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Inhibition of *Streptococcus mutans* biofilm formation using extracts from Assam tea compared to green tea

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ABSTRACT

Objective: Streptococcus mutans, a gram-positive oral bacterium, has been identified as one of the principal etiological agents of human dental caries. To clarify the nature of the difference anti-biofilm effect against *S. mutans* between Assam tea from *Camellia sinensis* var. *assamica*, partially fermented, and green tea from *Camellia sinensis*, non-fermented, active agents from the teas were purified.
Methods: Effects of Assam tea and green tea samples on biofilm were assessed by using the conventional titer plate method and the human saliva-coated hydroxyapatite discs. The purification and identification of inhibitors were performed by using ultrafiltration with centrifugal filter devices and high performance liquid chromatography.
Results: Assam tea has stronger biofilm inhibition activity against *S. mutans* than green tea. A substance of $<$10 kDa in mass in Assam tea had a high concentration of galloylated catechins and a stronger biofilm inhibiting activity than green tea. In contrast, substances >10 kDa in mass from green tea included higher concentrations of polysaccharides composed of galacturonic acid, such as pectin, that enhance biofilm formation.
Conclusions: The higher concentrations of galloylated catechins in Assam tea may assist in prevention of dental caries, whereas in green tea, this mode of inhibition was likely offset by the presence of pectin. Purification of catechins in partially fermented Assam tea with lower-molecular-weight polysaccharide than pectin may be useful for developing oral care products such as toothpaste and oral care gel pastes.

1. Introduction

Streptococcus mutans, a gram-positive oral bacterium, has been identified as one of the principal etiological agents of human dental caries (Kuramitsu, He, Lux, Anderson, & Shi, 2007; Mitchell, 2003). The high pathogenicity of *S. mutans* is attributed to its high biofilm-forming abilities, acid production, acid tolerance, and high affinity for the incorporation of diverse carbohydrate sources (Bowen & Koo, 2011; Mattos-Graner, Klein, & Smith, 2014). The formation of plaque biofilm on the tooth surface is an important step in the initiation and progression of dental caries (Bowden, 1990; Loesche, 1986). The contents in the biofilm include, in addition to microorganisms, extracellular polysaccharides and DNA, proteins, and lipids (Flemming & Wingender, 2010). *S. mutans* enzymes synthesize glucans that form the three-dimensional extracellular polysaccharide scaffold of the biofilm, which is responsible for surface adhesion and cohesion. Glucans are employed for bacterial survival in the severe environmental conditions of the oral cavity.

Controlling pathogenic bacteria, such as *S. mutans*, is important for the prevention of oral diseases, and the development of inhibiting agents effective against oral biofilm formation of microorganisms is necessary for oral disease prevention. Various types of antimicrobial agents targeting oral streptococci have been reported (Jeon et al., 2011; McBain et al., 2003a, 2003b; Walker, 1998). However, a general shift of the oral flora in the oral cavity after treatment with these agents may increase the chance of infection by opportunistic pathogens and other microorganisms (Tada et al., 2006). To prevent infection, safe inhibitory agents with anti-biofilm-formation activity without bactericidal effects are

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desirable for routine oral care in healthy and diseased people, particularly elderly and immunocompromised individuals including HIV-positive and bone marrow-transplant patients.

Tea is arguably the most popular beverage in the world (Sharma, Bhattacharya, Kumar, & Sharma, 2007). It is grown widely throughout the world, with India, Sri Lanka, and parts of China constituting prime growing areas. The three major types of tea are green, oolong and black. Green tea, a non-fermented tea, and oolong tea, a partially fermented tea, have strong bactericidal activities and inhibit bacterial attachment to some surfaces (Friedman, 2007; Lee et al., 2006; Otake, Makimura, Kuroki, Nishihara, & Hirasawa, 1991; Xu, Zhou, & Wu, 2011). Black tea and pu-erh tea, fully fermented teas, have less effective bactericidal activity but may inhibit attachment of bacteria to dental plaque (Wang, Chung, Lee, & Dykes, 2013). All of these teas include polyphenols as primary anti-bacterial agents. The polyphenol (−)-epigallocatechin-3-gallate (EGCg) accounts for 50–80% of the catechins in green tea; other catechins such as (−)-epicatechin-3-gallate (ECg), (−)-epigallocatechin (EGC), and (−)-epicatechin (EC) exist at lower concentrations (Fong, 2002). EGCg has beneficial therapeutic effects including anti-oxidant, anti-inflammatory, anti-cancer, and immunomodulatory effects (Abbott, Rutter, & Berkeley, 1983; Huffman, 2003; Miller, Leibowitz, & Newby, 2004). Furthermore, EGCg has an inhibitory effect on the attachment of bacteria to surfaces (Xu et al., 2011). However, during the oxidative fermentation process, enzymes present in the tea leaves convert catechins into theaflavins and thearubigins in black tea.

Assam tea is a common form of tea; one type is generally produced as a black tea after full fermentation from *Camellia sinensis* var. *assamica*. In this study, Assam tea was selected as a typical black tea because black tea may inhibit attachment of bacteria to dental plaque while having less effective bactericidal activity. To increase anti-microbial activity of catechins in Assam tea, partial fermentation was performed in *C. sinensis* var. *assamica*. The fermentation of *C. sinensis* var. *assamica* proceeded naturally for 1 day after tea leaf picking and was stopped by steaming as thoroughly as possible. Assam tea infusions were acquired from brewed tea leaves with partial fermentation. In contrast, for the Japanese green tea, fermentation of *C. sinensis* was immediately stopped by steaming after picking tea leaves, and the extracts were used as a control for catechins in extracts from Assam tea. The partial fermentation in Assam tea may provide inhibitory effects on biofilm formation as strong as oolong tea. To observe effects of Assam tea on biofilm formation compared with Japanese green tea and other black teas, Assam tea, Japanese green tea and other black teas were added into biofilm formation assays using a 96-well microtiter plate. The Assam tea infusion showed strong inhibitory activity against biofilm formation by *S. mutans* when compared to other teas. To determine the source of inhibition in the Assam tea infusion and to understand the component differences between infusions of Assam tea and Japanese green tea, purification of effective components and assessment of purified components with regard to biofilm formation were performed. We identified higher concentrations of polyphenols, including galloylated catechins, which inhibited biofilm formation, in Assam tea and exopolysaccharides, such as pectin, which increased biofilm formation, in Japanese green tea. These components might provide new insights into the preventive aspects of tea extracts for dental caries.

2. Materials and methods

2.1. Tea leaves

Camellia sinensis and *Camellia sinensis* var. *assamica* were used as Japanese green and Assam tea leaves in this study, respectively. Tea leaves were obtained from Maruei Co., Ltd. (Mie, Japan).

2.2. Catechins

Standard reagents of EC (≥98% purity), EGC (≥98%), ECg (≥98%), EGC (≥98%), and GCg (≥98%) were purchased from Nagara Science Co., Ltd. (Gifu, Japan).

2.3. Preparation of tea infusions

For Assam tea extracts, fermentation of *C. sinensis* var. *assamica* was allowed to occur naturally for 1 day after leaf picking and was stopped by steaming to deactivate enzymes before drying. For Japanese green tea extracts, fermentation of *C. sinensis* was stopped immediately after leaf picking to deactivate enzymes before drying. Nine grams of dried tea leaves were combined with 350 ml boiling water for 10 min, and the brewed infusion was incubated for 20 min at room temperature. The cooled infusion was filtered with filter paper (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and centrifuged at 6000*g* for 10 min. The supernatant was sterilized using a 0.2 μm sterile filter (Toyo Roshi Kaisha, Ltd.). The filtrate was used as the tea infusion.

2.4. Fractionation of tea infusion

Fractionation of tea infusions was performed using ultrafiltration with centrifugal filter devices. The infusion was applied to an Amicon Ultra-15 centrifugal filter device with a 10 kDa nominal molecular-weight limit (NMCL) (Millipore Co., Billerica, MA) and centrifuged for 40 min at 5000*g*. The retentate (>10 kDa) and flow-through (<10 kDa) were both collected. Each fraction was adjusted with distilled water to the original volume.

2.5. Biofilm formation assay on the 96-well microtiter plate

Biofilm formation was assayed by measuring the ability of cells to adhere to and grow on the wells of 96-well polystyrene microtiter plates (SUMILON Multi well plate, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) using a previously reported protocol with modifications (Motegi et al., 2006). *Streptococcus mutans* MT8148, UA159 and ATCC25175 were used to confirm the diverse effects of Assam tea on biofilm formation of *S. mutans* strains, and MT8148 was mainly used in all experiments. Bacterial strains were cultured in Brain Heart Infusion (BHI) (BBL Microbiological Co., Cockeysville, MD) under 5% CO_2 (AnaeroPack CO_2; Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) in a Brewer Jar at 37 °C for 24 h. Bacterial cells were collected by centrifugation at 4 °C for 10 min at 10,000*g*, washed twice with PBS, and resuspended to an absorbance of 0.5 at 550 nm in PBS. Twenty microliters of cell suspension and 180 microliters TSB without dextrose supplemented with 0.25% (w/v) sucrose and a range of 0–5% (v/v) tea sample, 16–1000 μg/ml catechins or 0.02–0.75% (w/v) pectin were inoculated into the 96-well microtiter plate wells. After inoculation, the plates were incubated under 5% CO_2 at 37 °C for 16 h. After incubation, the plates were rinsed twice with distilled water. Fifty microliters of a 1% safranin solution (Gram Hucker's stain solution III; Muto Pure Chemicals, Co., Ltd., Tokyo, Japan) was added to each well, and the plates were then incubated at room temperature for 15 min. Excess dye was removed by washing twice with distilled water and the dye was extracted from the safranin-stained biofilm with 100 μl of 70% ethanol for 30 min. The absorbance of the extract at 492 nm was determined using a plate reader (Multiskan Ascent; Thermo Electron Oy, Vantaa, Finland). Samples showing absorbance >1.0 were re-measured after appropriately diluting the solution to give an absorbance in the range of 0.1–0.9.