Anti-cariogenic Properties of a Water-soluble Extract from Cacao

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Received June 5, 2003; Accepted August 28, 2003

The addition of a water-soluble extract from cacao-extracted powder (CEPWS) to a cariogenic model food, a white chocolate-like diet that contains 35% sucrose, significantly reduced caries scores in SPF rats infected with Streptococcus sobrinus 6715, compared to control rats fed a white chocolate-like diet. CEPWS markedly inhibited water-insoluble glucan (WIG) synthesis through crude glucosyltransferases (GTFs) from Streptococcus sobrinus B13N in vitro. GTF-inhibitor(s) in CEPWS was prepared through three-step fractionation, and was termed CEPWS-BT, which is a high molecular weight (>10 kDa) heat-stable matrix of sugar, protein, and polyphenol. When the inhibitory effect of CEPWS-BT on glucan synthesis was examined using the purified GTF-I, GTF-T, and GTF-U enzymes from S. sobrinus B13N, significant reduction in GTF-I and GTF-T activity as a result of adding CEPWS-BT at low concentrations was observed. These results suggest that the addition of CEPWS to cariogenic food could be useful in controlling dental caries.

Key words: dental caries; glucosyltransferase; inhibitor; Streptococcus sobrinus; cacao

The mutants streptococci, Streptococcus mutans and Streptococcus sobrinus, have been implicated as primary causative agents of dental caries in humans.1,2 The abilities to colonize the tooth surface and to form cariogenic biofilms in the presence of dietary sucrose are important pathogenic properties of these organisms. These phenotypes are primarily dependent on the synthesis of adhesive water-insoluble glucans (WIG) from sucrose, which is mediated by extracellular glucosyltransferases (GTFs; EC 2.4.1.5).

To prevent dental caries, a number of attempts to find natural anti-GTF substances have been made, and these GTF-inhibitors have been isolated from various plant1-3 or microbial sources.4-5

Generally, confectionary such as chocolate, candy, and cake have been implicated as primary causative agents of dental caries in humans. The addition of anti-cariogenic substance(s) to cariogenic food is useful in preventing dental caries.

Cacao-extracted powder (CEP) is a commercially available ingredient, extracted from cocoa powder (alkalized defatted cacao mass) with hot water. The addition of a water-soluble extract from cacao-extracted powder (CEPWS) significantly reduced caries scores in SPF rats infected with S. sobrinus 6715 in this study.

The characteristics of GTF-inhibitors from cocoa have not been clarified although ’s-Gravenmade and Jenkins9 and Kashket and Paolino10 suggest the possibility that the anti-cariogenic factors in cacao might be polyphenol-like substances. CEPWS was prepared through three-step fractionation using GTF inhibitory activity as an indicator. The final fraction had a high molecular weight (>10 kDa) heat-stable matrix of sugar, protein, and polyphenol. These results are new findings that do not agree with the results of previous studies.6-9,10 This study describes the chemical characteristics and anti-GTFs properties of the CEPWS.

Materials and Methods

Bacteria. Streptococcus sobrinus strain 6715 (serotype g), which was made resistant to streptomycin (1 mg/ml) was primarily used in the in vivo experiment. S. sobrinus strain B13N(d) and S. mutans strain PS14(c) were used in the in vitro study.

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Abbreviations: WIG, water-insoluble glucan; GTF, glucosyltransferase; CEP, cacao-extracted powder; CEPWS, cacao-extracted powder water-soluble fraction; SPF, specific pathogen-free; CFU, colony-forming units; WC, white chocolate; CEPWS-WC, CEPWS added to white chocolate; CEPWS-L, cacao-extracted powder water-soluble low molecular fraction; CEPWS-H, cacao-extracted powder water-soluble high molecular fraction; CEPWS-HA, cacao-extracted powder water-soluble hydroxyapatite adsorbent fraction; CEPWS-BT, cacao-extracted powder water-soluble Butyl Toyopearl-extracted fraction; KPi, potassium phosphate buffer; p-APMSF, p-amidinophenylmethylsulfonyl fluoride hydrochloride; AAb, sodium acetate buffer; WSG, water-soluble glucan
Preparation of CEPWS. Cacao-extracted powder (CEP, Meiji Seika Kaisha, LTD., Tokyo, Japan) is a commercially available ingredient, extracted from cocoa powder (alkalized defatted cacao mass) with hot water. The CEP (1.5 kg) was suspended in 13.5 L of distilled water, stirred for 2 hours at room temperature, and then centrifuged. The supernatant was successively filtrated (5, 3, 1.2, 0.8, 0.3 μm, membrane filter), and the filtrate was lyophilized to produce 350 g of CEP water-soluble extract (CEPWS).

Experimental rat caries. Animal experiments were carried out based on the method of Ooshima et al. Specific pathogen-free (SPF) Sprague-Dawley rats (20 days of age; Japan Clea Laboratory, Tokyo, Japan) were treated with tetracycline (4 mg/g MF powdered diet, Oriental Yeast Co., Tokyo, Japan) and penicillin (4,000 U/ml drinking water) for 1 day to eliminate the microbial flora of the mouth. At 21 days of age, the rats were randomly separated into four experimental groups, each group containing 9 rats. All the rats of the three groups, except the non-infected control group, were infected daily for 6 consecutive days (21 to 26 days of age) with 0.1 ml of the cell suspension containing 50 μg streptomycin and about 4 × 10⁶ colony-forming units (CFU) of S. sobrinus 6715. All the rats were fed a cariogenic diet based on AIN-76. The rats in the non-infected control and infected control groups were fed a powder diet containing 35% sucrose, 24% soybean peptide (High nute PM, Fuji Oil, Osaka, Japan), 0.6% methionine, 24.9% cornstarch, 6% corn oil, 5% cellulose, 3.5% AIN-76 mineral mix (Oriental Yeast Co.), and 1% AIN-76 vitamin mix (Oriental Yeast Co.). The rats in the white chocolate (WC) group were fed the above diet without corn oil and with a reduced cornstarch ratio, and to which was added 24% cacao butter and 0.5% lecithin. The rats in the CEPWS added white chocolate (CEPWS-WC) group were fed the WC diet with a reduced cornstarch ratio and to which 0.5% CEPWS was added. Tap water was available ad libitum. The diet was administered based on the paired feeding method so that the consumption of sucrose was the same in all groups. At the end of the experiment (after 56 days), the rats were killed by CO₂ gas. The mandibles of the rats were removed and caries scores were measured using the method of Keyes. This study was approved by the Animal Committee of Meiji Seika Health & Bioscience Laboratories, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Meiji Seika Health & Bioscience Laboratories.

Preparation of GTF-inhibitor(s). CEPWS (18.5 g) was dissolved in 120 ml of distilled water and ultrafiltrated using an ultrafiltration system (Minitette, Filtron, Massachusetts, USA) and lyophilized. The high molecular weight substance (MW > 10 kDa) of CEPWS was termed CEPWS-H, and the residue was termed CEPWS-L (MW < 10 kDa). The CEPWS-H (3.7 g) was dissolved in 225 ml of 10 mM potassium phosphate buffer (K₆PB, pH 7.0) and applied to a column (15 × 210 mm) of Macro-prep ceramic hydroxyapatite type I 40 μm (Bio-Rad Laboratories, California, USA). The column was washed with 10 mM K₆PB (pH 7.0) and eluted with 0.4 M K₆PB (pH 7.0). The eluent in 0.4 M K₆PB was concentrated using an ultrafiltration system (Minitette, MW > 6000) and then lyophilized. This fraction was termed CEPWS-HA. CEPWS-HA (1.1 g) was dissolved in 55 ml of 10 mM K₆PB (pH 7.0) / 0.1 M (NH₄)₂SO₄, filtrated (0.4 μm) and then applied to a column (22 × 200 mm) of Butyl Toyopera 650M (Tosoh Corp., Tokyo, Japan). After being washed with 10 mM K₆PB (pH 7.0) / 0.5 M (NH₄)₂SO₄ for 36 min, the column was developed with a linear gradient from A to B (A: 10 mM K₆PB, pH 7.0 / 0.5 M (NH₄)₂SO₄; B: H₂O) for 15 min and then held at 100% B for a further 60 min, at a flow rate of 5.0 ml/min, monitored at 280 nm. The eluent from 50 min to 70 min was concentrated, desalted with an ultrafiltration system (Minitette, MW > 6000) and lyophilized. This final fraction was termed CEPWS-BT (0.36 g).

GTF preparation. Crude GTFs from S. sobrinus B13N and S. mutans PS14 were prepared from the culture fluids of both organisms as previously described. GTF-I (a primer-dependent 1,3-α-D-glucan synthase), GTF-U (a primer-dependent highly-branched 1,6-α-D-glucan synthase), and GTF-T (a primer-independent 1,6-α-D-glucan synthase) were isolated from the culture fluids of the B13N strain as previously described, except that 10 μM p-amidinophenylmethanesulfonyl fluoride hydrochloride (p-APMSF, Wako Pure Chemical Industries, Ltd., Tokyo, Japan) was added to the culture medium and all buffers for purification, and that each enzyme was further purified by re-chromatography with DEAE Bio-Gel A (Pharmacia Inc., New Jersey, USA) in the same manner. The homogeneity of the isolated GTF-I, GTF-U, and GTF-T preparations was confirmed by Western-blot analysis using monoclonal antibodies B17, B76 and B19, respectively, as previously described.

Enzyme assays. The WIG-forming activity of the crude GTFs was estimated by a colorimetric assay as previously reported, except that the reaction mixture (0.3 ml), containing 100 mM sodium acetate buffer pH 5.5 (AAB), 50 mM sucrose, and 5.2 μM enzyme was incubated at 37°C for 16 hours. The WIG- and water-soluble glucan (WSG)-forming activity of the purified GTFs was determined in the
same manner, except that the reaction mixture (0.3 ml), containing 100 mm AAb (pH 5.5), 50 mm sucrose, 33 mm dextran T10 (Pharmacia Inc., for the GTF-I and GTF-U assays) or distilled water (for the GTF-T assay), and about 5 mU enzyme was incubated at 37°C for 16 hours. The inhibitory effects of various compounds from CEPWS were estimated using the colorimetric assay with the crude B13N enzyme as described above, except that the compounds were pre-incubated at 37°C for 1 minute in the reaction mixture without sucrose. The IC50 value was defined as the concentration that gave a 50% inhibition of WIG synthesis under the assay.

Other analytical methods. Total carbohydrate was measured using the phenol-sulfuric acid method19 with D-glucose as the standard carbohydrate. Protein was measured using the method of Bradford20 using a protein assay kit (Bio-Rad Laboratories) with bovine serum albumin (Sigma Chemicals, St. Louis, MO, USA) as the standard protein. Polyphenol was measured using the prussian blue method21 with (-)-epicatechin (Sigma) as the standard polyphenol. The compositions of amino acids and carbohydrates were measured using the method of Hirayama et al.22 and Hosono et al.23 respectively. The effect of heat treatment on the anti-GTF activity of CEPWS-BT was tested after heating CEPWS-BT (10 mg/ml, 0.1 M KPB, pH 7.0) at 100°C for 20 min. The effects of enzymatic treatment on anti-GTF activity were tested after reacting CEPWS-BT (10 mg/ml) with 100 U/ml of hydrolytic enzyme at 37°C for 2 hours. The enzymes and reaction buffers were as follows: dextranase from Penicillium sp. (Sigma) in 0.1 M KPB, pH 6.0, pronase from Streptomyces griseus (protease type XIV, Sigma) in 0.1 M KPB, pH 7.5, and tannase from Aspergillus oryzae (Wako Pure Chemicals Co., Ltd.) in 0.1 M AAb, pH 5.5. The effect of polyphenol-adsorbent treatment on anti-GTF activity was tested as follows: 10 mg Polyclad 10 (polysulphonic pyrrolidone, Gokyo Trade Co., Tokyo, Japan) was added to 1 ml of CEPWS-BT solution (1 mg/ml water), mixed at room temperature, allowed to stand for 20 min, centrifuged, and the supernatant was used to measure the anti-GTF activity.

Statistical analysis. The caries scores were expressed as the mean ± standard error. Analysis was performed using SPSS for Windows Version 7.5.1 software (SPSS Japan Inc., Tokyo, Japan). When analysis of variance revealed a P-value < 0.05, the data were further analyzed using Tukey’s multiple range test. Differences were considered statistically significant at P < 0.05.

Results

Caries-reducing effect of CEPWS in SPF rats

In the experimental period (56 days), no significant difference in weight gain or food intake was found among any of the groups of rats. No notable symptoms of side effects were observed in the rats fed the CEPWS-added diet during the experiments. Figure 1 shows the total caries scores of the SPF rats infected with S. sobrinus 6715. The caries scores of the non-infected control group, the infected control group, the white chocolate (WC) group, and the CEPWS-added to WC (CEPWS-WC) group were 7.4 ± 1.2, 32.9 ± 1.8, 17.2 ± 1.9, and 10.8 ± 1.5, respectively. The caries scores of the CEPWS-WC group were 87% lower than those of the infected control group (P < 0.01). They were also 65% lower than those of the WC group (P < 0.05).

Inhibitory effects of cacao extract on WIG synthesis

When the inhibitory effects of CEPWS, CEPWS-L, and CEPWS-H against the crude GTF preparations from the culture fluids of S. sobrinus B13N and S. mutans PS14 were examined, CEPWS and its high molecular weight fraction (CEPWS-H) were found to significantly inhibit WIG synthesis by S. sobrinus enzymes. As shown in Fig. 2, both fraction CEPWS and CEPWS-H caused a 90% inhibition at low concentrations of 10 μg/ml and 3 μg/ml, respectively. In contrast, the inhibitory effect of CEPWS against S. mutans GTF was not as remarkable, with only a 19% inhibition at a concentration of 10 μg/ml.

Preparation of cacao GTF-inhibitor(s)

GTF-Inhibitor(s) in CEPWS-H was prepared by hydroxyapatite and Butyl Toyopearl column chromatographies. Table 1 shows the preparation profiles.
Chemical composition of CEPWS-BT

After the three-step preparation, the ratio of protein, polysaccharide, and polyphenol in the fractions increased 3.4-, 1.6-, and 2.5-fold, respectively (Table 1). The final fraction, CEPWS-BT, consisted of 40.1% protein, 28.4% carbohydrate, and 20.6% polyphenol. The main amino acid and sugar components were 6.05% glutamine (or glutamic acid) and 2.06% asparagine (or aspargic acid), and 4.7% D-glucose, respectively. When the effects of various enzymatic and chemical treatments on the anti-GTF activity of CEPWS-BT were examined, the anti-GTF activity was not reduced by heating, dextranase, pronase, or tannase treatments. It was, however, reduced by 75% as a result of treatment with Polyclar 10.

Table 1. Preparation and Chemical Compositions of Cacao GTF Inhibitors

<table>
<thead>
<tr>
<th></th>
<th>Dry Weight (g)</th>
<th>Total Protein (g)</th>
<th>Total Carbohydrate (g)</th>
<th>Total Polyphenol (g)</th>
<th>IC₅₀ (µg/ml)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEPWS</td>
<td>18.5</td>
<td>2.1</td>
<td>3.2</td>
<td>1.5</td>
<td>4.8</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>CEPWS-H</td>
<td>3.7</td>
<td>0.66</td>
<td>1.5</td>
<td>0.55</td>
<td>1.2</td>
<td>79</td>
<td>4</td>
</tr>
<tr>
<td>CEPWS-HA</td>
<td>1.1</td>
<td>0.25</td>
<td>0.23</td>
<td>0.14</td>
<td>0.4</td>
<td>72</td>
<td>12</td>
</tr>
<tr>
<td>CEPWS-BT</td>
<td>0.36</td>
<td>0.14</td>
<td>0.1</td>
<td>0.074</td>
<td>0.2</td>
<td>46</td>
<td>24</td>
</tr>
</tbody>
</table>
**Discussion**

The main findings of this study demonstrate that CEPWS can have an anti-caries effect by adding it to a cariogenic food model. In the *in vivo* experiment, CEPWS reduced the dental caries of rats infected with *S. sobrinus*, feeding on CEPWS added to a white chocolate-like diet containing sucrose. In the *in vitro* experiment, CEPWS, which includes a water-soluble, high molecular weight (>10 kDa) heat-stable matrix of sugar, protein, and polyphenol, inhibited GTF-I and GTF-T from *S. sobrinus*.

The effect of the cacao extract on caries development was also investigated through an *in vivo* experiment using *S. sobrinus*-infected rats. The caries-reducing effect of CEPWS was studied using a white chocolate-like diet containing 35% sucrose. Figure 1 illustrates the caries scores of the rats, showing a significantly lower cariogenic rate in the white chocolate-like diet with the CEPWS group than in the group without CEPWS (*P*<0.05). These results demonstrate the caries-reducing effect of CEPWS *in vivo*. The caries scores were significantly lower in the white chocolate-like diet group than in the control group (*P*<0.01).

The mutans streptococci, including *S. mutans* and *S. sobrinus*, are the principal cariogenic organisms among oral bacterial flora. They produce WIG from sucrose through GTF action, firmly adhering to the tooth surface, causing dental caries. On the other hand, it has been reported that *S. sobrinus* is more acidogenic and more cariogenic in animals than *S. mutans*. In addition, several epidemiological studies have shown that the presence of *S. sobrinus* is associated with high numbers of salivary mutans streptococci, with high caries prevalence. These results indicate the great significance of *S. sobrinus* as a cariogenic bacterium, and suggest that substances that inhibit *S. sobrinus* GTFs might be effective in suppressing dental caries. CEPWS markedly inhibited WIG synthesis by *S. sobrinus* GTFs, but not that by *S. mutans* GTFs, at a concentration of 10 μg/ml (Fig. 2).

GTF-inhibiting substance(s) in CEPWS were prepared using the inhibitory effect against crude GTFs from *S. sobrinus* as an indicator. The final preparation, CEPWS-BT, obtained after ultrafiltration and two steps of column chromatography, exhibited a potent inhibitory effect, reducing WIG synthesis by 50% at a low concentration of 0.2 μg/ml.

Componential and biochemical analyses revealed that CEPWS-BT is highly water-soluble despite being a high molecular weight polymer (>10 kDa). Furthermore, its water solubility and anti-GTF activity were retained during fractionation and after heat or enzymatic treatments, except for a treatment with Polyclar 10, which markedly reduced anti-GTF activity. These findings suggest that CEPWS-BT has a high molecular water-soluble element with polyphenol as its principal constituent. Polyphenol or polyphenolic polymers are indicated to be anti-caries ingredients of cocoa extract, green tea extract, and oolong tea extract. However, CEPWS-BT is likely to represent a new type of GTF-inhibiting substance, because it is a high molecular weight heat-stable matrix of sugar, protein, and polyphenol. Polyphenol forms water-insoluble complexes with protein, and the water solubility of these complexes is reported to be dependent upon the ratio of these two substances and on the condition of polymer formation. When p- (+)-catechin is enzymatically polymerized using horseradish peroxidase, an increase in polymerization leads to a marked increase in the inhibitory effect on *S. sobrinus* GTF. In the case of cacao beans, polyphenolic compounds such as epicatechin undergo oxidative polymerization by polyphenol oxidase from cacao and O₂ in the air, leading to the formation of a quinone product. It is assumed that it further polymerizes with protein and sugar during alkaline heat treatment to become a water-soluble polymer with GTF-inhibitory activity. Ooshima et al. found no significant caries-reducing effect of a hot water extract of cacao mass either *in vitro* or *in vivo*. On the other hand, the CEPWS used in this study was hot water extracted from cacao mass following alkaline treatment. Its strong caries-reducing effect is likely to result from polymerization of sugar, protein, and polyphenol during the alkaline treatment. Polymerization of polyphenol has been shown to be associated with the manifestation of a caries-inhibiting effect of oolong tea polyphenols and apple polyphenols, which is in agreement with the results of this study.

*S. sobrinus* strains produce, extracellularly, at least four kinds of GTFs consisting of one 1,3-α-d-glucan synthase, GTF-I, two 1,6-α-d-glucan synthases, GTF-U (referred to as GTF-S, or -Sd) and GTF-T
(referred to as GTF-S2 or -Si), and one oligo-isomaltosaccharide synthase, GTF-S (referred to as GTF-Si or -SiN), which are encoded by the gftI, gftU, gftT, and gftS genes, respectively.18,38 These enzymes form cariogenic dental plaque through adhesive WIG synthesis from sucrose, and it has been proposed that adhesive-WIG synthesis and subsequent sucrose-dependent colonization are mediated by the cooperative action of GTF-I and GTF-T.17,19,20 The roles of GTF-U and GTF-S in cariogenic dental plaque formation, however, are not yet clear.

This study examined the inhibitory effect of CEPWS-H and CEPWS-BT against three homogeneous preparations of the S. sobrinus GTFs, and it was found that the cacao GTF-inhibitor(s) strongly inhibits dextran-dependent WIG synthesis by the GTF-I and 1,6-α-d-glucan synthesis by the GTF-T, and slightly stimulated highly branched 1,6-α-d-glucan synthesis by the GTF-U (Fig. 3). The fact that the preparation from CEPWS effectively inhibited the activity of both the GTF-I and GTF-T, which are essential for adhesive WIG synthesis, is an important development as no other polyphenolic GTF-inhibitors with such specific properties have been identified. In fact, it has been reported that oolong tea polyphenols,45 ellagic acid,39 maillard reaction products,22 and mutainstein30 strongly inhibit only WIG synthesis by the GTF-I.

In conclusion, our findings indicate that the cacao GTF-inhibitor(s) prepared in this study is a novel anti-GTF substance(s) that differs from other polyphenolic GTF-inhibitors in structure, is a high molecular weight (>10 kDa) heat-stable matrix of sugar, protein, and polyphenol, and whose mechanism of action is an inhibitory effect not only on GTF-I but also on GTF-T. People have enjoyed cocoa for many centuries, and it is a very safe and natural ingredient that can be obtained in large quantities at low cost. The cocoa-derived extract used in this study can be highly beneficial as an anti-cariogenic ingredient.

Acknowledgments

This investigation was supported in part by a grant from the Ministry of Education, Culture, Sports, Science, and Technology to promote research between academia and industries.

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