recovered at several days after the start of feeding at the time when gurmarin-binding proteins were induced in saliva. This suggests that these diet-induced salivary proteins influence behavioral preference for sucrose in rats.

Materials and methods

Animals

Male Wistar rats (320–350 g body wt) were individually housed in plastic cages in a room maintained at 22–25°C with ~50% relative humidity. The room was lighted from 6.00 a.m. to 6.00 p.m. Animals were fed a commercial brand of non-purified diet (CE-2, Clea Japan) for 2 weeks prior to experiments. In feeding trials (14 days), animals were divided into two groups, one of which was fed the commercial diet (control diet group) and the other diet supplemented with 3% ground (10 mesh) *Gymnema* leaves (3% gymnema

Table 1 The composition of control diet used in the experiment

Ingredients	% of weight
Crude protein	25.4
Crude fat	4.4
Crude fiber	4.1
Crude ash	6.9
Soluble non-nitrogen compounds	50.3
Water	8.9

The diet consists of soybean, white fishmeal, flour, corn, yeast and supplements (vitamins and minerals).

diet group). In one experiment, animals fed diets containing 1 and 10% gymnema (1 and 10% gymnema diet groups) were also used. Table 1 gives the composition of the control diet.

Preference test for a sucrose–quinine mixture

Preference behavior was routinely measured with a 48 h, two-bottle preference test. Rats were presented with a choice between distilled water and a test solution for 48 h (Ninomiya *et al.*, 1989). The taste solutions (in distilled water) used were: 0.01, 0.03 and 0.1 M sucrose; 0.03 M NaCl; 0.03 M MSG; 0.003 mM quinine-HCl; and a mixture of 0.03 or 0.1 M sucrose and 0.03 mM quinine-HCl. Total intakes of each solution over 48 h were measured and used to calculate preference percentages according to the following formula:

$$\text{Preference percentage} = \text{volume of testing taste solution (ml)} \times 100 / \text{total volume of testing taste solution and water (ml)}.$$

Preference percentages were compared before and during a feeding trial.

Collection of submandibular saliva

After the preference test, rats were starved overnight and then the submandibular ducts were cannulated intraorally with polyethylene tubes (SP-8, Natsume, Japan) under pentobarbital anesthesia (i.p., 40–50 mg/kg). Saliva was collected into a tube containing ice-cold 10 mM citrate buffer (pH 4.5) for 1 h by evoking secretion with an i.p. dose of 20 mg/kg body wt of DL-isoproterenol–HCl (Ninomiya *et al.*, 1994). Saliva samples were stored at –80°C until analysis. The salivary glands were removed and weighed after saliva collection. This procedure was carried out in both groups of rats.

Inhibition of the gurmarin–antigurmarin mouse antiserum reaction by saliva

Saliva (0.25–1 μl) was added to a reaction mixture of gurmarin (10 ng) and antigurmarin mouse antiserum (15 μl), and incubated at room temperature for 60 min according to the method of Imoto *et al.* (Imoto *et al.*, 1992). The precipitates were treated with biotin-labeled antimouse IgG rabbit antiserum for 60 min and then with horseradish-peroxidase-labeled streptoavidin (Histofine, Nichirei, Japan) for 30 min. The peroxidase-labeled products were incubated with 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] (Boehringer Mannheim, Germany) as a substrate in the presence of 0.01% H_2O_2 at room temperature for 15 min. Amounts of the precipitates in reaction with gurmarin and antigurmarin antiserum were expressed as peroxidase activity (OD_{415nm}).

Affinity chromatography of salivary gurmarin-binding protein

Gurmarin (2 mg) was coupled to *N*-hydroxysuccinide-activated sepharose (1 ml, Pharmacia, Sweden) according to the manufacturer's instruction manual, except that 0.1 M phosphate buffer containing 0.5 M NaCl (pH 5.0) was used as a coupling buffer. Saliva samples were incubated with the gurmarin-coupled matrix at room temperature for 30 min. The column was washed with 50 ml of 0.1 M phosphate buffer containing 0.5 M NaCl (pH 5.0) and then with 0.1 M Tris–HCl buffer containing 0.5 M NaCl (pH 7.0) until the optical density at 280 nm of the eluate became zero. Gurmarin-binding proteins were eluted with 0.1 M Tris–HCl buffer containing 0.5 M NaCl and 2% hydroxypropyl β-cyclodextrin.

For an electrophoretic analysis, the eluate (10 μg) from an affinity column was applied to 15% SDS–polyacrylamide gels prepared by the method of Laemmli (1970) and run at 20 mA for 60–90 min. Protein bands were visualized by silver staining procedures using a commercial kit (Sil-Best Stain, Nakarai Co., Japan).

Results

Food intakes and increment of body wt in animals fed the 1, 3 and 10% gymnema diets were compared in order to choose the diet to be used for taste preference tests (Figure 1). There was no difference in daily intakes of diet between control diet groups (17.6 ± 1.5 g/day) and gymnema diet groups (1%, 20.1 ± 1.3; 3%, 18.0 ± 1.6; 10%, 20.9 ± 1.2 g/day) (Figure 1A). The intakes of any diet group remained unchanged from the beginning to the end of a trial. Increments of body wt in the 1 and 3% groups (58.7 ± 1.3 and 56.0 ± 3.3 g) were comparable to that (60 ± 5.8 g) in the control group in the 14 day feeding trials, but marked decreases were found in the 10% group (30.1 ± 4.5 g) (Figure 1B). In all of the following experiments, the 3% gymnema diet, having little or no effect on the growth of animals, was used as the experimental diet.

Figure 2 shows changes in preferences for various sucrose solutions in animals fed the gymnema diets. Preference percentages for 0.1 M sucrose did not significantly change after the start of the gymnema diet (Student's *t*-test, $P > 0.05$).

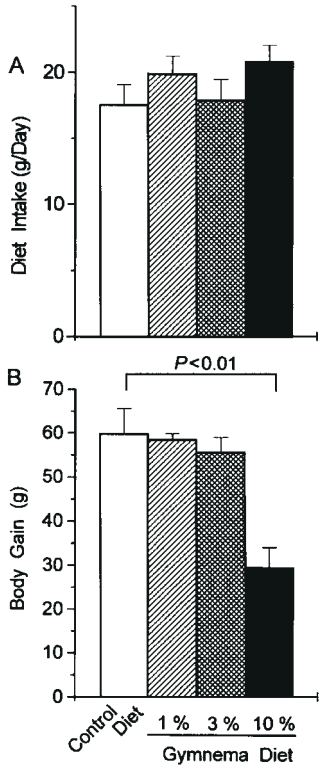

Figure 1 Daily food intakes (top) and body wt gain (bottom) of rats in a feeding trial, where 1, 3 and 10% gymnema diets were used. Open column: animals fed the control diet; striped column: animals fed the 1% gymnema diet; cross-hatched column: animals fed the 3% gymnema diet; solid column: animals fed the 10% gymnema diet. Results were analyzed using Student's *t*-test. Each column represents the average with SE of 8–12 animals.

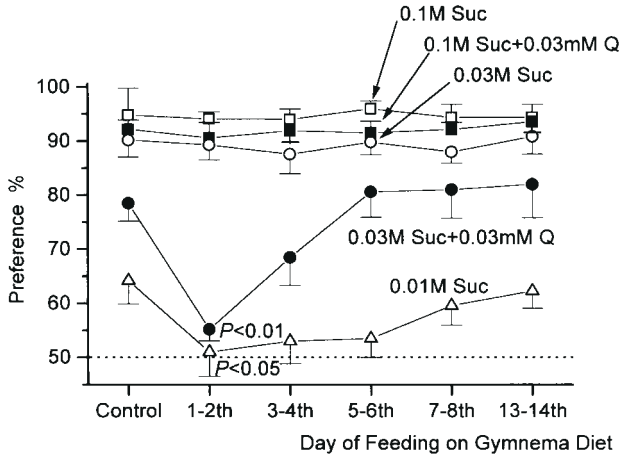

Figure 2 Preference percentages for various sucrose solutions in rats fed the 3% gymnema diet. 0.1 M Suc (□): 0.1 M sucrose; 0.03 M Suc (○): 0.03 M sucrose; 0.01 M Suc (△): 0.01 M sucrose; 0.1 M Suc + 0.03 mM Q (■): a mixture of 0.1 M sucrose and 0.03 mM quinine-HCl; 0.03 mM Suc + 0.03 mM Q (●): a mixture of 0.03 M sucrose and 0.03 mM quinine-HCl. Results were analyzed using Student's *t*-test. Each symbol represents the average of 8–12 animals.

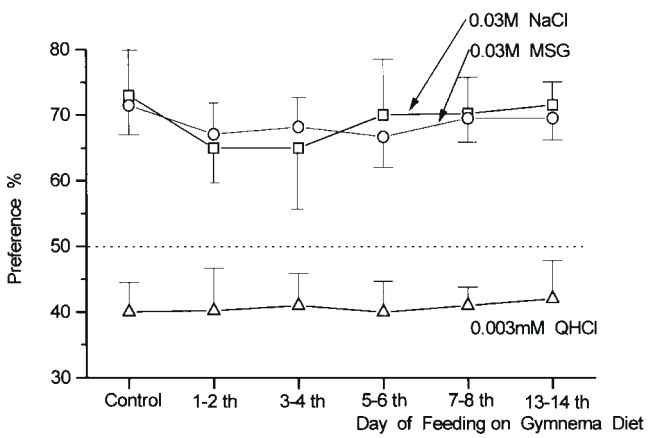

Figure 3 Preference percentages for 0.03 M NaCl (□), 0.03 M MSG (○) and 0.003 mM quinine (△) in rats fed the 3% gymnema diet. Results were analyzed using Student's *t*-test. Each symbol represents the average with SE of 8–12 animals.

Similarly, no change was found in preference percentages for 0.03 M sucrose and for a mixture of 0.1 M sucrose and 0.03 mM quinine-HCl, although preference percentages for the single sucrose solution and the mixture were somewhat low compared with those for 0.1 M sucrose (Student's *t*-test, $P > 0.05$). Preference percentages for 0.01 M sucrose and a mixture of 0.03 M sucrose and 0.03 mM quinine-HCl decreased by 13.2% (Student's *t*-test, $P < 0.01$) and 23.3% (Student's *t*-test, $P < 0.05$), respectively, from the control levels at 1–2 days after the start of the gymnema diet. Subsequently, preference for 0.01 M sucrose and a mixture of 0.03 M sucrose and 0.03 mM quinine-HCl returned closely to the control levels within a week. As shown in Figure 3, preference percentages for 0.03 M NaCl, 0.03 M MSG and 0.003 mM quinine were not affected by the gymnema diet (Student's *t*-test, $P > 0.05$). Animals showed aversion for quinine independently of diet regime.

Previous studies have revealed that salivary glands increase in weight and a new protein appears in their secretions in rats fed tannin- and papain-containing diet (Mehansho *et al.*, 1983; Ninomiya *et al.*, 1994). In the present study, however, there was no difference in the relative weight of the submandibular gland between animals fed the control and gymnema diets (Figure 4). No difference was also found in the flow rate of salivary secretion (not shown). Figure 5 shows inhibitory effects of rat submandibular saliva on immunoreaction between gurmarin and antigurmarin antiserum. Reduction of peroxidase activity shows inhibitory effects of saliva. When saliva collected at 4 and 14 days after start of the gymnema diet was added to the reaction mixture, activities of peroxidase binding to reaction products were low in the gymnema diet groups compared with the control diet group (*t*-test, $P < 0.05$), suggesting that the gymnema diet increases inhibitory effects of saliva on the immuno-

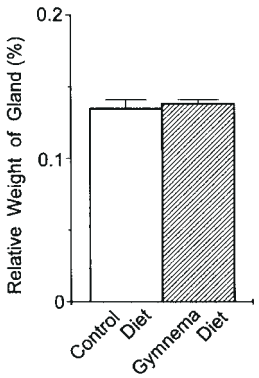

Figure 4 Relative weights of the submandibular glands of rats at the end of feeding trials. Open column: animals fed the control diet for 14 days; shaded column: animals fed the 3% gymnema diet for 14 days. Results were analyzed by Student's *t*-test. Each symbol represents the average with SE of eight animals.

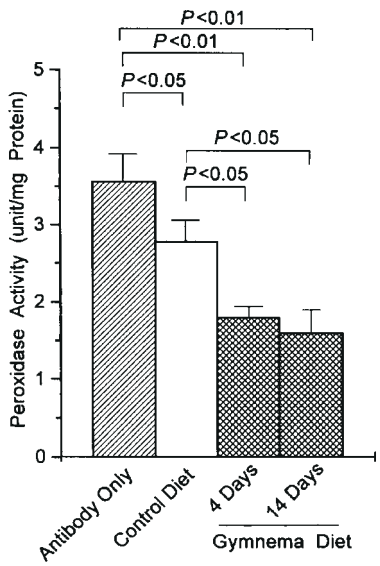

Figure 5 Inhibitory effects of rat submandibular saliva on immunoreaction between gurmarin and antigurmarin antiserum evaluated as levels of peroxidase activity in the ELISA method. Open column: peroxidase activity in a mixture of gurmarin (10 ng) and antigurmarin antiserum (15 μl); striped column: peroxidase activity in a mixture of gurmarin, antigurmarin antiserum and saliva (0.25–1 μl) of rats fed the control diet; shaded column: peroxidase activity in a mixture of gurmarin, antigurmarin antiserum and saliva of rats fed the 3% gymnema diet for 4 or 14 days. Results were analyzed using Student's *t*-test. Each symbol represents the average with SE of 8–12 animals.

reaction, but it does not increase the weight of the submandibular glands.

Gurmarin-binding protein (300 kDa), which suppresses the immunoreaction, is included in rat saliva (Imoto *et al.*, 1992). In order to confirm the presence of this salivary protein, saliva collected from rats fed the gymnema diet was eluted from a gurmarin-coupled Sepharose column and

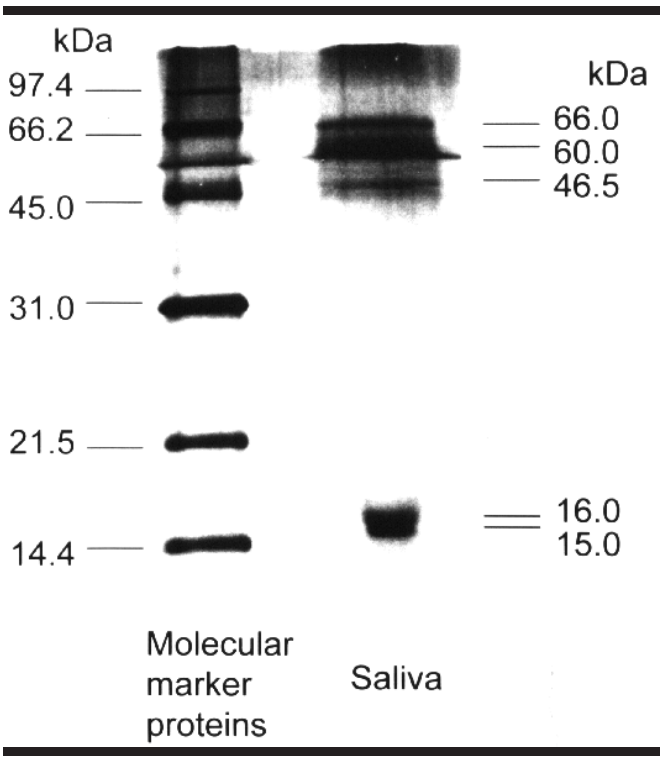

Figure 6 Electrophoretogram of the eluate from a gurmarin-coupled Sepharose column to which submandibular saliva of rats fed the experimental diet had been applied. The proteins (10 μg) were applied to 15% SDS-acrylamide gels prepared by the method of Laemmli (1970) and run at 20 mA for 60–90 min. Protein bands were visualized by silver staining procedure using a commercial kit (Sil-Best Stain, Nakarai, Japan). Molecular marker proteins used (Bio-Rad, USA) were rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and hen egg lysozyme (14.4 kDa).

subjected to electrophoretic analysis (Figure 6). Proteins tightly absorbed on the resin were effectively eluted from the affinity chromatography column with buffer with 2% hydroxypropyl β-cyclodextrin added, but not with the same buffer alone (data not shown). The eluate was separated by SDS electrophoresis into five protein bands and their mol. wts were estimated at 15, 16, 45, 60 and 66 kDa. The 300 kDa, gurmarin-binding protein was not found in the eluate. These findings suggest that there are at least five proteins with affinity for gurmarin (putative gurmarin-binding proteins) in saliva of rat fed gymnema diet and that they are different from the gurmarin-binding protein normally found in rat saliva.

Discussion

Preference percentages for a lower concentration of sucrose (0.01 M) transiently decreased immediately after the start of the gymnema diet, although those for higher concentrations of sucrose (0.03 and 0.1 M) and other taste solutions were not significantly affected by the gymnema diet (Figures 2 and 3). *Gymnema sylvestre* leaves contain a water-soluble

peptide (gurmarin) which selectively suppresses responses of the chorda tympani nerve to sweet taste stimuli in rats (Imoto *et al.*, 1991; Miyasaka and Imoto, 1995) and certain strains of mice (Ninomiya and Imoto, 1995; Ninomiya *et al.*, 1997, 1998). Even when the tongue is continuously rinsed with water, the suppressive effects of this peptide last for several hours. Gurmarin has been suggested to inhibit interaction between sweeteners and their receptor proteins on the taste-cell membrane, because sucrose response suppressed by gurmarin is recovered by rinsing the tongue with antigurmarin serum (Miyasaka and Imoto, 1995) and with β-cyclodextrin which could form inclusion complexes with gurmarin and reduce its effect (Ninomiya *et al.*, 1998). The decreases in preference for lower concentrations of sucrose in the present study, therefore, are probably due to the action of gurmarin on the taste-cell membrane and its inhibitory effects on sweetener responses. The fact that gurmarin did not affect preference for higher concentrations of sugar (0.03 and 0.1 M) may be due to the amount of gurmarin in the diet being barely enough to suppress neural responses to sucrose at lower concentrations. Gurmarin content in dry *G. sylvestre* leaves is about 10 p.p.m., which is estimated to be about 0.1 μM when the leaves are used in the 3% gymnema diet. On the other hand, in an electrophysiological study, gurmarin could suppress sucrose responses of the chorda tympani nerve at >0.5 μM (Imoto *et al.*, 1991).

In the present study, preference at 0.03 M sucrose was clearly decreased by the gymnema diet when quinine was added, but not when the single solution was used (Figure 2). Suppression of sucrose responses by gurmarin contained in the diet was probably not strong enough to override peripheral neural responses that induce preference behavior to 0.03 and 0.1 M sucrose. However, when quinine is added to the solution, the central nervous system would become susceptible to reduction in preference behavior, because the neural responses to quinine are not affected by gurmarin (Imoto *et al.*, 1991) and the quinine solutions induce avoidance behavior (Figure 3).

The present study showed that decreased preference for sucrose returned to a control level within a week or two after cessation of the gymnema diet (Figure 2) and that rat saliva after the diet has an inhibitory effect on immunoreaction between gurmarin and antigurmarin serum (Figure 5). The time course of restoration of the preference was comparable with that of increases in such an inhibitory effect of saliva, as shown in Figures 2 and 5. Inhibition of the immunoreaction by saliva was suggested to be attributed to the gurmarin-binding protein (>300 kDa), which is normally included in rat saliva at concentrations of >100 p.p.m. (Imoto *et al.*, 1992). Also in the present study, electrophoresis showed five proteins with affinity for gurmarin (gurmarin-binding proteins, GBPs) in saliva of rats fed the gymnema diet. Their mol. wts (15–66 kDa), however, were clearly different from that of the gurmarin-binding protein normally included in rat saliva, suggesting that the lower mol. wt proteins were newly induced by the gymnema diet. Probably, GBPs decrease concentrations of free gurmarin in the diet and suppress the activity of gurmarin, and, as a result, preference for sucrose returns to the control levels. Reasons for the absence of the 300 kDa gurmarin-binding protein will be investigated in a future study.

The parotid glands of rats maintained on tannin-containing diets show dramatic increases both in weight and in amounts of proline-rich proteins (Mehansho *et al.*, 1983). Because the overall responses to tannins closely resemble the effects of isoproterenol (a β-adrenergic agonist) treatment, release of catecholamines induced by dietary tannins has been suggested to trigger production of proline-rich proteins in the salivary glands. Similarly, enlargement of the submandibular gland and induction of cystatin S in rat saliva by dietary papain was mimicked by isoproterenol treatment (Ninomiya *et al.*, 1994). In the case of cystatin S, induction by dietary papain was suppressed by section of the glossopharyngeal nerve. Therefore, it has been suggested that chemosensory information for papain is conveyed to the sympathetic center for salivation via the glossopharyngeal nerve and then sympathetic impulses from there stimulate production of cystatin S in the submandibular gland. On the other hand, weights of the submandibular glands remained unchanged after the gymnema diet, although the PGBTs were induced. Therefore, the mechanism of induction of the PGBTs could be different from those of proline-rich proteins and cystatin S.

Tannins contained in a diet induce proline-rich proteins in saliva of the rat (Mehansho *et al.*, 1983) and mouse (Mehansho *et al.*, 1985), which bind to tannins and suppress a variety of antinutritional effects [e.g. inhibition of activity of alpha-amylase (Zhang and Kashket, 1989)]. Also, our previous study (Ninomiya *et al.*, 1994) suggested that cystatin S is induced in saliva of rats fed papain-containing diets. Cystatin S is known to suppress activities of papain (a cysteine protease) contained in the diet. Thus, certain salivary proteins may be induced in response to ingestion of aversive substances, such as tannin and papain, contained in food, and resultant suppression of detrimental effects of the substances at oral level would serve as a defense for the animals. Induction of the salivary GBPs may also represent a line of defense against unfavorable reduction in the ability to discriminate nutrients such as sucrose. Purification and further investigation of the salivary GBPs is in progress.

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