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Original Research

Absence of inhibitory effects of two new glucanases on *streptococcus mutans* growth

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Glucanohydrolases have shown promise in degrading exopolysaccharides in cariogenic biofilms, making them a potential strategy for biofilm control without disrupting the oral microbiota. However, their direct antimicrobial effects remain unclear. **Aim:** To determine the antimicrobial activity on *S. mutans* of two newly discovered glucanases characterized by our group, *Pm*GH87 (mutanase) from *Prevotella melaninogenica* and *Co*GH66 (dextranase) from *Capnocytophaga ochracea*, using a commercial dextranase from *Penicillum sp.* as a control. **Methods:** Their effects on growth were assessed using a luciferase reporter system coupled with the promoter of the *ldh* gene in *Streptococcus mutans*. **Results:** Quantification of optical density and luminescence over a 10-hour growth period revealed that the commercial dextranase exhibited inhibitory effects on *S. mutans* growth. However, these effects were neutralized by heat treatment, suggesting the presence of a heat-sensitive contaminant or an additional antimicrobial property associated with the commercial dextranase from *Penicillum sp*. On the other hand, the purified mutanase and dextranase enzymes had no inhibitory effect on *S. mutans* growth. **Conclusion:** In conclusion, the absence of inhibitory effects on *S. mutans* growth by the newly discovered enzymes emphasizes their potential for biofilm control while preserving the delicate balance of the oral microbiota and preventing the emergence of resistance.

Keywords: *Streptococcus mutans.* Dextranase. Anti-Infective agent.

Introduction

Glucanohydrolases have demonstrated potential in degrading biofilms by specifically targeting the polysaccharides within the extracellular matrix[1]. In the oral cavity, biofilms are constantly formed on tooth surfaces, and due to the frequent consumption of sucrose, large amounts of extracellular polysaccharides (EPS) are synthesized, mainly glucans, such as α(1→3)-linked (mutans) and α(1→6)-linked (dextrans) glucans[2]. EPS favor the adhesion and accumulation of biofilm, and if not removed, contribute to the development of dental caries. Therefore, the degradation of dextrans and mutans by the enzymes α(1→3)- and α(1→6)-glucanases (mutanase and dextranase, respectively) have shown promise in degrading exopolysaccharides in oral biofilms[3].

Furthermore, these enzymes exhibit substrate-specificity, making them a potential strategy for controlling biofilms without disrupting the oral microbiota[4]. This differentiates them from traditional antimicrobial therapies, which may harm the commensal microbiota and contribute to antimicrobial resistance. As an alternative approach, they have been explored in combination with conventional antimicrobials to investigate potential synergistic anti-biofilm effects[4-6]. By breaking down the EPS barrier, glucanases facilitate the penetration of antimicrobials deeper into biofilms to prevent bacterial regrowth. However, it is crucial to consider the possible impact of these enzymes on bacterial growth, as they may carry antimicrobial compounds from the microorganisms used for their isolation or other impurities that could inhibit bacterial growth, and thus result in anti-biofilm effects not solely related to the direct activity of glucanases in degrading EPS. Of note, this aspect of glucanase activity is often overlooked in studies investigating their efficacy as anti-biofilm agents[4-9].

Therefore, we investigated the antimicrobial activity of two newly discovered glucanases characterized by our group, *Pm*GH87 (mutanase) from *Prevotella melaninogenica* and *Co*GH66 (dextranase) from *Capnocytophaga* ochracea, using a commercial dextranase from *Penicillum* sp. as a control. Previously, our group demonstrated the potential of such enzymes on *S. mutans* biofilm inhibition and degradation[10-11]. Here, we assessed the impact of the enzymes on the growth and metabolism of *Streptococcus mutans* cultures in real-time using both optical density and a luciferase *ldh* gene reporter, respectively.

Material and Methods

Expression and purification of cloned mutanase (PmGH87) and dextranase (CoGH66)

The nucleotide sequences encoding the GH87 mutanase from *Prevotella melaninogenica* (*Pm*GH87) (GenBank ID: WP_004358976.1) and the GH66 dextranase from *Capnocytophaga ochracea* (*Co*GH66) (GenBank ID: WP_128091406.1) were amplified from the respective genomic DNA and cloned, as previously described by Cortez et al.[10] (2023). Heterologous protein expression was performed in Luria-Bertani (LB) broth containing 50 μg/mL of kanamycin at 37°C and 180 rpm until OD 0.6

(600 nm) followed by induction with 1 mM IPTG at 18°C for approximately 16 h. Then, the cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM PMSF buffer and stored at 4 °C before purification.

Purification of expressed mutanase (PmGH87) and dextranase (CoGH66)

Enzyme purification was carried out following the protocol described by Camilo and Polikarpov[12] (2014). Briefly, the resuspended cells were disrupted performing six cycles of sonication on ice bath using a Sonic Dismembrator Sonifier (Fisher Scientific, Hampton, USA) at 40% amplitude. The resulting lysate was then clarified by centrifugation at 20,200 x *g* at 4°C for 30 min to remove cell debris, and the resulting supernatant was used for mutanase or dextranase purification. The supernatant from each sample was loaded in a column containing Ni-NTA Superflow resin (Qiagen, Hilden, Germany), previously equilibrated with 50 mM Tris-HCl (pH 8.0) and 200 mM NaCl buffer. Elution was performed using an imidazole gradient. The resulting eluted protein fractions were combined and concentrated using a 50 kDa molecular cut-off concentrator to reduce imidazole concentration to less than 20 mM. The resultant sample was incubated with recombinant TEV protease at 4°C for 16 h for 6xHis-thioredoxin tag removal from the target enzyme. Finally, a second Ni^{2+} affinity chromatographic step was used for separation of tag-free mutanase or dextranase from contaminants and TEV protease. The protein sample's purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. The purified enzymes were freeze-dried and, previously to start the biofilm assay, enzymes were resuspended Phosphate-Buffer Salive (PBS) solution 1 X, pH 8.0. The concentration was determined using Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, EUA), measuring absorbance at 280 nm and applying the calculated extinction coefficient (ε = 165180 M^{-1} cm^{-1} for mutanase and ε = 122000 M^{-1} cm^{-1} for dextranase)[12].

Experimental treatments

In addition to mutanase (*Pm*GH87) from *Prevotella melaninogenica* and dextranase (*Co*GH66) from *Capnocytophaga ochracea*, both of which were expressed and purified, a commercially available dextranase (α-(1→6) glucanase, EC3.2.1.11, from *Penicillium* sp.; Product number: D4668-1KU; Batch number: 0000136817) was evaluated as control. The three enzymes were also evaluated in their denatured form by heating them at 100°C for 15 min[10]. The antimicrobial chlorhexidine digluconate (CHX), commonly used in mouthwash, was used as the positive control, while only pure CDM medium as the negative control.

Bacterial growth and luciferase reporter bioassay

Lactate dehydrogenase (*ldh*) gene is constitutive in streptococci and plays a crucial role in the ATP-generating pathway, providing information into both viability and metabolic status[13]. In this study, an isogenic mutant strain of *Streptococcus mutans* UA159 containing a luciferase reporter for *ldh* gene (designated as SM120) as described by Dornelas-Figueira et al.[14] (2023), was used in the bioassay. Briefly, bacterial inoculum of *S. mutans* SM120 was prepared in Chemical Defined Medium

(CDM) containing 1% glucose[15]. Overnight culture of *S. mutans* SM120 reporter strain was adjusted to an OD 0.7 (600 nm). The enzymes, active or denatured, were prepared at a concentration of 0.125 mg/mL (w/v) in the bacterial inoculum. This enzyme concentration was chosen based on our previous study[3], where the lower dosage to cause a significant *S. mutans* biofilm degradation was 0.125 mg/mL (w/v). The positive control was prepared with 0.12% chlorhexidine digluconate in bacterial inoculum, as well as a group with 0.12% chlorhexidine digluconate in pure medium. A negative control group consisting of the inoculum without any treatment and a blank containing only pure medium were also prepared. A group containing inoculum without added luciferin was also included as a control in the luminescence assay. Aliquots of 200 µL of the mixtures were added in 96-well flat bottom plates (Nunc Thermo Scientific), followed by the addition in each well of 10 µL of 1.0 mM luciferin solution (Synchem, Felsberg-Altenberg, Germany) at room temperature. The plates were incubated at 37°C, 10% CO_2 for 10 h. The optical density (OD), as an indicative of bacterial growth, and relative light unit (RLU), as an indicative of bacterial metabolism, were measured every 30 min after a 10-seconds elliptical agitation in a microplate reader (Synergy HT; BioTek, Winooski, VT, USA). The data of CHX in inoculum was subtracted from that in the pure medium, as the antimicrobial produced a whitish color in the medium, which affected OD measurement. RLU and OD data were log transformed and the area under the curve (AUC) for OD or RLU x h were calculated. Three independent experiments were conducted in triplicate, with each experiment carried out on different days.

Statistical Analyses

The data were analyzed by comparing treatment groups to the positive control group (CDM + *S. mutans* for OD data, or CDM + *S. mutans* + luciferin for RLU data). Each time point for OD and RLU curves was analyzed by two-way Anova followed by Dunnett's Test (α=5%), considering as factors treatments and time. The AUC (OD or RLU x h) was analyzed by one-way Anova, Dunnett's Test (α=5%). Statistical analysis was conducted using GraphPad Prism 9, and a p-value < 0.05 was considered statistically significant.

Results

Quantification of optical density (OD) and luminescence (RLU) over the 10-h incubation period revealed that the commercial dextranase exhibited inhibitory effect on the growth of *S. mutans*. The commercial dextranase, chlorhexidine and negative control groups presented no growth quantified by OD or *ldh* activity by RLU when compared to the respective positive control groups after 5 h-growth (Fig 1.A) and 1 h-growth (Fig 2.A), respectively. However, this effect was not observed for the commercial dextranase submitted to the heat-denaturation procedure (Fig 1.A and 2.A). Additionally, cultures in the presence of the other enzymes (whether denatured or not) exhibited similar growth and metabolic patterns to their respective positive control groups over the incubation period (Fig 1.A and 2.A).

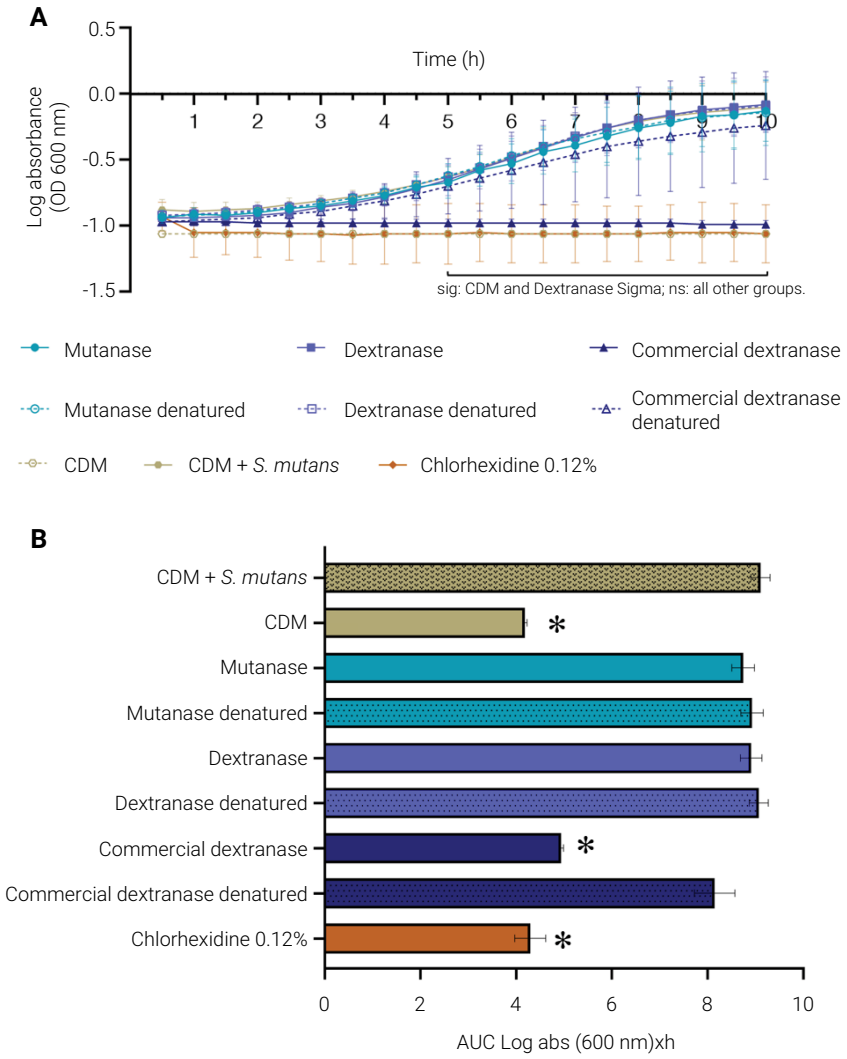

Figure 1. Optical density (OD) quantification (Log). A. *S. mutans* 10 h-growth curve (n=3; mean ± SD) according to the treatments. B. Area under the curve for OD x h. * means significant differences against CDM + *S. mutans* control group.

The AUC data for both OD and RLU compile the results observed at each time point throughout the entire period of the experiment. The active commercial dextranase exhibited lower values for both growth (AUC-OD; $p<0.05$; Fig. 1B) and metabolism (AUC-RLU; $p<0.05$; Fig. 2B) of *S. mutans* compared to the positive control. However, the commercial heat-denatured dextranase did not differ significantly from the positive control for both analyses ($p > 0.05$; Fig. 1.B and 2.B). Mutanase and dextranase, whether denatured or not, showed higher AUC values for OD ($p<0.05$) and RLU ($p<0.05$) compared to the positive control.

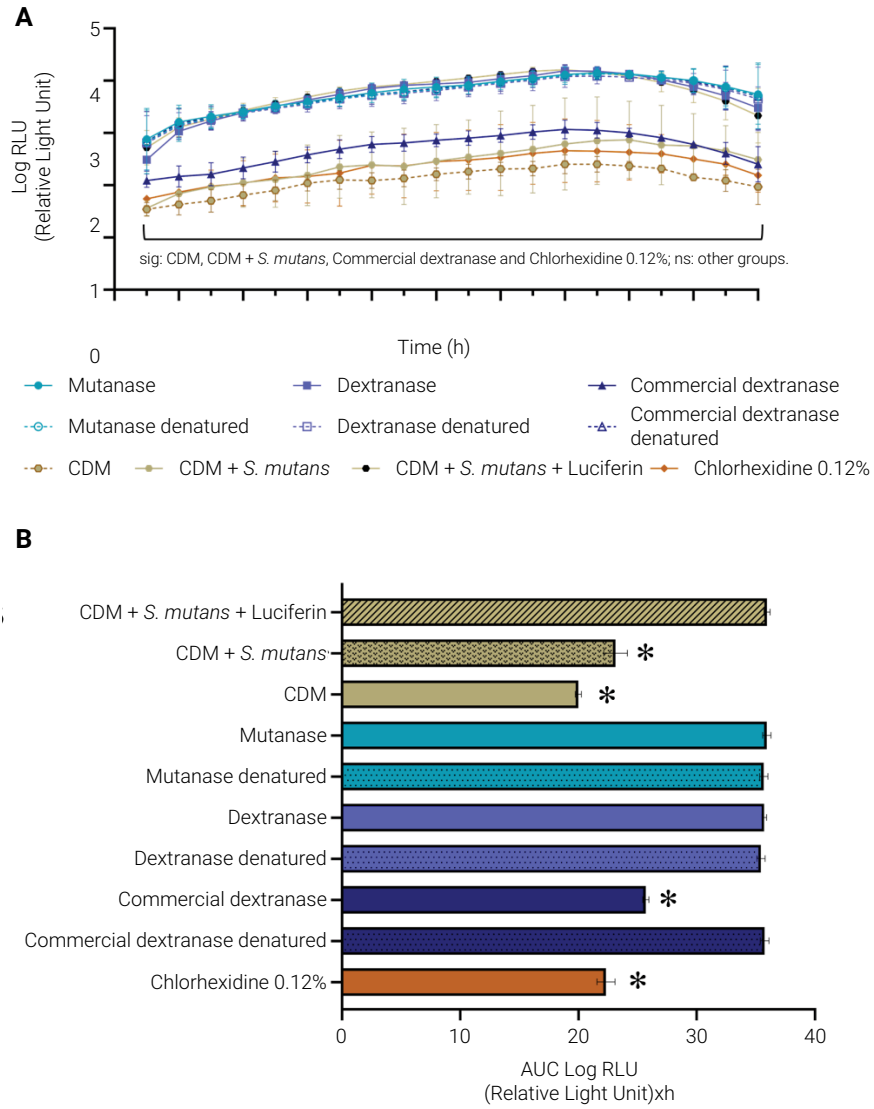

Figure 2. Relative light unit (RLU) quantification (Log). A. *S. mutans* 10 h-growth curve (n=3; mean ± SD) according to the treatments. B. Area under the curve for RLU x h. * means significant differences against CDM + *S. mutans* + luciferin control group.

Discussion

The great advantage of employing enzymes for biofilm control lies in their ability to target specific molecules, thereby preventing the formation or disruption of the biofilm structure, without directly interfering with microbial viability. In the context of oral biofilms, it is expected that mutanases target mutans, while dextranases target dextrans. Therefore, the use of such enzymes to control oral biofilms could offer a beneficial therapy without disrupting the biological balance in the oral microbiota through antimicrobial effects. The newly discovered and characterized mutanase from *Prevotella melaninogenica* and the dextranase from *Capnocytophaga ochracea* have been used

by our group to degrade *S. mutans* biofilms[10-11]. In this study, we confirmed that these enzymes do not exhibit antimicrobial effects on *S. mutans* cultures, highlighting their potential to degrade oral biofilms solely through specific hydrolytic action which was verified previously[10-11].

The lack of antimicrobial effect for the recombinant enzymes aligns with findings by Rikvold et al.[16] (2024) that also evaluated the antimicrobial effects of mutanase, DNAse, and beta-glucanase. In this context, the observed reduction of viability in enzymatically treated biofilms[4,6] may be attributed to the degradation of extracellular polymeric substances, which impairs bacterial aggregation during biofilm formation or detaches bacterial cells from already formed biofilms. Our study supports this hypothesis, as the recombinant enzymes did not affect the planktonic growth of *S. mutans* (Fig. 1 and 2), which has been considered one thousand more susceptible to antimicrobials in its planktonic than in biofilms[17,18].

Differently, commercial dextranase exhibited a similar inhibition of bacterial growth as digluconate chlorhexidine (Fig. 1 and 2). However, when the commercial dextranase was heated-denatured, bacterial growth occurred similarly to the untreated group. Thus, it could be speculated a possible antimicrobial effect of commercial dextranase. However, the substrate-specificity action of such enzymes for the recombinant enzymes, as observed in our study and the reported by Rikvold et al.[16] (2024), do not support this hypothesis. Additionally, *S. mutans* cultures were grown in a chemically defined medium (CDM) supplemented with glucose instead of sucrose, which cannot lead to the synthesis of exopolysaccharides such as dextran and mutan that comprise the biofilm matrix. As a result, the antimicrobial effect could also not be due to EPS protection. Thus, the absence of enzymatic activity observed in this experiment could be attributed both to the lack of available substrate and the enzymes' inability to exert antibacterial effects. A more plausible explanation would be contaminants in the lyophilized enzyme product that could affect the bacterial viability.

Indeed, the certificate of analyses of the commercial dextranase does not offer details regarding the enzyme's production or purification process. In fact, it states that the protein content is 20.85% of the whole product. However, it is unclear whether this percentage specifically refers to the dextranase, as the total protein content was quantified using Lowry's method[19]. Additionally, fungal dextranases are known to be produced by induction methods[3] where the enzyme is secreted extracellularly along with many others fungal products that may not be removed if enzymes are not properly purified. Considering that the commercial dextranase was produced by *Penicillium spp.*, it is possible to speculate that the presence of penicillin as a contaminant could have contributed to the observed antimicrobial effect. In contrast, in our study, we used a well standardized protocol for intracellular heterologous expression of the mutanase and dextranase, as well as for protein purification[10-12].

It is worth noting that the commercial dextranase used in our study has been widely utilized in studies as a test or control enzyme in biofilm assays[4-5,8]. Upon reviewing the literature, we found one study[7] in which another commercial enzyme was employed to specifically test its effect on the growth of *S. mutans* in culture, with notable inhibitory results. While this may be a true effect, the purification grade of such commercial enzyme is not informed and may have an impact on its observed effects.

Overall, our study highlights the necessity of implementing a purification step after enzyme production to ensure the removal of any contaminants that may not only affect microbial viability but also pose toxicity risks to humans, particularly in the context of enzyme application in oral care products. Enzymes can also be a desirable strategy when antimicrobials are needed. Despite biofilms being resistant to antimicrobials[17], enzymes may play a crucial role in degrading EPS, facilitating antimicrobial diffusion, and enhancing bacterial killing[4-6]. Thus, enzymes contaminated with antimicrobials may lead to an overestimated reduction in bacterial viability. Besides, the presence of antimicrobials in biofilms can result in secondary effects[18] that may also impact other evaluated outcomes.

Some limitations of our study should be highlighted. While the oral cavity hosts hundreds of different species[20], our study focused only on *S. mutans*. However, *S. mutans* is a relevant species due to its ability to produce an EPS-rich matrix and its association with biofilms, which contribute to dental caries[21-23]. Since EPS is the target substrate for dextranases and mutanases, the use of this microorganism was appropriate. Regarding the impact on other bacterial species, Rikvold et al.[16] (2024) did not observe any antimicrobial effects. Therefore, studies evaluating commercially available enzymes aimed at degrading components of the biofilm matrix must include bacterial viability controls, as contaminants within the contents may interfere with bacterial growth or metabolism. In addition, the inhibitory effects of the commercial dextranase are not clear and require further investigation.

In conclusion, the absence of inhibitory effects on *S. mutans* growth by the newly discovered and characterized enzymes emphasizes their potential for biofilm control while preserving the delicate balance of the oral microbiota and mitigating the emergence of resistance. In addition, this study highlights the potential of biofilm reduction previously demonstrated by our group to be solely attributable to the enzymatic specific action on the EPS substrate. Furthermore, it is crucial to further investigate the underlying mechanisms responsible for the observed inhibitory effects of the commercial dextranase, as it is commonly employed in research to evaluate anti-biofilm properties. Additionally, future research should explore the broader applications of the newly discovered enzymes to harness their full potential in oral health interventions.

Declarations

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Conflict of Interest

The authors have no conflict of interest to disclose.

Data availability

Datasets related to this article will be available to the corresponding author upon request.

Author Contribution

Mateus Xavier de Queiroz: study conception and design, experimental work, data acquisition, data acquisition, data analyses, writing and manuscript review.

Pedro Ricardo Viera Hamann: experimental work, data acquisition.

Heidi Aarø Åmdal: experimental work, data acquisition, data analyses.

Igor Polikarpov: study conception and design, data analyses, writing and manuscript review, funding acquisition.

Fernanda Cristina Petersen: study conception and design, data analyses, writing and manuscript review, funding acquisition.

Antonio Pedro Ricomini Filho: study conception and design, data analyses, writing and manuscript review, funding acquisition.

All authors reviewed the final version of the manuscript.

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