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Note

Effect of chlorhexidine on molecular weight distribution of fructans produced by fructosyltransferase in solution and immobilized on surface

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Abstract

The effect of chlorhexidine (CHX), a potent antibacterial agent, was tested on the molecular weight distribution (MWD) of fructans synthesized by cell-free fructosyltransferase (FTF) in solution in comparison to FTF immobilized onto hydroxyapatite (HA). Size-exclusion chromatography (SEC) analysis has shown that cell-free FTF, both in solution and immobilized on HA, produces both low MW (1.9–2.2 kDa) and high MW (913–1047 kDa) fructans. CHX at a concentration of 0.02% altered the MWD of the fructans by reducing the polydispersity ratio and changing the MWD of the fructans synthesized both by immobilized FTF and by FTF in solution. These changes of the fructans in the presence of CHX adds a new prospective to the anticaries effect of CHX in addition to its antibacterial properties.

Keywords: Fructans; Chlorhexidine; Fructosyltransferase; Biofilm; Hydroxyapatite; Immobilization

Dental caries is an oral disease highly associated with the presence of cariogenic bacteria. One of the major virulence factors of cariogenic bacteria has been attributed to their ability to produce extracellular polysaccharides, which facilitate their adhesion to tooth surfaces, and act as a reservoir of nutrition for the bacteria.[1,2]

β-D-Fructosyltransferases (FTF, EC 2.4.1.9) are extracellular enzymes that synthesize fructan polymers from sucrose.[3] Mutans streptococci lacking FTF have been shown to be less cariogenic than native strains.[4,5] Fructans in the oral cavity serve as carbohydrate reservoirs for bacteria in periods of shortage in exogenous carbohydrate supply.[6] Degradation of fructans by fructanase to fermentable sugars serves as a source for acid production by oral bacteria, thus inducing the cariogenic challenge in the oral cavity.[1,7] Recently, it has been suggested that fructans may also serve as binding sites for oral bacteria in the dental plaque biofilm.[8]

Chlorhexidine (CHX) is one of the most potent antibacterial and antiplaque drug used in dentistry. [9,10] Recently, it has been shown that CHX inhibits production of fructan synthesis by cell-free FTF.[11] It was further documented that CHX alters the molecular structure of fructans synthesized by FTF.[11]

The purpose of this study was to further investigate the effect of CHX on fructans production by FTF. In this study we investigated whether the molecular weight distribution (MWD) of fructans synthesized by FTF in solution phase compared with those synthesized on hydroxyapatite (HA) surface is altered in the presence of CHX.

Our results show a bimodal molecular weight distribution profile for fructans synthesized from sucrose by cell-free FTF originated from *Streptococcus mutans* V-1995 (Fig. 1). FTF, both in the solution phase and immobilized on the HA surface, produced both high and low molecular weight fructans (Table 1).

In the presence of 0.02% CHX, FTF in solution produced 50% low MW (LMW) and 50% high MW (HMW) fructans (Fig. 1(A)). The LMW fructans dis-

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Table 1
Effect of Chlorohexidine on weight average (M_w) and number average (M_n) molecular weights and polydispersity (M_w/M_n) of fructans synthesized by cell-free FTF in solution and on hydroxyapatite surface

		FTF in solution		FTF immobilized on HA			
		Fructans produced by FTF in solution		Fructans produced by FTF on HA-released to supernatant fluid		Fructans produced by FTF on HA-bound to surface	
		LMW [b]	HMW [c]	LMW [b]	HMW [c]	LMW [b]	HMW [c]
Sucrose [a] with 0.02% CHX	M_w	2.7	1760	2.7	1510	3.3	1499
	M_n	2.3	510	2.3	310	2.9	280
	M_w/M_n	1.16	3.45	1.17	4.86	1.11	5.35
Sucrose [a] only (control)	M_w	2.2	915	2.1	1047	1.9	913
	M_n	1.9	67	1.8	96	1.7	99
	M_w/M_n	1.16	13.58	1.15	10.93	1.15	9.19

M_w/M_n = polydispersity.
[a] Sucrose 100 mM was used as substrate in all experiments.
[b] Average low molecular weight fructan (kDa).
[c] Average high molecular weight fructan (kDa).

played a higher weight average MW (2.7 kDa) than the control (2.2 kDa), but similar polydispersity index as the control (Table 1). The HMW fructans also had a higher weight average MW (1760 kDa) than control (915 kDa), although the polydispersity index for HMW fructans synthesized in the presence of CHX was lower (3.45) than in the control (13.58).

In the presence of 0.02% CHX, the fructans synthesized by FTF on HA, which were detached and released into supernatant fluid demonstrated also LMW (25%) and HMW (75%) fructans (Table 1). The LMW fructans had a higher weight average MW (2.7 kDa) than the control (2.1 kDa), while the polydispersity index of these LMW fructans (1.17) was comparable to that of

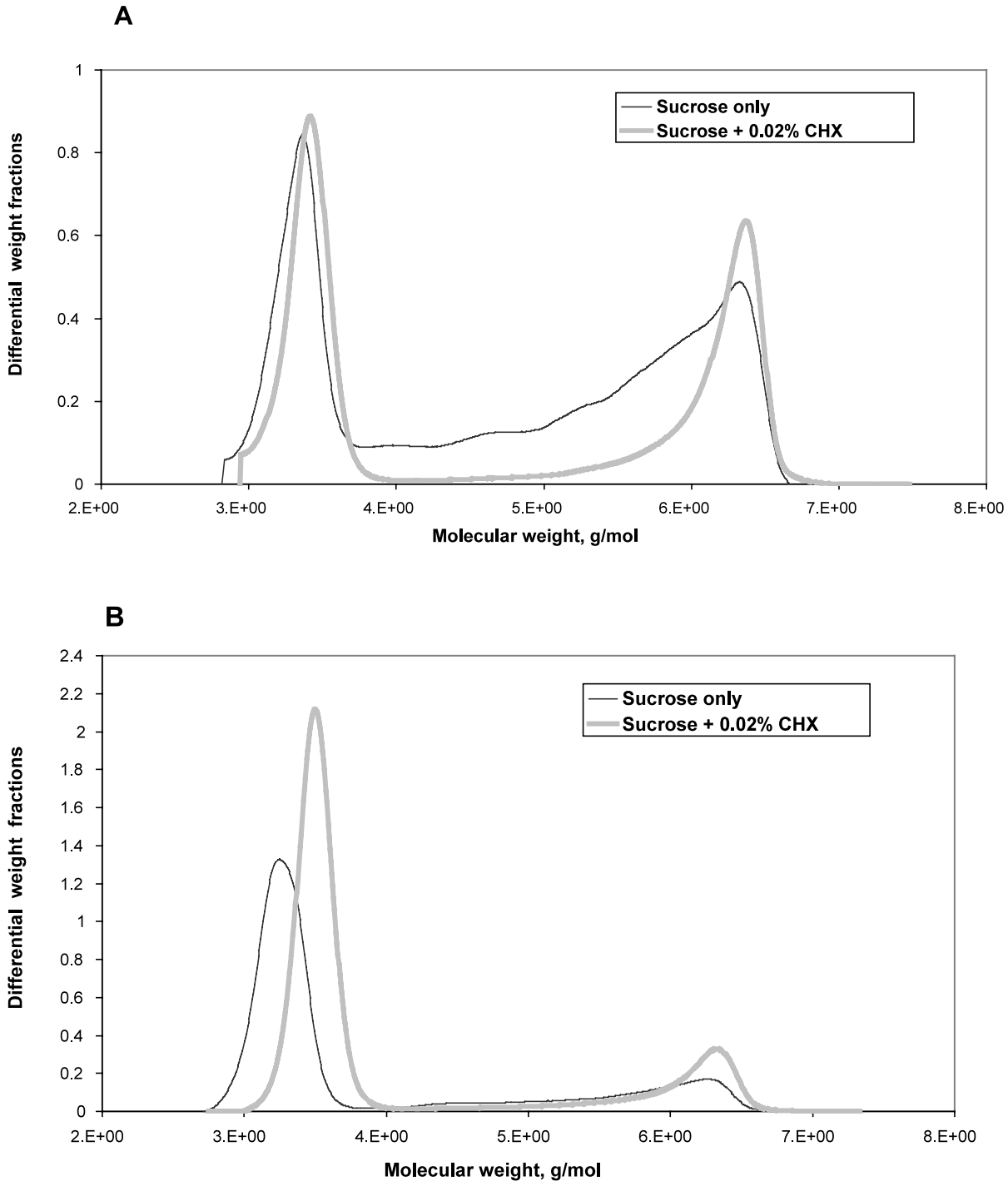

Fig. 1. The effect of 0.02% chlorhexidine (CHX) on molecular weight distribution of fructans synthesized by cell-free FTF. (A) Fructans produced by FTF in solution. (B) Fructans bound to hydroxyapatite (HA), which were produced by FTF immobilized on surface.

the control (1.15). Under the same conditions, HMW fructans displayed a weight average MW of 1510 kDa compared to 1047 kDa of control, but the polydispersity index of these HMW fructans (4.86) was lower than that of the control (10.93).

In the presence of 0.02% CHX, fructans which were synthesized by immobilized FTF and remained attached onto the surface, demonstrated also LMW (23%) and HMW (77%) fructan (Fig. 1(B)). The LMW fructans had a weight average MW of 3.3 kDa compared to control of 1.9 kDa. The polydispersity index of these LMW fructans was lower (1.11) than control (1.15). Under the same conditions, HMW fructans had a weight average MW (1499 kDa) which was comparable to that of the control (913 kDa). The polydispersity index of these HMW fructans was lower (5.35) than that of the control (9.19).

CHX is an anti-plaque agent commonly used in dentistry. Recently, we found that CHX has a significant inhibitory effect on FTF activity in solution and on FTF immobilized onto HA surface.[11] This inhibitory effect was more profound in the solution phase than on HA surface. Fourier transform infrared spectroscopy and circular dichroism analysis have shown that CHX effects fructan synthesis, by altering the chemical structure.[11] It may be postulated that some of the structural changes of fructans in the presence CHX results in a change in the MW of the synthesized fructans. In this study we found that FTF, both in solution and immobilized onto HA surface, synthesized two very different types of fructans, low MW (1.9–2.1 kDa) and high MW (913–1047 kDa). Various MW's have been described in the literature regarding the size of fructans synthesized by FTF in solution phase. Ebisu et al.[13] and Birkhed et al.[14] found that different strains of *S. mutans* synthesized high MW inulin in the range of $1.24–2 \times 10^7$, while *S. salivarius*, *S. sanguis* and *Actinomyces viscosus* synthesized levan with MW's between 2.7×10^6 and 2.16×10^7. Inulin polymers with an even higher MW (7×10^7) were found by Hayer et al.[15]

Our results show that in the presence of CHX a shift in the MWD and polydispersity of fructans is recorded in comparison to control. In addition changes were recorded between the immobilized and the solution states. These changes reflects modifications in properties of cell-free enzymes between the solution phase and the immobilized phase.[12,18] The changes in FTF activity can be attributed to a change in enzyme conformation upon adsorption to HA, or to the unique surface microenvironment which differs from the microenvironment to which the enzyme is expose to solution. Our results show that the MWD of fructans differs between FTF immobilized on HA and FTF in solution, indicating a difference in properties of FTF in the two environments. This notion is also supported by Chambert and Petit-Glatron.[16] which have found that FTF of *Bacillus subtilis*, immobilized on HA synthesized higher MW fructans than FTF in solution.

Results from this study add to the existing knowledge on CHX as an anti-plaque agent. Based on our results, CHX alters the size of the fructans synthesized by FTF. This change affects the properties of the synthesized fructans which may result in a change of the virulence of the dental plaque biofilm.

1. Experimental

1.1. Purification of FTF

Fructosyltransferase was prepared from a culture fluid of *S. mutans* V-1995, FTF hyperproducing strain V-403 with inactivated *gtf* genes[5] by the same method as described by Steinberg et al.[17] This preparation was free of fructanase activity. The specific activity of this FTF preparation was 45 U/mg protein (U = μmol of fructosyl incorporated into fructans/min).

1.2. Preparation of fructans from FTF in solution

Synthesis of fructans in solution phase was conducted in by modified method of Steinberg et al.[17] FTF (100 mU) in solution were transferred to 12,000–14,000 dialysis tubing (Spectrum Industrum Inc., CA, USA) containing 100 mM sucrose solution (control) or 100 mM sucrose solution supplemented with 0.02% CHX-gluconate (Sigma, St. Louis, MO, USA). Pervious results have shown that, CHX at a 0.02%, partially inhibited FTF activity both in the solution and when immobilized on HA surface.[11] The dialysis tube was immersed in 100 mM sucrose solution. After 24 h of incubation at 37 °C, fructans synthesized in the dialysis tubing were precipitated by adding ice-cold EtOH to a final concentration of 80% for 18 h at 4 °C. The precipitated fructans were recovered by centrifugation at $5000 \times g$ for 15 min, washed twice with absolute ice-cold EtOH, centrifuged at $5000 \times g$ for 15 min and dried by lyophilization.

1.3. Preparation of fructans from FTF on hydroxyapatite (HA) surface

Two fractions of fructans synthesized by FTF on the HA (fructan bound to the HA surface, and fructan released from the HA into the supernatant fluid) were prepared in a modified method of Steinberg et al.[17] 500 mg HA beads (80 μm, surface area 40 m^2/g, Bio-Rad, Hercules, USA) were equilibrated by two washes with phosphate buffer (pH 6.5). The washed HA beads were incubated with FTF (100 mU) for 2 h at 37 °C, while being rotated on a shaker. HA beads coated with FTF were transferred to the dialysis tubing under the same

conditions as described above for FTF in solution. After 24 h incubation, the enzymatic reaction was stopped by removing the HA beads from the supernatant fluid in dialysis tubing. Fructans released into the supernatant fluid were isolated using the same precipitation procedure as described for fructans in solution. Fructans bound to the HA surface were detached by a mild dissolution of the HA beads with 100 mM EDTA followed by precipitation procedures similar to those described above.

1.4. Size-exclusion chromatography measurements

The MWD of fructans was determined using aqueous size-exclusion chromatography (SEC) by the same method as described by Steinberg et al.[17] The weight average molecular weight (M_w), number average molecular weight (M_n) and polydispersity (M_w/M_n) for each fructans sample were calculated.

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