Effects of Phosphoryl-Oligosaccharides (POs) on Remineralization of Enamel Lesions in vitro

Daisuke INABA, Kentaro MINAMI, Hiroshi KAMASAKA*, Masami YONEMITSU
Department of Preventive Dentistry, School of Dentistry, Iwate Medical University
(Chief : Prof. Masami YONEMITSU)
*Biochemical Research Laboratory, Ezaki Glico Co., Ltd.
[Received : October 23, 2002 ; Accepted : November 18, 2002]

Abstract : We reported that phosphoryl-oligosaccharides (POs) prepared from potato starch hydrolysates strongly solubilize calcium and inhibit formation of calcium phosphate precipitates. The aim of this study was to examine the effects of POs on remineralization of caries-like lesions in enamel in vitro. Bovine enamel slabs were demineralized in a 0.1 M lactic acid gel (pH 5) at 37℃ for 3 w, and subsequently remineralized in a mineral solution (20 mM Hepes, 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, pH 7) with addition of 2 ppm F^- as NaF or various concentrations of POs (0.07-4%) at 37℃ for 7 d. The mineral distributions in enamel were quantified by transversal microradiography. The mineral loss values (ΔZ, vol%·μm) in the samples treated by the mineral solution containing 0.07-0.2% POs were significantly lower ($p<0.001$) compared with demineralized enamel samples and were statistically similar to that in the samples in 2 ppm F group. Remineralization rates of the samples in the POs groups were inversely correlated with the logarithmic values of POs concentration with a significant correlation coefficient ($r=0.99$, $p<0.0001$). The present results suggested that POs may be a novel and unique substance to enhance enamel remineralization, and could be utilized for caries prevention by nutritional approach.

Key words : Phosphoryl Oligosaccharides (POs), Remineralization, Enamel, Microradiography

Introduction

It is known that starches from various sources contain ester phosphorus[1], and that phosphoryl groups bind covalently to a potato starch molecules in which one in 200-500 glucosyl residues are phosphorylated on the average[2]. Preparation of phosphoryl-oligosaccharides (POs) from potato starch hydrolysates has been established previously[3]. In brief, POs can be extracted as indigestible components after amylolytic treatment because the activity of the amylolytic enzymes are covered by phosphoryl groups linking to the glucosyl residues.

The POs are fractionated into two fractions called PO-1 and PO-2[3,4]. The PO-1 is the major component of POs consisting of maltotriose, maltotetraose and

Effects of Phosphoryl-Oligosaccharides (POs) on Remineralization of Enamel Lesions in vitro
Daisuke INABA, Kentaro MINAMI, Hiroshi KAMASAKA*, Masami YONEMITSU
Department of Preventive Dentistry, School of Dentistry, Iwate Medical University
1-3-27 Chuo-dori, Morioka, Iwate 020-8505, Japan.
*Biochemical Research Laboratory, Ezaki Glico Co., Ltd., Osaka,555-8502 Japan.

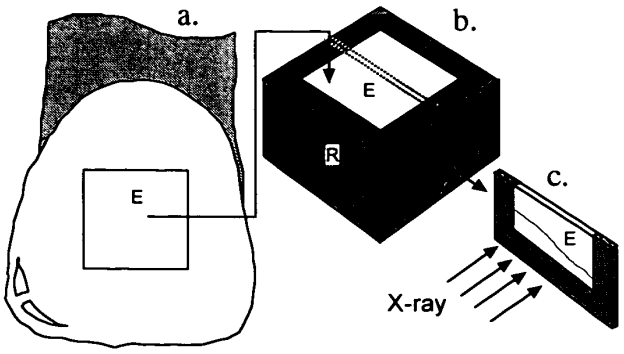

Fig. 1. Diagrams of sample preparation. a. preparation of an enamel block from buccal surface of a bovine incisor, b. embedition of an enamel block in an acrylic resion for experimental treatments and final slicing, c. planoparall thin section for transversal microradiography. E=enamel, R=resin.

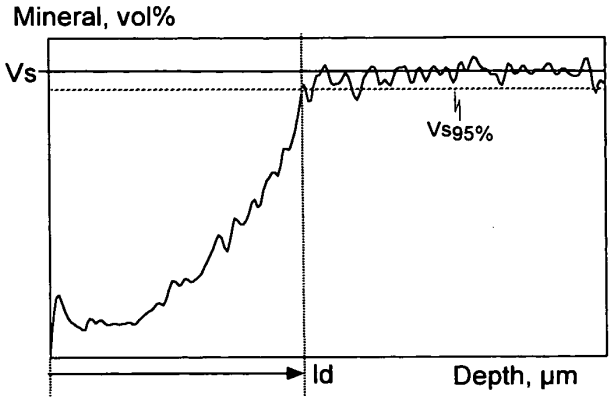

Fig. 2. Schematical mineral distribution and the mineral parameter measured. Vs = mineral vol% level of sound tissue, ld= the lesion depth (μm) defined as the distance from the outer surface position to the location in the lesion at which the mineral content reaches to the 95% level of mineral content in sound tissue ($Vs_{95\%}$), ΔZ = integrated mineral loss value in vol%•μm.

maltopentaose to which one phosphoryl group attaches. The fraction PO-2 is predominantly composed of maltopentaose and maltohexaose to which at least two phosphoryl groups bind. The average degrees of polymerization of dephosphorylated PO-1 and PO-2 were evaluated to be 4.02 and 5.82, respectively. It was found recently that POs remarkably solubilize calcium[5,6]. Based on the previously revealed features of POs, this study examined the effects of POs on remineralization of artificial early caries lesions in bovine enamel *in vitro*.

Materials and methods

Sample preparation.

The outline of sample preparation was illustrated in Fig. 1. Sound enamel slabs (about 7 x 7 x 1.5 mm) were cut from crown parts of freshly extracted bovine incisors. The enamel samples were embedded in a cold curing acrylic resin (Unifast Trad, GC, Japan) except for the buccal surfaces. After setting, the buccal surfaces were slightly abraded on a wet abrasive paper (grit 800) to expose fresh and flat planes of enamel.

De- and remineralization.

To produce early caries-like lesions in enamel, the enamel slabs were demineralized by immersion in a 0.1 M lactic acid gel (pH 5) containing 6 wt% carboxymethyl-cellulose (CMC) at 37℃ for 3 w. The gel volume was always 100 ml per 6 samples.

The demineralized enamel samples were remineralized in a mineral solution (20 mM Hepes, 1.5 mM Ca^{2+}as $CaCl_2$, 0.9mM phosphate as KH_2PO_4, pH 7) with addition of 2 ppm F^- as NaF or various concentrations of POs (0.07 - 4%) at 37℃ for 7 d (n= 6 per treatment). The volume of the remineralization solution was always 500 ml per 6 samples. POs were prepared from potato starch hydrolysates as described in detail by Kamasaka et al[3].

Microradiography and data analysis.

After remineralization, planoparallel sections of about 500 μm thickness were cut

Table 1. Mineral parameter values (Mean±SD) in enamel after initial demineralization and remineralization in mineral solutions containing fluoride or various concentrations of POs.

Treatment	Mineral loss value ΔZ, vol%.μm	Lesion depth ld, μm
Demin.	5,530 ±1,158	112 ±16
Mineral soln.	5,066 ±2,347	114 ±26
4% POs	4,665 ±1,509	102 ±23
1% POs	3,714 ±1,613	93 ±31
2 ppm F	3,224 ±566**	103 ±13
0.2% POs	2,580 ±490**	73 ±21*
0.12% POs	1,812 ±169**	64 ±10*
0.07% POs	1,865 ±292**	64 ±12*

Data connected by vertical bars were not significant by Tukey-Kramer multiple comparison test at $p=0.05$; *$p<0.05$, **$p<0.01$ compared with the values after initial demineralization (Demin.) by Dunnet test.

from the enamel samples using a water-cooled diamond coated saw (Isomet, Buhler, USA) under water supply (Fig. 1). These sections were ground planoparallely on a wet 800-grit abrasive paper to a thickness of about 200 μm. The sections were fitted on a high resolution positive film (Fuji, Japan) together with an aluminum step wedge (15 μm x 15 steps) and microradiographed (PW-1830, Philips, The Netherlands) by Cu-Kα x-ray generated at 25 kV and 25 mA for 24 sec. The films were developed, fixed, and rinsed under standardized conditions.

The mineral distributions were quantified on digitized microradiographic images by computer-assisted videodensitometry (CAV)[7] and a mineral distribution analysis software (MDA)[8]. The lesion depth ld (μm) and mineral loss value ΔZ (vol%·μm) were measured as shown in Fig. 2. The data were analyzed statistically by one-way ANOVA followed by the Dunnett test or Tukey-Kramer test for multiple comparison.

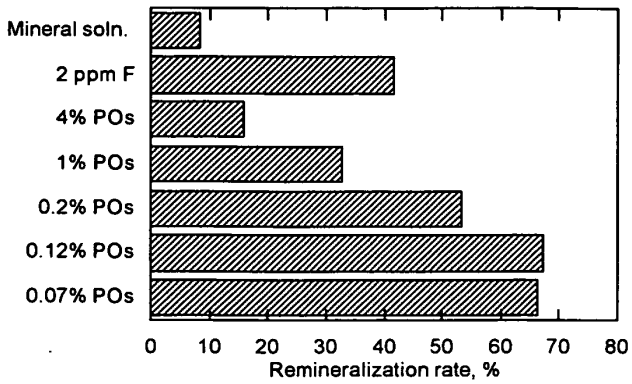

Fig. 3. Remineralization rate (percentage reduction of ΔZ reduction with respect to the initial ΔZ value after demineralization).

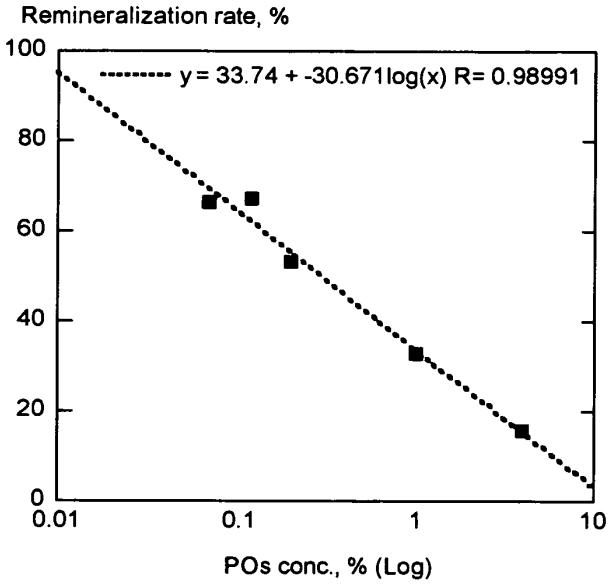

Fig. 4. Correlation between logarithmic values of POs concentrations in mineral solutions and remineralization rates.

Results

The mineral parameter values were compiled in Table 1. Compared with the demineralized enamel samples (Demin.) or the samples exposed to the mineral solution without fluoride (Mineral soln.), addition of 0.07-0.2% POs into the mineral solution showed significant reduction of ΔZ values in enamel ($p<0.001$). In addition, it was found that ΔZ values in the 0.07-1% POs groups were statistically identical with that in 2 ppm-F group indicating POs had

comparable effectiveness on remineralization with environmental low fluoride ions (Table 1). The ld values in the samples treated by mineral solution containing 0.07-0.2% POs were significantly lower than that in the samples remineralized under the presence of 2 ppm F ($p<0.001$).

Remineralization rates (percentage of ΔZ reduction with respect to the ΔZ value after initial demineralization) in the samples treated with 0.07-0.2% POs were in the order of 53-67% exceeding that of samples exposed to the mineral solution containing 2 ppm F (42% ; Fig. 3).

As presented in Fig. 4, the remineralization rates of the samples in the POs groups were inversely correlated with the logarithmic values of POs concentration in the mineral solution with statistically significant correlation coefficient ($r=0.99$, $p<0.0001$).

Discussion

In nutritional sciences, oligosaccharides have been so far revealed as one of beneficial ingredient to improve the intestinal microflora and calcium absorption, to reduce acidogenicity, and to decreased calorie effects of foods[9-12]. In addition to these advantageous features, this study newly demonstrated an unique function of the substance that the presence of relatively low contents of POs (0.07-0.2%) in the mineral solutions enhanced remineralization of enamel significantly without fluoride and, furthermore, the effects of POs on remineralization exceeded that of low fluoride ions (2 ppm as NaF) in the solution. It was also interestingly found that the beneficial effects of POs on enamel remineralization was inversely correlated with logarithmic values of POs concentrations. The optimum concentration of POs seemed to be in the order of 0.05-0.1% for remineralization of early caries lesions in enamel (Table 1 and Fig.4).

Although available information about the role of POs in caries process is limited at present, the enhanced remineralization of enamel lesions by POs could be explained as following so far. The pH of the mineral solution was adjusted to 7 in this study. This pH is suitable for remineralization, but is not preferable for Ca and phosphate to be ionized 3). In fact, the mineral solution itself had no substantial effects on remineralization in this study despite the solution contained enough amounts of Ca and phosphate (Table 1 and Fig. 3). Therefore, it was implied from the present results that small amounts of POs as low as 0.1% in the mineral solution could maintain solubility of mineral ions even at pH 7 and, therefore, contributed for soluble Ca to recrystallize onto the residual hydroxyapatite crystals in enamel lesions. Hence, POs may play an important role in caries prevention as an ingredient to regulate mineral solubility in the oral environment (saliva and/or dental plaque).

The inhibitory effect of POs on formation of calcium phosphate precipitate was suggested to be derived by the feature of POs to form a soluble complex with Ca dependent upon the covalently bound phosphoryl groups in the molecule[3,5,13]. In addition to the present findings, it was suggested in vitro that POs are not fermented by both of *Streptococcus mutans* MT1848 and *Streptococcus sobrinus* 6715, and inhibit the pH fall ascribed to the fermentation of sucrose by these

microorganisms in dose-dependent manner[14]. The inhibition of pH drop in the culture was considered to be facilitated by buffering action of phosphoryl groups in POs.

Thus, POs is expected not only to enhance remineralization by acting as a mineral ion stabilizer at neutral pH but also to be classified as one of non-cariogenic carbohydrates. In conclusion, it was suggested that POs may be a novel and unique substance produced from potato starch to enhance enamel remineralization by solubilizing environmental Ca, and could be utilized for caries prevention by nutritional approach. To examine the possibility of POs as a dietary ingredient for caries prevention, further studies on the intraoral effects of POs on remineralization of enamel are in progress at present.

Acknowledgement

This study was supported in part by Grants-in-Aid for High Performance Bio-Medical Materials Research by the Ministry of Education, Science, Sports and Culture (2001), Japan.

References

1) Takeda, C., Takeda, Y. and Hizukuri, S. : Structure of the amylopectin fraction of amylomaize. *Carbohydr. Res.* 246 : 273-281, 1993.

2) Takeda, Y. and Hizukuri, S. : Location of phosphate groups in potato amylopectin. *Carbohydr. Res.*, 102 : 321-327, 1982.

3) Kamasaka, H., Uchida, M., Kusaka, K., Yamamoto, K., Yoshikawa, K., Okada, S. and Ichikawa, T. : Inhibitory effect of phosphorylated oligosaccharides prepared from potato starch on the formation of calcium phosphate, *Biosci. Biotech. Biochem.* 59 : 1412-1416, 1995.

4) Kamasaka, H., To-o, K., Kusaka, K., Kuriki, T., Kometani, T., Hayashi, H. and Okada, S. : The structures of phosphoryl oligosaccharides prepared from potato starch, *Biosci. Biotech. Biochem.* 61 : 238-244, 1997.

5) Kamasaka,, H., To-o, K., Kusaka, K., Kuriki, T., Kometani, T. and Okada, S. : A way of enhancing the inhibitory effect of phosphoryl oligosaccharides on the formation of calcium phosphate precipitate by using the coupling reaction of cyclomaltodextrin glucanotransferase. J. Appl. Glycosci. 44 : 285-293, 1997.

6) Kamasaka, H., To-o, K., Kusaka, K., Kuriki, T., Kometani, T. and Okada, S. : Inhibitory effect on the formation of calcium phosphate precipitate by the conjugates of ovalbumin and phosphoryl oligosaccharides. *J. Appl. Glycosci.* 44 : 295-302, 1997.

7) Inaba, D., Takagi, O. and Arends, J. : A computer-assisted videodensitometric method to visualize mineral distributions in in vitro and in vivo formed root caries lesions. *Eur. J .Oral .Sci.* 105 : 74-84, 1997.

8) Inaba, D., Tanaka, R., Takagi, O., Yonemitsu, M. and Arends, J. : Computerized measurements of microradiographic mineral parameters of de- and remineralized dental hard tissues. *J. Dent. Hlth.* 47 : 67-74, 1997.

9) Okazaki, M., Fujikawa, S. and Matsumoto, N. : Effect of xylooligosaccharide on the growth of bifidobacteria. *Bifidobact. Microflora.* 9 : 77-86, 1990.

10) Terada, A., Hara, H., Kataoka, M. and Mitsuoka, T. : Effect of lactulose on the composition and metabolic activity of the human faecal flora : Microb. Ecol. Hlth. Di.s 5 : 43-50, 1992.

11) Kaneko, T., Matsukubo, T., Yatake, T., Muramatsu, Y. and Takaesu, Y. : Evaluation of acidogenicity of commercial isomaltooligosaccharides Mixture and its hydrogenated derivative by measurement of pH response under human dental plaque. *Biosci. Biotech. Biochem.* 59 : 372-377, 1995.

12. Oku, T. : Available energy of nondigestible and/or nonabsorbable saccharides. *Jpn. J. Nutr.* 54 : 143-150, 1996.

13) Kamasaka, H., To-o, K., Kusaka, K., Kuriki, T., Kometani, T. and Okada, S. : Effect of phosphoryl oligosaccharides on iron solubility under neutral conditions, *Biosci. Biotech. Biochem.* 61 : 1209-1210, 1997.

14) Kamasaka, H., Imai, S., Nishimura, T., Kuriki, T. and Nishizawa, T. : Effect of phosphoryl oligosaccharides from potato starch on acid fermentation by mutans streptococci. *J. Dent. Hlth.* 52 : 66-71, 2002. [in Japanese]

エナメル質齲蝕病巣のin vitro再石灰化におよぼすリン酸化オリゴ糖（POs）の効果

稲葉　大輔，南　健太郎，釜阪　　寛*，米満　正美
岩手医科大学歯学部予防歯科学講座
（主任：米満　正美　教授）
*江崎グリコ㈱生物化学研究所

（受付：2002年10月23日）
（受理：2002年11月18日）

抄録：著者らは，馬鈴薯澱粉の加水分解物より調製されるリン酸化オリゴ糖（*POs*）がカルシウムの溶解を強く促進し，カルシウムーリン酸の沈殿形成を阻害することを報告した。本研究の目的はエナメル質の齲蝕様病巣の再石灰化におよぼす*POs*の効果を*in　vitro*で検討することである。ウシエナメル質試料を0.1*M*乳酸ゲル（*pH*5）で脱灰後，*NaF*として2*ppmF*または様々な濃度の*POs*（0.07－4%）を添加したミネラル溶液（20*mM Hepes*, 1.5*mM* $CaCl_2$, 0.9*mM* KH_2PO_4, *pH*7）に37℃で7日間，浸漬した。エナメル質のミネラル濃度分布は*transversal microradiography*により定量化した。0.07－0.2%の*POs*を含むミネラル溶液で処理した試料のミネラル喪失量（ΔZ, *vol*%・μm）は，脱灰エナメル質よりも有意に低く（$p<0.001$），しかも2*ppmF*群と統計学的に同等の値であった。*POs*群試料の再石灰化率は*POs*濃度の対数値と有意な負の相関を示した（$r=0.99$, $p<0.0001$）。本研究の結果から，*POs*は新規かつユニークなエナメル質の再石灰化促進物質であり，栄養学的なアプローチにより齲蝕予防に活用しうる可能性が示唆された。

キーワード：リン酸化オリゴ糖（POs），再石灰化，エナメル質，マイクロラジオグラフィ