Effect of a Novel Type of Propolis and Its Chemical Fractions on Glucosyltransferases and on Growth and Adherence of Mutans Streptococci

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Received August 20, 2002; accepted November 21, 2002

Flavonoids have been considered the main biologically active components in propolis. However, a new variety of flavonoid-free propolis was recently found and chemically classified as type 6. Because it showed activity against oral microorganisms, this study evaluated the effects of the crude ethanolic extract of this propolis and its chemical fractions on the activity of purified glucosyltransferases (GTFs) and on the growth and adherence of mutans streptococci. The inhibitory effect of propolis extracts on GTF activities was determined either in solution or adsorbed onto saliva-coated hydroxyapatite. *Streptococcus mutans* Ingbritt 1600, *Streptococcus sobrinus* 6715, and two clinical isolates of each species were used for antibacterial assays. Susceptibilities to the test extracts were analyzed using the agar diffusion method and by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC); the effect on bacterial adherence to a glass surface was also assessed. The activity of GTFs in solution was effectively inhibited by the ethanolic extract of propolis type 6 (EEP) (>80% inhibition at 0.5 mg/ml), hexane, and chloroform fractions (60—90% inhibition at 100 μg/ml); their inhibitory effects on surface enzymes were less pronounced. The EEP, hexane, and chloroform fractions also showed significant antibacterial activity. The data showed that propolis type 6 remarkably reduced GTF activity and inhibited mutans streptococci growth and adherence; these biological activities are associated with its nonpolar components.

Key words propolis; glucosyltransferase; antimicrobial activity; mutans streptococci; adherence

Propolis is a nontoxic natural substance collected by *Apis mellifera* bees from various plant sources and has been used in folk medicine for centuries.[1)] It is known that propolis exhibits several biological activities, such as antimicrobial, antinflammatory, anesthetic, cytostatic, and cariostatic properties.[1—3)] The chemical composition of propolis is complex; flavonoids and (hydroxyl) cinnamic acid derivatives have been considered the primary biologically active compounds.[1)]

Previous *in vitro* studies have shown that propolis inhibits the growth of mutans streptococci *in vitro*.[2,4)] These microorganisms, especially *Streptococcus mutans*, have been implicated as primary etiological agents of dental caries in animal and humans.[5—7)] Furthermore, propolis in drinking water[2)] or applied topically reduced the incidence of dental caries in rats.[3)] However, the chemical composition of propolis is highly variable and depends on its geographic origin.[8—10)]

Twelve chemically distinct types of Brazilian propolis have been characterized to date.[11)] Two (one from southeastern Brazil, type 12; and the other from southern Brazil, type 3), flavonoid- and cinnamic acid-rich types, showed remarkable inhibitory effects on the activity of several purified glucosyltrasnferases (GTFs) *in vitro*.[12)] GTFs are of central importance in adhesive interactions with *S. mutans* and are essential in the expression of virulence by these microorganisms.[13—16)] The glucans synthesized by GTFs not only promote the accumulation of cariogenic streptococci on the tooth surface, but also contribute significantly to the bulk of dental plaque.[17,18)] *S. mutans* produces at least three GTFs: GTF B, which synthesizes mostly insoluble α1,3-linked glucan; GTF C, which synthesizes a mixture of insoluble and soluble α1,6-linked glucan; and GTF D, which synthesizes soluble glucan. Among them, GTFs B and C appear to be the most important GTFs related to dental caries.[16)] Clearly, GTF should be a primary focus of any strategy based on the prevention of either dental caries or the formation of dental plaque. In addition, most of the currently commercially available oral hygiene products are not effective inhibitors of GTF enzymes.[19,20)]

Recently, a novel type of propolis was reported, classified as type 6 (from northeastern Brazil, Atlantic forest, Bahia state), in which neither flavonoids nor cinnamic acid derivatives were detected.[21)] However, the ethanolic extract of propolis type 6 (EEP) showed remarkable antimicrobial activities against several oral pathogens, including mutans streptococci. Its inhibitory effects were more pronounced than those of any other Brazilian propolis tested in our laboratory.[22)] Because the effects of this novel propolis and its chemical fractions on the activity of GTFs as well as on the growth and adherence of clinical isolates of mutans streptococci were unknown, we conducted this study.

MATERIALS AND METHODS

Propolis Samples and Fractionation Crude samples of *A. mellifera* propolis were obtained from the Atlantic forest region of Bahia state, northeastern Brazil, and were classified as type 6.[11)] The EEP at 20% (w/v) in aqueous ethanol (80% v/v) was prepared as detailed elsewhere.[10)] The EEP was subjected to chemical fractionation, based on a polarity gradient, and the hexane, chloroform, ethyl acetate, and ethanol fractions were obtained. Each fraction was monitored by paper chromatography and developed under UV light (λ=254, 366

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nm).[23] The ethanolic extracts of these fractions (10% w/v) in ethanol (80% v/v) were used.

Bacterial Strains The bacterial strains used for the production of GTFs were: 1) *Streptococcus milleri* KSB8, which harbors the *gtfB* gene from *S. mutans* GS-5 (for GTF B production); and 2) *S. mutans* WHB 410 (54), in which the genes for GTF B and D and fructosyltransferase were deleted (for GTF C). For susceptibility and adherence assays, the following bacterial strains were used: 1) *S. mutans* Ingbritt 1600; 2) *S. mutans* 1 (clinical isolate); 3) *S. mutans* 2 (clinical isolate); 4) *S. sobrinus* 6715; 5) *S. sobrinus* 1 (clinical isolate); and 6) *S. sobrinus* 2 (clinical isolate). The cultures were stored at −80 °C in brain-heart infusion (BHI) or tryptic soy broth (TSB) containing 20% glycerol. The *S. milleri* constructs were a kind gift from Dr. Howard K. Kuramitsu (SUNY, Buffalo, NY, U.S.A.).

Assays of Activity of GTFs in Solution and Adsorbed onto Hydroxyapatite Beads The GTF B and C enzymes were obtained from culture supernatants and purified to near homogeneity by hydroxyapatite column chromatography as described by Venkitaraman *et al.*[24] and Wunder and Bowen.[25] GTF activity was measured by the incorporation of [^{14}C]glucose from labeled sucrose (NEN Research Products, Boston, MA, U.S.A.) into glucans.[12,24] The GTF enzyme added to each sample for all assays was equivalent to the amount required to incorporate 1 to 1.5 μmol of glucose over the 40-h reaction.

For solution assays, GTF B or C was mixed with a two-fold dilution series of the EEP (concentrations ranging from 0.078 to 5.0 mg/ml) or the fractions (concentration ranging from 25.0 to 400.0 μg/ml) and incubated with ^{14}C-(glucosyl)-sucrose substrate (0.2 μCi/ml; 200.0 mmol/l sucrose, 40 μmol/l dextran 9000, 0.02% sodium azide in adsorption buffer, pH 6.5) to a final concentration of 100 mmol/l sucrose (200 μl final volume). For the control, the same reaction was performed with ethanol (final concentration of 8%, v/v) replacing the test extracts. The samples were incubated at 37 °C with rocking for 4 h. After incubation, ice-cold ethanol (1.0 ml) was added and the samples were stored for 18 h at 4 °C for precipitation of glucans. Radiolabeled glucan was determined by scintillation counting.[12,24]

For surface assays, the GTFs were adsorbed onto hydroxyapatite beads (Macro-Prep Ceramic Hydroxyapatite Type I, 80 μm, Bio-Rad) coated with clarified whole saliva (sHA) (free of GTF activity) as described by Schilling and Bowen[15] and Venkitaraman *et al.*[24] Following adsorption of the enzyme, the beads were washed three times with absorption buffer to remove the loosely bound material and exposed to 300 μl of the two-fold dilution series of the test extracts (or control) for 30 min at the concentrations described above. The beads were washed and exposed to 300 μl ^{14}C-(glucosyl)-sucrose substrate (100.0 mmol/l sucrose, final concentration). The radiolabeled glucan formed was collected and quantified by scintillation counting.[12,24] All of the solution and surface assays were performed in triplicate in at least three different experiments.

Susceptibility Testing The antimicrobial activity was determined using the agar diffusion method and by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in accordance with the NCCLS guidelines and Koo *et al.*[22] For the agar diffusion method, the inoculum procedures were appropriate to provide a semiconfluent growth of the microorganisms tested (1—2×10^8 colony-forming units (CFUs)/ml) onto a brain heart infusion agar plate. Six sterilized steel cylinders of 8.0×10.0 mm (inside diameter 6.0 mm) were placed on the inoculated agar plates. The test extracts (400 μg/ml) or control (80% ethanol, v/v) (40 μl) was applied inside the cylinders. The plates were incubated at 37 °C for 24 h in a 10% CO_2 incubator. The zones of inhibition of microbial growth around the cylinder containing the extracts were measured. The inhibitory zone was considered the shortest distance (mm) from the outside margin of the initial point of microbial growth.[26] Six replicates were made for each microorganism. Data were initially evaluated using the F-test followed by the Tukey test ($p<0.05$).[26] For MIC determination, the starting inoculum was 5×10^5 CFU/ml, and the concentration of test extracts ranged from 25 to 1600 μg/ml (for EEP) and 6.25 to 800 μg/ml (for propolis fractions). To determine the MBC, an aliquot (50 μl) of all incubated tubes with concentrations higher than the MIC was subcultured on BHI agar supplemented with 5% defibrinated sheep blood with a spiral plater (Whittley Automatic Spiral Plater). The MBC was defined as the lowest concentration that allowed no visible growth on the agar.[22] Six replicates were made for each concentration of the extracts.

Inhibition of Adherence of Growing Cells to a Glass Surface To assess the bacterial adherence to a glass surface, organisms were grown at 37 °C 10% CO_2 with an angle of 30° for 18 h in test tubes as detailed in Koo *et al.*[26] and Hamada and Torii.[27] The microorganisms (the same as described above) were grown in BHI broth plus 1% sucrose (w/v) containing sub-MIC concentrations of the test extracts or control (80% ethanol, v/v). The sub-MIC concentrations were relevant to the present study because those concentrations demonstrate bacterial growth. After incubation, the adhering cells were washed and resuspended in an ultrasonic bath (Vibracell, Sonics & Material Inc.). The amount of adherent cells was measured spectrophotometrically at 550 nm.[25] The concentration for total bacterial adherence inhibition (TBAI) was defined as the lowest concentration that allowed no visible cell adherence on the glass surface ($p<0.05$). Six replicates were made for each concentration of the test extracts.

RESULTS

The effects of the propolis extracts on the activity of GTF B and C are shown in Figs. 1, 2, and 3. The EEP effectively reduced the activity of all enzymes tested in solution (85 to 95% inhibition) and on a glass surface (65 to 70% inhibition) at the concentration of 0.5 mg/ml. Among the propolis fractions, the hexane and chloroform fractions displayed the most potent inhibition of GTF activity (Figs. 2, 3). They inhibited 50 to 85% of the activity of GTF B and C in solution at a concentration as low as 100 μg/ml. The inhibitory effect of these fractions on surface-adsorbed enzymes was not as potent as that observed when the same enzymes were in solution. Nevertheless, the fractions were effective inhibitors (40 to 50% inhibition at a concentration of 100 μg/ml).

The mean bacterial growth inhibition zones induced by propolis extracts are shown in Table 1. Among the test ex-

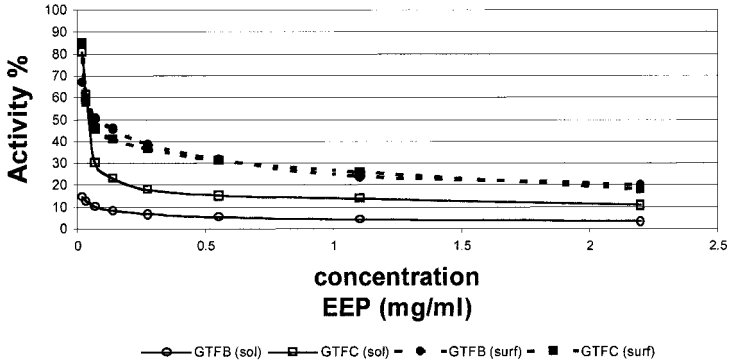

Fig. 1. Effect of Ethanolic Extract of Propolis (EEP) Type 6 on the Activities of Glucosyltransferases (GTF B and C) in Solution (sol) and Adsorbed onto sHA Surface (surf)

For the control, ethanol (8.0%, final concentration) was added instead of EEP. The percentage of GTF activity was calculated considering the control as maximum enzymatic activity.

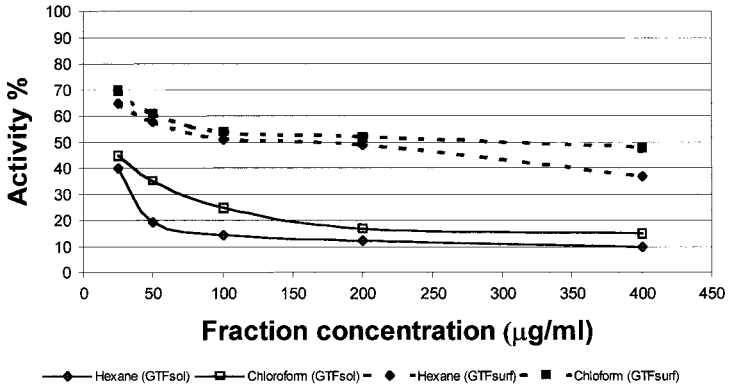

Fig. 2. Effect of Propolis Type 6 Fractions on the Activity of GTFB in Solution (sol) and Adsorbed onto sHA Surface (surf)

For the control, ethanol (8.0%, final concentration) was added instead of EEP. The percentage of GTF activity was calculated considering the control as maximum enzymatic activity.

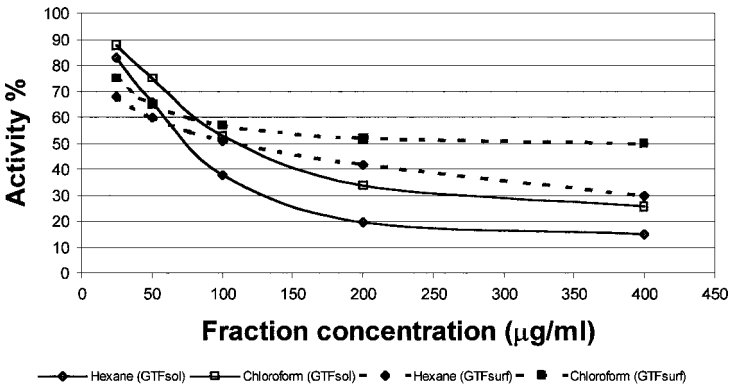

Fig. 3. Effect of Propolis Type 6 Fractions on the Activity of GTFC in Solution (sol) and Adsorbed onto sHA Surface (surf)

For the control, ethanol (8.0%, final concentration) was added instead of EEP. The percentage of GTF activity was calculated considering the control as maximum enzymatic activity.

Table 1. Means (±S.D.; $n=6$) of Growth Inhibition Zone (mm) by Ethanolic Extract of Propolis Type 6 and Its Fractions against *S. mutans* and *S. sobrinus*

Treatment	*S. mutans* Ingbritt 1600	*S. mutans* 1	*S. mutans* 2	*S. sobrinus* 6715	*S. sobrinus* 1	*S. sobrinus* 2
Crude propolis (EEP)	$6.30\pm0.44^{a\,A}$	$5.70\pm0.32^{a\,AB}$	$5.51\pm0.51^{ab\,B}$	$5.04\pm0.55^{a\,A}$	$4.79\pm0.69^{a\,B}$	$4.31\pm0.69^{a\,C}$
Hexane fraction	$6.72\pm0.74^{b\,A}$	$6.31\pm0.38^{b\,AB}$	$5.99\pm0.56^{a\,B}$	$5.99\pm0.57^{b\,A}$	$5.33\pm1.00^{b\,B}$	$5.42\pm0.50^{b\,C}$
Chloroform fraction	$5.78\pm0.78^{a\,A}$	$5.04\pm0.50^{a\,AB}$	$5.39\pm0.57^{b\,B}$	$4.84\pm0.44^{a\,A}$	$4.76\pm0.72^{a\,A}$	$4.29\pm0.36^{a\,B}$
Ethyl acetate fraction	$0.0^{c\,A}$	$0.0^{c\,A}$	$0.0^{c\,A}$	$0.0^{c\,A}$	$0.0^{c\,A}$	$0.0^{c\,A}$
Ethanol fraction	$0.0^{c\,A}$	$0.0^{c\,A}$	$0.0^{c\,A}$	$0.0^{c\,A}$	$0.0^{c\,A}$	$0.0^{c\,A}$
80% Ethanol (negative control)	$0.0^{c\,A}$	$0.0^{c\,A}$	$0.0^{c\,A}$	$0.0^{c\,A}$	$0.0^{c\,A}$	$0.0^{c\,A}$

Lower case letters (vertical comparison) indicate that mean values for treatment were statistically different. Upper case letters (horizontal comparison among *S. mutans* or *S. sobrinus* alone) indicate that mean values for microorganisms were statistically different ($p<0.05$).

Table 2. Values of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Total Bacterial Adherence Inhibition (TBAI) of Ethanolic Extracts of Propolis Type 6 and Its Fractions against Mutans Streptococci

Microorganism	Crude propolis (EEP)			Hexane fraction			Chloroform fraction		
	MIC	MBC	TBAI	MIC	MBC	TBAI	MIC	MBC	TBAI
S. mutans Ingbritt 1600	100	>1600	50	50	800	>25[a)]	100	>800	>50[a)]
S. mutans 1	100	1600	50	50	800	25	100	>800	50
S. mutans 2	100	>1600	50	50	800	25	100	>800	50
S. sobrinus 6715	50	800	25	25	200	>12.5[a)]	50	>800	>25[a)]
S. sobrinus 1	100	800	25	50	400	25	100	>800	50
S. sobrinus 2	100	800	50	50	400	25	100	>800	50

The values are expressed in μg/ml. *a*) The concentration of TBAI is higher than the MIC. Chloroform and ethyl acetate fractions showed no MIC of inhibition of cell adherence.

tracts, only the EEP and the hexane and chloroform fractions displayed antibacterial activity, producing inhibitory zones against all the microorganisms tested ($p<0.05$). The hexane fraction showed significantly higher inhibitory activity against the growth of mutans streptococci strains (inhibitory zones ranging from 5.33 to 6.72) than other extracts ($p<0.05$). In general, clinical isolates had significantly lower inhibitory zones values than the laboratory strains ($p<0.05$). The control (80% aqueous ethanol, v/v) did not form an inhibitory zone with any of the strains tested, although it showed inhibition when in direct contact with them.

The MIC and MBC values of the propolis extracts shown in Table 2 indicate that the extracts showed the following order of potency for *S. mutans* and *S. sobrinus* strain inhibition: Hexane fraction>EEP>chloroform fraction. The MBC of the EEP and hexane fraction was 4 to 8 times higher than the MIC values; the chloroform fraction did not display any bactericidal effect at the concentrations tested in this study. *S. sobrinus* strains were more susceptible to the propolis fractions than *S. mutans*, and the clinical isolates were more resistant than the respective laboratory strains, especially of *S. sobrinus*. Furthermore, the EEP and the hexane and chloroform fractions inhibited the adherence of growing *S. mutans* and *S. sobrinus* cells to a glass surface at sub-MIC levels (Table 2). The hexane fraction again showed better results than the EEP and chloroform fraction by inhibiting bacterial adherence at concentrations as low as 12.5 μg/ml for most microorganisms tested.

The ethyl acetate and ethanol fractions did not display any inhibitory activity on the *in vitro* parameters tested in this study.

DISCUSSION

The development of therapeutic agents aimed at disrupting both colonization of the teeth by dental pathogens and the subsequent formation of dental plaque is one of the prime strategies to reduce the incidence of tooth decay.[20)] Among the different targets, GTF enzymes have been shown to be one of the most important virulence factors since they are involved in both colonization of oral bacteria and formation of the plaque matrix. These enzymes are present in the soluble fraction of saliva and are also found in the salivary pellicle formed on the dental surface. The surface-adsorbed GTFs synthesize glucans *in situ*, providing binding sites for cariogenic streptococci and contributing to the formation of plaque matrix.[15,20)] Thus it is desirable to determine the effects of potential inhibitors of GTFs, but mainly the surface-adsorbed enzymes.

The data obtained in the present study showed that propolis type 6 effectively reduced the activities of GTFs B and C, irrespective of whether the enzymes were exposed before or after adsorption to the sHA surface. This level of inhibition has not been observed for any other synthetic or natural agent, including previously tested propolis (types 3 and 12). In addition, the inhibition of surface-adsorbed enzymes is particularly important because most of the currently commercially available compounds fail to affect surface GTF B and C significantly.[19,25)] These GTFs appear to be the most important enzymes related to dental caries, consistent with a reduction in smooth-surface caries observed with mutants of *S. mutans* defective in the production of either or both GTFs.[16)] The possible biological active compound(s) of propolis type 6 that modulates GTF inhibition is(are) unknown. The subsequent fractionation of the EEP and further biological assays suggested that the active compounds are present in the nonpolar chloroform and especially hexane fractions; none of the more polar fractions (ethanol and ethyl acetate) showed inhibitory activities. It has been suggested that flavonoids and other related compounds are the active compounds of propolis involved in enzyme inhibition[1)] and some of them, *e.g.*, flavonols and flavones, inhibited the activity of GTFs.[28)] Propolis type 6 clearly contains different chemical groups responsible for the inhibitory activities against GTF enzymes.

The antimicrobial assays demonstrated that propolis type 6 has significant activity against mutans streptococci growth, showing MIC values between 25 to 100 μg/ml and MBC values between 400 and 1600 μg/ml. The clinical isolates were somewhat more resistant to propolis extracts than the laboratory strains. This observation is important because the laboratory strains, which have been commonly used to determine susceptibility to antimicrobials, may not express the same virulence or resistance level compared with strains recently isolated from the oral cavity. Nevertheless, propolis type 6 was efficient against bacteria isolated recently.

Our findings are relevant since according to Rios *et al.*[29)] natural crude extracts that have activity at concentrations lower than 100 μg/ml could have great antimicrobial potential, since the active compounds can be isolated and used at lower concentrations. Several studies have shown that flavonoids, hydroxycinnamic acid derivatives, and some ter-

penoids are the compounds related to the antimicrobial activity of propolis.[2,30,31] The results in our study indicate that the antibacterial compounds in propolis type 6 have nonpolar characteristics as judged by the inhibitory effects of the chloroform and hexane fractions.

Propolis type 6 also inhibited the adherence of growing cells of mutans streptococci to a glass surface at sub-MIC levels. This result is consistent with the effective inhibition of GTFs B and C by propolis extracts; the sucrose-dependent adherence and accumulation of cariogenic streptococci is mediated by water-insoluble glucans synthesized by these enzymes.[13—15] Furthermore, only the hexane and chloroform fractions exhibiting anti-GTF activity were able to reduce the bacterial adherence, confirming the importance of these enzymes for the colonization of hard surfaces by mutans streptococci.

Propolis type 6 showed multiple inhibitory activities at concentrations as low as 25 μg/ml. The effective inhibition of GTFs B and C by propolis type 6 may affect the process of dental caries and plaque formation. The active compounds of propolis type 6 appear to have nonpolar characteristics and could be a valuable resource for the exploration of novel bioactive compounds in propolis.

Acknowledgments The authors are grateful to Sr. José Elpídio Cerqueira for providing the propolis samples and to Dr. Reginaldo Bruno Gonçalves for providing the clinically isolated microorganisms. This research was supported by FAPESP (#99/11994-8) and by CNPq (#466879/00-8).

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