INHIBITION BY COCOA EXTRACTS OF BIOSYNTHESIS OF EXTRACELLULAR POLYSACCHARIDE BY HUMAN ORAL BACTERIA

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Summary—Extracts of defatted cocoa inhibited the biosynthesis of extracellular polysaccharide by both cell-free and cell-associated streptococcal glucosyltransferases, but did not affect growth or acid production. Both water-insoluble and water-soluble polysaccharide syntheses were inhibited, and the net result was a shift away from adherence-supportive polysaccharide. Inhibition was non-competitive with respect to sucrose. The inhibition of polysaccharide biosynthesis was observed also with Actinomyces species. The findings suggest that the inhibitory effects of cocoa on plaque accumulation and caries formation are due to inhibition of bacterial polysaccharide production.

INTRODUCTION

Stralfors (1966a-c, 1967) showed that the incorporation of cocoa powder, defatted cocoa, or chocolate in hamster diets led to a suppression of caries development. A similar effect of chocolate on caries in adult people was suggested by Gustafsson et al. (1954); Grenby (1974) found that, young people on a chocolate-skim milk diet for five days had less dental plaque than those on a normal diet. Kinkel and Cremer (1960) attributed the anti-caries effect to the minerals in cocoa, but Wynn, Haldi and Law (1960) were unable to confirm this. Palenik et al. (1979) found that 1-4 per cent hot-water extracts of cocoa virtually eliminated plaque formation on glass slides by Streptococcus mutans.

We have examined the effects of water-soluble components of cocoa on growth, acidogenesis and biosynthesis of extracellular polysaccharide by selected plaque-forming microorganisms. Our findings were presented in preliminary form (Paolino et al., 1979; Paolino, 1982).

MATERIALS AND METHODS

Bacterial cultures

Strep. mutans strains GS-5, IB 1600 and 6715, Streptococcus sanguis H7PR3 and F90A, Lactobacillus fermenti ZPLG, Actinomyces naeslundii 12104 and Actinomyces viscosus M100, and Actinomyces naeslundii 12104 were obtained from the culture collection of Forsyth Dental Center. Cultures were grown from 0.2 per cent inoculum in brain heart infusion (BHI; Difco Laboratories, Detroit, Michigan) at 37°C, for 18-20 h, under a N2-H2-CO2 (80: 10: 10) atmosphere.

Enzyme preparation

Cell-free enzyme was prepared by centrifuging the cultures at 10,000 g for 20 min at 4°C. The supernatant media were removed and used in the assay system. The preparations were stored at −20°C. Enzyme from Strep mutans strain GS-5 was partially purified by precipitation with (NH4)2SO4 (60 per cent of saturation). The precipitate was dissolved and brought to the original volume of culture medium with 0.067 M phosphate buffer, pH 7.0, then dialysed against 200 vol of the same buffer to remove any remaining (NH4)2SO4. Cell-associated enzyme was prepared by washing and resuspending the cells to the original volume with the phosphate buffer.

Cocoa preparation

Cocoa powder (Hershey Foods, Hershey, Pennsylvania) was extracted twice with petroleum ether (100 ml/30 g) and the solids dried under infra-red heat lamps. The resulting defatted powder was suspended in distilled water (100 mg/ml) and refluxed at 100°C for 1 h. Solids were removed by centrifugation at 10,000 g for 10 min at 4°C. The resulting clear extract contained about 30 mg of dissolved solids/ml. The extracts were assayed for fluoride with a F-specific electrode (Orion Research, Cambridge, Massachusetts), calcium was determined with a flame emission spectrophotometer (Perkin-Elmer Corporation, Norwalk, Connecticut) and inorganic phosphate was measured according to the method of Penniall (1966). The extracts contained about 0.1 μg F, 0.5 μg calcium, and 1.5 μg inorganic phosphate/ml, none of which affected GTF activity when tested at the stated concentrations.

Studies of growth and acid production

Cultures were grown anaerobically in BHI, with or without cocoa extract, and the turbidity and culture pH were followed with time. For these experiments, the cocoa extract was concentrated 10-fold by lyophilization and reconstitution with 0.1 vol of H2O. The concentrate was added to growth medium to give 30 mg solids/ml. Culture pH was monitored with a standard-pH electrode, and lactic acid was determined by an enzyme-coupled assay employing lactate dehydrogenase (Sigma Chemical Company, St Louis, Missouri). Turbidity was determined at 625 nm using a Spectronic 20 spectrophotometer (Bausch and Lomb, Incorporated, Rochester, New York). For these measurements, the cells were harvested by centrifugation at 10,000 g for 20 min at 4°C, washed, and resuspended with 0.067 M phosphate buffer, pH 7.0, to remove coloured material from the cocoa extract and diluted 1:4 in buffer.
Assay of extracellular polysaccharide (EPS) biosynthesis

EPS synthesizing activity was determined by following the incorporation of label from $[14C]$-sucrose into polysaccharide according to the method of Montville, Cooney and Sinskey (1977). The assay system contained 10 µl of 1 per cent dextran T-10 (Pharmacia Fine Chemicals, Piscataway, New Jersey), 10 µl of 0.35 M NaF, 50 µl of 1 M sodium acetate buffer, pH 5.5, 10 µl of 0.5 M sucrose and 30 µl (1 µCi) $[14C]$-sucrose (400-700 Ci/mol; New England Nuclear, Boston, Massachusetts), up to 500 µl of either whole culture, washed cells, or cell-free culture medium and, when indicated, up to 500 µl of cocoa extract, plus sufficient H$_2$O to bring the total volume to 1.0 ml. Incubation was at 37°C. At 0 and 100 min, 6 replicate samples of 50 µl each were withdrawn and adsorbed on Whatman 3MM discs (2.5 cm). Total EPS was determined by batch-washing three discs three times in absolute methanol (10 ml solvent/disc). Water-insoluble EPS was determined by washing the three remaining discs once with methanol, twice with water and again with methanol. Water-soluble activity was taken as the total minus the water-insoluble activity. The discs were dried under 250 W infra-red lamps and placed in a liquid scintillation cocktail [4 g, 2,5-diphenyl oxazole (PPO) and 0.5 g dimethyl-POPOP in 1 litre toluene] for the determination of radioactivity. Reaction rates were constant for at least 100 min; the coefficient of variation for the assay was 4.5 per cent.

RESULTS

Cocoa powder

Preliminary experiments showed that the addition of a suspension of defatted cocoa powder to growing cultures of Strep. mutans GS-5 led to the immediate inhibition of polysaccharide biosynthesis (Fig. 1). The degree of inhibition was proportional to the concentration of added powder and, at 10 per cent (w/v), the inhibition of GTF activity was complete.

![Fig. 1. Effect of the addition of cocoa powder on the synthesis of polysaccharide by a growing culture of Strep. mutans GS-5. Samples of the culture were incubated and cocoa powder was added at 30 min. Control (○); plus cocoa powder at the indicated concentrations (w/v) (●).](image)

Water extraction of cocoa powder

The GTF inhibitory factor was water-soluble and was extractable at room temperature. However, better extraction was obtained following refluxing at 100°C for 40-60 min. Some inhibitory activity remained in the cocoa particles under these conditions but, with repeated extractions, this could be reduced to zero. Removal of lipid from cocoa powder with petroleum ether, prior to the water extraction, did not affect the subsequent extraction of the GTF inhibitors.

Cocoa extract did not affect growth or acid-production rates of some oral microorganisms. Figure 2 demonstrates the lack of effect on growth and lactate production by Strep. mutans GS-5. Measurements of culture pH during growth of this strain, Strep. sanguis strains H7PR3 and F90A, and l. fermenti 2PLG, revealed no significant effect of addition of the extract.

Table 1. Inhibition of GTF activity from Strep. mutans GS-5

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Cocoa extract</th>
<th>Total</th>
<th>Water-insoluble</th>
<th>Water-soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole culture</td>
<td>12,039</td>
<td>2544</td>
<td>9495</td>
<td></td>
</tr>
<tr>
<td>7489 (37.8)</td>
<td>392 (84.6)</td>
<td></td>
<td></td>
<td>7097 (25.3)</td>
</tr>
<tr>
<td>Cell-free culture</td>
<td>11,791</td>
<td>2055</td>
<td>9735</td>
<td></td>
</tr>
<tr>
<td>medium</td>
<td>7246 (38.5)</td>
<td>289 (86.1)</td>
<td>6957 (28.5)</td>
<td></td>
</tr>
<tr>
<td>Washed cells</td>
<td>1983</td>
<td>476</td>
<td>1107</td>
<td></td>
</tr>
<tr>
<td>medium</td>
<td>794 (49.9)</td>
<td>63 (86.8)</td>
<td>731 (34.0)</td>
<td></td>
</tr>
<tr>
<td>Partially purified</td>
<td>2956</td>
<td>731</td>
<td>2225</td>
<td></td>
</tr>
<tr>
<td>medium</td>
<td>836 (71.7)</td>
<td>141 (80.7)</td>
<td>695 (68.8)</td>
<td></td>
</tr>
</tbody>
</table>

*Concentration of cocoa extract was 7.5 mg/ml.
†Numbers in brackets indicate percentage inhibition.
$Culture medium was fractionated with ammonium sulphate and the enzyme was assayed with 15 mg cocoa extract/ml.
Cocoa inhibits EPS synthesis

Cocoa extract inhibited the formation of both water-insoluble and water-soluble polysaccharides produced by either cell-free or cell-associated enzymes (Table 1). However, the inhibition of water-insoluble EPS was greater than for water-soluble polysaccharide. The inhibitory effect on partially-purified GTF was similar to that on the cell-free culture medium.

The inhibition of GTF increased with increasing concentrations of extract (Fig. 3). The curve indicated saturation kinetics and a double-reciprocal plot of enzyme activity versus sucrose concentration was linear (Fig. 4). The intersecting straight lines obtained in the presence of two concentrations of cocoa extract indicated a non-competitive type of inhibition.

Effects of different microbial strains

The inhibitory effect of cocoa extract was observed with two additional strains of Strep. mutans and two strains of Strep. sanguis (Table 2). Polysaccharide

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cocoa extract</th>
<th>Water-insoluble (counts/min per ml per h)</th>
<th>Water-soluble</th>
</tr>
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<tbody>
<tr>
<td><em>Strep. mutans</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS-5</td>
<td>†</td>
<td>2335</td>
<td>7882</td>
</tr>
<tr>
<td>IB 1600</td>
<td>†</td>
<td>5714</td>
<td>22,529</td>
</tr>
<tr>
<td>6715</td>
<td>†</td>
<td>8703</td>
<td>11,990</td>
</tr>
<tr>
<td><em>Strep. sanguis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H7PR3</td>
<td>†</td>
<td>1794</td>
<td>5802</td>
</tr>
<tr>
<td>F90A</td>
<td>†</td>
<td>9084</td>
<td>43,665</td>
</tr>
<tr>
<td><em>A. viscosus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M100</td>
<td>†</td>
<td>3323</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1386 (58.3)</td>
<td>372</td>
</tr>
<tr>
<td><em>A. naeslundii</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12104</td>
<td>†</td>
<td>1324</td>
<td>664</td>
</tr>
</tbody>
</table>

*Concentration of cocoa extract was 15 mg/ml.
†Numbers in brackets indicate percentage inhibition.
†GTF activities were measured in cell-free culture media except for the *Actinomyces* species, where samples of whole culture were used.
inhibitors of GTF in tea, coffee and a commercial preparation of tannic acid (Sigma Chemical Co.). We have found that organic solvents (e.g. methanol) are effective inhibitors of streptococcal GTF; serum and salivary phospholipids have been reported to stimulate the enzyme (Schachtele and Harlander, 1983; Ciardi et al., 1978). The cocoa inhibitor is another modulator of GTF activity; however, it is the only one that is a constituent of a common food. Furthermore, the concentrations used in our experiments are similar to those expected to be attained in the mouth after the ingestion of cocoa in the form of chocolate drinks or candy. At these concentrations, the extract brings about a 60–70 per cent inhibition of GTF and so is likely to have a significant effect on EPS biosynthesis in vivo. Commonly-encountered cocoa-containing foods, however, possess high levels of sucrose. The non-competitive inhibition by the cocoa components would favour the reduction of EPS biosynthesis in spite of the high concentrations of GTF substrate.

The mechanisms of inhibition of GTF by various inhibitors are complex and not fully understood. Several glucosyltransferase are elaborated by oral streptococci (reviewed by Ciardi, 1983) and Montville, Cooney and Sinskey, 1979; Fukui et al., 1982) and the nature of the polysaccharide products can change following the addition of dextran (Germains et al., 1977; Montville et al., 1977) or specific antibody to GTF (Taubman et al., 1982; Linzer and Slade, 1976). We found that the biosyntheses of both soluble and insoluble GTF were inhibited by cocoa extract. However, the greater sensitivity of insoluble GTF to inhibition by the extract led to a shift to a predominantly soluble type of product. Thus, calculations from the data in Table 2 showed that the EPS formed by the uninhibited GTF from Strep. mutans GS-5 consisted of 77 per cent soluble and 23 per cent insoluble polysaccharide; the inhibited enzyme yielded polysaccharides that were only 7 per cent in the insoluble form. Strain IB 1600 gave similar results. The shift in product type with strain 6715 was not as pronounced as for the other two strains.

The overall inhibition of EPS biosynthesis and the shift from insoluble to soluble products may explain the reported effects of cocoa on plaque formation and accumulation. Furthermore, Bozzola, Johnson and Schechmeister (1980). Johnson et al. (1977) and Tanzler et al. (1974) showed that virulence of Strep. mutans is directly related to the ability to form insoluble EPS; strains of Strep. mutans which produced low levels of insoluble dextran could not adhere to hard surfaces and were unable to produce smooth-surface caries in laboratory animals (reviewed by Hamada and Slade, 1980). Thus the inhibitory effects of cocoa extract may be highly important in determining cariogenicity of diets containing cocoa. Similarly, the anti-caries effect of teas (Rosca et al., 1984) may be related to the inhibition of GTF. We suggest that a wide variety of foods contain GTF-inhibitory constituents and that these may influence not only the cariogenicity of the foods in which they are found, but may also modulate the effects of other, highly-cariogenic foods in the diet.

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Cocoa inhibits EPS synthesis

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