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Glucanhydrolase from *Lipomyces starkeyi* KSM 22 as Potential Mouthwash Ingredient

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Abstract A glucanhydrolase (a DXAMase exhibiting both dextranolytic and amylolytic activities) from *Lipomyces starkeyi* KSM 22 hydrolyzed polysaccharides having α-(1→3)-, α-(1→4)-, and α-(1→6)-D-glucosidic linkages. The oral hygiene benefits of DXAMase-containing mouthwash were examined in relation to human experimental gingivitis during a 3-week period without brushing. The DXAMase-treated group exhibited a lower increase in plaque accumulation and gingival index score than the chlorhexidine-treated group. The DXAMase-treated group also showed less tongue accumulation, bad taste, and tooth staining, thus indicating a positive role for DXAMase as an antiplaque agent ingredient.

Key words: Glucanhydrolase, *Lipomyces starkeyi*, dextranase, dental plaque, chlorhexidine

Dental plaques, bacterial films that adhere to the tooth surfaces, are composed of closely packed bacteria and noncellular materials [16]. Carbohydrates make up approximately 20% of the dry weight for dental plaque. A major polysaccharide component of some dental plaques is a water-insoluble glucan or mutan. Microbes are able to synthesize this sticky polymer that appears to be one of the major determinants of cariogenicity [12]. Structural studies of extracellular glucans produced *in vitro* have shown that they contain mainly α-(1→3)-, α-(1→4)-, and α-(1→6)-D-glucosidic linkages [24, 27]. Thus, mutanolytic, amylolytic, and dextranolytic activities are required for the efficient removal of dental plaque. The combined activities of a dextranase and an amylase (DXAMase) from *Lipomyces starkeyi* KSM 22, a constitutive and DXAMase hyper-producing strain, that is produced from starch fermentation, have been found to inhibit or prevent dental plaque formation [7]. In previous studies, we demonstrated that DXAMase is an effective inhibitor of the glucan-mediated aggregation of *Streptococcus mutans* and formation of insoluble glucan. The sucrose-dependent adherence of *Streptococci* to a glass surface was also significantly reduced by adding DXAMase [7]. The DXAMase was found to be stable with 0.29% (w/v) EDTA, 20% (v/v) ethanol, 0.05% (w/v) fluoride, and 0.05% (w/v) SDS [7]. In mouthwash, DXAMase was able to partially remove preformed films of glucan-bound *S. mutans* cells [7], and the addition of 0.1 U/ml DXAMase in mouthwash prevented the formation of insoluble-glucan [7]. The present report describes the properties of a purified DXAMase that was prepared from *L. starkeyi* as a dental plaque control agent, along with its oral hygiene benefits in human experimental gingivitis.

The *L. starkeyi* KSM 22 was maintained on a slant of LW medium containing 1% (w/v) soluble starch and 0.05% (w/v) 2-deoxy-D-glucose. The LW medium consisted of 0.3% (w/v) yeast extract and 0.3% (w/v) KH_2PO_4. The pH of the medium was adjusted to 4.5 with HCl [7, 26]. The DXAMase was produced in a 10-l fermenter (Han-Il Co., Korea) with 8.0 l of the LW medium containing 1% (w/v) starch. The pH was maintained at 4.0 by adding 3.0 M NaOH. The aeration rate, temperature, and stirring rate were 3.0 vvm, 28°C, and 250 rpm, respectively. The inoculum was 1.5% (v/v) culture grown for 48 h in the LW medium containing 1% (w/v) starch. One unit of dextranase or amylase was defined as the amount of enzyme that liberated 1 μmole of isomaltose or maltose equivalents in one minute at 37°C by using 2% (w/v) dextran or soluble

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starch (Sigma Chemical Co. S9765) as the enzyme substrate. The reducing value was determined by following the copper-bicinchoninate method [3]. Eight liters of the culture supernatant was concentrated to 500 ml with a 50 K cut-off microfiltration module (Pall, Japan). A DXAMase was purified as reported previously with some modification [7]. A DEAE-Sepharose column (2.5 cm×25 cm) was prepared and equilibrated with 20 mM potassium phosphate buffer (pH 6.4). The 50 K cut-off concentrate (1.5 ml-20 mg protein/ml) was applied to the column, which was then eluted with a linear NaCl gradient (0– 2.0 M) in the potassium phosphate buffer. The active fractions were pooled and concentrated by the isopropanol precipitation method. The dextranase fractions from the DEAE-Sepharose column were size-fractionated by Gel Permeation Chromatography with a BIO-RAD A-0.5 m column (70 cm×2.6 cm) which was prepared and equilibrated with 50 mM citrate phosphate buffer (pH 5.5). Three ml of the DEAE-Sepharose concentrate fraction was applied to this column (4 mg protein/ml). The fractions exhibiting the activity were pooled.

The insoluble-glucan [93% α-(1→3)-glucosidic linkages, and 7% α-(1→6)-linkages] was prepared by using mutansucrase from *S. mutans* 6715 [7, 22]. The strain was grown in 4 l stirred batch cultures with pH maintained at 7.0. The culture medium was LW containing 2% (w/v) glucose. After fermentation, the culture was harvested and centrifuged. The supernatant (1 l) was mixed with 1 l of sucrose (200 mM) dissolved in 20 mM phosphate buffer (pH 7.0). After the sucrose was exhausted, the remaining insoluble material was collected by centrifugation, washed with water (3 times), and titurated with acetone and ethanol. The glucans were dissolved or suspended in citrate-phosphate buffer (20 mM, pH 5.5) at 10 mg/ml concentration for the soluble-glucans and 5 mg/ml for the insoluble-glucans. Five IU of the enzyme was mixed with 1 ml of the glucans at 37°C. The end products were identified after 48 h hydrolysis reactions [15, 20]. The samples were then analyzed by a thin-layer chromatography. Aliquots (1 μl) were placed on Whatman K5F TLC plates. The TLC plate was developed at room temperature with nitromethane/1-propanol/water, 2/5/1.5 (v/v/v) [5, 6]. The compounds were developed on the plate by dipping the plate into a solution containing 3 g of N-(1-naphthyl) ethylenediamine and 50 ml of concentrated H_2SO_4 in 1 l of methanol. The quantitative determination of the hydrolysis product on the TLC plate was achieved by scanning the plate with a Bio-Rad Imaging Densitometer, model GS-670 (Bio-Rad Laboratories, Hercules, CA, U.S.A.) [8, 11, 19, 21].

Dextranase and amylase are enzymes that hydrolyze the α-(1→6)- and α-(1→4)-glucosidic linkages, respectively, in glucan, which is the main constituent of dental plaque as a contaminant. Commercially, dextranase is produced by either *Penicillium* sp. or *Chaetomium* sp. Both genera are known to produce antibiotics and toxins [9, 10]. *L. starkeyi* also produces dextranase and/or amylase. In addition, the mutant, *L. starkeyi* KSM 22, produces dextranase and amylase (referred to as DXAMase for both activities) with a high yield on starch [7]. Only one activity band was exhibited on nondenaturing SDS-PAGE for both dextranase and amylase, in which they are always co-purified [7, 23]. The purified DXAMase had the same molecular mass of 100 kDa with either nondenaturing or denaturing SDS-PAGE (10%) [7, 23], and always showed both dextranase and amylase activities. The purified DXAMase hydrolyzed the polysaccharides including α-(1→3)-, α-(1→4)-, and α-(1→6)-D-glucoside linkages (Table 1). It hydrolyzed 94.8% of the soluble potato starch [containing α-(1→4)-D-glucoside linkages] and 99% of the linear dextran [α-(1→6)-D-glucoside linkages]. In the case of an insoluble mutan [containing α-(1→3)-D-glucoside linkages], it made the insoluble glucan soluble and produced glucose. Alternan, composed of alternating α-(1→3)- and α-(1→6)-glucosidic linkages, was also hydrolyzed by approximately 63.6%. In a model system using *S. mutans*, DXAMase was able to inhibit or prevent plaque formation and partially remove preformed plaque. Furthermore, it showed elevated activity on insoluble glucans when compared to dextranase from *P. funiculosum* [7, 23]. Accordingly, since the structure of the insoluble glucans produced by cariogenic oral streptococci includes predominantly linear chains with α-(1→3)-, α-(1→4)-, and α-(1→6)-glycosidic linkages [24], the above hydrolysis characteristics are valuable as a dental plaque control agent. DXAMase and *P. funiculosum* both extensively hydrolyzed a linear dextran from *L. mesenteroides* B-512FMC. Water-insoluble glucans are somewhat resistant to purified *Penicillium* dextranase, yet found to be susceptible to α-(1→3)-glucanohydrolases [2]. Degradation of *S. mutans* insoluble glucan by DXAMase was 3.75-fold greater than that produced by the dextranase from *P. funiculosum* [23].

A 3-week clinical trial was carried out in a double-blind design to evaluate and compare the oral hygiene benefits and side effects of DXAMase (1 U of dextranase activity equivalent/ml) or 0.12% chlorhexidine-containing mouthwash.

Table 1. Hydrolysis of polysaccharides using DXAMase.

Polysaccharides	Percentage of hydrolysis [a]
Starch	94.8
Dextran	99.0
Mutan	89.3
Alternan	63.6

[a]Hydrolysis percentage was calculated as [Hydrolysis product/(Hydrolysis product+residual substrate)]×100.
Each glucan was dissolved or suspended in a citrate-phosphate buffer (20 mM, pH 5.5) at concentrations of 10 mg/ml for the soluble-glucans and 5 mg/ml for the insoluble-glucans. Five IU of the enzyme was mixed with one ml of the glucans at 37°C. The end products were identified after 48 h hydrolysis reactions. The samples were then analyzed by using a thin-layer chromatography as described in the text.

Table 2. The experimental design used to determine the plaque and gingival indexes.

Pre-prophylaxis	Baseline	Week 1	Week 2	Week 3
GI, PI, TQHI, Professional prophylaxis	GI, PI, DCI, TQHI	GI, PI	GI, PI	GI, PI, DCI TQHI Professional prophylaxis, Toothbrushing

Abbreviations: GI, Gingival index by Löe and Silness; PI, Plaque index [14]; TQHI, Turesky modified Quigley-Hein plaque index [24]; DCI, Discoloration index [14]; Pre-prophylaxis, two weeks before baseline examination.

A total of 39 systematically healthy subjects with moderate levels of plaque participated in the clinical trial [25]; whole mouth Turesky-Quigley-Hein plaque index score >1.5 and gingivitis [16]; whole mouth Löe-Silness gingival index >1.0. Two weeks after a professional prophylaxis, the subjects were provided with a specific mouthwash and instructed to use 20 ml volumes for 30 s, twice a day without brushing. The subjects were grouped according to the mouthwash allocated (DXAMase-treated group, using 1 U of dextranase that is equivalent to activity/ml in sodium phosphate buffer at pH 7.0 and chlorhexidine-treated group, using 0.12% of chlorhexidine digluconate). At the baseline prior to the experiment, then after the first, second, and the third week of the experiment, the subjects were scored for plaque thickness by using the Silness and Löe plaque index, and for gingival inflammation by using the Löe and Silness gingival index system [16]. Table 2 shows the experimental design used to determine the plaque and gingival indices. The subjects were evaluated for the plaque area at the baseline, and after the 3-week experimental period, for the Turesky modified Quigley-Hein plaque index along with plaque severity index, and for tooth staining using the area and severity index system [13]. The oral mucosa was also examined and every subject was questioned regarding tongue accumulation, altered taste, and other side effects. For comparison between the baseline values and the post-experiment data for each week among the groups, an ANOVA test was used. Duncan grouping was performed on the differences for each parameter among the groups and periods at a probability level of 0.05.

DXAMase binds strongly to hydroxylapatite, thereby raising the possibility that it can easily bind to teeth in the mouth [23]. Accordingly, the current study determined the oral hygiene benefits of DXAMase-containing mouthwash for human experimental gingivitis over a 3-week period.

Chlorhexidine is antimicrobial agent against Gram-positive and Gram-negative bacteria and yeast [4]. Plaque accumulation increased significantly in all three groups in the first week of the trial. Over a 3-week period, the DXAMase-treated group exhibited lower plaque accumulation than the chlorhexidine-treated ($p<0.05$) (Table 3).

After three weeks, both the chlorhexidine or DXAMase-treated groups showed increases in plaque accumulation, yet the DXAMase-treated group score was still lower than the scores seen prior to professional prophylaxis and the

Table 3. Changes of plaque and gingival score index or prevalence (%) of side effects during experimental period.

Score		Group[e,f]					
		Chlorhexidine-treated			DXAMase-treated		
		Pre-prophylaxis	Baseline	Week 3	Pre-prophylaxis	Baseline	Week 3
Plaque thickness[a]		–[g]	0.137±0.011	0.769±0.019	–	0.151±0.011	0.535±0.016
Plaque area[b]		2.408±0.274	0.643±0.023	1.969±0.042	2.275±0.179	0.609±0.022	1.697±0.039
Gingival index[c]		1.344±0.070	0.167±0.008	0.694±0.042	1.278±0.071	0.171±0.061	0.442±0.013
Prevalence (%) of side effects	Tongue accumulation	–	–	44.4 ±0.89	–	–	8.3 ±0.580
	Bad taste	–	–	13.8 ±0.28	–	–	2.8 ±0.112
	Desquamation	–	–	16.6 ±0.33	–	–	5.5 ±0.22
Discoloration[d]		–	8.369±0.688	15.877±0.487	–	8.538±0.784	12.569±0.765

[a]Determined by using Silness and Löe plaque index.
[b]Changes in plaque area score by using the Turesky modified Quigley-Hein plaque index.
[c]Changes in gingival index score.
[d]Changes in discoloration index score.
[e]Number of subjects in each group was 13.
[f]Values are expressed as Mean±SE.
[g]Not applicable.

plaque area was smaller than that in the chlorhexidine-treated group ($p<0.05$) (Table 3).

As for gingival inflammation, both groups showed a significant increase during 1 to 3 weeks of the experiment. The inflammation scores after 3 weeks of the treatment were still lower than the pre-prophylaxis scores for both groups. The DXAMase-treated group exhibited lower increase in gingival inflammation, compared to the chlorhexidine-treated group ($p<0.05$) (Table 3). In particular, the appearance of gingival inflammation was much less in the DXAMase-treated group than in the chlorhexidine-treated group during the first and second weeks of the experiment ($p<0.05$).

The long-term use of chlorhexidine can cause certain problems due to local side effects, including increased staining of the teeth, restorations, or tongue and calculus formation. Some patients have also reported inconvenience due to bitter taste and dryness of the mouth [1]. In the current study, tongue accumulation, bad taste, and desquamation were less prevalent in the DXAMase-treated group compared to the chlorhexidine-treated group (Table 3). Tooth stain increased significantly in both DXAMase and chlorhexidine-treated groups compared to the baseline. However, the stain score for the DXAMase-treated group was still lower than for the chlorhexidine-treated group ($p>0.05$) (Table 3).

Based on the above results, mouthrinsing with *Lipomyces starkeyi* KFCC 11077 DXAMase was comparable to 0.12% chlorhexidine mouthwash in regards to inhibition of plaque accumulation and gingival inflammation. Local side effects were, if any, less frequent and less intense in the DXAMase-treated group than in the chlorhexidine-treated group. Thus, all data provided positive benefits for the use of DXAMase as an antiplaque agent, thereby suggesting further development of DXAMase formulations for plaque control. Recently, there has been considerable interest in prebrushing rinses as agents to loosen plaque and facilitate its mechanical removal [17], and DXAMase therefore may perform such functions. In addition, DXAMase can be produced in large amounts on a cheap carbon source, starch [7]. Further studies are necessary to evaluate and compare the effectiveness of prebrushing or postbrushing with a DXAMase mouthrinse for plaque control.

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REFERENCES

1. Ashley, F. P., A. Skinner, P. Y. Jackson, and R. F. Wilson. 1984. Effect of a 0.1% cetylpyrimidium chloride mouthrinse on the accumulation and biochemical composition of dental plaque in young adults. *Caries Res*. **18:** 465–471.
2. Bramstedt, F. and C. J. Lusty. 1979. The nature of the intracellular polysaccharides synthesized by *Streptococci* in the dental plaque. *Caries Res*. **2:** 201–213.
3. Fox, J. D. and J. F. Robyt. 1991. Miniaturization of three carbohydrate analyses using a microsample plate reader. *Anal. Biochem*. **195:** 93–96.
4. Grenier, D. 1996. Effect of chlorhexidine on the adherence properties of *Porphyromonas gingivalis*. *J. Clin. Periodontol*. **23:** 140–142.
5. Kim, C. Y., J. H. Lee, B. H. Kim, S. K. Yoo, E. S. Seo, K. S. Cho, D. F. Day, and D. Kim. 2002. Production of mannitol using *Leuconostoc mesenteroides* NRRL B-1149. *Biotechnol. Bioprocess Eng*. **7:** 234–236.
6. Kim, D., K. H. Park, and J. F. Robyt. 1998. Acarbose effect for dextran synthesis, acceptor and disproportionation reactions of *Leuconostoc mesenteroides* B-512FMCM dextransucrase. *J. Microbiol. Biotechnol*. **8:** 287–290.
7. Kim, D., S. J. Ryu, S. J. Heo, D. W. Kim, and S. H. Kim. 1999. Characterization of a novel carbohydrolase from *Lipomyces starkeyi* KSM 22 for dental application. *J. Microbiol. Biotechnol*. **9:** 260–264.
8. Kim, H. S., D. Kim, H. J. Ryu, and J. F. Robyt. 2000. Cloning and sequencing of the α-1→6 dextransucrase gene from *Leuconostoc mesenteroides* B-742CB. *J. Microbiol. Biotechnol*. **10:** 559–563.
9. Koenig, D. W. 1988. The dextranase of *Lipomyces starkeyi* and its use in sugarcane process. PhD thesis Louisiana State University, LO, U.S.A.
10. Koenig, D. W. and D. F. Day. 1989. Induction of *Lipomyces starkeyi* dextranase. *Appl. Environ. Microbiol*. **55:** 2079–2081.
11. Kim, Y. M., H. J. Ryu, S. O. Lee, E. S. Seo, S. Y. Lee, S. K. Yoo, D. L. Cho, D. Kim, A. Kimura, S. Chiba, and J. H. Lee. 2001. Production of maltopentaose and biochemical characterization of maltopentaose-forming amylase. *J. Microbiol. Biotechnol*. **11:** 636–643.
12. Koga, T. and M. Inou. 1978. Cellular adherence, glucosyltransferase adsorption, and glucan synthesis of *Streptococcus mutans* AHT mutants. *Infect. Immun*. **19:** 402–410.
13. Lang, N. P., P. Hotz, H. Graf, A. H. Geering, and U. P. Saxer. 1982. Effects of supervised chlorhexidine mouthrinses in children. *J. Period. Res*. **17:** 101–111.
14. Lang, N. P. and K. Raber. 1981. Use of oral irrigators as vehicle for the application of antimicrobial agents in chemical plaque control. *J. Clin. Peridont*. **8:** 177–188.
15. Lee, H. S., S. H. Park, J. H. Lee, and H. K. Lee. 2001. Effect of aeration rates on production of extracellular polysaccharides, EPS-R by marine bacterium *Hahella chejuensis*. *Biotechnol. Bioprocess Eng*. **6:** 359–362.
16. Löe, H. and J. Silness. 1963. Periodontal disease in pregnancy. *Acta Odontologica Scandinavia* **21:** 533–551.
17. Marsh, P. D. and D. J. Bradshaw. 1995. Dental plaque as a biofilm. *J. Ind. Microbiol*. **15:** 169–175.
18. Mukasa, H. and H. D. Slade. 1974. Mechanism of adherence of *Streptococcus mutans* to smooth surfaces. II. Nature of the binding site and the adsorption of dextran-levan synthetase enzymes on the cell-wall surface of the *Streptococcus*. *Infect. Immun*. **9:** 419–429.

19. Mukerjea, R., D. Kim, and J. F. Robyt. 1996. Simplified and improved methylation analysis of saccharides, using a modified procedure and thin-layer chromatography. *Carbohydr. Res*. **292:** 11–20.
20. Park, J. G., J. H. Sohn, H. W. Park, and Y. H. Lee. 2001. Encapsulation of whole cell CGTase from concentrated broth solution. *Biotechnol. Bioprocess Eng*. **6:** 67–71.
21. Park, M. R., H. J. Ryu, D. Kim, J. Y. Choe, and J. F. Robyt. 2001. Characterization of *Leuconostoc mesenteroides* B-742CB dextransucrase expressed in *Escherichia coli*. *J. Microbiol. Biotechnol.* **11:** 628–635.
22. Robyt J. F. and J. M. Paula. 1983. Mechanism of synthesis of D-glucans by D-glucosyltransferase from *Streptococcus mutans* 6715. *Carbohydr. Res*. **113:** 301–315.
23. Ryu, S. J., D. Kim, H. J. Ryu, S. Chiba, A. Kimura, and D. F. Day. 2000. Purification and partial characterization of a novel glucanhydrolase from *Lipomyces starkeyi* KSM 22 and its use for inhibition of insoluble glucan formation. *Biosci. Biotechnol. Biochem*. **64:** 223–228.
24. Tsumuraya, Y. and A. Misaki. 1979. Structure of the water-insoluble α-D-glucan of *Streptococcus salivarius* HHT. *Carbohydr. Res.* **74:** 217–225.
25. Turesky, S., N. D. Gilmore, and I. Glickman. 1970. Reduced plaque formation by the chloromethyl analogue of vitamin C. *J. Periodontol.* **41:** 41–43.
26. Yoo, S. K., D. Kim, and D. F. Day. 2001. Highly branched glucooligosaccharide and mannitol production by mixed culture fermentation of *Leuconostoc mesenteroides* and *Lipomyces starkeyi*. *J. Microbiol. Biotechnol*. **11:** 700–703.
27. Yoo, S. K., D. Kim, and D. F. Day. 2001. Co-production of dextran and mannitol by *Leuconostoc mesenteroides*. *J. Microbiol. Biotechnol*. **11:** 880–883.